The ecology and evolution of larval competitive ability in laboratory populations of *Drosophila*

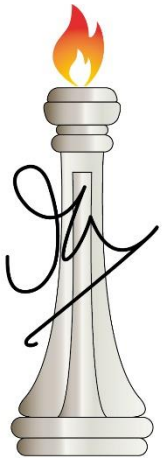
A Thesis

submitted for the degree of

Doctor of Philosophy

By

Srikant Venkitachalam



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This thesis is dedicated to all those studies which helped me understand the ecology of competition and the evolution of competitive ability.

In particular, it is dedicated to two studies: Bakker 1961, and Sang 1949, which gave me the path ahead for my research.

Declaration

I hereby declare that the thesis titled "**The ecology and evolution of larval competitive ability in laboratory populations of *Drosophila***", submitted towards the partial fulfilment of the Ph.D. degree, is the result of investigations carried out by me, Srikant Venkitachalam, at the Evolutionary and Organismal Biology Unit of the Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India, under the supervision of Prof. Amitabh Joshi. The work incorporated in this thesis has not been submitted for any other degree elsewhere.

In keeping with reporting scientific observations, this study has taken due care to acknowledge and cite other investigators' work. Any omission, which may have occurred due to oversight or misjudgement, is regretted.

Srikant Venkitachalam

Bengaluru, India

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CERTIFICATE

This is to certify that the work described in the thesis entitled "**The ecology and evolution of larval competitive ability in laboratory populations of *Drosophila***" is the result of investigations carried out by Mr. Srikant Venkitachalam in the Evolutionary Biology Laboratory, Evolutionary and Organismal Biology Unit of Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Amitabh Joshi

(Professor)

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Ph.D. Thesis Synopsis

My studies are broadly concerned with the experimental testing of the theory of density-dependent selection, which predicts that population density may differentially affect the Darwinian fitness of organisms with differing phenotypes and hence potentially shape evolutionary trajectories. There have been several decades of theoretical and empirical studies on density-dependent selection, leading to much progress in understanding the role of population density as a potential selective agent. Empirically, some of the most rigorous testing has been carried out using selection experiments on laboratory populations of *Drosophila* adapted to chronic conditions of crowding at the larval stage. The details of each of these selection experiments have been summarised in **Chapter 1**. Briefly, a series of early selection studies on crowding-adapted laboratory *Drosophila* populations saw very consistent results regarding the traits evolved when selecting for high larval density conditions. This led to a 'canonical' view of adaptation to larval crowding in laboratory *Drosophila* populations, which persisted for 15-20 years. However, recent studies in our lab have since dismantled this canonical view – three selection studies on different species of *Drosophila* showed that the traits that could evolve as a response to selection for chronic larval crowding were very different from what the canonical view had predicted. It was also inferred that the primary reason for the differences seen between the recent and the earlier studies was due to the precise manner in which larval crowding was implemented (i.e., egg number, food volume). Traditionally, experimental studies on density-dependent selection treated density, as defined by the total eggs per unit volume of total food, as a perfect descriptor of the ecological effects of crowding. However, several recent studies showed that this measure of density was not enough to understand the ecology of a given crowded culture, nor was it enough to predict the evolutionary trajectory of a population adapted to larval crowding in the given type of culture.

My PhD studies are aimed at understanding: a) the evolution of traits in different sets of populations adapted to being reared in various types of crowded cultures (each set reared at a different combination of egg number and food volume); and b) what factors beyond the total eggs/food density contribute to differences in larval ecologies and evolutionary trajectories of populations adapted to different types of crowded cultures. My work was primarily done using three sets of crowding-adapted populations: MCU (crowded at 600 eggs in 1.5 mL food), CCU (crowded at 1200 eggs in 3 mL of food – at the same eggs/food density as MCU), and LCU (crowded at 1200 eggs in 6 mL of food). These were contrasted with MB, a set of low-density reared populations, ancestrally related to MCU, CCU and LCU.

In **chapter 2**, I describe the evolution of increased larval competitive ability in the MCU, CCU and LCU populations, as contrasted with the MB. While the evolution of increased larval competitive ability has been seen in most laboratory *Drosophila* crowding-adaptation studies, those have typically used only pre-adult survivorship as a measure of competitive ability. In my study, I used pre-adult survivorship, pre-adult development time, dry mass at eclosion per fly and dry biomass as outcomes of competitive ability. I also further partitioned larval competitive ability into proactive (effectiveness) and reactive (tolerance) components. According to my results, all crowding-adapted populations had evolved greater larval competitive ability than the MB controls, but this increase in competitive ability was dependent on the type of crowded culture as well as the measure of competitive ability used. Among the crowding-adapted populations, the MCU showed the most consistently high larval competitive ability. However, depending on the food volume used and the measure of competitive ability examined, even the CCU could have greater competitive ability. The results of this study also indicated that larval competition likely unfolds in a very nuanced, time-dependent manner within a culture.

In **chapter 3**, I examine if the crowding-adapted populations had evolved traits that could confer head starts in competition – i.e., increased egg size and shorter hatching time. The results indicated that all crowding-adapted populations had evolved greater egg length and egg width than MB (with LCU having the largest eggs). Only LCU populations had evolved a shorter mean hatching time than the MB, whereas MCU and CCU had non-significantly shorter mean hatching time than the MB.

Chapter 4 outlines a study wherein I asked if the MCU, CCU and LCU populations could fare better than MB in competition against an age disadvantage imposed on them in larval competition, via a head start to a common competitor. The results indicated that crowding-adapted populations each coped better against competitive disadvantages than MB. However, the benefit gained by the head start to the common competitor was maximum against LCU, and minimum against the low-density reared MB populations, suggesting that larger egg size and shorter egg hatching time did not translate into an unequivocal benefit in terms of dealing with a head start to the common competitor.

In **chapter 5**, I report results from a study of the effects of changing egg number, food column height and the surface area of food in contact with air on the outcome of a crowded culture (pre-adult survivorship or development time), in a fully factorial design. It was inferred from the results that the 'effective' density of a culture, or the density of eggs in the feeding band (a volume of food close to the surface having air contact, to which larval feeding is restricted), predicts the outcome of a crowded culture much better than the culture's 'total' density (eggs per unit volume total food).

In **chapter 6**, I describe the development of, and results from, an individual-based simulation framework to understand the ecology and mechanistic underpinnings of larval competition in crowded cultures of *Drosophila*. This involved the construction of a 'base' model, which

captured the understanding of the ecology of larval crowding before the recent studies, as well as an 'extended' model, which incorporated effects of a) food column height and b) cross-section surface area of food, on larval competition, in order to see if the model could capture the patterns seen in the experimental results described in chapter 5.

Finally, I briefly list the conclusions from each chapter, and put forth some ideas for future studies in **chapter 7**.

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List of publications

1. Venkitachalam, S., Temura, C., Kokile, R., and Joshi, A. 2023. The evolution of competitive effectiveness and tolerance in populations of *Drosophila melanogaster* adapted to chronic larval crowding at varying combinations of egg number and food volume. bioRxiv. https://doi.org/10.1101/2023.07.24.550285
2. Venkitachalam, S., Das, S., Deep, A., and Joshi, A. 2022. Density-dependent selection in *Drosophila*: evolution of egg size and hatching time. Journal of Genetics, *101*(1), 13. https://doi.org/10.1007/s12041-021-01355-6
3. Venkitachalam, S., Deep, A., Das, S., and Joshi, A. 2023. The role of greater competitive ability in countering age disadvantages in larval competition in *Drosophila melanogaster*. bioRxiv. https://doi.org/10.1101/2023.07.25.550469
4. Venkitachalam, S., V.S. Sajith, and Joshi, A. 2023. More than just density: the role of egg number, food volume and container dimensions in mediating larval competition in *Drosophila melanogaster*. bioRxiv. https://doi.org/10.1101/2023.07.26.550621
5. Venkitachalam, S. and Joshi, A. 2023. An individual-based simulation framework exploring the ecology and mechanistic underpinnings of larval crowding in laboratory populations of *Drosophila*. bioRxiv. https://doi.org/10.1101/2023.07.30.551144

Chapter I

Introduction

NOTE

The detailed background for each problem tackled in this thesis has been laid out in chapters 2-6, respectively. These chapters can largely be read independently, as they are stand-alone publications or preprints of manuscripts prepared for journal submission. Additionally, each chapter follows a different formatting and citation style, in order to fit the particular journal in which it is published or planned to be submitted for review. I would nevertheless recommend reading chapter 4 after reading chapters 2 and 3, and reading chapter 6 after reading chapter 5, as the impetus for studies done in both chapters 4 and 6 are linked to the results discussed in their corresponding preceding chapter(s). The premise of the overall problem is common amongst all the chapters, and primarily stems from the experimental testing of the theory of density-dependent selection, which I have reiterated briefly in this introduction. Furthermore, I have elaborated herein the role which I hope my thesis may play in furthering this field of rigorous study.

Experiments on density-dependent selection – a brief introduction

(See Introductions in chapters 2 and 3 for detailed background)

The theory of density-dependent selection (first formally described by MacArthur 1962; MacArthur and Wilson 1967; reviewed in Mueller 1997, 2009; Joshi et al. 2001) predicts that the fitness landscape can change across population densities, and thus integrates the fields of population genetics and population dynamics. The theory has seen several expansions since its inception, both theoretical (reviewed in Mueller 1997; Joshi et al. 2001) and experimental (reviewed in Mueller 1997; Sarangi et al. 2016).

An overview of empirical studies

NOTE: The studies covered in this chapter, and the overall thesis, focus on intraspecific density-dependent selection. There are several studies that have explored the evolution of competitive ability in inter-species competition experiments, under density-uncontrolled (e.g., Moore 1952; Ayala 1969) or density-controlled (e.g., Joshi and Thompson 1995) setups. Similarly, inter-strain trans-generational competition experiments have also been carried out in house flies *Musca domestica* (Bryant and Turner 1972) and *D. melanogaster* (Sulzbach 1980). In inter-species set-ups, only those species have been used which can feed on the same resource (i.e., usually closely related species). The effects of multiple species in the ecology of competition and the evolution of competitive ability have been briefly conjectured in the Discussion section of Chapter 2.

This section covers some empirical studies on density-dependent selection briefly. For a review of theoretical investigations, see Mueller (1997) and Joshi et al. (2001) (also briefly summarised in Chapter 2).

Several early studies, some carried out even before the crystallisation of density-dependent selection theory, showed that different inbred strains, and thus different genotypes, could perform differently at high vs. low density conditions. Lewontin (1955), Bakker (1961), Seaton and Antonovics (1967), Mather and Caligari (1981), de Miranda et al. (1991), Santos et al. (1992), to name a few, respectively carried out extensive experiments on differences in competitive ability of two different strains of *Drosophila melanogaster* under various 'high density' scenarios (also see similar studies on *D. busckii* by Lewontin and Matsuo (1963) and on *Musca domestica* by Sokal and Sullivan (1963)).

While this a good first step in showing the possibility of density-dependent selection, the best proof would be expected to be revealed in long term selection experiments. Several experimental evolution studies have indeed been carried out. Birch (1955) showed that different chromosomal arrangements were favoured at low vs. high larval density conditions in a selection experiment spanning 3 generations using a UU, CU and UC design (see Mueller et al. 1993, Joshi and Mueller 1996, and the section below) in *D. pseudoobscura*. Luckinbill (1979) found that selection for increased rate of growth also leads to increased saturation density (K), in multiple species of protozoans. In addition, the study demonstrated that those species having lower saturation density had increased competitive ability, and concluded that selection for increased saturation density was not the same as selection for increased competitive ability. Barclay and Gregory (1981) tested different models of age-specific density dependence in *Drosophila* using various *r*-selected (low density, primarily density independent mortality) and *K*-selected (high density, primarily density dependent mortality) regimes. Their study concluded that "The simple model of *r*- and *K*-selection based on the effects of density alone seems inadequate to explain variation in life-history features of complex organisms". Bolnick (2001) found that populations of *D. melanogaster* flies reared in high population density scenarios for up to 4 generations evolved greater tolerance to Cadmium laced food than

low density reared populations. Agashe and Bolnick (2010) found that populations of red flour beetle *Tribolium castaneum* evolved niche use with respect to the food eaten when reared in relatively high population density conditions.

Some field studies on density-dependent selection also exist. Bassar *et al.* (2013) showed that Trinidadian guppies *Poecilia reticulata* raised in low predation environment are likely to face high population densities and undergo density-dependent natural selection. This likely results in a lower sensitivity of their population growth rate towards density, as compared with the guppy populations raised in high predation environments. Bradshaw and Holzapfel (1989), on studying the pitcher plant mosquito *Wyomia smithii*, found that there was no correlation between rearing density in different natural zones with estimates of either the r or K components of any population reared under uniform laboratory conditions. However, when two visually distinct populations of mosquitoes, originating from two different geographical zones, were reared together, there were differences in estimates of larval competitive ability. Moorcroft et al. (1996) found evidence of density-dependent natural selection associated with variation for two simple polymorphic traits in a free-living Soay sheep *Ovis aries* population at the Hirta island of the St. Kilda archipelago, across a study period of 1985-1992. The sheep population had been measured to go through repeated density-dependent mortality cycles every three years since 1959, and the authors found an association of coat morph and horn type with density-dependent mortality, respectively. Furthermore, the extent of differences seen in differential survival of the morphs also appeared to depend on sex.

Some studies which have cited the existence of density-dependent selection in their respective systems, may be measuring density-dependent ecological effects through a single generation of observation on target phenotypes. Examples include studies on density dependence of sociability in common lizards *Zootoca vivipara* (Le Galliard et al. 2015), consequences of phenotypic plasticity in stem elongation on density dependent fitness in the plant *Impatiens*

capensis (Dudley and Schmitt 1996) and density-dependent fitness in reproductive-effort-selected lines of bank voles *Myodes glareolus* (Mappes et al. 2008).

Finally, the most extensive empirical studies on density-dependent selection, and of which this Ph.D. thesis is a continuation, involves a series of long-term experimental evolution studies on crowded laboratory populations of fruit flies *Drosophila melanogaster*. These are discussed in the following sections.

Selection experiments on laboratory Drosophila populations

Experimentally, some of the most rigorous testing of the theory of density-dependent selection was done through selection experiments using outbred laboratory *Drosophila* populations (reviewed in Sarangi et al., 2016; also see Chapter 3). Selected populations were reared under chronically crowded conditions, in order to explore the evolution of traits compared to their respective low-density reared control populations. Several such selection experiments have been carried out since the early 1980s. Summaries of each selection experiment are listed below, along with some of their important contributions in furthering the understanding of density-dependent selection:

1. *r* and *K* populations (first described in Mueller and Ayala 1981):

   The first density-dependent selection experiment on *D. melanogaster* populations was done using the *r* and *K* populations. Each *K* population was maintained at a high population density in both larval and adult phases using the serial transfer method (first described in Ayala 1965). The *r* populations were instead maintained at low population density by culling. Compared to the *r* populations, the *K* populations evolved greater larval competitive ability (Mueller 1988*a*), along with increased larval feeding rate (Joshi and Mueller 1988), but with an increased minimum food requirement for pupation, indicating the evolution of decreased food to biomass conversion efficiency (Mueller 1990). Chapter

3 contains further details on the evolution of traits in *K*- relative to *r*-populations and related studies, as well as the drawbacks of this selection regime.

2. UU and CU populations (CU first described in Mueller et al. 1993; UU in Joshi and Mueller 1996):

Given that the *K* populations confounded selection for crowding at both larval and adult stages, the next selection study sought to separate the larval and adult phases of crowding. This resulted in the creation of the CU populations, selected for Crowding at the larval stage, but Uncrowded at the adult stage. These were compared with the low-density control populations, the UU, which were Uncrowded at the larval stage and Uncrowded at the adult stage. Additionally, the UC populations were also derived, crowded only at the adult stage (see Mueller et al. 1993; Joshi et al. 1998). Similar to the *K*-populations, the CU populations also evolved increased larval feeding rate, along with a reduction in food to biomass conversion efficiency, as compared to the UU populations (Joshi and Mueller 1996). The UU and CU populations were also tested for tolerance to metabolic waste products, which were known to build up in crowded larval cultures (Botella et al. 1985; Borash et al. 1998). Compared to their low-density controls, the CU populations evolved both increased pre-adult urea tolerance as well as ammonia tolerance (Shiotsugu et al. 1997; Borash et al. 1998). The CU populations also maintained an interesting temporal polymorphism: offspring of early eclosing CU flies exhibited greater larval feeding rate but with lower waste tolerance, whereas those of late eclosing CU flies exhibited lower larval feeding rate but with increased waste tolerance (Borash et al. 1998).

Given that the results from the studies of *r*- and *K*-populations, as well as of the UU and CU populations, were broadly consistent with respect to the larval traits evolved in the crowding-adapted populations (also see Guo et al. 1991; Mueller et al. 1991, for further

verification of the results), there was a 'canonical' understanding which arose in the context of adaptation to larval crowding (Mueller 1997; Joshi et al. 2001; Prasad and Joshi 2003; Mueller and Barter 2015). According to this canon, laboratory *Drosophila* populations adapted to chronically crowded larval conditions were expected to evolve increased larval competitive ability compared to low-density control populations, primarily via increased larval feeding rate, foraging path length (Sokolowski et al. 1997) and increased tolerance to ammonia and urea. These adaptations would be accompanied by a supposed trade-off with food to biomass conversion efficiency. Furthermore, the positive relationship between larval feeding rate and pre-adult competitive ability was also prevalent across other independent studies. Evolution of increased feeding rate in populations selected for faster feeding also resulted in an associated increase in larval competitive ability (Sewell et al. 1974; Burnet et al. 1977). The evolution of decreased larval competitive ability was seen in populations wherein feeding rate had evolved to lower values, such as those selected for increased parasitoid resistance (Fellowes et al. 1998, 1999) or those selected for faster pre-adult development followed (Prasad et al. 2001; Shakarad et al. 2005; Rajamani et al. 2006).

3. ACU/AB, NCU/NB and MCU/MB populations (Nagarajan et al. 2016; Sarangi et al. 2016):

Over the last two decades, three selection studies upturned this canonical model for adaptation to larval crowding. These involved selection for high larval density conditions in *D. ananassae* (ACU crowded, AB uncrowded), *D. nasuta nasuta* (NCU crowded, NB uncrowded) as well as *D. melanogaster* (MCU crowded, MB uncrowded – the ancestry of these populations traced back to the UU populations). Compared to their respective low-density ancestral control populations, each set of crowding-adapted populations showed the evolution of increased larval competitive ability without any change in larval feeding rate

or tolerance to urea or ammonia. Moreover, these populations evolved faster pre-adult development (seen across both low- and high-density conditions) and an increased time efficiency of food to biomass conversion compared to their controls (Nagarajan et al. 2016; Sarangi et al. 2016). The major factor that differed between these selection studies and the previous ones was the ecological details of how larval crowding was imposed (Sarangi et al. 2016). While the CU populations were crowded in relatively high volumes of food, with approx. 1500 eggs in 6-7 mL food, the ACU and MCU populations were selected for crowding at very low food volumes, at approx. 600 eggs in 1.5 mL food (Nagarajan et al. 2016; Sarangi et al. 2016). The NCU populations had slightly lower density at approx. 400 eggs in 2 mL food, in order to account for their larger size (Nagarajan et al. 2016).

4. LCU, MCU, CCU and MB populations (Sarangi 2018):

Given the understanding that the exact manner in which high larval density was imposed was likely to affect the ecology of a crowded culture and influence the evolutionary trajectory of a population selected for chronic larval crowding, the next step was to study the ecology of two crowded cultures having the same high density, but achieved via different combinations of egg number and food volume. When the low-density reared MB populations (as well as the crowding-adapted MCU populations) were subjected to crowding at either 600 eggs in 1.5 mL food or 1200 eggs in 3 mL food, the outcome of the ensuing competition in terms of pre-adult survivorship, development time and mass at eclosion were different (Sarangi 2018). This suggested that the underlying fitness functions of two cultures with the same high-density conditions achieved in different ways could be different.

Consequently, in addition to the pre-existing MCU and MB populations, two new population sets were derived from the MB populations, each subjected to chronic larval

crowding imposed differently from the MCU (Sarangi 2018). The LCU populations were meant to broadly capture the ecology of the CU populations and were reared at approx. 1200 eggs in 6 mL food, in narrower vials than MB. The CCU populations had the same high density as the MCU, but had double the number of eggs (1200) and double the food volume (3 mL). Like the MCU populations, the LCU and CCU populations also evolved greater larval competitive ability than the MB populations (Sarangi 2018; also see Chapter 2). Also similar to the MCU populations, there was no evolution of pre-adult ammonia or urea tolerance in the LCU and CCU sets of populations (Sarangi 2018). However, unlike the MCU populations, and similar to the CU populations, both the LCU and CCU population sets evolved greater larval feeding rate (Sarangi 2018). Further complicating the understanding of the relationship between larval competitive ability and feeding rate were experiments done to measure feeding rate in culture vials mimicking the specific crowded conditions of MCU, LCU and CCU populations (Sarangi 2018). In all previous studies, the measurement of feeding rate was carried out on single larvae in small agar-layered plates smeared with yeast-water solution. However, under the native crowded conditions in vials, the MCU populations actually had the highest feeding rate across all three types of cultures, followed by CCU and LCU, with MB populations showing the lowest feeding rates (Sarangi 2018). Furthermore, population dynamics experiments revealed that the MCU populations had evolved greater stability in terms of both constancy ("staying essentially unchanged" *sensu* Grimm and Wissel 1997) and persistence ("persistence through time of an ecological system" *sensu* Grimm and Wissel 1997), whereas LCU populations only evolved greater persistence stability, as compared to the MB populations (Pandey and Joshi 2022*a*, 2022*b*).

Given the myriad complexities and complications arising in our understanding of the adaptation to larval crowding, it has become clear that a better understanding of the ecology of

Drosophila larval competition is required. This would help predict the traits that could evolve in differently-crowded cultures, promote better selection experiments, and ultimately refine and strengthen predictions from the theory of density-dependent selection.

Previous studies on the ecology of crowded larval cultures

(See Introduction in chapter 5 for detailed background)

Other than the experimental testing of predictions from the theory of density-dependent selection, the outcome of larval crowding has also been studied quite extensively, primarily to untangle the nuanced ecology of competition using the pliable laboratory *Drosophila* system. While many such studies originally focused on providing evidence for the existence of larval crowding through effects on fitness related traits (Sang 1949; Chiang and Hodson 1950; Bakker 1961; Ohnishi 1976), subsequent studies led to a better understanding of the mechanisms through which the outcome of competition was shaped. These included the role of larval feeding rate in competition (Bakker 1961; Sewell et al. 1974; Burnet et al. 1977), food to biomass conversion and the passage of food through the gut (de Miranda and Eggleston 1988), indirect interference primarily through metabolic waste products (Weisbrot 1966; Dawood and Strickberger 1969; Botella et al. 1985), the cessation of larval development under crowded conditions (Ménsua and Moya 1983), yeast availability (Klepsatel et al. 2018), and transciptomic changes under crowding (Morimoto et al. 2022). The outcome of crowding has been used to study thermal stress (Sørensen and Loeschcke 2001; Henry et al. 2018), and effects on sexual selection (Morimoto et al. 2016; Mital et al. 2021). Several theoretical models, both verbal and mathematical, were also created, either exclusively or in part, to predict the mechanisms and/or outcomes of crowding (Bakker 1961; Bentvelzen 1964; De Jong 1976; Nunney 1983; Moya and Castro 1986; Mueller 1988*b*; Jansen and Sevenster 1997; Tung et al.

2019). It's interesting to note that all but one of the models developed in these cited studies (i.e., Moya and Castro 1986) were directly or indirectly inspired by the rigorous experiments carried out by Bakker (1961). Chapter 6 contains some additional discussion regarding the consequences of these models.

The measurement of larval competitive ability has also received a lot of focus, with studies implementing experimental designs of varying complexity (Bakker 1961, 1969; Gale 1964; Kearsey 1965; Seaton and Antonovics 1967; Mather and Caligari 1981; de Miranda et al. 1991). The further partitioning of larval competitive ability into 'effectiveness' and 'tolerance' components has also been helpful in some contexts, and is reviewed in chapter 2 (also see Joshi et al. 2001).

As in the crowding adaptation work described in the previous section, most studies on the ecology of competition did not focus on the importance of differences in ecology at the same high density experienced in different ways – with the exceptions primarily being Sang 1949 and Bakker 1961. Bakker extensively discussed the possibility of different ecological consequences at the same high density when a) egg number was increased while keeping food constant, vs. b) when egg number was kept constant while increasing food volume (Bakker 1961). However, he was unable to find these predicted differences empirically, either due to his usage of relatively low densities where such differences might not have been apparent, or due to his unique culture implementation of using a layer of agar with a yeast solution on top (discussed in chapter 6). In contrast, J. H. Sang discovered that crowded cultures had observable differences in survivorship, development time and dry mass when larval crowding was implemented through two different means: a) changing food volume through food column height, vs. b) Changing food volume through changing food cross-section surface area – in both a) and b) the number of larvae were kept constant (Sang 1949). This finding was the

primary impetus for the study carried out in chapter 5, and the possible mechanisms for the same have been extensively discussed in chapter 6.

The role of my Ph.D. thesis

My studies primarily aim to incorporate past understanding the ecology of larval competition into the recently changing framework of the evolution of larval competitive ability, in order to ultimately add greater nuance and predictive strength to the theory of density-dependent selection, at least as it pertains to larval crowding in *Drosophila*. I hope that some of the insights gained will also be generalizable beyond the domain of crowded *Drosophila* cultures.

Populations used in my studies

In the studies described in this thesis, four sets of long-term laboratory populations of *D. melanogaster* were used. Each set consisted of four replicate populations, as briefly described below. The detailed description of the ancestry and maintenance of all four population sets can be found in Sarangi, 2018.

MB 1-4: These four low density reared populations served as ancestral controls to the three sets of crowding-adapted populations. They were maintained at a relatively low density of approx. 70 eggs in 6 mL of cornmeal-sugar-yeast medium, in cylindrical Borosilicate glass vials with inner diameter of 2.2-2.4 cm, and height of approx. 9.5 cm (8 dram vials).

MCU 1-4: This set of populations experienced larval crowding at ~600 eggs in ~1.5 mL of cornmeal medium, in the same vial type as MBs.

CCU 1-4: These populations experienced larval crowding at ~1200 eggs in ~3 mL of cornmeal medium, in the same type of vials as MBs. MCU and CCU populations were reared at the exact same overall eggs/food density.

LCU 1-4: These populations were selected for adaptation to larval crowding at ~1200 eggs in ~6 mL of cornmeal medium, in Borosilicate glass vials of ~2 cm inner diameter and ~9 cm height (6 dram vials), in conditions similar to those the CU populations (Mueller et al. 1993) were reared in.

OE (Orange Eye): In addition to the four sets of focal populations, some studies also employed a population of *D. melanogaster* with an orange eye colour mutation. These were used as visual markers in competition experiments described in chapters 2 and 4. The OE population was reared in similar fashion to the MB populations (Sarangi et al. 2016; Sarangi 2018).

Premise of each chapter

Chapter 2: Larval competitive ability has evolved to increase in response to every selection experiment done under chronic larval crowding so far, found either through direct measurement (Mueller 1988a, Nagarajan et al 2016, Sarangi et al 2016) or via indirect inference (Joshi and Mueller 1996). However, given that our laboratory now has three sets of populations each adapted to larval crowding experienced in different ways, there is a need to elaborate how they might differ in larval competitive ability. Until now, only preliminary experiments had been done at around 50 generations of selection of LCU and CCU (Sarangi 2018). Moreover, it is known that besides pre-adult survivorship, changes in both pre-adult development time and body mass at eclosion are robust indicators of larval competition (Sang 1949; Bakker 1961; Ohnishi 1976; Sarangi 2018). Chapter 2 outlines an extensive larval competitive ability experiment carried out in order to study competitive ability along the axes of survivorship,

development time and dry body mass at eclosion, with the dry biomass per type per culture also measured. We further partitioned larval competitive ability into 'effectiveness' and 'tolerance' components, following a suggestion from a previous study (Joshi et al. 2001), whose argument was based on earlier research which had taken that approach (e.g., Mather and Caligari 1983; Hemmat and Eggleston 1988; Joshi and Thompson, 1995).

Chapter 3: One of the primary requirements for understanding larval competitive ability is to elucidate its component traits under various regimes of larval crowding. Previously, feeding rate, waste tolerance and development time (partly via time to convert food to biomass) have emerged as some of the major traits involved in conferring increased competitive ability (Borash et al. 1998; Sarangi et al. 2016; Sarangi 2018). However, an aspect of competition not yet studied in this regard was to study traits possibly conferring improved head starts in larval competition. Head starts imposed in competition are known to confer competitive advantages to larvae (Bakker 1961, 1969). This prompted the study of the evolution of hatching time and egg size in crowding adapted populations, two traits that may confer improved head starts to competing larvae.

Chapter 4: In this study, we combined both the evolution of competitive ability and the role of head starts in competition in crowding-adapted populations to ask the following question: *How well do competitively superior populations tackle age disadvantages in competition?*

We asked this by providing larvae of the OE population various durations of head starts against those of the crowding-adapted populations, under crowded conditions, and compared the response achieved with that seen for the uncrowded control populations (MB).

Chapter 5: In chapter 5, we broke down the implementation of larval crowding to its fundamentals, with the goal of finding out what the primary determinants of larval crowding were. We explored the implementation of larval crowding along three axes while keeping all

else equal – i) changing egg number, ii) changing food volume via food column height and iii) changing food volume via vial cross-section surface area. We implemented a three-way, fully-factorial design with three levels each of starting egg number, food column height and vial cross-section surface area, in the MB populations. We also challenged the importance of 'total density' (the overall eggs/food density) in predicting the outcome of a crowded culture.

Chapter 6: This chapter presents the development of an individual based-simulation framework, which explores the ecological consequences and the mechanistic underpinnings of crowding in *Drosophila* larvae and attempts to capture the results seen in Chapter 5. We further make some predictions for future experiments, and lay down the most important questions that need to be asked in order to improve our understanding of the ecology of larval competition, the evolution of larval competitive ability, leading to a nuanced expansion of predictions from density-dependent selection theory.

Chapter 7: This chapter serves as an epilogue for the thesis, containing summarised conclusions from each chapter, while also laying down the path forward from the findings of this thesis.

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Chapter II

The evolution of competitive effectiveness and tolerance in populations of *Drosophila melanogaster* adapted to chronic larval crowding at varying combinations of egg number and food volume

NOTE

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Introduction

The theory of density-dependent selection is based on the notion that different genotypes or phenotypes may have a selective advantage at low vs. high population density conditions, and forms an important bridge between the fields of population genetics and population ecology (reviewed in Mueller 1997, 2009; Joshi et al. 2001). Some of the early ideas about the fitness of a type varying with population density were put forward by Charles Elton (Elton 1927), followed by Theodosius Dobzhansky (Dobzhansky 1950), J. B. S. Haldane (Haldane 1956), and Robert MacArthur (1962). The most well-known treatment of this concept was laid out in the book 'The Theory of Island Biogeography' by MacArthur and Wilson (1967). The authors defined *r*- and *K*-selection as the evolutionary consequences of selection under low and high population densities, respectively (MacArthur and Wilson 1967). Many models, both verbal and mathematical, stemmed from this work, and have been reviewed extensively (Mueller 1997; Joshi et al. 2001). The salient prediction coming from several of these models, including the one proposed by MacArthur and Wilson (1967), was that under *K*-selection, evolution of higher equilibrium population size *K* (also called 'carrying capacity' by some authors – critically reviewed in Mallet 2012) would occur (MacArthur 1962; MacArthur and Wilson 1967; Gadgil and Bossert 1970; Anderson 1971; Roughgarden 1971; Pianka 1972). Furthermore, the evolution of higher *K* would be consequent on the increase in the efficiency of resource utilisation of *K*-selected individuals (MacArthur and Wilson 1967; Pianka 1970). Over the last four decades, this prediction regarding efficiency was tested and subsequently rejected through a series of rigorous long-term selection experiments using populations of fruit flies *Drosophila melanogaster* adapted to high-density conditions (Mueller 1990; Joshi and Mueller 1996). These studies failed to find the evolution of greater food-to-biomass conversion

efficiency as a correlate of selection for adaptation to chronically high population density or larval crowding.

Several alternate models of density-dependent selection predicted the evolution of increased competitive ability as a consequence of adaptation to high-density (Gill 1972, 1974; Asmussen 1983; Mueller 1988*a*; reviewed in Joshi et al. 2001; Mallet 2012). Some models also conflated or subsumed competitive ability under *K*-selection (MacArthur 1962; Pianka 1972; Gilpin et al. 1976; reviewed in Bradshaw and Holzapfel 1989; Joshi et al. 2001). Competitive ability, defined here as the sum of traits relevant to an individual's performance in competition (Bakker 1961), could be equated to fitness in conditions of resource limitation (Prasad and Joshi 2003). The prediction that increased competitive ability evolves under adaptation to high-density was observed in several *Drosophila* selection studies, each of which saw the evolution of increased larval competitive ability for limited food (Mueller 1988*b*; Mueller et al. 1991). Moreover, some studies of adaptation to crowding in *Drosophila* saw an increase in larval feeding rate (Joshi and Mueller 1988, 1996), which had been shown to be a strong positive correlate of larval competitive ability (Burnet et al. 1977), leading eventually to a view that adaptation to larval crowding in *Drosophila* would almost invariably involve the evolution of increased larval feeding rate (Prasad and Joshi 2003).

In the last fifteen years, this canonical view of adaptation to larval crowding via increased larval feeding rate was challenged by three selection studies on adaptation to larval crowding in *Drosophila* species, including *D. melanogaster*, with the high densities implemented by varying combinations of egg amount, food volume and container dimensions (Nagarajan et al. 2016; Sarangi et al. 2016). In these studies, crowding-adapted populations did evolve greater larval competitive ability, but through increased efficiency with respect to development time, and not increased larval feeding rate as seen in the previous studies (Nagarajan et al. 2016; Sarangi et al. 2016).

What became apparent from these studies was that populations adapted to high larval densities at varying egg number, food amount and container dimensions actually experienced different ecologies in the culture vials, which likely altered their evolutionary trajectories toward increased larval competitive ability (Sarangi 2013, 2018; Sarangi et al. 2016). Thus, efficiency may or may not evolve in populations adapted to high-density conditions depending on the manner in which the high density was imposed. Moreover, the details of the high-density rearing also influenced the evolution of population stability parameters – with increased constancy stability evolving in populations subjected to selection for larval crowding in low food amounts (Dey et al. 2012; Pandey and Joshi 2022*a*), but not in populations selected for crowding with relatively higher food amounts, with higher food columns (Mueller et al. 2000; Pandey and Joshi 2022*b*). While the exploration of traits correlated with different types of high-density conditions is still underway (Sarangi 2018; Chapter 3), the prediction regarding the evolution of increased competitive ability under chronic crowding has been directly or indirectly met in every *Drosophila* selection study seeking to test it (reviewed in Chapter 3).

Here, we draw on the findings of the studies suggesting that there is more to crowding than just density (eggs per unit volume of food) as well as an earlier argument for thinking about the consequences of density dependent selection in terms of the effectiveness and tolerance components of competitive ability rather than just efficiency (Joshi et al. 2001), in order to see whether viewing the outcomes of adaptation to crowding experienced in different ways in terms of effectiveness and tolerance might provide deeper insights into the ecology and evolution of competitive ability in *Drosophila*.

We define effectiveness as the average degree of potential population growth inhibition imposed by individuals of a focal group on those of a competitor group, as compared to the inhibition imposed by competitor group individuals on each other. Tolerance is the average degree of reduction of such inhibition by the focal group, inflicted by the competitor group, as

compared to the inhibition observed in the focal group individuals when competing amongst themselves. These definitions are very similar to those used by Joshi et al. (2001). This approach of splitting competition into a proactive and a reactive component has been discussed for several decades, albeit with varying terms being used for the two components.

Early work focused on components of competition in plant species – called aggressiveness (similar to effectiveness) and sensitivity (inverse of tolerance) (Breese and Hill 1973), or effect and response (Peart 1989; Goldberg and Landa 1991), respectively. Peart (1989) found little or no relationship between colonising ability (of seeds) and inhibition ability (of adult plants), and equated the terms to competitive effect and response, respectively. Goldberg and Landa (1991) found competitive effect and response to be uncorrelated in several species of herbaceous plants.

In *Drosophila*, some of the earliest studies on this concept were carried out by Mather and Caligari (1983). The authors studied competitive pressure (or aggressiveness) and response (used in lieu of sensitivity) in intraspecific larval competition between two strains of *Drosophila*, across three experiments, studying these aspects for both pre-adult survivorship and body weight of eclosed adults. While no difference was found in the response component of either strain, aggressiveness was found to differ among strains in all three experiments. The authors concluded that aggressiveness and response appeared to be independent in terms of their variation, and could combine additively to result in the competitive ability of a genotype (Mather and Caligari 1983). A continuation of the above-mentioned study found that competition in trio-cultures was a reflection of the competitive ability of each strain in duo-cultures (Caligari and Mather 1984). Paul Eggleston (Eggleston 1985) speculated that the aggressiveness and response of genotypes "must depend upon factors such as larval feeding rate, critical weights, food conversion efficiency, the secretion and excretion of various substances into the medium and even the exploitation of behavioural characteristics".

An early selection experiment was also carried out on a *D. melanogaster* population, for low aggression and for high response (response here was equated with sensitivity, or the inverse of tolerance) – both markers of lowered competitive ability(Hemmat and Eggleston 1988). Both trajectories in response to selection led to a decrease in the average competitive ability of the population. However, selection for low aggression led to a clearer result than for high response (Hemmat and Eggleston 1988). This study, thus, showed that the two components of competitive ability could evolve independently, and this was explored in greater detail in a later study on interspecific competition (Joshi and Thompson 1995).

Joshi and Thompson (1995) tracked changes in competitive effectiveness and resistance (i.e., tolerance) over 11 generations in 3 sets of populations of *D. melanogaster* vs. *D. simulans*, competing in 3 different environments, respectively. At the end of the experiment, almost all population sets saw an increase in the overall competitive ability of *D. simulans*, initially the weaker competitor. However, this increased competitive ability was likely achieved via alternate routes in each population – different combinations of effectiveness and resistance were seen to evolve in each population (Joshi and Thompson 1995).

To summarise, the *Drosophila* studies on effectiveness and tolerance highlighted two things – a) that effectiveness and tolerance can be affected independently by ecological factors in a given population, and need not evolve together, and b) that effectiveness and tolerance can be defined using readouts of competition other than just survivorship, such as body size of adults. However, the limitations of some of these studies have been their reliance on inbred strains (which may greatly limit inferential reach: Rose 1984), or on using populations relatively naïve to larval competition (e.g., Mather and Caligari 1983; Eggleston 1985).

These limitations can be overcome by selection experiments such as those that established that competitive ability can evolve in outbred populations adapted to chronic larval crowding, and

that this evolution can happen through the correlated evolution of different underlying traits, depending on the exact combination of egg number, food amount and container dimensions used to implement the larval crowding. However, despite the advances made in the understanding of effectiveness and tolerance in competitive ability, and their predicted usefulness in studying density-dependent selection, populations adapted to larval crowding experienced in different ways have yet to be examined for effectiveness and tolerance. In this study, we undertake a systematic examination of effectiveness and tolerance with regard to four different parameters that can be used as surrogates for evaluating larval competitive ability in several population sets of *D. melanogaster*, each adapted to larval crowding experienced in different ways. The measures of effectiveness and tolerance employed are similar to those used by Joshi and Thompson 1995 (see Methods).

Thee four parameters of competition we use – pre-adult survivorship, pre-adult development time, total dry biomass, and dry weight per fly – are each representative of the potential growth of a population. Pre-adult survivorship is the most straightforward readout (used in Bakker 1961; Mueller 1988*b*; Nagarajan et al. 2016; Sarangi et al. 2016) – only survivors to adulthood get a chance to reproduce and have possible non-zero fitness. Pre-adult development time may influence reproductive success in many ways (discussed in González-Candelas et al. 1990). In nature, the earliest developers can reproduce faster than late developers, and may get the advantages of a shorter generation time (Cole 1954). Even in discrete-generation laboratory cultures such as ours, where the generation time is fixed (see Methods), early eclosing individuals may have greater opportunities for mating (reviewed in Mital et al. 2022). Moreover, crowded *Drosophila* cultures tend to accumulate metabolic waste over time (Botella et al. 1985; Borash et al. 1998). In such scenarios, early eclosing flies, assuming they also pupate earlier, may partly escape the toxic build-up that could otherwise prove lethal or reduce the capacity to reproduce in some way (Borash et al. 1998; Mueller and Barter 2015). Dry

weight per fly is another potential measure of fitness – smaller flies often have some general loss of fitness (reviewed Mital et al. 2021), besides some mating-related disadvantages. Smaller males may have lower ability to manipulate females for mating (Mital et al. 2021), and females from crowded cultures, which are smaller in size than those from uncrowded cultures, may have lower fecundity than the latter (Pandey et al. 2022). It is, however, unknown if any variation in dry weight among flies within a crowded culture (as seen in Sarangi 2018, and in fig 5 of the current study) also results in size-based differences in fecundity. It may be reasonable to assume that the smallest flies from a crowded culture may also have the lowest fecundity, simply because they cannot provision the same number of eggs as larger flies. Additionally, there may not be much difference in the egg size of flies from crowded cultures compared to those from uncrowded cultures (Chapter 3). Ideally, we would prefer to directly measure fecundity and count the resulting offspring produced by surviving flies in our competition studies, as was done by Gale (1964). However, due to logistical reasons we used indirect proxies such as dry weight per fly instead. The total dry biomass of eclosed flies from a culture is a result of both the dry weight per fly as well as pre-adult survivorship. Unlike the dry weight of individual flies, biomass data can be analysed more readily if some populations don't show any eclosion (see fig 3, 4, 5 for examples). Biomass was earlier used as a readout of competition in several experiments by Bakker (1961, 1969). Finally, each of the four outcomes of competition used in the current study have previously been shown to be affected by larval crowding (Sang 1949; Bakker 1961; Ohnishi 1976).

Methods

Populations used

We used three sets of four replicate outbred *D. melanogaster* populations each, subjected to chronic larval crowding experienced at different combinations of egg number and food volume per rearing vial, along with the four ancestral control populations routinely maintained at moderate larval density. The four control MB populations are maintained at approx. 70 eggs in 6 mL cornmeal food, in cylindrical glass vials (8-dram vials: 2.2–2.4 cm inner diameter and approx. 9.5 cm height) (Nagarajan et al. 2016; Sarangi et al. 2016). Each replicate (i = 1 to 4) of all three sets of crowding-adapted populations is derived from replicate i of the MB set. This allows the implementation of a block design in the analysis of variance (see 'statistical analysis' section below). For all four sets of populations, matched replicate populations were assayed at the same time.

The details of the crowding-adapted population sets are as follows:

MCU: selected for larval crowding at 600 eggs in 1.5 mL food in the same vial type as MB. At the time of assaying, they had undergone selection for at least 229 generations. Each replicate population was assayed at a different generation – 229, 230, 231, 232 for replicates 4, 1, 3, 2 respectively.

CCU: selected for larval crowding at 1200 eggs in 3 mL food in the same vial type as MB. These are maintained at the same total eggs/food density as the MCU, but with twice the egg number and food volume. At the time of assaying, they had undergone at least 108 generations of selection.

LCU: selected for larval crowding at 1200 eggs in 6 mL food in slightly narrower 6-dram vials (see Sarangi 2018). This maintenance regime was somewhat similar to that used in earlier

studies for the CU populations (Mueller et al. 1993). At the time of assaying, they had undergone at least 107 generations of selection.

Additionally, we used a common competitor population with an orange eye (OE) mutation as a distinct visual marker in duo-cultures (see below). The OE population was maintained at low density, in a manner similar to the control MB populations.

Extensive descriptions of the ancestry and maintenance of these populations can be found in recent publications (Sarangi et al. 2016; Sarangi 2018; also see Chapter 3). In brief, all sixteen populations are maintained on a 21-day generation cycle. Eggs are collected into vials at the respective selection density on day 1. Once the flies eclose, they are transferred to Plexiglas cages ($25 \times 20 \times 15$ cm^3). This transfer takes place daily from around day 8 to day 20 in crowding-adapted populations, owing to a prolonged eclosion period in crowded cultures (see fig. 3 for an example). In MB as well as OE, transfer occurs only once, on day 11, by which time almost all eclosions are over. On day 18, the flies of all populations are provided food plates with a generous supplement of a paste containing live yeast mixed with water and a few drops of glacial acetic acid. On day 20, they are provided a food plate for egg laying for a duration of about 18 hours. On day 21, the next generation commences, starting with a new round of egg collection.

Standardisation

Prior to starting the experimental assay, we reared each population in a common environment to minimise potentially confounding effects of non-genetic inheritance. This was done by rearing each population in the MB type, low density regime of approx. 70 eggs in 6 mL cornmeal medium. The eggs of each population were collected in vials and reared for 11 days, and thereafter the eclosing flies were transferred to a Plexiglas cage. These populations were provided a Petri plate with cornmeal medium and a generous smear of live yeast paste made

with water and a few drops of glacial acetic acid. After around 60 hours, we removed the yeast-laden plates and added a cornmeal plate with vertical surfaces provided for egg laying. This plate was provided for one hour, and served to remove fertile eggs previously incubated inside the female flies. This ensured that most eggs laid for the experiment were more or less freshly fertilised, and no artificial head starts were being provided to larvae from previously incubated eggs hatching several hours earlier than others. The assay was started after this step.

Competition experiment: start

After the initial egg laying plate was removed, a fresh plate was added for 5 hours to each population's cage. This was a harder plate for easier egg removal, made with 2% agar along with yeast and sugar (Chapter 3). After 5 hours, each plate was removed from its respective cage. The eggs on the plate were transferred to a surface of 1% agar. We kept two cages for the OE population, owing to a larger requirement for eggs. The eggs from both the cages were mixed on the 1% agar surface after plate removal.

Egg collection

At this stage, eggs were exactly counted up to the required amount using a soft bristle brush and collected in bunches for transfer to experimental vials. These 8-dram vials (same as the ones used to rear MB) contained either 1, 1.5 or 2 mL of cornmeal food. They were further divided into two categories: mono-culture, which received 400 eggs of a focal population or OE; and duo-culture, which received 200 eggs of a focal population and 200 eggs of OE. Thus, each vial had a total of 400 eggs, in either 1, 1.5 or 2 mL of food. There were five replicate vials per culture type, per food level, per selection regime, per block. We additionally set up low density vials with 70 eggs in 6 mL cornmeal food in a similar fashion. In this case, duo-culture vials had 35 eggs focal population + 35 eggs OE. Each replicate set (MBi, MCUi, CCUi, LCUi, i = 1…4), along with OE, was assayed together per maintenance generation. A total of

228,600 eggs, spread over 720 vials, were thus used for the experiment. After egg collection, the vials were kept at 25°C in constant light in 70-90% relative humidity. The position of the racks (containing vials of various selection regime and density combinations) were shuffled daily to randomise the rearing environment as much as possible.

Collection of eclosing flies: Pre-adult survivorship and development time measurements

On the 8th day from egg collection, observations were started in order to collect eclosing flies from the culture vials. In each observation check, all vials were visually scanned for any eclosed flies. These checks were done 12 hours apart on the night of the 8th day, morning of the 9th day, night of the 9th day and morning of the 10th day, respectively. Afterwards, the checks were carried out 24 hours apart (10th day, 11th day, 12th day…and so on). This was done until all eclosion ceased.

In each check, eclosed flies from each vial were transferred to a corresponding empty transfer vial. These flies were then frozen by dousing the transfer vials in liquid nitrogen for approx. 2 minutes. After this, flies from each vial were taken out onto a stereomicroscope platform for separation and counting. Flies from mono-cultures were segregated and counted on the basis of sex (female or male). Those from duo-cultures were segregated and counted on the basis of eye colour (wild type or orange) and sex. Any flies that inadvertently escaped during the transfer, freezing or counting process were noted (if noted before escape, sex and eye colour were recorded too).

For each vial, the pre-adult survivorship of a population was obtained by summing up the counts of all eclosed flies per eye colour (male and female) and taking its proportion to the total eggs of the respective population added into the culture. The pre-adult development time was taken as the average time of eclosion per eye colour, per sex.

After counting, we transferred the flies to 1.5 mL centrifuge tubes kept in respectively labelled sachets, in order to freeze and store them for subsequent weight measurements.

Biomass and dry weight collection

Flies eclosing from each vial were transferred into their respective packages and stored at -20°C for weighing later (after conclusion of the entirety of survivorship and development time assays). In these transfers, there were exceptions made as to the flies that could not be weighed, although they were noted for survivorship and development time. These exceptions included:

1. Any flies found stuck in food. These were discarded from weighing due to the likely presence of an additional confounding mass of leftover food.
2. Any flies that moved deep inside the cotton plug during eclosion, and got crushed in the process.
3. Any flies that were otherwise crushed during counting.
4. Any inadvertently escaped flies.
5. Any human error regarding sachet identity during fly transfer. The packet receiving the wrong transfer was discarded (see supplementary material for a detailed list of such exceptions).

These exceptions meant that the biomass was usually a small underestimate of the actual biomass (see supplementary material).

As an additional step for increased rigour, in order to control for any inadvertent transfers or losses, we also approximately re-counted all the flies from each tube before weighing. Any tube that had flies outside of ±15% of the number expected from survivorship counts was discarded from biomass readings. For dry weights, this percentage was relaxed to -30% of the expected counts for the lower limits, as lower than expected counts could still provide enough

flies for obtaining the dry weight per fly. This discarding process resulted in some treatments having less than 5 replicate vials (at least 1 replicate vial was present in every case).

Flies were transferred according to their development time into one of three different time interval bins. These bins were:

T1: all flies eclosing up to day 11 (approx. 270 hours development time) were binned into the T1 sachets.

T2: all flies eclosing between day 11 and day 15 (approx. 270-370 hours development time) were assigned as T2.

T3: all flies eclosing after day 15 were kept in the T3 marked sachets. For T3, since the number of flies eclosing was generally lower, all vials were pooled together per combination of block × food level × population × culture type × sex.

The first time-interval bin, T1, was assigned such that it captured the vial duration of MB maintenance (which are transferred to cages on day 11), and could be classified as containing the early eclosing flies. The end of the second time bin, T2, completed a week of eclosion from the vials, and was set as such for logistical convenience. The bins are visually represented in the eclosion profiles in fig. 3.

Dry biomass and dry weight measurement

Dry biomass measurements were taken to capture the weight of all eclosing flies from a particular centrifuge tube (containing a combination of block × food level × population × culture type × sex). Dry weight measurements were taken for a fixed sample of these flies, to obtain more accurate per fly weights, as was done in previous experiments (Sarangi 2018). By taking a constant number of flies per measurement, machine error per fly was also standardised. Thus, for dry weight per fly, a total of 10 flies (max.) were taken per reading. In case 10 or less

flies were present in the tube, all the flies were weighed for both biomass and dry weight (lower limit set at 5 flies per tube). The weighing process was as follows:

a) Remove tube from freezer. Keep in convection oven (tube lid open) and dry at 70°C for 36-42 hours.
b) Weigh the tube along with the flies stored within.
c) Remove all the flies and weigh the empty tube.
d) Add 10 flies randomly sampled from the removed flies, into the tube, and weigh again.

(The weight of all flies + tube) – weight of tube = resulting dry biomass.

((The weight of 10 flies + tube) – weight of tube) ÷ 10 = resulting dry weight per fly.

Effectiveness and Tolerance: calculations and properties

The measurement of both effectiveness and tolerance was broadly inspired by the definitions used by Joshi and Thompson (1995). The values were calculated as follows:

Effectiveness of a focal (i.e., MB, MCU, CCU, or LCU) population =

(Competitive outcome of OE in duo-culture vs. focal population - Competitive outcome of OE in mono-culture) ÷ Competitive outcome of OE in mono-culture

Tolerance of a focal population =

(Competitive outcome of focal population in duo-culture vs. OE - Competitive outcome of focal population in mono-culture) ÷ Competitive outcome of focal population in mono-culture

Competitive outcome was represented by either pre-adult survivorship, pre-adult development time, total vial dry biomass of eclosing adults (pooling time windows and sex), or dry weight per fly at eclosion (averaged across time windows and sex). With the exception of pre-adult development time, a greater negative value of effectiveness implied greater competitive ability of the focal population. A population with high effectiveness would suppress the common competitor's mean competitive outcome more than the common competitor could suppress its own outcome in competition. For development time, a lower absolute value confers greater fitness on average (see introduction). Thus, positive values of effectiveness for development time signify greater competitive ability of the focal population.

For tolerance, the patterns are reversed. For all outcomes except development time, a positive tolerance value suggests greater competitive ability. High tolerance values mean that the 200 larvae of the focal population had a more beneficial outcome when competing against 200 OE larvae than vs. 200 additional focal population larvae. As with effectiveness, pre-adult development time tolerance is inversely related to the competitive ability of the focal population. Negative tolerance values imply greater focal population competitive ability for development time.

Statistical Analyses

We performed type 3 (mixed effects model) factorial ANOVA on both effectiveness and tolerance for pre-adult survivorship, pre-adult development time, dry weight per fly and total dry biomass, respectively. Selection was treated as a fixed factor with four levels (MB, MCU, CCU, LCU). Starting food volume was also considered a fixed factor with three levels (1 mL, 1.5 mL, 2 mL – 1 mL excluded for development time and dry weight per fly, see below). Block was a random factor with four levels, representing common ancestry and handling during assays (replicates 1-4). In addition, we also carried out ANOVA on the raw data for each trait,

before calculating effectiveness or tolerance. For pre-adult survivorship, the raw data analysis included 2 additional fixed factors as compared to effectiveness or tolerance for survivorship – these were eye colour (2 levels - wild type (MB, MCU, CCU or LCU) and orange eye) and culture type (2 levels – mono-culture and duo-culture). The survivorship data were also arcsine-square-root transformed to verify the statistical differences observed in the untransformed data. For pre-adult development time, there was no development time data in some blocks for orange eye at 1 mL in duo-culture vs. MCU and CCU, due to them having 0% survivorship. Thus, the 1 mL treatment was excluded from the raw data analysis as well as effectiveness analysis. The ANOVA for the raw data for pre-adult development time also included sex as a factor (2 levels – female, male), in addition to selection, starting food volume, block, eye colour and culture type.

For total biomass, as in pre-adult survivorship, the factors included selection, starting food volume (1 mL, 1.5 mL and 2 mL), block, eye colour and culture type. An additional fixed factor, the time window of eclosion, was also included, with three levels (T1, T2, T3, see text above). Female and male biomass were pooled per replicate vial.

Dry weight per fly was not analysed in its entirety due to several points of data being missing, due to lack of eclosion, at 1- and 1.5-mL food, in OE, at different time windows, in different sexes. Dry weight effectiveness analysis was carried out for the 1.5 mL and 2 mL cultures only.

All analyses were conducted in STATISTICA for windows (StatSoft 1995). Tukey's HSD was used for post-hoc pairwise comparisons, calculated manually in each case. All results were plotted in R version 4.1.3 (R Core Team 2022) using the ggplot2, forcats and tidyverse packages (Wickham 2016; Wickham et al. 2019).

Results

Pre-adult Survivorship

The tolerance and effectiveness of the MB, MCU, CCU and LCU populations for pre-adult survivorship are shown in figures 1a and 1b, respectively. The raw survivorship values for the data of crowded cultures containing 400 eggs in 1-, 1.5- and 2-mL food, from which the effectiveness and tolerance were computed, are given in supp figure 1a-c, respectively.

Increasing food level increased mean survivorship for all selection regimes and culture types (compared across supp fig 1a, 1b, 1c); main effect of food volume ($F_{2, 6} = 33.80$, $P = 0.0005$, supp table 3). The differences between selection regimes were the largest at 1 mL. Both MCU and CCU populations showed a gain in survivorship in duo-cultures as compared with mono-cultures. This was reflected in a mean tolerance significantly greater than 0 for both the population sets (fig 1a, interaction effect selection × food volume, $F_{6, 18} = 3.15$, $P = 0.0273$, supp table 1). CCU tolerance declined to values no different from 0 at both 1.5 mL and 2 mL food. However, MCU tolerance remained greater than zero when the starting food volume was increased to 1.5 mL food, although they too showed nearly zero mean tolerance at 2 mL food volume (fig 1a). Both MCU and CCU were significantly different from MB populations in mean tolerance at only 1 mL food. LCU tolerance values were, on average, between MB and CCU at 1 mL food, and nearly equal to CCU at higher food volumes (fig 1a). As a result, LCU tolerance was not different from MB, MCU or CCU at any food volume, nor was it significantly different from 0.

All three crowding-adapted population sets (MCU, CCU, LCU) had a significantly more severe competitive effect on the survivorship of the common competitor OE, than they did on themselves. This was reflected in the mean effectiveness data, with a significant main effect of selection (supp table 2, $F_{3, 9} = 24.92$, $P = 0.0001$). These patterns of differences were mainly

driven by mean effectiveness at 1 and 1.5 mL food, with each of the MCU, CCU and LCU population sets differing significantly in effectiveness from the MB set, which was itself not significantly different from 0 effectiveness (supp table 2, interaction effect Selection × Food Volume, $F_{6, 18} = 4.13$, $P = 0.0088$). It is worth noting that the average effectiveness of both MCU and CCU at 1 mL food, likely the highest intensity of competition, was almost equal to -1 (fig 1b). This was due to OE survivorship against them being nearly 0% at the highest intensity of competition (supp fig. 1a). At 1.5 mL food, effectiveness for survivorship generally declined as compared to 1 mL, but this decline was more severe for CCU and LCU than for MCU, with both showing lower effectiveness than MCU at 1.5 mL food (fig 1b). MCU effectiveness was significantly negative even at 2 mL food – OE in duo-culture vs. MCU were performing poorly compared to OE in mono-culture at every single high-density food level. At 2 mL food, CCU and LCU effectiveness values were indistinguishable from 0. OE showed better survivorship in duo-culture vs. MB at 2 mL than in OE mono-culture, as can be seen from a positive effectiveness value for MB (fig 1b).

Pre-adult development time (hours)

As mentioned in the methods section, the relationship of development time, all else being equal, tends to be inversely related to fitness – a greater development time implies lower fitness, on an average. Consequently, this also implies reversed patterns of tolerance (more negative value implies greater tolerance), as well as effectiveness (ascribed to greater positive values) for development time, as compared to survivorship.

Overall, we observed a pattern of increase of mean development time, as well as its variance, with food level, although these could not be tested for significance at the 1 mL food level due to lack of any surviving OE flies in duo-cultures vs. MCU and CCU.

Differences between mono- and duo-cultures tended to be larger at higher food levels for the crowding-adapted populations (fig3). However, the ANOVA did not show a significant interaction effect for selection × food volume for tolerance (supp table 4). There was a significant main effect of selection for tolerance ($F_{3, 9} = 7.36$, $P = 0.0085$, supp. table 4). Both MCU and CCU showed significantly negative tolerance (fig. 1c). They were also significantly different from MB, which was itself not significantly different from 0 tolerance. LCU showed no significant difference from any other selection regime, or from 0, for tolerance in development time (fig. 1c).

Effectiveness at 1 mL food could not be tested – the OE showed 0% pre-adult survivorship in competition against some MCU and CCU blocks, which meant that there was no development time to test in those cases. The ANOVA showed no significant main or interaction effect for starting food volume, implying an absence of statistical differences in effectiveness values at 1.5 and 2 mL food levels. The ANOVA did show a significant main effect of selection regime ($F_{3, 9} = 8.05$, $P = 0.0064$, supp. table 5), and the resulting pairwise comparisons are plotted in fig 1d, which pools both food levels per selection regime. All three sets of crowding-adapted populations were significantly more detrimental to OE than OE themselves, on average. This can be seen from the significantly 'positive' effectiveness values of MCU, CCU and LCU in fig 1d, whose means are not different from each other either. Average MB effectiveness was not significantly different from 0, nor was it significantly different from that of LCU, whereas both MCU and CCU had greater effectiveness than MB (fig. 1d).

Average variance for development time did not show any significant differences between mono- and duo-cultures (data not shown).

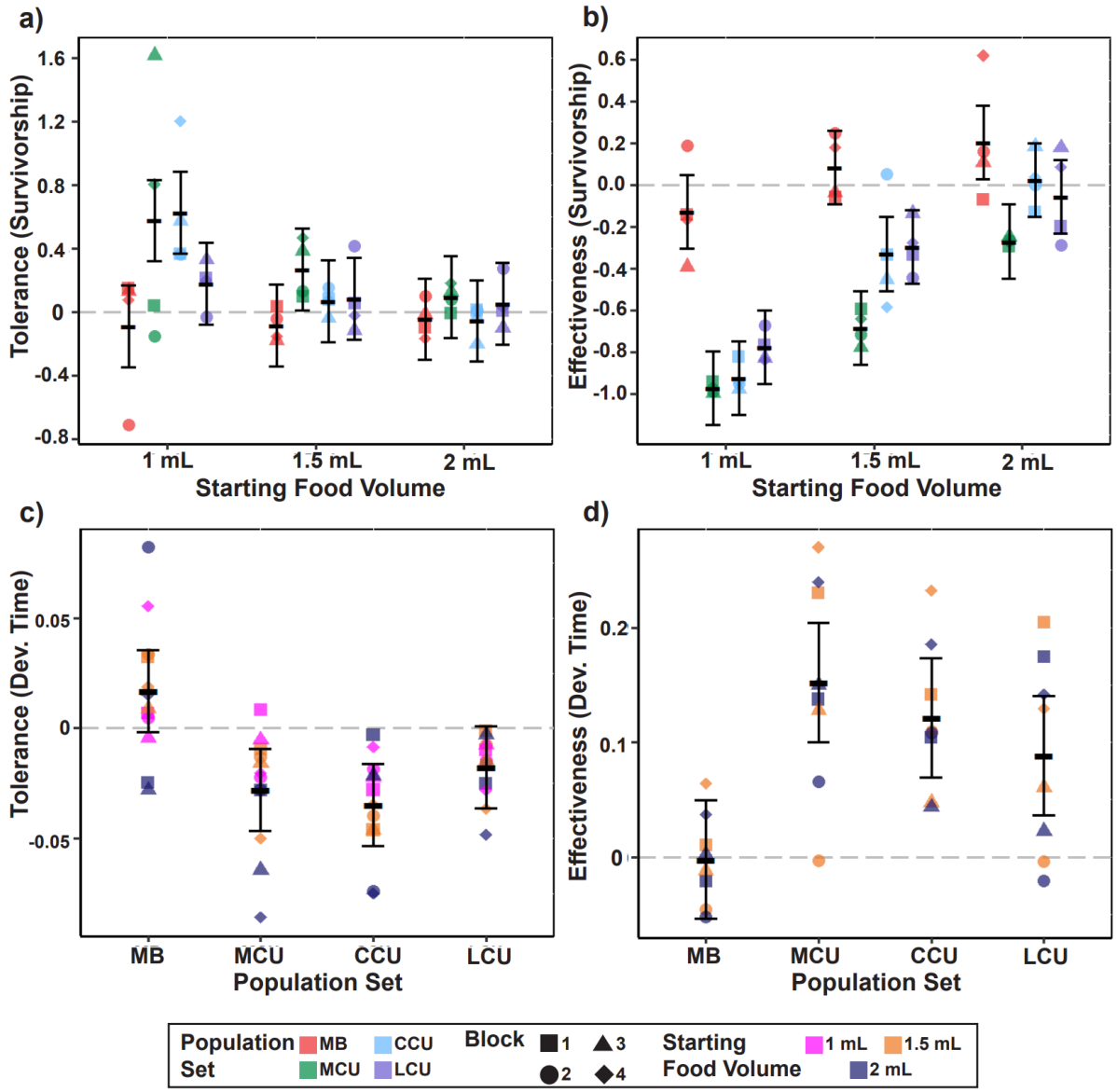

Figure 1: Tolerance and effectiveness of the three crowding-adapted population sets (MCU, LCU and CCU) as well as their low-density ancestral controls (MB). Error bars show ±95% confidence intervals around the mean of four replicate populations, based on the ANOVA. The individual plots are a) Tolerance for survivorship (interaction effect of starting food volume × selection, supp table 1); b) Effectiveness for survivorship (interaction effect of starting food volume × selection, supp table 2); c) tolerance for development time (main effect of selection, supp table 4); d) effectiveness for development time (main effect of selection, supp. table 5). Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Biomass

Average tolerance for biomass is plotted in fig. 2a. At the lowest food level, all three crowding adapted population sets (MCU, CCU, LCU) showed, on an average, increased biomass in competition with OE than in competition with themselves (interaction effect Selection × Food volume $F_{6, 18} = 4.02$, $P = 0.0099$, supp table 7). This was indicated by their significantly positive mean tolerance levels at 1 mL food (fig. 2a). All three population sets also showed higher tolerance than MB. CCU tolerance was significantly greater than LCU tolerance at 1 mL food (fig. 2a). At 1.5 mL food, both MCU and CCU maintained this pattern, but LCU tolerance dropped to an average that was positive, though not statistically different from 0, nor from MB tolerance. Finally, at 2 mL food, only MCU populations retained a positive mean tolerance, although this was not statistically different from MB (fig. 2a).

For biomass-based effectiveness, only the main effects of selection and food level were significant in the ANOVA. The interaction between the two factors was not significant. All three crowding-adapted populations reduced OE biomass significantly compared to OE themselves, when averaged across all food levels (fig. 2b, $F_{3, 9} = 20.98$, $P = 0.0002$, supp. table 8). These were also different from MB, which was itself not significantly different from 0 in effectiveness. Additionally, all populations, on average, showed greater effectiveness at 1 mL food than at 1.5 and 2 mL food ($F_{2, 6} = 41.32$, $P = 0.0003$, supp table 8).

Dry weight per fly at eclosion

The ANOVA for tolerance with respect to dry weight did not reveal any significant differences for selection regime (supp table 10). However, the patterns of gain in dry weight were largely similar to those seen for biomass (fig. 2a). Moreover, there was a significant main effect of starting food volume ($F_{2, 6} = 7.11$, $P = 0.0262$, table 10). Both 1 and 1.5 mL food had average tolerance greater than 0, which appeared to be driven mainly by the crowding-adapted

populations (fig. 2c). There was nearly 0 tolerance on average at 2 mL food, which was also significantly different from average tolerance at 1 mL (fig. 2c).

Patterns for effectiveness of dry weight per fly were also similar to that seen in biomass. OE flies were smaller, on average, in competition against all three crowding adapted populations as compared to competition against OE (main effect of selection, $F_{3,9} = 8.76$, $P = 0.0049$, supp. table 11). This can be seen from the non-zero effectiveness of MCU, CCU and LCU in fig 2d. Additionally, MB effectiveness was also significantly different from zero, implying that OE flies also lost dry body mass when competing against MB. This difference, however, was smaller than the reductions in dry body mass of OE flies when competing against the crowding-adapted populations – MCU, CCU as well as LCU were significantly more effective than MB in competition against OE (fig. 2d).

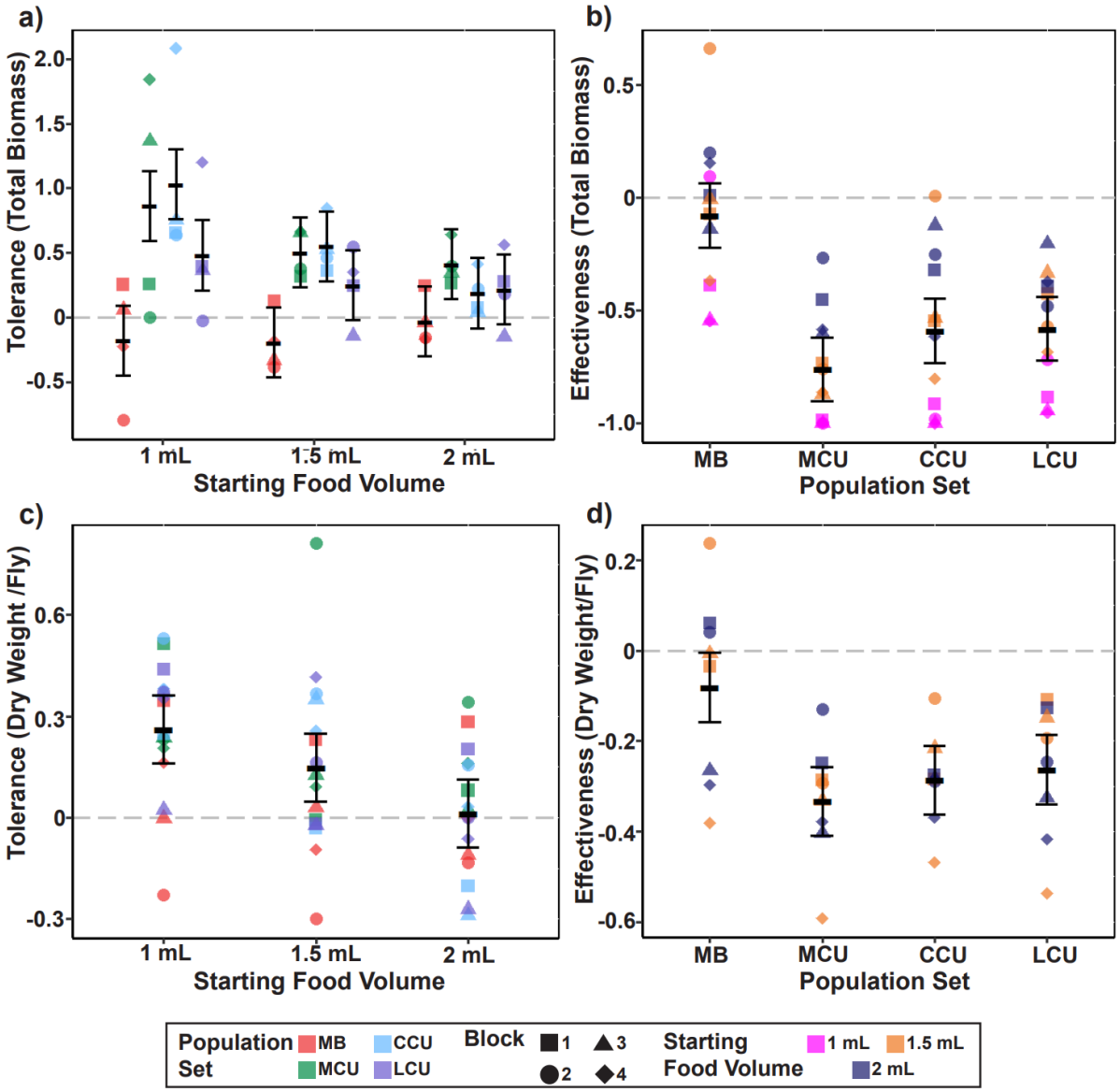

Figure 2: Tolerance and effectiveness of the three crowding adapted population sets (MCU, LCU and CCU) as well as their low-density ancestral controls (MB). Error bars show ±95% confidence intervals around the mean of four replicate populations, based on the ANOVA. The individual plots are a) Tolerance for dry biomass (interaction effect of starting food volume × selection, supp table 7); b) Effectiveness for dry biomass (main effect of selection, supp table 8); c) Tolerance for dry weight per fly (main effect of starting food volume, supp. table 10); d) Effectiveness for dry weight per fly (main effect of selection, supp. table 11). Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Eclosion profiles

Figure 3 shows the profiles for eclosion of the different population sets across different culture types and starting food volumes. The number of eclosing flies is shown per 200 eggs in case of mono-cultures, in order to compare the values with the 200 eggs of the respective duo-cultures. Moreover, each time point on the X-axis is an average across replicate populations of the development time checks done over similar time windows per block.

MB and OE showed largely similar eclosion profiles over time (fig. 3a-c). The peak of eclosion increased in amplitude as food level increased, although this change was more pronounced from 1 mL to 1.5 mL than from 1.5 to 2 mL. Most 1 mL eclosions ended by the end of T2 time window, whereas they continued well into T3 at higher starting food volumes. There was a large gain in survivorship of OE at 2 mL food in duo-cultures when compared with mono-cultures (fig. 3c). This was also exemplified by the positive effectiveness for survivorship of MB at 2mL food (fig. 1b). MCU flies appeared to showed a relatively greater peak of eclosion in duo-cultures at both 1 mL and 1.5 mL food than in mono-cultures (fig. 3d-e). At 2 mL starting food (fig. 3f), the peak of MCU duo-culture eclosion was closer to mono-culture, and appeared lower than the peaks at 1 and 1.5 mL food. OE had zero (or very close to zero) survivorship against MCU at 1 and 1.5mL food duo-cultures, respectively (fig. 3d-e). At 2 mL food (fig. 3f), there were a steady number of OE duo-culture eclosions across a hundred hour period, although the relative peak of eclosion at mono-culture was greater. There was a significant difference between mono- and duo-cultures in the total eclosion of OE at 2 mL vs. MCU, however, as also reflected in negative effectiveness (fig. 1b). CCU eclosion profiles largely resembled MCU at 1mL (fig. 3d, 3g), as can also be seen from the tolerance and effectiveness plots (figures 1 and 2). At 1.5 mL food (fig. 3h), CCU populations lost the survivorship advantage in duo-culture vs. mono-culture (see fig. 1a), but continued to suppress OE survivorship (fig. 3h, 1b). Finally, at 2mL food (fig. 3i), survivorship differences between CCU mono- and duo-cultures

disappeared, but a pattern for advantage in development time was present (fig. 1c). LCU populations followed a largely similar pattern to the CCU, with an overall smaller peak of eclosion, in both mono- and in duo-cultures (fig. 3j-l). There was also a small peak of eclosion that OE showed in duo-culture vs. LCU at 1mL food (fig. 3j) – a feature that was absent against the MCU and CCU populations.

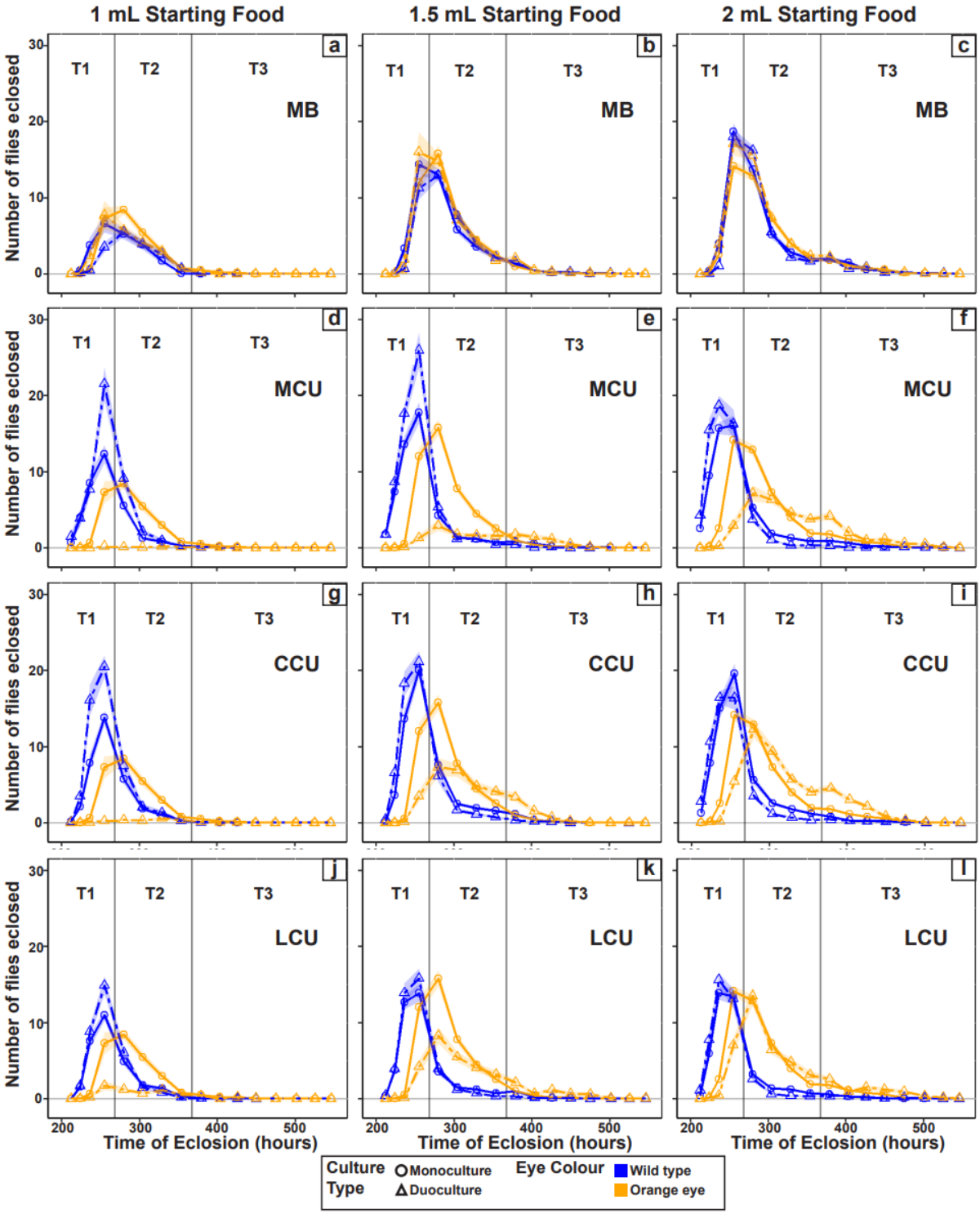

Figure 3: Eclosion over time for each focal population set across three starting food volumes (Top labels). Each row represents a population set; each column represents a starting food volume. Solid lines with circular points represent mono-culture (per 200 eggs); dashed lines with triangular points represent duo-culture. The shaded regions around the data points show S.E.M. The 3 time windows (T1, T2, T3; see methods) are also marked to aid visual interpretation of data in figures 4 and 5.

Dry biomass across time windows

The biomass measurements were divided across three time windows: T1, T2, T3 (up to 270 hours, 270-370 hours, post 370 hours from egg collection, respectively) (fig. 4). As in the eclosion profiles, mono-cultures are plotted per 200 eggs, to be matched with the 200 eggs seeded in the respective duo-cultures. These biomass values were also statistically tested for differences (supp table 9). There was a general pattern of biomass increasing with food volume (fig. 4). MB populations largely showed no differences from OE at any time point or food volume (fig. 4a-c). In duo-cultures, MCU populations consistently showed greater biomass than the mono-cultures at T1 across all food levels (fig. 4d-f) (fig. 4d). These large differences were also likely represented by a significantly positive total biomass tolerance of the MCU at each food volume (fig. 2a). At 2 mL, the T1 duo-culture MCU biomass was almost double that of the biomass of the (scaled to 200 eggs) mono-culture MCU populations (fig. 4f). Dry biomass in both T2 and T3 was significantly reduced compared to T1 across all food levels, in both mono- and duo-cultures in MCU (fig. 4d-f). The biomass of OE in duo-cultures vs. MCU was not significantly different from 0 at both 1 and 1.5 mL food (fig. 4d-e). Duo-culture biomass of OE vs. MCU was also significantly lower than the mono-culture OE at T1, at both 1.5 and 2 mL food (fig. 4e-f). However, in the most delayed time window from egg collection, T3, OE showed a non-significant pattern of greater biomass in duo-culture vs. MCU than in mono-cultures at 2 mL food (fig. 4f). In CCU, the patterns were largely similar to MCU (fig. 4g-i). There were some exceptions of note – the difference between duo-culture and mono-culture biomass of CCU at 1 mL, T1, appeared to be greater than the pattern seen in MCU(fig. 4g), and this was likely also reflected in the non-significant pattern of slightly higher tolerance for biomass in CCU vs. MCU at 1 mL food (fig. 2a).. However, the difference between the two culture types in CCU largely remained similar across food levels (fig. 4g-i), while this pattern of differences increased in the MCU (fig. 4d-f). Additionally, OE biomass in duo-culture was

more comparable to mono-culture at both 1.5 mL as well as 2 mL food at T2 (fig. 4h-i). At T3 in 2 mL food, OE duo-culture biomass was nearly twice the value (scaled for 200 eggs) of the mono-culture biomass in competition against CCU, although this difference was not significant (fig. 4i).

LCU duo-culture biomass did not show much difference compared to that of mono-culture at both 1 mL and 1.5 mL food (fig. 4j-k). However, the duo-culture biomass at 2 mL was significantly greater than that of mono-culture at T1 (fig. 4l). The extent of this difference, however, was slightly lower than CCU, and much lower than MCU. T2 and T3 biomass of the LCU at 1.5 mL and 2 mL were not significantly different from 0 in duo-culture (fig. 4k-l). OE biomass values at duo-culture vs. LCU were (either significantly or with a non-significant pattern) reduced from those of mono-culture at both T1 and T2 across all food volumes (fig. 4j-l). It is also worth noting that there was very little difference in biomass of OE at T3 in duo-culture vs. LCU, as compared to the scaled biomass of OE in mono-culture (fig. 4l).

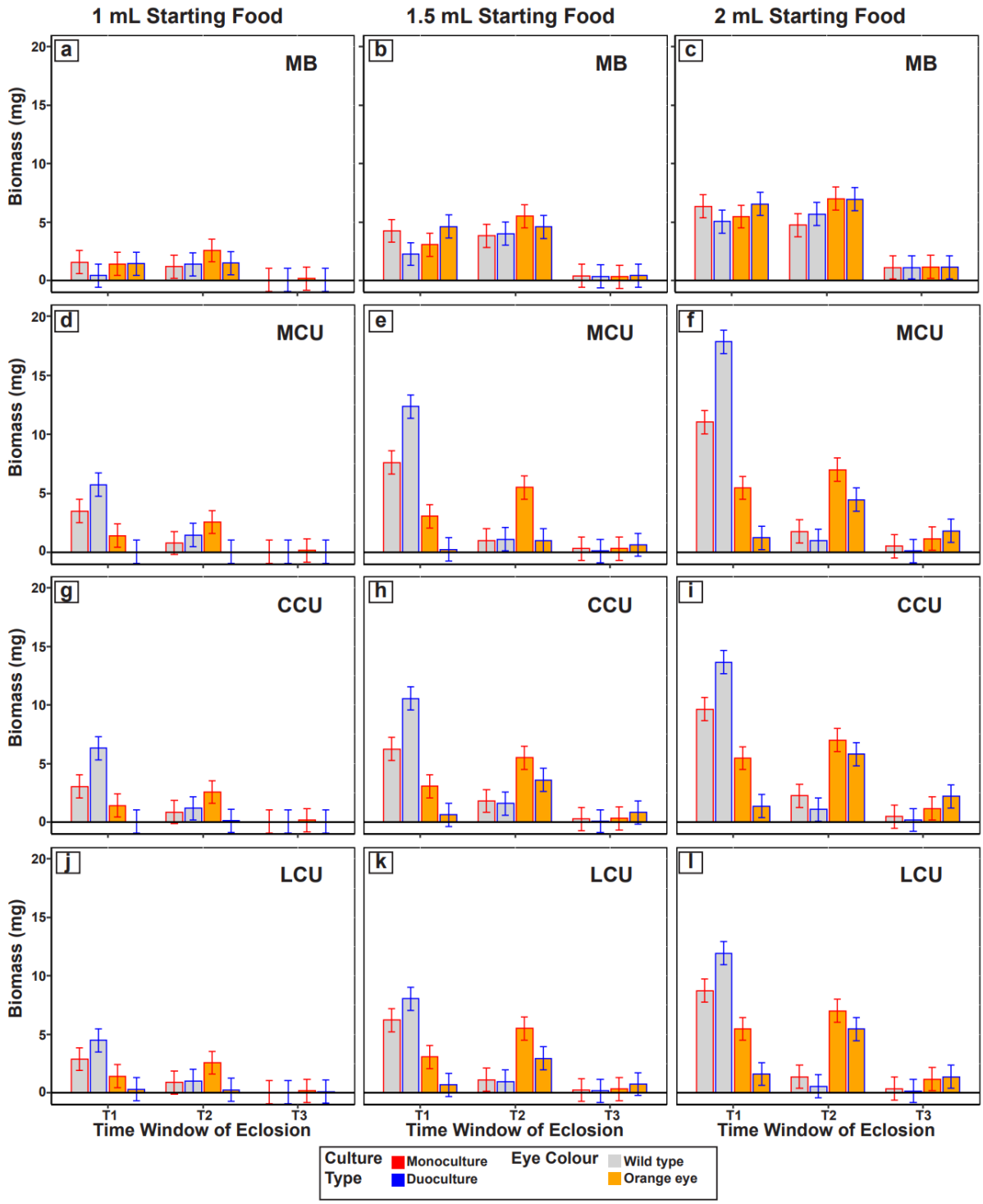

Figure 4: Biomass per population set per starting food volume, across the three time windows (T1, T2, T3; see methods). Error bars indicate ±95% Confidence Intervals around the means of four replicate populations (interaction effect selection × starting food volume × time window × culture type × eye colour, $F_{12, 36} = 4.85$, $P < 0.001$, supp. table 9). Each figure label (a-l) corresponds to the same culture as labelled in figure 3.

Dry weight per fly across time windows

The dry weight per fly data could not be analysed in its entirety due to the lack of data for OE in 1 mL cultures across time steps (see fig. 1b, supp. fig. 1a). Nevertheless, the data are presented in figure 5 in order to see the overall patterns of dry weight over time, per population, per culture type. There were a few prominent patterns observable in the data. While OE in mono-cultures appeared larger than all focal population flies across all time windows, their dry weights in duo-cultures were reduced vs. the crowding adapted populations, especially in T1 (fig. 5d-l). In the dry weight distributions of 1 mL cultures, the flies eclosing in the T1 time window were generally larger than flies from T2 (fig. 5a, 5d, 5g, 5j). In the mono-culture distributions of 2 mL (fig. 5c, 5f, 5i, 5l), T1 flies as well as T3 eclosing flies appeared larger than T2 eclosing flies. This pattern changed however, in the duo-cultures of crowding-adapted populations. OE and (more prominently) MCU, CCU and LCU population flies were reduced or plateaued in size at T3 in duo-cultures as well as at T2, unlike in mono-cultures, which appeared to show an increase in average size from T2 to T3 (fig. 5c, 5f, 5i, 5l).

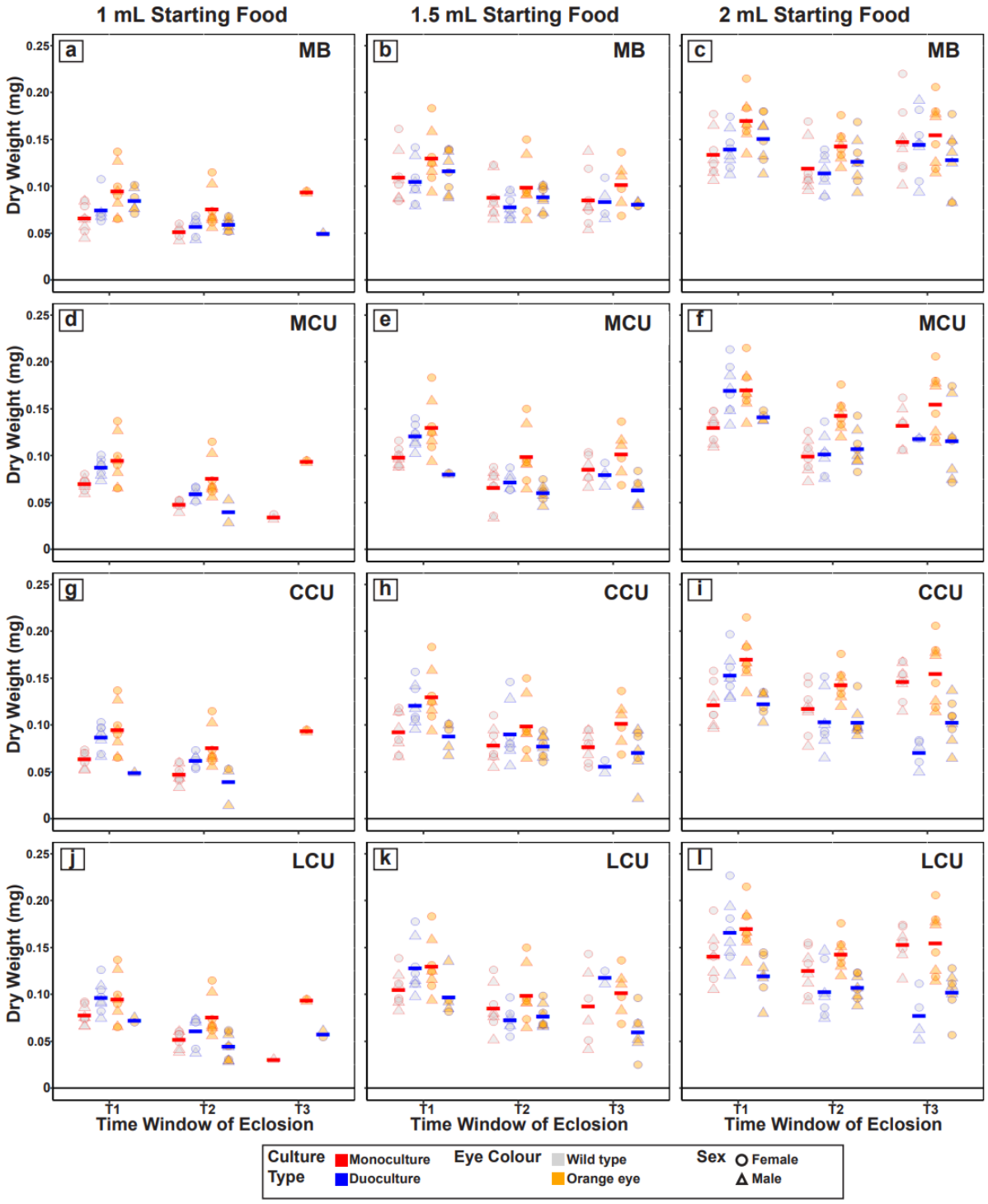

Figure 5: Dry weight per fly, for each combination of population set × starting food volume, across the three time windows (T1, T2, T3; see methods). Bold coloured bars represent mean under each category, respectively. Each figure label (a-l) corresponds to the same culture as labelled in figure 3.

Low-density cultures

Data from the low-density cultures containing 70 eggs in 6 mL food are shown in supp fig. 3a-d. These show the potential values each of the traits used as outcomes of competition under crowding can take without the influence of crowding. Both mono- and duo-culture data are plotted.

Discussion

Our results reinforce earlier findings that increased larval competitive ability is a consistent outcome of selection for adaptation to larval crowding. Moreover, our results highlight many nuances that become apparent upon splitting competitive ability along the axes of effectiveness and tolerance, for different measures of competitive outcome.

Starting food volume, even at relatively high densities, can be a major determinant of whether differences in competitive ability are seen between crowding-adapted and control populations. At 1 mL food volume, with a total density of 400 eggs/mL food, survivorship was lowest for all mono-cultures (supp fig 1). Furthermore, effectiveness and tolerance were most visibly different at this food volume for survivorship as well as biomass and dry weight per fly (fig. 1a-b and fig. 2). Out of the three crowding-adapted population sets, the MCU and CCU populations were practically identical in their effectiveness and tolerance at 1 mL along every axis of measurement (with perhaps CCU having a slight advantage in tolerance for development time (fig. 1c) as well as the biomass of early eclosing flies (figures 4d, 4g)). At higher food levels, however, CCU populations had relatively lower effectiveness and tolerance, and were similar to the LCU. In contrast, the MCU populations remained at average non-zero effectiveness and tolerance, at least at 1.5 mL food (figures 1 and 2). This is surprising, given that the MCU and CCU populations were similar in competitive ability at 1 mL food. However,

the MCU populations had undergone over 100 more generations of selection than the CCU populations at the time of the current study. If we assume that the selection response has plateaued in both sets of populations, we should expect MCU and CCU to show similar competitive ability across all food levels. Alternatively, the MCU populations could, as they did against the LCU, show greater competitive ability than the CCU at every high-density condition examined. In contrast to both assumptions, the CCU populations competed just as well as the MCU when the total (eggs/food) density was close to their rearing density, but not when food levels were higher (and closer to the rearing food volumes of MCU). This suggests the involvement of some food column height effects in competitive ability as well.

LCU populations showed the lowest competitive ability in most conditions (as compared with MCU and CCU). This is not surprising, given that the LCU populations are reared at the lowest eggs/food density (200 eggs/mL for LCU vs. 400 eggs/mL in case of MCU and CCU). In the current experiment, the treatment containing 400 eggs in 2 mL food is the same total density as the one in which LCU is reared (200 eggs/mL food). Even at that treatment, however, LCU populations still performed, at best, on par with the MCU and CCU populations (figures 1 and 2). One possible exception was in the biomass of T3 (i.e., the last eclosing) OE flies, which showed a pattern of being the lowest against LCU at 2 mL duo-culture (fig. 4l). This might indicate the presence of greater effectiveness of the LCU larvae towards the later part of a culture, when any remaining food is likely riddled with high concentrations of metabolic waste (Borash et al. 1998). This is a scenario that is very likely to happen in the LCU native rearing cultures, wherein relatively high volumes of food are used. It remains to be seen if the LCU populations display any greater competitive advantages at the exact container dimensions, food volume and egg number quantities as their native rearing conditions (i.e., 1200 eggs, 6 mL food, 6 dram vials). While earlier experiments have examined competitive ability at the exact rearing conditions of LCU, they were too early in the selection process to give any clear results

(Sarangi 2018). Moreover, it has recently been demonstrated by us that the density at the feeding band (a narrow volume of food, close to the surface, where the larvae feed) is an important determinant of the outcome of competition, more so than the total eggs/food density (Chapter 5). Given this knowledge, it cannot be ruled out that the LCU populations may have greater larval competitive ability close to the feeding band density at which they have been reared. Experiments are underway to determine if such specific competitive ability exists.

These results are also in agreement with the data of Pandey et al. (2022), who saw that MCU in mono-cultures had greater survivorship at higher densities of crowding compared to LCU. Moreover, the sensitivity of MCU survivorship to increased crowding was lower than LCU (Pandey et al. 2022). With the current study, we further add that MCU populations also show greater competitive ability across a wider range of crowding densities than LCU. In mono-cultures, Pandey et al. (2022) observed differences primarily at the highest density tested (300 eggs in 1 mL food). In contrast, in the current study, the effectiveness and tolerance of MCU show differences from LCU even at lower crowding densities. This indicates that larvae in duo-cultures can potentially show greater sensitivity to the outcomes of competition than mono-cultures.

Furthermore, while both average effectiveness and tolerance for pre-adult survivorship of each crowding-adapted population set changed similarly across crowding densities, there were some notable exceptions. MCU tolerance for survivorship was not significantly different from 0 at 2 mL food (fig. 1a), while the effectiveness was different (fig. 1b). This was true for CCU at 1.5 mL food (fig. 1a-b). LCU populations never showed a difference from 0, or from MB, in tolerance, but showed a difference in effectiveness (fig. 1a-b). The MB populations showed poor effectiveness at 2 mL but no change in tolerance (fig. 1a-b). These results together indicate that tolerance and effectiveness are likely independent in their evolution, as suggested

previously (Mather and Caligari 1983; Caligari and Mather 1984; Eggleston 1985; Hemmat and Eggleston 1988; Joshi and Thompson 1995).

The role of development time

While differences in survivorship and dry weight (and hence biomass) can be modelled to result from the simplest possible forms of exploitation competition (Bakker 1961; Chapter 6), those in development time are likely to rise from more complex processes. In a crowded culture, there are two ways in which development time of a larva can be delayed – a) high concentrations of metabolic waste products building up and slowing developmental rate (Botella et al. 1985), and b) lack of space to feed, leading to lower overall feeding rate (discussed in Sang 1949; Bakker 1961). The phenomenon by which the development of a larva is arrested in a crowded culture, wherein it presumably faces severe competition and high concentrations of waste, has been termed 'larval stop' (Ménsua and Moya 1983). This process would be expected to lead to an extended development time distribution, as larval stop duration can be quite variable (Ménsua and Moya 1983). While the build-up of waste products such as urea, ammonia would be expected in all the food levels tested in the current study, the higher volumes of food would allow larvae to ingest more waste before running out of food, slowing overall development time, and possibly giving rise to larval stop (Botella et al. 1985). This could lead to a long tail of the development time distribution, which could further extend if more food were present, as larvae that were facing high levels of waste due to low initial feeding rate could still feed slowly in waste ridden food (see Borash et al. 1998). This is in overall agreement with what was observed in our study – while the 1 mL cultures showed a truncation in development time at around the T2 stage (fig. 3a, 3d, 3g, 3j), likely due to severe food shortage, the 2 mL cultures could sustain feeding, and thus eclosion, for a much longer duration – well into the T3 stage (fig. 3c, 3f, 3i, 3l). The crowded cultures with higher initial volumes of food also appeared to have a number of larvae feeding after the bulk of pupation

was over, and had some moist food left over after all the eclosions were done (S. Venkitachalam, personal observation). This was in contrast to the 1 mL culture, in which food got depleted much faster and the remnants turned to dried powder by around day 10 from egg collection (S. Venkitachalam, personal observation). It is worth speculating here that the overall moisture of food in a culture can be an important factor in determining the volume of accessible food remaining in a culture. An additional noteworthy point is that the development times of every crowded culture are several hours longer than their respective low-density cultures (supp. fig. 2 vs. supp. fig. 3b), possibly suggesting some action of larval stop in each crowded food level. A previous study from our research group found the existence of some duration of larval stop in the MB populations, but not in the MCU populations (Pandey 2022).

The second possibility for delayed larval development has found some discussion in earlier papers – the existence of crowding for limited space, leading to reduced access to food, when a large number of larvae feed together in limited spatial constraints of the feeding band (Bakker 1961). A recent study by us also demonstrated that the larval density in the feeding band is a more important determinant of the outcome of competition than overall density (Chapter 5). However, while some initial overcrowding of larvae may cause space shortages in the feeding band in the cultures used in the current study, these shortages are unlikely to be sustained to nearly the levels that are seen in the regular maintenance cultures of LCU or CCU, or even the MCU, which are each reared with higher absolute numbers of eggs. Thus, it is likely that the development time delays at moderately high feeding band densities, as used in the current study, are primarily through the consequences of metabolic waste build-up. This argument has some support from earlier studies as well, assayed at high feeding band densities (Borash et al. 1998; Mueller and Barter 2015). Given the argument developed above, it is also worth noting the pattern that 1 mL cultures appear to result in lower tolerance than 2 mL cultures (fig. 1c).

These results can also be compared with another study we conducted (Chapter 4), wherein MB, MCU, CCU and LCU populations were each competitively handicapped by providing a temporal head start in age to the OE eggs in competition against them (200 eggs focal population + 200 eggs OE in 2 mL food). When competing against each of the crowding-adapted population sets, head starts to OE gave greater development time advantages to OE adults against both CCU and LCU, than against MCU (Chapter 4). This was another example of MCU competitive superiority in 2 mL food volume, similar to the effectiveness and tolerance measurements in the current study, which once again suggests that the food column height at which the larvae of the population are adapted may play a major role in determining larval competitive ability in food columns of varying height.

Distribution of dry weight over development time

The elongated development time distribution in crowded cultures with higher volumes of food also has consequences on the distribution of dry weight of eclosed flies. It has been shown before that in a crowded culture, early eclosing flies are usually larger than later eclosing flies (Hughes 1980; Sarangi 2018). This can be seen in the dry weight per fly distributions of 1 mL cultures, with T1 flies generally being larger than T2 flies (figure 5a, d, g, j). Sarangi (2018) also showed that in crowded cultures with higher food levels, early eclosing flies were larger than flies that eclosed in the middle of the distribution, but the flies eclosing towards the tail-end of the distribution were again quite large. This was observed in the mono-culture distributions of 2 mL (fig. 5c, 5f, 5i, 5l), wherein T1 as well as T3 eclosing flies appeared larger than T2 eclosing flies. This pattern changed, however, in the duo-cultures of crowding-adapted populations (fig. 5f, i, l). Instead of showing an increase in size as in the mono-cultures, OE and (more prominently) focal population flies appeared to reduce (or plateau) in size at T3, compared to T2. A future experiment might explore this aspect in greater detail, with greater replication for vials and a greater resolution of binning in time windows.

Surprisingly, at 2 mL food, the smallest flies appeared to come from the crowding-adapted populations in duo-culture, in T3 (fig. 5f, 5i, 5l). Given the eclosion and biomass distributions (fig. 3 and 4, f, i, l, respectively), these were likely to be lower in number. However, this indicates the possible existence of crowding-induced late-eclosing variants in the MCU, CCU and LCU populations that can survive at smaller body masses than MB (see also Nagarajan et al. 2016, Sarangi et al. 2016).

Fitness-functions – a matter of competitive ability distributions?

In the current study, the MCU, CCU and LCU populations had generally higher competitive ability than OE as seen by their increased effectiveness and tolerance across different starting food volumes (figures 1, 2) and the nuanced time-based expression of competitive ability displayed in biomass distributions (fig. 4). The duo-cultures of crowding-adapted populations also differed (or appeared to differ) from mono-cultures in eclosion, biomass and dry weight per fly distributions (figures 3, 4, 5). Given these two observations, we can speculate that the overall distribution and the variance of competitive ability likely determine the development time and dry weight distribution in crowded cultures. Duo-cultures containing eggs from crowding-adapted populations vs. OE are likely to furnish bimodal distributions in competitive ability, as opposed to unimodal distributions in mono-cultures, and this uniquely shapes dry weight, development time and biomass distributions in the crowded cultures.

This difference in competitive ability distributions becomes relevant when we compare interspecific and intraspecific competition experiments. In interspecific competition experiments using populations of two closely related *Drosophila* species which can be reared on the same food resource (e.g., Moore 1952; Ayala 1969; Joshi and Thompson 1995), we may be able to compare the competitive ability distributions of the two competing species. In some previous experiments, there have been large starting differences in competitive ability between

the two competing species (Moore 1952; Ayala 1969; Joshi and Thompson 1995). Such large differences in competitive ability distributions would be akin toa bimodal starting distribution for competitive ability seen in MCU vs. OE competition in the current study. This is in contrast to a selection experiment on intraspecific competition, which is more likely to start with a unimodal competitive ability distribution. Given the differences in competitive ability distributions, interspecific competition may result in very different patterns of survivorship, body size and development time for each species in the culture compared to their density-matched mono-culture counterparts. This would suggest that each competitor species could experience different underlying fitness-functions depending on whether they are in inter- or intraspecific competition. Consequently, these differences may lead to the evolution of very different traits for a given species in inter- vs. intraspecific competition, depending on the competitive ability of the species as well as the details of crowding under which the selection is carried out. In interspecific competition, different competitive strategies may be selected by the different species if there is more starting food, as there is likely to be enough survivorship of both species with a development time and size difference (as seen with MCU vs. OE in fig. 3-5). An interesting question also arises as to the rate of evolution of competitive ability (in the broad sense, as described in Joshi and Thompson 1996) in duo-species vs. mono-species systems.

Conclusions

The evolution of competitive ability is a fundamental outcome of density-dependent selection in crowded conditions, and constitutes an important conceptual bridge between ecology and evolution. Our results indicate that outbred *D. melanogaster* populations can evolve increased competitive ability to different degrees depending on the precise nature of crowded rearing conditions. Furthermore, competitive ability, when split along the axes of effectiveness and tolerance, and measured for four different outcomes of competition, shows a staggering depth

of nuance in its evolution, even in a seemingly simple laboratory culture system. Larvae of crowding-adapted populations can be superior competitors depending on the starting food volume, and show patterns of time-dependence in their competitive superiority, as seen from eclosion, biomass and dry weight per fly distributions over time (fig 3, 4, 5). While there have been some recent advances, both theoretical and experimental, in the study of density-dependent selection (Fronhofer et al. 2022; Bertram and Masel 2019; see also Than et al. 2020), our results highlight the need for understanding the ultimate consequences, as well as the mechanistic bases, of the depth of nuance visible in the process of competition and evolution of competitive ability. This requires greater exploration both in simple laboratory systems as used in the current study, as well as complex natural ones as discussed by other authors (Travis et al. 2013; Morimoto and Pietras 2020). Where logistically possible, the experiment design used in the current study can be further expanded in order to better study the ecology and evolution of larval competition. Such expansions include the use of multiple marked competitors (e.g., de Miranda et al. 1991; Santos et al. 1992), as well as more complex experiments incorporating replacement series or substitution methods (De Wit 1960; Seaton and Antonovics 1967; Mather and Caligari 1981).

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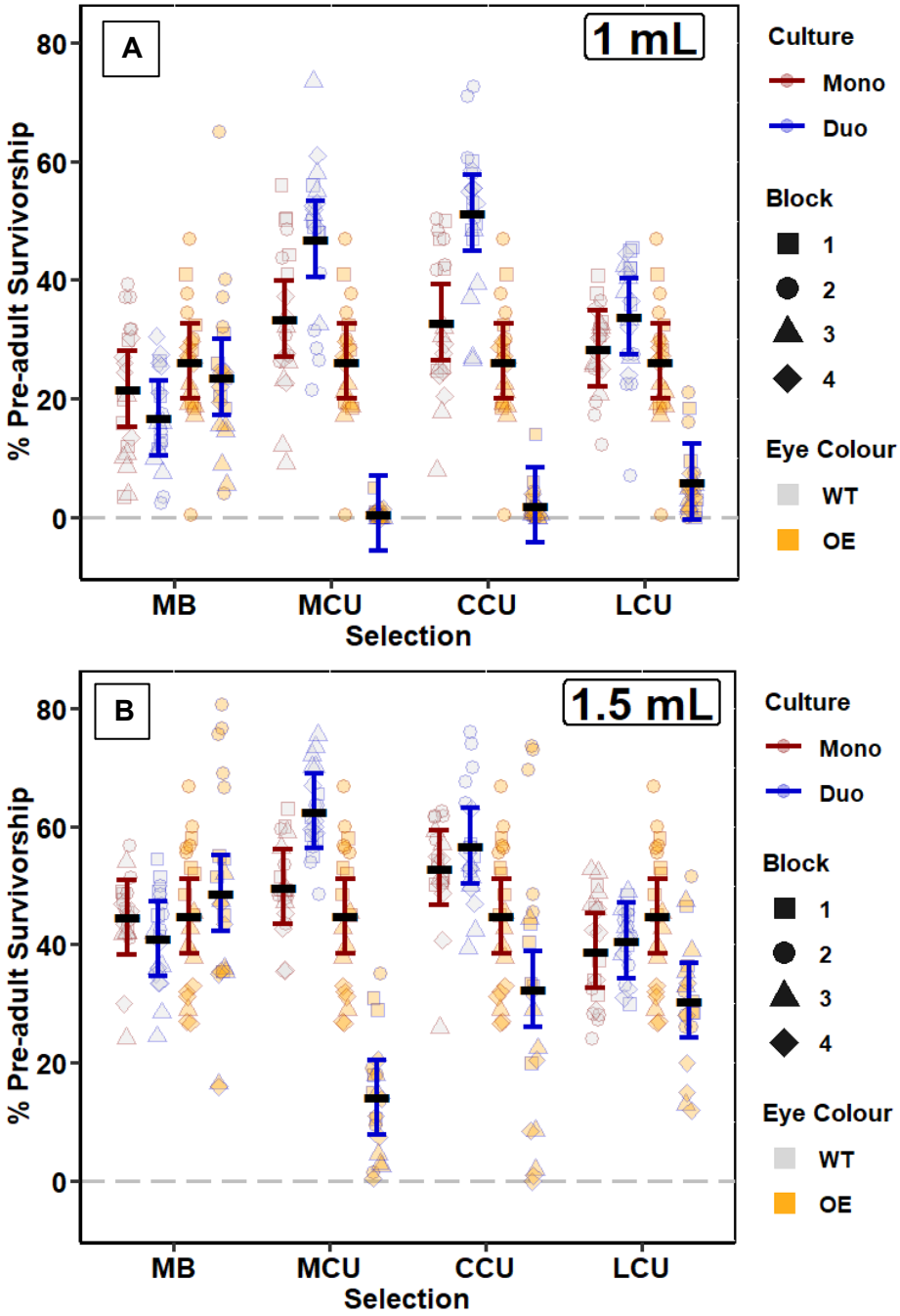
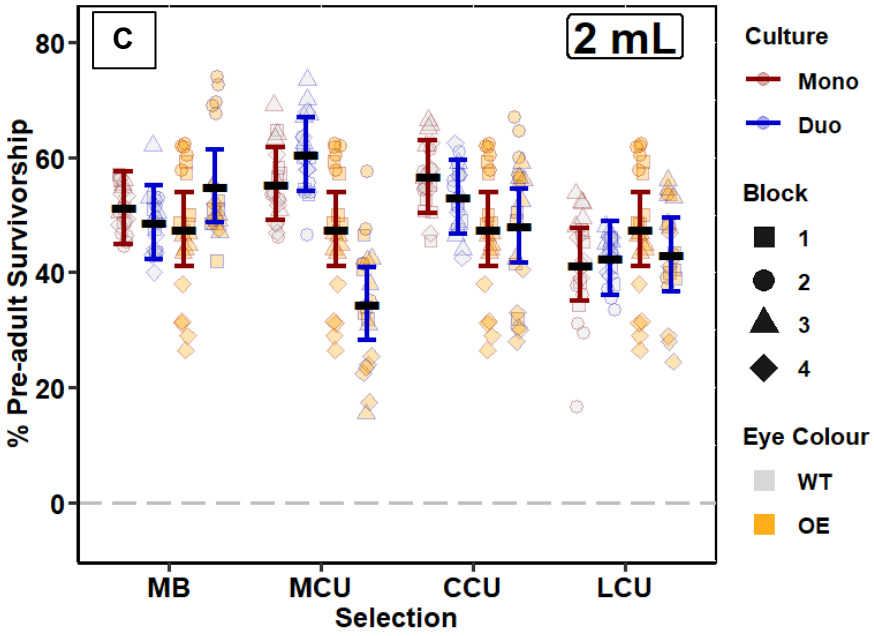
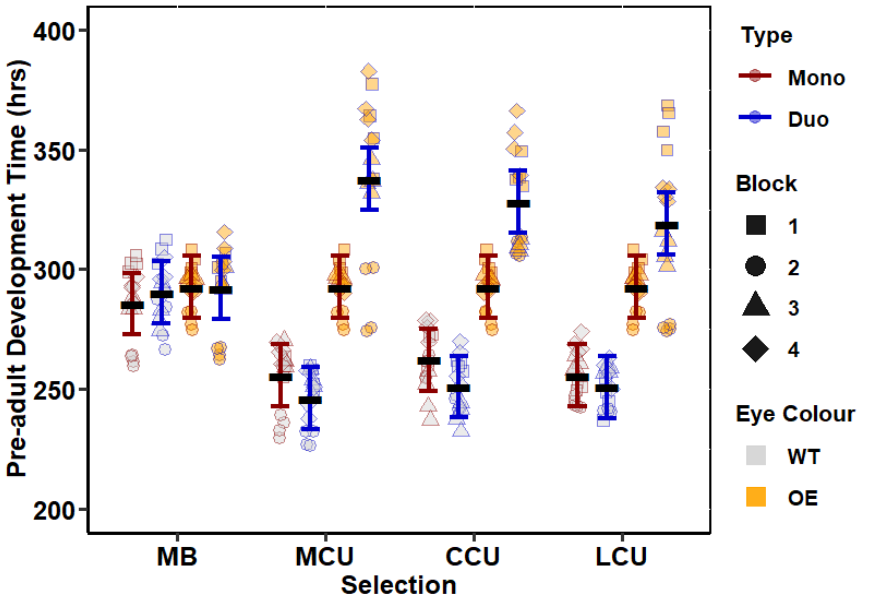
Supplementary material for chapter 2

The evolution of effectiveness and tolerance in populations of *Drosophila melanogaster* adapted to chronic larval crowding at varying combinations of egg number and food volume

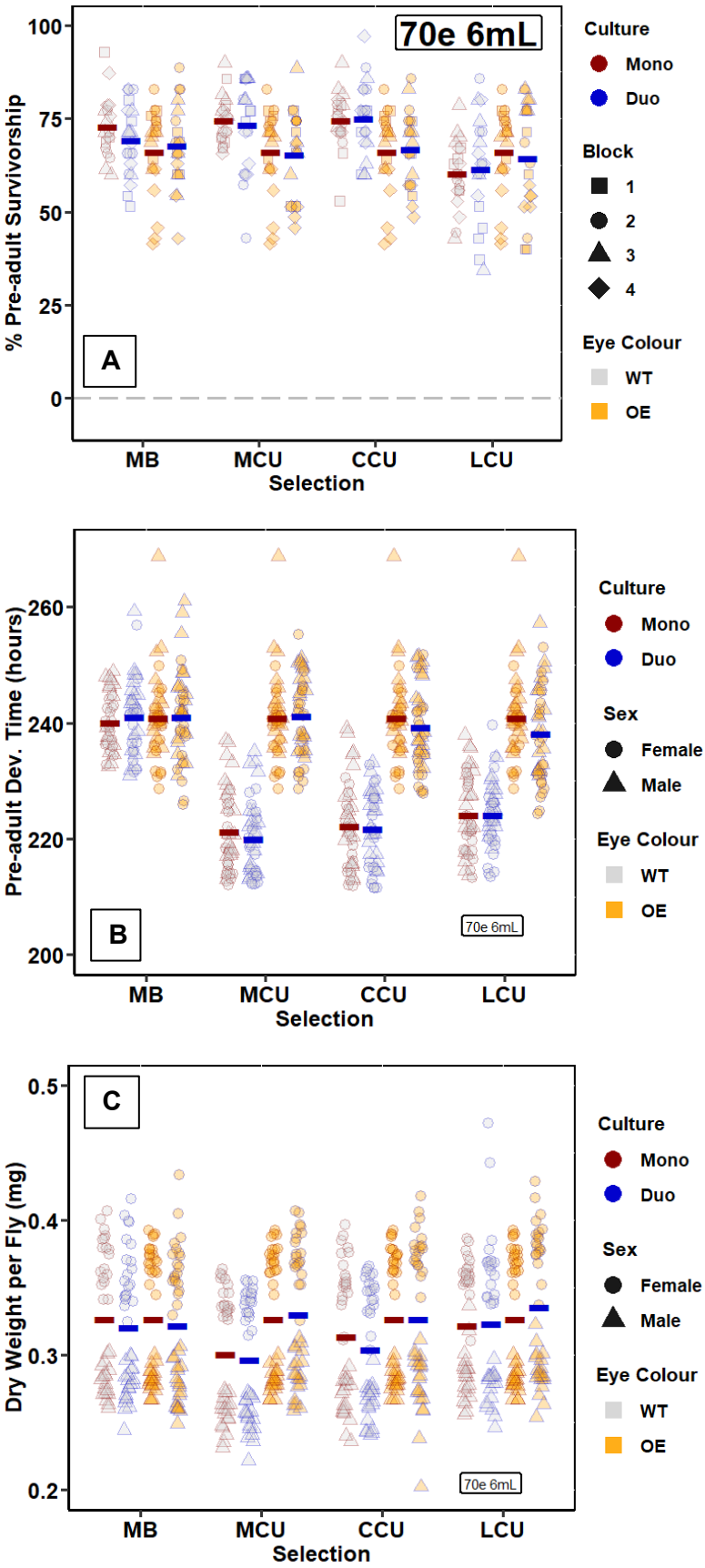




Supp. Fig. 1. Pre-adult survivorship in A) 1 mL; B) 1.5 mL; C) 2 mL cultures. Black bars in each group represent the mean. Error bars show 95% C.I. for the post hoc test using the relevant within-group error from ANOVA. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.



Supp. Fig. 2. Pre-adult development time (hours) pooling 1.5 mL and 2 mL cultures, as well as male and female data. Black bars in each group represent the mean. Error bars show 95% C.I. for the post hoc test using the relevant within-group error from ANOVA.



Supp. Fig. 3. Outcomes at 70 eggs in 6 mL cultures (non-crowded). A) Pre-adult survivorship. B) Pre-adult development time (hrs). C) Dry weight per fly (mg). Coloured bars represent means of each group.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	2.728070	0.106245
Food	2	6	1.763133	0.249854
Selection*Food	6	18	3.149238	0.027261

Supp. Table 1. ANOVA on tolerance for survivorship.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	24.9151	0.000108
Food	2	6	38.9512	0.000366
Selection*Food	6	18	4.1277	0.008830

Supp. Table 2. ANOVA on effectiveness for survivorship.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	2.1178	0.168128
Food	2	6	33.7987	0.000542
Type	1	3	10.6024	0.047269
Eye	1	3	7.1108	0.075910
Selection*Food	6	18	4.6312	0.005166
Selection*Type	3	9	1.8019	0.216846
Food*Type	2	6	2.2254	0.189241
Selection*Eye	3	9	43.8494	0.000011
Food*Eye	2	6	4.2396	0.071156
Type*Eye	1	3	262.3098	0.000512
Selection*Food*Type	6	18	0.8149	0.572306
Selection*Food*Eye	6	18	6.0920	0.001263
Selection*Type*Eye	3	9	12.2814	0.001560
Food*Type*Eye	2	6	8.1230	0.019620
Selection*Food*Type*Eye	6	18	6.1507	0.001198

Supp. Table 3. ANOVA on the raw survivorship data (without effectiveness and tolerance calculations).

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	7.36425	0.008532
Food	2	6	3.30806	0.107566
Selection*Food	6	18	0.84211	0.553924

Supp. Table 4. ANOVA on tolerance for development time.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	8.052838	0.006449
Food	1	3	1.407017	0.320936
Selection*Food	3	9	0.132010	0.938569

Supp. Table 5. ANOVA on effectiveness for development time.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	2.778	0.102499
Food	1	3	0.546	0.513442
Type	1	3	6.427	0.085042
Eye	1	3	103.760	0.002016
Sex	1	3	0.002	0.969169
Selection*Food	3	9	0.531	0.672565
Selection*Type	3	9	3.750	0.053606
Food*Type	1	3	3.025	0.180370
Selection*Eye	3	9	23.034	0.000147
Food*Eye	1	3	11.999	0.040522
Type*Eye	1	3	8.600	0.060877
Selection*Sex	3	9	3.142	0.079614
Food*Sex	1	3	0.373	0.584592
Type*Sex	1	3	2.027	0.249737
Eye*Sex	1	3	4.733	0.117847
Selection*Food*Type	3	9	0.053	0.982687
Selection*Food*Eye	3	9	0.105	0.954966
Selection*Type*Eye	3	9	10.701	0.002520
Food*Type*Eye	1	3	0.123	0.748556
Selection*Food*Sex	3	9	0.153	0.925020
Selection*Type*Sex	3	9	1.842	0.209904
Food*Type*Sex	1	3	17.001	0.025863
Selection*Eye*Sex	3	9	2.431	0.132202
Food*Eye*Sex	1	3	0.879	0.417537
Type*Eye*Sex	1	3	0.070	0.808865
Selection*Food*Type*Eye	3	9	0.377	0.771984
Selection*Food*Type*Sex	3	9	0.103	0.956520
Selection*Food*Eye*Sex	3	9	0.191	0.899642
Selection*Type*Eye*Sex	3	9	1.256	0.346357
Food*Type*Eye*Sex	1	3	2.703	0.198687
Selection*Food*Type*Eye*Sex	3	9	0.234	0.870290

Supp. Table 6. ANOVA on the raw development time data (without effectiveness and tolerance calculations).

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	6.535786	0.012243
Culture	2	6	1.851222	0.236489
Selection*Culture	6	18	4.024106	0.009896

Supp. Table 7. ANOVA on tolerance for dry biomass.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	20.98280	0.000212
Culture	2	6	41.31956	0.000310
Selection*Culture	6	18	1.13016	0.384548

Supp. Table 8. ANOVA on effectiveness for dry biomass.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	0.7619	0.543299
Culture	2	6	56.4226	0.000129
Culture Type	1	3	95.1140	0.002290
Time Period	2	6	19.5251	0.002362
Eye Colour	1	3	9.8346	0.051823
Selection*Culture	6	18	1.8671	0.142237
Selection*Culture Type	3	9	0.9895	0.440505
Culture*Culture Type	2	6	0.0926	0.912818
Selection*Time Period	6	18	6.3127	0.001038
Culture*Time Period	4	12	20.8850	0.000025
Culture Type*Time Period	2	6	27.5558	0.000946
Selection*Eye Colour	3	9	19.9492	0.000258
Culture*Eye Colour	2	6	0.3575	0.713340
Culture Type*Eye Colour	1	3	32.9023	0.010521
Time Period*Eye Colour	2	6	81.5255	0.000045
Selection*Culture*Culture Type	6	18	1.8061	0.154470
Selection*Culture*Time Period	12	36	4.9308	0.000093
Selection*Culture Type*Time Period	6	18	4.2259	0.007936
Culture*Culture Type*Time Period	4	12	0.6102	0.663168
Selection*Culture*Eye Colour	6	18	9.7010	0.000078
Selection*Culture Type*Eye Colour	3	9	10.9354	0.002340
Culture*Culture Type*Eye Colour	2	6	1.1565	0.376003
Selection*Time Period*Eye Colour	6	18	18.7577	0.000001
Culture*Time Period*Eye Colour	4	12	36.6022	0.000001
Culture Type*Time Period*Eye Colour	2	6	29.7227	0.000771
Selection*Culture*Culture Type*Time Period	12	36	1.9379	0.062373
Selection*Culture*Culture Type*Eye Colour	6	18	2.5536	0.057390
Selection*Culture*Time Period*Eye Colour	12	36	15.4230	0.000000
Selection*Culture Type*Time Period*Eye Colour	6	18	4.6180	0.005237
Culture*Culture Type*Time Period*Eye Colour	4	12	8.1356	0.002058
Selection*Culture*Culture Type*Time Period*Eye Colour	12	36	4.8455	0.000109

Supp. Table 9. ANOVA on the raw dry biomass data (without effectiveness and tolerance calculations).

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	1.14488	0.382530
Culture	2	6	7.10533	0.026165
Selection*Culture	6	18	1.76215	0.163953

Supp. Table 10. ANOVA on tolerance for dry weight per fly.

Effect	*df*	*df* Error	*F*	*p*
Selection	3	9	8.762882	0.004915
Culture	1	3	0.463798	0.544711
Selection*Culture	3	9	2.823193	0.099270

Supp. Table 11. ANOVA on effectiveness for dry weight per fly.

Exceptions for measurements

The following section marks occasions when eclosing flies could not be used for measurement of pre-adult survivorship, pre-adult development time, dry mass at eclosion or dry biomass.

The first section marks 'general' exceptions, which were prevalent throughout the culture vials.

The second section marks specific known exceptions, wherein the contents of the particular replicate were discarded due to some issue. In cases where the contents were not discarded, these issues represent some confounding error.

General Exceptions:

1. Some flies were crushed or drowned or escaped. Those were noted down. In case they could be sexed and the eye colour could be identified, they were used for survivorship and development time. None of these flies could be used for weight measurements.
2. Some flies (<5%) in CCU 2 resembled the OE phenotype even under mono-cultures. This was likely to be a preserved mutation in the CCU 2 population and unlikely to be a contamination since CCU flies are maintained under intense selection pressure, under which OE larvae are unlikely to survive.
3. Several pupae failed to eclose, or drowned.
4. Freezer switched off for 12 hours from 5-6 March. The temperature was generally cold inside at opening. All flies were tightly sealed in tubes, which were sealed inside two levels of Ziplock bags. Thus, there was unlikely to be significant loss of dry mass due to decomposition.
5. Some tube sets were left at room temperature for a few days (tightly sealed in centrifuge tubes, kept inside oven (switched off)). Thus, there was unlikely to be significant loss of dry mass to due to decomposition. This was unavoidable due to COVID-19-related quarantine procedures.

Specific Exceptions:

Block 1:

a) MB 1, 2 mL mono-cultures:
    - Vials 2 and 5 T2 flies exchanged on 22 to 23 Feb, 2020, reshuffled back with correct numbers. however, the vial identity was lost, thus they could not be used for biomass calculations.
    - Vials 1 and 4 same as above.
b) LCU 1, 2 mL mono-cultures:
    - Vials 2 and 3: 3 males, 1 female transferred to vial 2 from vial 3 on 24 Feb, 2020 (T2), this count was reshuffled back. These could not be used for biomass calculations.
c) MCU 1, 1.5 mL mono-cultures: T3, 27 Feb 2020, 3 male and 3 female of 2mL added to T3 tube.

Block 2:

a) MB 2 1mL duo-cultures 1: vial accidentally frozen on 10 April, 2020. Most eclosions were done. Most pupae had either eclosed or were dried. No further eclosions seen in this vial but also very few eclosions overall in duo-cultures after this.
b) CCU 2 2mL duo-culture vial 5: frozen on 18th April, 2020. Eclosions were more or less over at this point.
c) MB 1.5mL duo-culture vial 4: frozen on 14th April, 2020. Eclosions were more or less over by this point.
d) LCU 2 1.5mL duo-culture: 10th April, 2020 : vials 2, 3 and 4 reshuffled into each other.

Block 3:

a) OE 3 1.5mL, 2mL all vials: 18th march vials added to T2 (should have been T3)
b) MCU 3 2mL duo-cultures: vial 1 and vial 5 reshuffled in T1.

Block 4:

a) LCU 4 1mL mono-culture all vials: first (very few) flies eclosing from all vials were inadvertently discarded.
b) MCU 4 2mL duo-culture: Vial 2 and Vial 5 reshuffled on 6th Feb, 2020. Not used for biomass calculations.

Chapter III

Density-dependent selection in *Drosophila*: evolution of egg size and hatching time

NOTE

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Introduction

Populations adapted to high density conditions are expected to evolve greater competitive ability, a prediction highlighted by the theory of density-dependent selection, first formulated by MacArthur (1962) and MacArthur and Wilson (1967) (see Mueller 1997, 2009 for reviews on subsequent developments in this area). Several rigorous long-term selection experiments on populations of *Drosophila* reared under high larval density conditions subsequently validated this prediction, showing the evolution of increased pre-adult competitive ability in the crowding adapted populations when compared to their low density controls (Mueller 1988; Nagarajan *et al.* 2016; Sarangi *et al.* 2016). However, the traits that evolved as correlates of the increased pre-adult competitive ability differed widely across the studies (Nagarajan *et al.* 2016; Sarangi *et al.* 2016; Sarangi 2018).

The first of these experiments was done using two sets of replicate populations: the *K*-populations, maintained at high population density (larval and adult) by serial transfer, and the *r*-populations, maintained at low population density by culling (Mueller and Ayala 1981). Compared to the *r*-populations, the *K*-populations evolved greater larval competitive ability (Mueller 1988), increased larval feeding rate (Joshi and Mueller 1988), greater pupation height (Mueller and Sweet 1986; Joshi and Mueller 1993), greater larval foraging path length (Sokolowski *et al.* 1997), increased adult dry weight and pre-adult viability at high density (Bierbaum *et al.* 1989), and increased minimum larval food requirement for completion of development (Mueller 1990).

The next selection study sought to validate the results from the *r*- and *K*-populations, as the earlier selection regime confounded the effects of larval and adult crowding. Moreover, the *r*-populations were maintained on discrete generations, whereas the *K*-populations were maintained on overlapping generations (Mueller *et al.* 1993). Consequently, populations of *D.*

melanogaster, originally derived from a different geographical region than the ancestors of the *r*- and *K*-populations, were used in a selection experiment that differentiated the effects of larval and adult crowding, and in which all selected populations and controls were maintained on a three-week discrete generation cycle (Mueller *et al.* 1993). The populations reared at high larval, but not adult, density were called the CU (Crowded as larvae, Uncrowded as adults), and the low density controls were called UU (Uncrowded as larvae, Uncrowded as adults) (Mueller *et al.* 1993). Similar to what was seen earlier in the *K*-populations, the CU populations evolved increased larval feeding rate and minimum larval food requirement for completion of development (Joshi and Mueller 1996), and larval foraging path length (Sokolowski *et al.* 1997). Moreover, the CU populations evolved increased pre-adult urea tolerance (Shiotsugu *et al.* 1997; Borash *et al.* 1998) and ammonia tolerance (Borash *et al.* 1998). The CU populations, however, did not evolve increased pupation height than controls (Joshi and Mueller 1996), unlike the *K*-populations; possible explanations are discussed by Joshi *et al*. (2003).

The broadly consistent results from the *r*- and *K*-populations and the CU and UU populations, together with similar results from the *rK* and *r*×*rK* populations (Guo *et al*. 1991), resulted in the canonical model for adaptation to larval crowding in *D*. *melanogaster* populations: these populations would exhibit increased pre-adult competitive ability and larval feeding rate, foraging path length, and tolerance to ammonia and urea, but would show reduced food to biomass conversion efficiency as a trade-off (Mueller 1997; Joshi *et al.* 2001; Prasad and Joshi 2003; Mueller *et al.* 2005; Mueller 2009; Mueller and Cabral 2012; Mueller and Barter 2015; Bitner *et al*. 2021). The canonical model was strengthened by observations in *D*. *melanogaster* of greater pre-adult competitive ability in populations selected for increased larval feeding rate (Burnet *et al*. 1977), and the evolution of reduced pre-adult competitive ability in populations that evolved reduced larval feeding rate due to selection for either rapid pre-adult development

(Prasad *et al*. 2001; Shakarad *et al*. 2005; Rajamani *et al*. 2006) or for increased parasitoid resistance (Fellowes *et al*. 1998, 1999).

The canonical model was, nevertheless, challenged later by three selection studies involving adaptation to larval crowding, in *D*. *ananassae*, *D*. *nasuta nasuta* and *D*. *melanogaster*, respectively (Nagarajan *et al.* 2016; Sarangi *et al*. 2016). In all three studies, crowding adapted populations did evolve greater larval competitive ability compared to their respective low density controls, but did so through a suite of traits different from the canonical model. No evolution of increased feeding rate was seen, nor were there any changes in urea tolerance, compared to controls. Instead, the crowding-adapted populations seemed to evolve greater larval competitive ability primarily through a decrease in pre-adult development time, expressed even when assayed at low density, and an increase in the time efficiency of food to biomass conversion, relative to controls (Nagarajan *et al.* 2016; Sarangi *et al*. 2016). It then became apparent that the major difference between these studies and the earlier work that had given rise to the canonical model was in the ecological details of the context in which larvae in selected populations experienced crowding (Sarangi 2018). Specifically, the populations used by Nagarajan *et al*. (2016) and Sarangi *et al*. (2016) had very low amounts of food per vial, whereas the earlier studies had used larger amounts of food and a greater number of eggs. For example, the MCU populations of Sarangi *et al*. (2016) were maintained at a density of about 600 eggs per vial containing 1.5 mL food whereas the CU populations (Mueller *et al*. 1993) were reared in vials containing about 1500 eggs in 6-7 mL of food. Subsequently, altering the amount of food and number of eggs while keeping overall eggs per unit food density the same was shown to affect pre-adult survivorship and development time, as well as the weight distribution of eclosing flies (Sarangi 2018). Therefore, in order to examine this phenomenon further, two new sets of *D*. *melanogaster* populations were subjected to selection for adaptation to larval crowding. One set of four populations, called LCU, was maintained at around 1200

eggs in 6 mL food, and this regime was meant to approximate the CU populations of Mueller *et al*. (1993). The other set of four populations, called the CCU, and was maintained at twice the number of eggs and twice the volume of food, and thus an identical overall density, as the MCU populations (Sarangi 2018). Thus, a system of 16 populations was created: ancestral controls (MB), MCU, CCU and LCU, with four replicate populations in each regime (Sarangi 2018).

Interestingly, although the LCU and CCU populations did evolve greater pre-adult competitive ability compared to the MB controls (Sarangi 2018; Chapter 2), they did so via an increased larval feeding rate, unlike the MCU populations (Sarangi 2018). However, as in the MCU populations, no evolution of pre-adult urea or ammonia tolerance was seen in the CCU and LCU populations (Sarangi 2018). The overall picture that emerges is, thus, one of 'unity in ends, diversity in means', with even populations experiencing identical larval density in slightly different ecological contexts exhibiting the evolution of increased pre-adult competitive ability with or without a concomitant increase in larval feeding rate (Sarangi 2018).

Here, we show that there is nevertheless a commonality in evolutionary trajectories across the MCU, CCU and LCU populations in that they all seem to have evolved a shorter egg hatching time and a greater egg size than the MB controls. These traits may be important for larval competitive ability, as together they can effectively provide a temporal head-start and initial size advantage in competition. A shorter egg hatching time may be important for larval competitive ability as it can provide a temporal head start in competition (Bakker 1961). Earlier hatching larvae will likely encounter lower density conditions early on, before the density increases due to the presence of later hatching larvae (Sokolowski *et al.* 1997). Experiments providing a few hours head start to larvae with poor competitive ability showed an enhancement in the ability of those larvae to compete against otherwise superior competitors (Bakker 1961, 69; Chapter 4). A similar example comes from the colonising of *Morinda* fruit

by larvae of various species of *Drosophila*. A species with poorer larval competitive ability, *D. sechellia*, may be able to successfully colonise the *Morinda* fruit in its earliest, toxin-rich phases of rotting, likely due to its vastly shorter egg hatching time compared to other competitors, as well as its greater tolerance to the toxins found in the early phase of the fruit (Mueller and Bitner 2015). A day's head start to *D. sechellia* larvae would be expected to allow them to grow to sufficient size to survive in the presence of the later arriving, superior competitors of other *Drosophila* species (Mueller and Bitner 2015). A greater egg size would also be expected to provide an initial size advantage in larval competition. This advantage, however small, may give a proportional weight advantage at the end of growth to a larva against otherwise equal competitors (Bakker 1961).

Materials and methods

Experimental populations

We used four sets of long-term laboratory populations of *D. melanogaster*, with each set consisting of four replicate populations, as briefly described below. The derivation and maintenance of all these populations have been discussed in detail by Sarangi (2018).

MB 1-4: These are four low density reared populations that serve as ancestral controls to the three sets of crowding-adapted populations. They are maintained at a relatively low density of approx. 70 eggs in 6 mL of cornmeal-sugar-yeast medium, in cylindrical Borosilicate glass vials of 2.2-2.4 cm inner diameter and 9.5 cm height.

MCU 1-4: These populations experience larval crowding at ~600 eggs in ~1.5 mL of cornmeal medium, in the same type of vials as MBs. At the time of assaying, the MCUs had undergone at least 218 generations of selection (replicate populations 1, 2 assayed at gen. 218; replicate populations 3, 4 assayed at gen. 219).

CCU 1-4: These populations experience larval crowding at ~1200 eggs in ~3 mL of cornmeal medium, in the same type of vials as MBs. It should be noted that MCU and CCU have the exact same overall eggs/food density. At the time of assaying, the CCUs had undergone at least 97 generations of selection (replicate populations 1, 2 assayed at gen. 97; replicate populations 3, 4 assayed at gen. 98).

LCU 1-4: These populations experience larval crowding at ~1200 eggs in ~6 mL of cornmeal medium, in Borosilicate glass vials of ~2 cm inner diameter and ~9 cm height (approx. 6-dram volume, to mimic the CU populations of Mueller *et al*. (1993)). At the time of assaying, the LCUs had undergone at least 96 generations of selection (replicate populations 1, 2 assayed at gen. 96; replicate populations 3, 4 assayed at gen. 97).

While the pre-adult stages of each population are maintained in vials, the adults are transferred to Plexiglas cages ($25 \times 20 \times 15$ cm^3) on the day of eclosion. Given the low larval density of MB populations, they are transferred to cages on the 11^{th} day from egg collection. In the crowding-adapted populations, there is a large amount of variation in eclosion time and, therefore, transfer of eclosing adults to cages is done daily from day 8 to day 21 from egg collection. Fresh cornmeal food plates are given (following a fresh plate given on initiation of transfers) on day 10, 12, 14 and 17 from egg collection. On day 18 from egg collection, the flies in the cages are provided a food plate with a generous smear of a paste of live yeast mixed with water and a few drops of glacial acetic acid. On day 20 from egg collection, the flies are provided cornmeal food plates with vertical edges present for egg laying, for 18 hours. Finally, eggs laid by the flies on these plates are used to initiate the next generation, with eggs being transferred to fresh vials containing the respective food volume assigned to each population. All populations are maintained under constant light, at 25 ± 1°C and 70-90% relative humidity.

Standardisation of populations

Prior to assays, all populations were subjected to one generation of standardisation (rearing in a common low larval density environment), to eliminate any non-genetic parental effects. Eggs from each population were collected at approx. 70 eggs in 6 mL of food per vial, for a total of 40 vials per population. The flies eclosing in these vials were transferred to cages on day 11 from egg collection, following which they were provided a food plate, with a generous smear of the live yeast-water-acetic acid paste, for approx. 48 hours. On day 13 from egg collection, the flies were provided a food plate for egg collection for around 18 hours, and two rearing environments for the assay were set up on day 14 from egg collection. All assays were conducted in constant light, at 25 ± 1°C and 70-90% relative humidity.

Rearing environments

For each population, the eggs collected from the previous standardised generation were used to form two sets of assay populations, reared at two larval densities.

***Low density rearing*:** The first set was kept at a relatively low eggs/food density of ~70 eggs in 6 mL cornmeal medium per vial, with a total of 40 vials per replicate population. As in the standardisation, the adults eclosing in the vials were transferred to a cage on day 11 from egg collection. On day 17 from egg collection, the flies were provided a food plate with a generous smear of live yeast paste for ~48 hours. Following this, a "dummy" egg collection cornmeal plate was provided for an hour, which was for the laying of any eggs previously incubating inside the females. Relatively synchronized eggs for the hatching time and egg size assays were then obtained by providing a harder plate with double the usual agar and different composition (only yeast, sugar added), for 45 minutes. This composition ensured easier egg removal for counting.

***High-density rearing*:** Eggs for the second set were collected into vials at a relatively high eggs/food density – approx. 300 eggs in 2 mL cornmeal medium per vial, with a total of 12 vials per population. This simple density change was done as a first pass to obtain reduced adult size without impacting survivorship greatly. Unlike in the low density rearing conditions, adults emerging from the vials were transferred to cages daily from the day of the start until the end of eclosion, usually day 15-16. The protocol from day 17 onwards was the same as in the low density reared populations.

Egg hatching time

The assay was carried out in plastic Petri plates (90 mm diameter × 14 mm height), in which a thin layer of 12 g/L agar solution (containing 2.4 g/L methyl 4-hydroxybenzoate, as preservative) was spread. A 6 × 6 square grid (36 square cells, each having 3 mm sides) was pasted on the bottom of each Petri plate, which was visible through the transparent layer of agar. A total of 5 Petri plates were used per selection × rearing density × block combination, with 36 eggs per Petri plate – one egg per cell of the grid (Figure 1). Observations for egg hatching were done at 13, 15, 17, 18, 19, 20, 21, 22, 24, 26, 28 and 30 hours from egg laying, respectively. At each time-point, the number of eggs that had hatched in the time interval between the current and previous observation was noted.

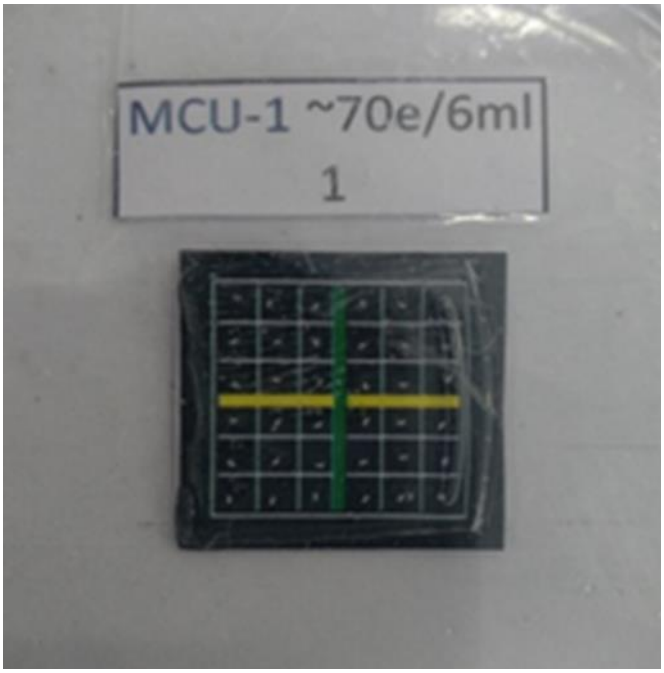

Figure 1: Apparatus for egg hatching time and hatchability measurements. The 6 × 6 grid is pasted on the bottom of a Petri plate containing a thin layer of agar solution. Each cell of the grid contains an egg, as can be seen in the image. The label denotes the selection × rearing density × block combination used, along with the replicate plate number ('1' in this case).

Egg hatchability

From the hatching time assay, we also recorded how many eggs hatched within 48 hours from egg laying. The egg hatchability was calculated as the number of eggs hatched divided by the total number of eggs. Earlier hatchability experiments on populations with relatively close ancestry to our MB populations did not use clear eggs due to their infertility (Chippindale *et al.* 1997). However, we have found that some clear eggs in our populations can lead to viable adults (S. Venkitachalam, *pers*. *obs*.), and thus we used all but the visibly damaged eggs for our experiments.

Egg length and width

For size measurements, a total of 30 eggs (obtained as 10 eggs each in 3 replicates) were measured per selection × rearing density × block combination. The eggs were placed on a Neubauer haemocytometer and photographed under a stereo-microscope. The parallel lines on the haemocytometer, which were a known distance apart (200 µm or 250 µm, depending on the set of lines used; see Figure 2) provided a scale with which to measure the eggs. Egg length (estimate of polar axis) and egg width (estimate of minor axis) were measured from the photographs (Figure 2) using ImageJ (Rasband 1997-2018).

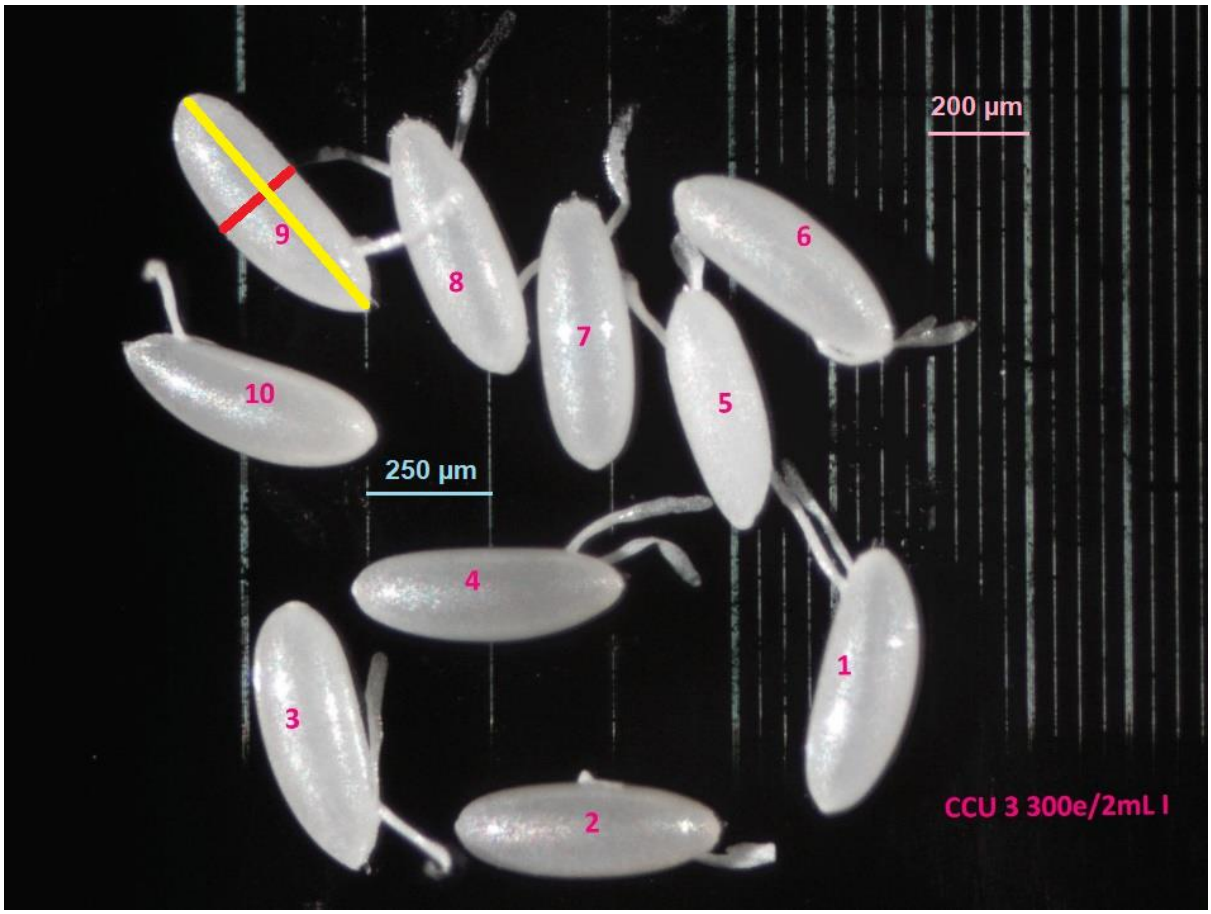

Figure 2: Egg size measurement setup for a replicate containing 10 eggs. There were three such replicates for each selection × rearing density × block combination. Eggs were numbered from 1 through 10. The egg labelled '9' has two lines of measurement drawn for demonstration: the yellow line denotes egg length and the red line, egg width. The background is that of a Neubauer haemocytometer, which

contains parallel lines set a known distance apart, and can thus be used to determine the scale in the image (parallel lines set either 250 µm or 200 µm apart could be used, as marked in the figure).

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, CCU 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. Assays were conducted concurrently on all populations of a block. The data were subjected to a mixed model ANOVA (type III) in a fully factorial design, with block (4 levels) treated as a random factor. Selection (4 levels) and rearing density (2 levels) were treated as fixed factors. For hatchability, the analysis was repeated after performing an arcsine square root transformation on the data, to check for any differences in the statistical significance of the fixed factors. All ANOVAs were done using STATISTICA™ Windows release 5.0 (Statsoft 1995). Tukey's HSD was used for post-hoc pairwise comparisons at α = 0.05. The image measurements for egg size were done using ImageJ (Rasband 1997-2018). Pearson's product-moment correlation coefficients were calculated pairwise for population means of egg hatching time, egg length and egg width.

Results

Egg hatching time

Mean egg hatching time across all four types of selected and control populations was close to 20 hours, and the range of variation among means was only about 30 min (Figure 3). However, all three sets of selected populations had shorter mean hatching times than the MB controls,

the shortest being in the LCU populations, followed by CCU, and then by MCU (Figure 3). The ANOVA revealed a significant main effect of selection ($F_{3,9}$ = 4.142, P = 0.042) on egg hatching time, but post-hoc pairwise comparisons showed a significant difference only between LCU and MB (Figure 3). There neither a significant main effect of rearing density ($F_{1,3}$ = 0.631, P = 0.485), nor a significant selection × rearing density interaction ($F_{3,9}$ = 1.871, P = 0.205).

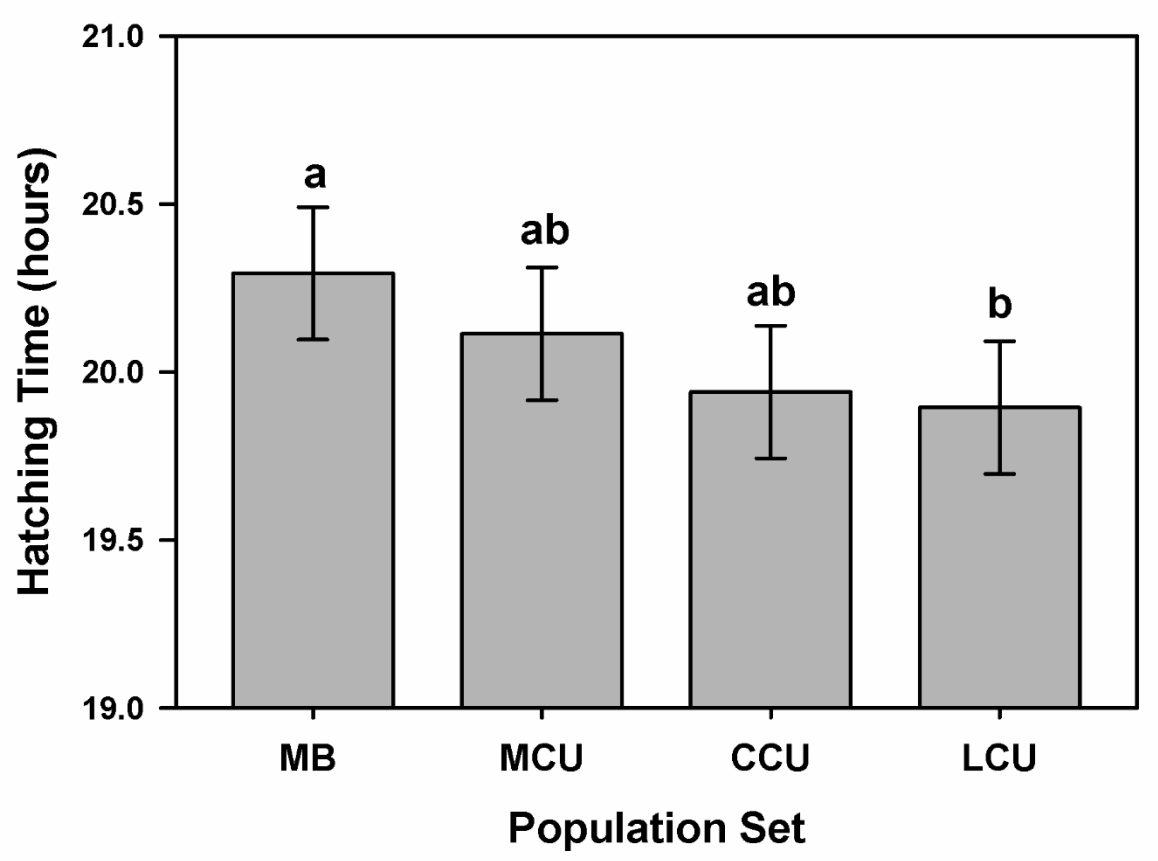

Figure 3: Mean egg hatching time in hours for the four levels of selection, averaged over all levels of rearing density and block. The error bars show 95% confidence intervals, calculated from post-hoc Tukey's HSD, and allow for visual hypothesis testing – identical superscript letters denote means that did not differ significantly, whereas different letters denote means that differed significantly. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Egg hatchability

Mean egg hatchability ranged from about 75-90% across selection × rearing density combinations, with flies reared as larvae at high density (300 eggs in 2 mL food) tending to lay more viable eggs than those reared at low density (70 eggs in 6 mL food), most markedly so in the MCU populations (Figure 4). The ANOVA revealed no significant main effect of selection ($F_{3,9} = 1.067$, $P = 0.410$). There was, however, a significant main effect of rearing density ($F_{1,3} = 12.484$, $P = 0.039$), as well as a significant selection × rearing density interaction ($F_{3,9} = 4.236$, $P = 0.040$). Post-hoc comparisons revealed that only MCU showed significantly higher hatchability when flies were reared as larvae at high versus low density. Similar but non-significant differences were also seen in the MB and LCU, whereas mean hatchability of CCU reared at low versus high larval density was very similar (Figure 4). The pattern of significant ANOVA effects was unaffected by whether untransformed or arcsine transformed data were used.

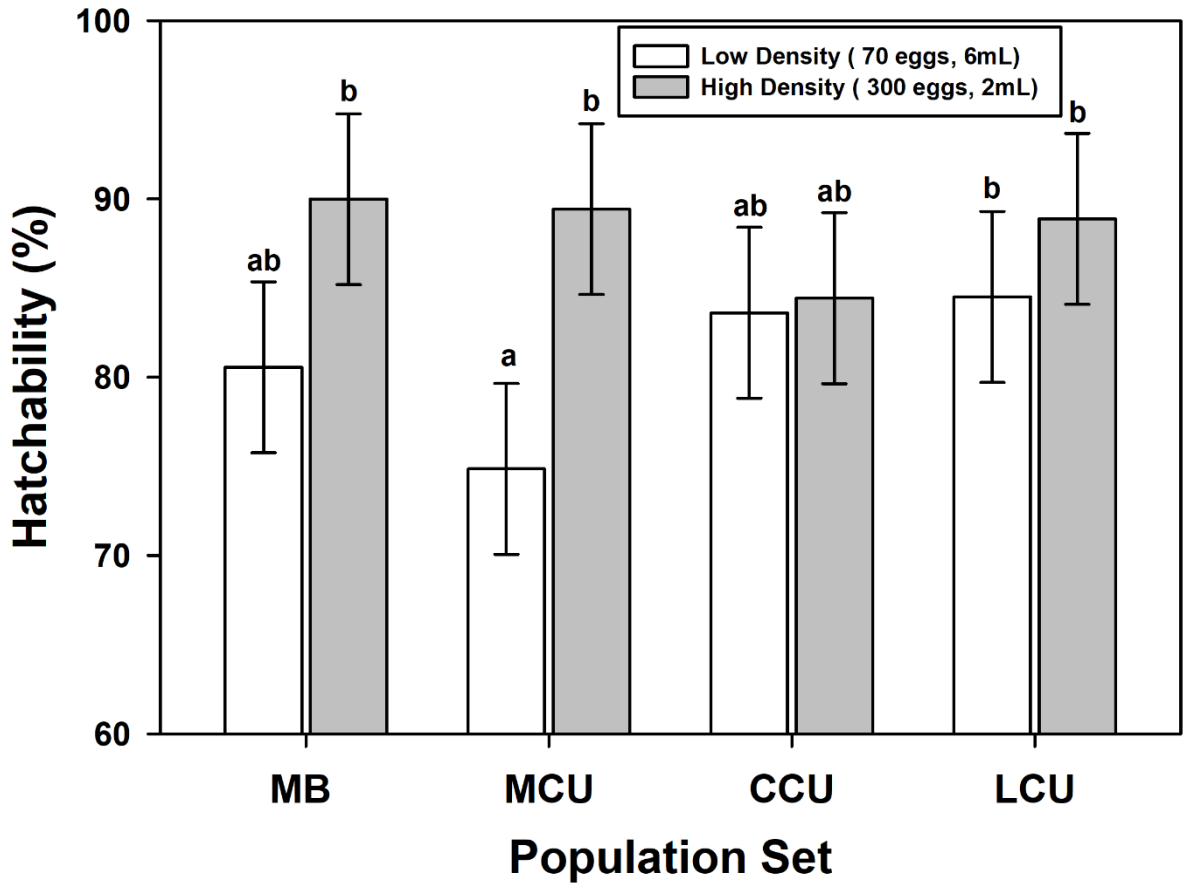

Figure 4: Mean hatchability (%), for all combinations of four levels of selection and two levels of rearing density, averaged across all blocks. The error bars show 95% confidence intervals, calculated from post-hoc Tukey's HSD, and allow for visual hypothesis testing – identical superscript letters denote means that did not differ significantly, whereas different letters denote means that differed significantly. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Egg length (µm)

Mean egg length in MB populations was significantly less than any of the sets of populations selected for larval crowding (Figure 5), driving a significant ANOVA main effect of selection ($F_{3,9}$ = 22.104, P < 0.001). Egg length, on an average, did not differ significantly between rearing densities (main effect of rearing density: $F_{1,3}$ = 8.109, P = 0.065; Figure 5). LCU eggs were longer than those of MCU across both rearing densities, but longer than CCU eggs only at high rearing density. On the other hand, MCU eggs were shorter than CCU eggs at low density, but of similar length at high density (Figure 5), and these rearing density-specific differences among various crowding adapted sets of populations drove a significant ANOVA selection × rearing density interaction ($F_{3,9}$ = 4.830, P = 0.029). This pattern of differences between CCU and the other crowding adapted populations was likely due to an average of 9 µm longer eggs laid by CCU females when reared at low as compared to high density, although this difference itself was not statistically significant (Figure 5).

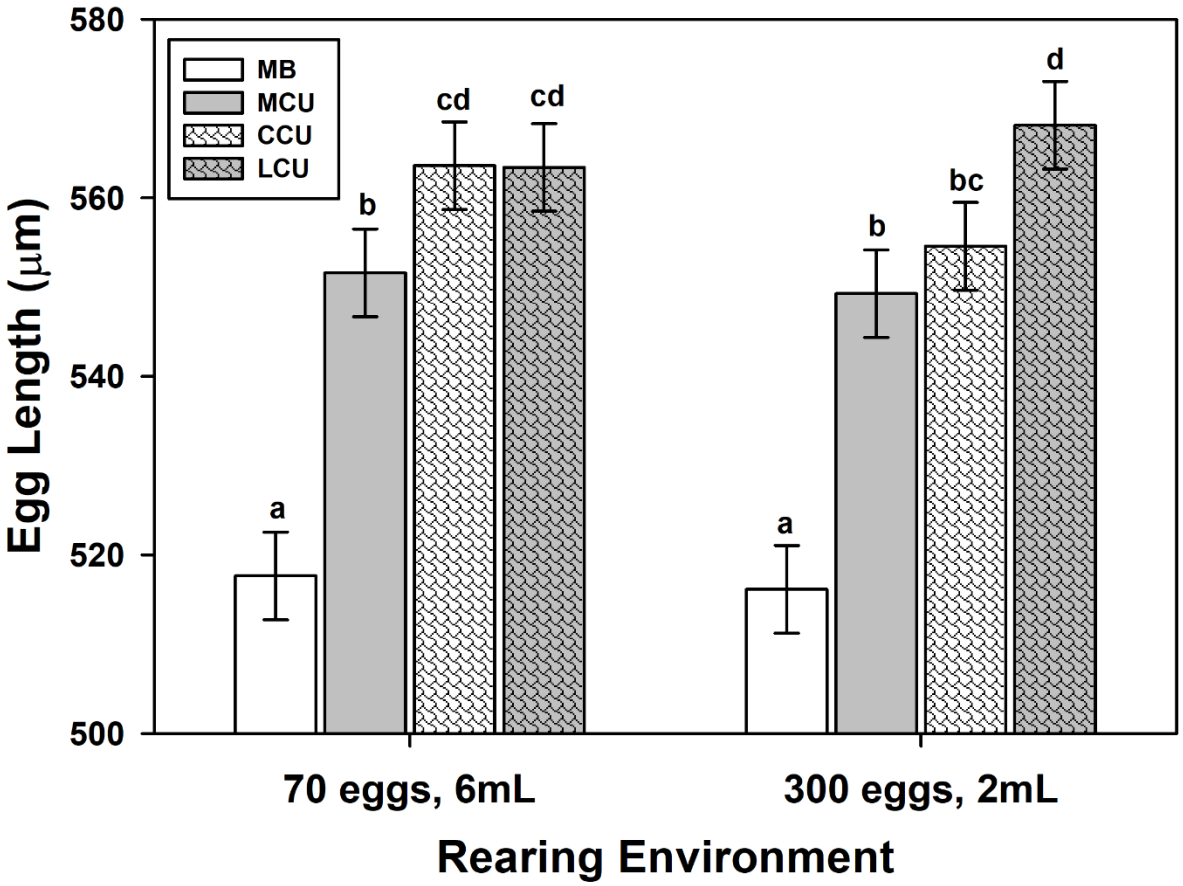

Figure 5: Mean egg length (μm), for all combinations of four levels of selection and two levels of rearing density, averaged across all blocks. The error bars show 95% confidence intervals, calculated from post-hoc Tukey's HSD, and allow for visual hypothesis testing – identical superscript letters denote means that did not differ significantly, whereas different letters denote means that differed significantly. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Egg width (µm)

Overall, the egg width data were fairly similar to those for egg length (Figures 5,6), with egg width being considerably lower in MB populations compared to all crowding adapted populations (ANOVA main effect of selection: $F_{3,9} = 5.496$, $P = 0.020$), and not differing, on an average, between rearing densities (main effect of rearing density: $F_{1,3} = 0.584$, $P = 0.500$) (Figure 6). Eggs laid by MCU, CCU and LCU flies reared at low density did not differ much in mean width, whereas at high rearing density LCU females laid the widest eggs, and the MCU and CCU did not significantly differ in egg width (selection × rearing density interaction: $F_{3,9} = 7.971$, $P = 0.007$; Figure 6).

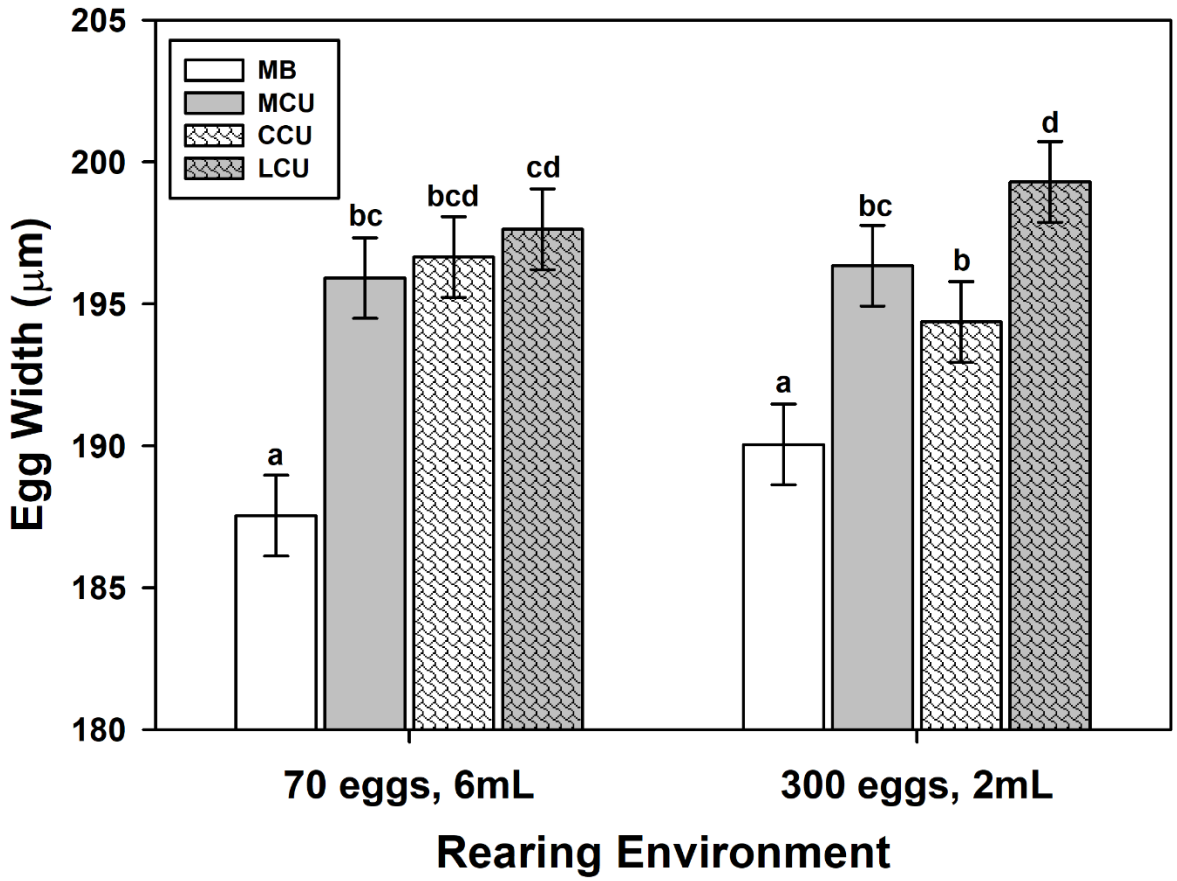

Figure 6: Mean egg width (μm), for all combinations of four levels of selection and two levels of rearing density, averaged across all blocks. The error bars show 95% confidence intervals, calculated from post-hoc Tukey's HSD, and allow for visual hypothesis testing – identical superscript letters denote means that did not differ significantly, whereas different letters denote means that differed significantly. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Trait correlations

There was a strong, positive correlation across population means between egg length and width (r = +0.771, P < 0.001; Figure 7), indicating that populations with longer eggs also tended to have wider eggs, and vice versa. The correlations for mean hatching time and mean egg length (r = -0.395, P = 0.025), and for mean hatching time and mean egg width (r = -0.548, P = 0.001) were both negative, although the strength of the correlation was moderate in both cases, being stronger for hatching time with egg width (Figure 7). There were no discernible patterns for within population correlations between egg length and egg width, with the mean correlation coefficient being around 0.11, and no selection × rearing density combination exceeding a correlation coefficient of 0.3 (data not shown).

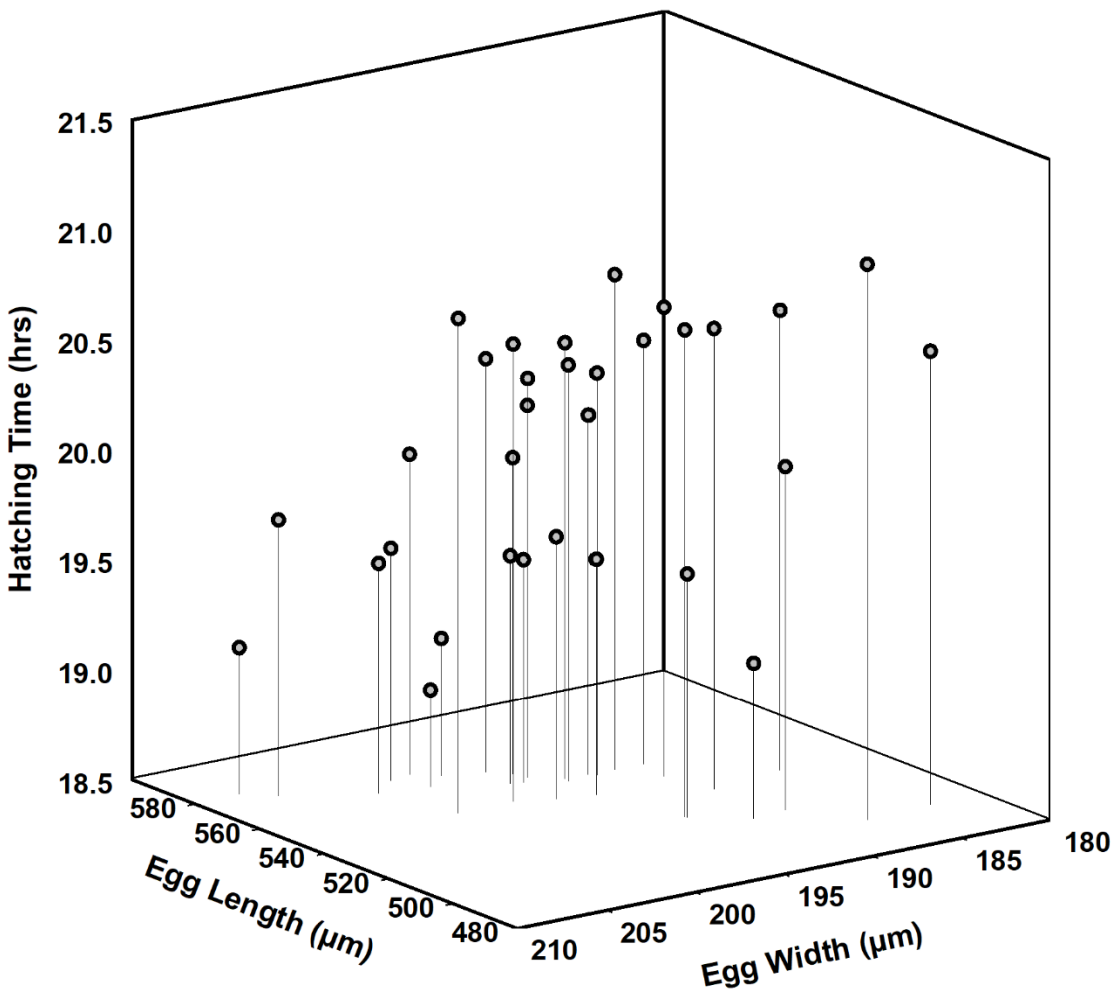

Figure 7: The relationship between mean egg length (µm), mean egg width (µm) and mean hatching time (hours) across the four sets of populations. Each data point represents the mean trait value for the three traits in one combination of selection × rearing density × block. Note the orientation of the x and y axes.

Discussion

Despite differences in the traits that underlie the evolution of greater pre-adult competitive ability in *Drosophila* populations experiencing larval crowding under slightly varying conditions (reviewed in Sarangi 2018), our results suggest one common adaptation across at least three such selection regimes covering a range of egg number and food amount combinations that more or less mimics the range of previous studies. Adults from the MCU, CCU and LCU populations laid eggs with greater length and width compared to the MB populations, when assayed at low (70 eggs in 6 mL food) or relatively high (300 eggs in 2 mL) density rearing conditions (Figures 5 and 6). Along with the strong positive correlation seen between the mean egg length and mean egg width across populations (Figure 7), these results indicate an increase in overall egg size of all these three sets of crowding adapted populations compared to the ancestral controls. Our results are also in agreement with an earlier study from a different laboratory, which demonstrated an increase in egg size, relative to controls, in crowding adapted populations derived from our MCU populations and maintained on a similar regime (Kumar 2014).

The eggs laid by LCU females were larger than those laid by MCU at both low- and high-density rearing conditions, with CCU eggs being intermediate in size (Figures 5 and 6). The differences among the MCU, CCU and LCU populations themselves are perhaps just as important as the consistent difference between the egg size of the crowding-adapted and MB populations. While previous comparisons of results from selection studies in differently crowded cultures have focused on the repeatability of qualitative differences found between a single set of crowding adapted populations against its controls (Joshi and Mueller 1996; Nagarajan *et al.* 2016; Sarangi *et al.* 2016), our study system permits more nuanced, quantitative comparisons between multiple types of high-density selection regimes.

The importance of plasticity in egg size has been studied extensively from the perspective of non-genetic maternal effects, in the contexts of both competition and malnutrition (Kawecki 1995; Azevedo et al. 1997; Prasad et al. 2003; Vijendravarma et al. 2010; Yanagi et al. 2013). In our study, egg size did not show any statistically significant difference between parents reared at low or high larval density (Figures 5,6). However, the CCU populations did show a consistent trend of smaller eggs when crowded.

These results indicate that the populations in our lab, control or crowding adapted, do not show any significant sensitivity to density with respect to egg size, at least up to the 'mild' crowding imposed in this experiment. It is likely that this trades off with fecundity, as seen in a recent study comparing MCU and LCU reared at 3 different densities, which showed that fecundity decreases when flies are reared at higher density (Pandey et al. 2022). An early study by Warren showed that flies made smaller through food limitations nevertheless laid eggs of the same size as optimally fed, larger flies (Warren 1924 Genetics).

Given the pattern of differences seen for the CCU populations compared to the rest, It is possible that either the relative scaling of egg size with female body size, or the sensitivity of female size to larval crowding, differs across the MCU, CCU and LCU populations.

It is also known that crowding more severe than what we used can further decrease body size (Sang 1949; Bakker 1961; Chapter 2), observed effects of rearing density on egg traits in these populations may change under more extreme crowding, when size at eclosion and pre-adult survivorship are more severely impacted than they were in this study.

Thus, future studies can aim to study the crowding adapted and control populations with respect to their egg size and fecundity across a large gradient of densities (or effective densities – see Chapter 5), which may shed light on the reaction norm of egg size with rearing density with

respect to larval crowding adaptation, and unravel the relationship between egg size and fecundity of adults from differently crowded cultures, respectively.

If we compare our egg size results with those obtained in a comparison of populations selected for rapid pre-adult development (FEJ) relative to their controls (JB), which are similar to the MB populations (first described in Prasad *et al*. 2000), there are some interesting similarities and differences. Although the MCU, CCU and LCU populations all have reduced pre-adult development time compared to MB controls (Sarangi 2018), the FEJ populations had undergone a far greater reduction in pre-adult development time, relative to their controls, as that was the primary trait under selection (Prasad et al. 2000; Prasad and Joshi 2003). On an average, eggs laid by FEJ females, after rearing at low density as larvae, were 3.8% longer, 7% wider, and 11% heavier than those of their controls (B.M. Prakash and A. Joshi, *unpubl. data*). The MCU, CCU and LCU populations in this study exhibited length increases of 6.5%, 8.2% and 9.5%, respectively, compared to the MB controls, and the corresponding width increases were 3.9%, 3.6% and 5.1%. From this comparison, we might conclude that MCU, CCU and LCU eggs are likely to be about 10-15% heavier than MB eggs. Interestingly, in the FEJ populations, the increase in width was greater than in length; it is just the opposite in the MCU, CCU and LCU populations. At this point, we cannot say why this may be so, although, given the very different selection pressures (rapid development vs. larval crowding), the mechanisms underlying the response could differ. The difference is unlikely to be explained by female size differences, since flies of FEJ, as well as MCU, CCU and LCU populations tend to be quite small relative to controls.

In the light of increased egg size and reduced hatching time having evolved in the crowding adapted populations, it is pertinent to inquire whether these populations also exhibit reduced fecundity as a tradeoff. We do not have data on fecundity for the MCU, LCU and CCU populations, but we do know that eclosing adults in these three sets of populations have reduced

dry weight compared to the MB controls (Sarangi 2018; Chapter 2). Derivatives of our MB and MCU populations are also studied in another lab and their data show that MCU females are slightly more fecund than MB females, but not significantly so (N.G. Prasad, *pers*. *comm*.). Thus, the MCU, CCU and LCU populations are likely to have evolved increased egg size and reduced hatching without a concomitant reduction in fecundity.

Although eggs from all crowding-adapted populations hatched faster than those of the controls, only the difference between mean egg hatching time between the LCU and MB populations was statistically significant (Figure 3). Moreover, the difference between LCU and MB mean egg hatching time was only ~30 minutes. However, given the egg size results (Figures 5,6), the pattern of MB > MCU > CCU > LCU for egg hatching time (Figure 3), and the negative correlation between mean egg length and mean hatching time, as well as between mean egg width and mean hatching time, we might expect crowding adapted populations that evolve increased egg size and decreased hatching time to benefit from a potent head-start in conditions of high pre-adult competition. Thus, we might expect LCU larvae to have a greater head-start in terms of pre-adult competition, compared to MCU larvae, and much greater still compared to MB larvae. This does not, however, necessarily imply that LCU larvae will have greater pre-adult competitive ability than MCU larvae, as differences in growth rates, efficiency and waste tolerance may also play a major role in determining pre-adult competitive ability (Bakker 1961; Joshi and Mueller 1996; Santos *et al.* 1997; Borash *et al.* 1998; Nagarajan *et al.* 2016; Sarangi *et al.* 2016).

The evolution of a greater potential head-start in the LCU populations could be driven by the fact that, compared to the MCU and CCU populations, the LCU larvae experience the highest density within the feeding band (the few mm deep zone below the food surface within which larvae feed), even though their overall eggs/food density is lower than that in the other two selection regimes.

Overall hatchability was lower in our study than usually observed in related populations (e.g. over 90% in Chippindale *et al.* (1994)). This might be attributed to reduced humidity due to the very thin layer of agar used by us – future experiments using a thicker agar layer or regular cornmeal food might alleviate the survivorship, if this explanation is correct. We also observed reduced hatchability of eggs laid by MCU flies reared under low density conditions (Figure 4). It is not clear if this is due to an increase in infertile or unviable eggs (e.g., Chippindale *et al.* 1994, 1997), and whether it is driven by some correlated response(s) to evolution under larval crowding for over 200 generations of selection in the MCU populations, much longer than their CCU and LCU counterparts.

In conclusion, our results highlight increased egg size as being a consistent evolutionary correlate of greater pre-adult competitive ability across three differently crowded selection regimes that otherwise differ in the traits they have evolved in response to chronic larval crowding. Moreover, adults from populations crowded with the lowest eggs/food density, but the highest feeding band density, laid the largest eggs with the fastest hatching times, thus potentially allowing for a substantial head-start in the context of pre-adult competition. The study system we describe allows the comparison of adaptations to different crowding scenarios, highlighting quantitative differences that may otherwise not be possible to see when comparing qualitative results between different long-term selection experiments.

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Chapter IV

The role of greater competitive ability in countering age disadvantages in larval competition in *Drosophila melanogaster*

NOTE

This chapter is a reproduction of a preprint on bioRxiv with the following citation:

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Introduction

Competition holds a central place in the theory of density-dependent selection (MacArthur, 1962; MacArthur and Wilson, 1967), which is one of the few interfaces between ecology and evolution (reviewed in Mueller, 1997, 2009). A fundamental prediction of this theory is that a population adapted to crowded conditions will evolve greater average competitive ability (Mueller, 1988a). We define competitive ability as the sum of traits relevant to the individual's performance in competition (Bakker, 1961): essentially, fitness under competitive conditions.

The traits contributing to competitive ability can be teased apart by studying laboratory populations adapted to high density conditions. Such long-term experiments would be expected to lead to the evolution of greater competitive ability over generations of selection. Over the past four decades, experimenters have used populations of *Drosophila* extensively for such selection experiments. The results from several selection experiments have explored the evolution of increased larval competitive ability and its contributing traits (reviewed in Mueller, 1997, 2009; Prasad and Joshi, 2003; Nagarajan *et al.*, 2016; Chapter 3).

However, over the decades, different selection experiments have shown very contrasting results regarding which traits underlie the greater competitive ability (Nagarajan *et al.*, 2016; Sarangi *et al.*, 2016; Sarangi, 2018). Due to this, we do not yet have a comprehensive understanding of the relative importance of different traits in the context of larval competition.

The early selection studies in the 1980s and 1990s did show some consistent results among themselves and led to a canonical understanding of which traits typically mediate the evolution of larval competitive ability in *Drosophila*. The first studies were done on *D. melanogaster* populations selected for high population densities at all life-stages (the *K*-populations), compared to their low population density controls (the *r*-populations) (Mueller and Ayala,

1981). The selection experiment met the predictions made by models of density-dependent selection. The *K*-populations evolved increased larval competitive ability compared to the *r*-populations (Mueller, 1988b). The *K*-population larvae also evolved increased larval feeding rate as well as increased larval foraging path length (Joshi and Mueller, 1988; Sokolowski *et al.*, 1997). Both these traits could be essential to larval competition. However, the *K*-populations showed reduced food to biomass efficiency (Mueller, 1990), which was a trait predicted to evolve under adaptation to high-density conditions (MacArthur and Wilson, 1967; Mueller, 1988a). This reduction gave the first indication that efficency of food acquisition versus utilization might trade off in optimising larval competitive ability: he *K*-population larvae were possibly sacrificing efficiency for a greater increase in feeding rate (Mueller, 1990, 1991).

This study was later repeated with greater rigour and gave rise to similar results once more (Guo *et al.*, 1991; Mueller *et al.*, 1991). Subsequently, another selection study sought to replicate these results using only larval crowding. The CU set of populations were maintained at high larval density, in contrast to their low larval density controls, named UU (Mueller *et al.*, 1993; Joshi and Mueller, 1996). Additionally, their geographical origin also differed from that of the *r*- and *K*-populations. Similar to the *K*-populations, the CU populations also evolved increased larval feeding rate and foraging path length, compared to the UU (Joshi and Mueller, 1996; Santos *et al.*, 1997; Sokolowski *et al.*, 1997), and they showed a reduction in food to biomass conversion efficiency as well (Joshi and Mueller, 1996). Additionally, the CU populations evolved increased larval tolerance to urea and ammonia (Shiotsugu *et al.*, 1997; Borash *et al.*, 1998) – both known to be components of larval metabolic waste, which could build up in crowded larval cultures (Botella *et al.*, 1985; Borash *et al.*, 1998).

Thus, three selection experiments yielded consistent patterns of adaptation to high larval density conditions, leading to the canonical view (Mueller, 1997, 2009; Joshi *et al.*, 2001; Prasad and Joshi, 2003; Mueller *et al.*, 2005; Mueller and Cabral, 2012; Mueller and Barter, 2015; Bitner *et al.*, 2021), which predicted that laboratory populations of *D. melanogaster* selected for adaptation to larval crowding would evolve increased larval competitive ability via:

a. Increased larval feeding rate.
b. Increased tolerance to metabolic waste products.
c. Increased larval foraging path length.

This adaptation would come at the cost of reduced food to biomass conversion efficiency. This view was reinforced by observations from other studies. *Drosophila* populations selected for increased larval feeding rate also evolved greater larval competitive ability (Burnet *et al.*, 1977). Another study demonstrated that *Drosophila* larvae evolved decreased competitive ability as well as decreased feeding rate as correlates of the evolution of increased parasitoid resistance (Fellowes *et al.*, 1998, 1999). Selection for rapid development, with or without concurrent selection for early reproduction, in populations descended from UU also led to reduced larval competitive ability along with a reduction in larval feeding rate (Prasad *et al.*, 2001; Shakarad *et al.*, 2005; Rajamani *et al.*, 2006).

This canonical view was subsequently challenged by three selection studies. The first two selection studies involved selection for adaptation to larval crowding in populations of *D. ananassae* and *D. nasuta nasuta*, respectively (Nagarajan *et al.*, 2016). The third study, of which the current study is a continuation, involved *D. melanogaster* populations named MCU (Sarangi *et al.*, 2016). These were descended from the UU populations. All three studies involved selection for larval crowding at slightly different egg and food combinations

compared to the *K*-populations or the CU populations. The results from all these three experiments were inconsistent with the canonical view, but consonant with one another (Nagarajan *et al.*, 2016; Sarangi *et al.*, 2016). The larval-crowding-adapted populations evolved greater larval competitive ability via a very different set of traits than those seen to evolve in the earlier studies. Compared to their low-density controls, they evolved reduced pre-adult development time and increased time efficiency of food to biomass conversion. They did not evolve greater larval feeding rate. Nor did they evolve greater urea tolerance.

It was clear that exactly how the crowding was imposed could affect the evolutionary trajectory of a population experiencing chronic larval crowding (Sarangi, 2018). More recently, our research group has carried out a long-term selection experiment with three different sets of larval-crowding-adapted populations (Sarangi, 2018). Each set is adapted to different crowding regimes. The details of these populations are as follows:

a) MCU – reared at high larval densities similar to those of *D. ananassae* and *D. n. nasuta* (Nagarajan *et al.*, 2016).
b) CCU – reared at the same larval density and vial dimensions as the MCU, but with double the eggs and food amount.
c) LCU – reared at high larval densities similar to the CU populations (Joshi and Mueller, 1996), in vials with narrower dimensions than MCU (see methods).

All three sets of crowding-adapted populations evolved greater larval competitive ability compared to their low-density controls, called MB (Sarangi, 2018; Chapter 2). When compared with MB populations, the LCU and CCU populations, but not the MCU populations, showed an increase in larval feeding rate (Sarangi, 2018). No evolution of urea or ammonia tolerance was seen in these populations (Sarangi, 2018).

We have recently shown that evolution of larger egg size is a consistent correlate of increased larval competitive ability in these populations (Chapter 3). Although all three crowding adapted population sets evolved increased egg size, LCU had greater egg length and egg width than the other two. Only LCU populations evolved a significantly shorter egg hatching time than the low-density controls, with the MCU and CCU populations showing a hatching time shorter than MB, but not significantly so (Chapter 3).

A shorter hatching time and greater egg size would likely confer increased competitive ability mainly through providing an initial advantage, or a head start, in the context of larval competition (Bakker, 1961, 1969; Mueller and Bitner, 2015). Thus, we can expect the larvae of the LCU populations to show the most potent head starts in competition. Even CCU and MCU can be expected to show greater larval head starts compared to the control MB populations.

In the current study, we have asked if these differences in egg size and hatching time are important for countering a head start imposed *against* the larvae of each of these populations. For this purpose, we have provided a common marked competitor population various durations of head start in competition against each focal population (MB, MCU, CCU, or LCU). This equates to an age disadvantage imposed on the respective focal populations. In the absence of artificially provided advantages, the common competitor is of roughly similar competitive ability as the low-density controls MB (Sarangi *et al.*, 2016; Sarangi, 2018; Chapter 2).

We assessed the effects of providing competitive disadvantage in the following two ways:

a) To what extent did the magnitude of the age disadvantage affect the outcomes of competition for each focal population?
b) How much did each focal population affect the competitive outcomes of the head-start-receiving common competitor?

We made the following predictions –

a) *All crowding adapted populations would be less affected by a competitive disadvantage than the low-density controls (MB).*
    - This would be expected, given the evolution of greater larval competitive ability and head start mechanisms in MCU, CCU and LCU.

b) *The common competitor would also gain the most competitive performance when given a head start against MB.*
    - Following from prediction 1, we can predict that the crowding-adapted populations cover up the growth lag soon and challenge the unabated growth of the common competitor. We do not expect to see this for MB populations.

c) *The populations which evolved the largest eggs and the fastest hatching time (LCU) would be least affected by the age disadvantage.*
    - Within the crowding-adapted populations, greater head start mechanisms should be most important for closing the initial time gap between the larvae of the focal population and the common competitor.

d) *The common competitor would gain the least competitive performance when given a head start against LCU.*
    - From prediction 3, LCU larvae should be at the lowest time disadvantage at emergence. Thus, we expect that they can start feeding and growing sooner, thereby challenging the growth of the common competitor before MCU or CCU.

We measured pre-adult development time as the indicator of competitive performance. It can be more sensitive to competition in *Drosophila* than pre-adult survivorship (Sang, 1949a; Ohba 1961 as cited by González-Candelas *et al.*; 1990; González-Candelas *et al.*, 1990; Chapter 2).

Methods

Populations used

We used three sets of populations undergoing selection for adaptation to larval crowding, experienced at different combinations of egg number and food amount (MCU, CCU and LCU). We also used one set of control populations (MB), undergoing maintenance at relatively low larval densities, and ancestral to all the selected populations. A population (OE) carrying an eye colour mutation was used as a common competitor.

Each set (MB, MCU, CCU and LCU) contains four replicate populations, ancestrally linked by replicate subscript. The replicate *i* of each set of selected populations is derived from the population MB-*i*, allowing us to treat replicate populations as randomised blocks in our analyses.

The maintenance regime of each population set is as follows:

a) **MB 1-4:** low density ancestral controls. Reared at ~70 eggs, ~6 mL cornmeal-sugar-yeast food medium, in cylindrical borosilicate glass vials of 2.2-2.4 cm inner diameter and 9.5 cm height.
b) **MCU 1-4:** larval crowding with lowest food amount. Reared at ~600 eggs, ~1.5 mL food. Same vial and food type as MB. At the time of the current study, they had undergone 218-219 generations of selection.
c) **CCU 1-4:** same density as MCU, but egg number and food amount doubled. Same vial and food type as MB. Reared at ~1200 eggs, ~3 mL food. At the time of the current study, they had undergone 97-98 generations of selection.
d) **LCU 1-4:** larval crowding with the highest food amount, meant to mimic the larval crowding protocol of the earlier used CU populations (Mueller *et al.*, 1993). Reared in ~1200 eggs, ~6mL food, in borosilicate glass vials of ~2 cm inner diameter and ~9 cm

height (approx. 6-dram volume). Same food type as MB. At the time of the current study, they had undergone 96-97 generations of selection.

Common competitor: OE (Orange Eye) is a population of *D. melanogaster* with an eye colour mutation. The mutation arose spontaneously in one of our populations ancestral to an MB population (Sarangi, 2018). We used it as a marker in the competition experiment of the current study. We rear this population at a similar egg number, food volume and vial dimensions as the MB populations.

The ancestry and maintenance of all these populations have been described in detail earlier (Sarangi, 2018; Chapter 3). Figure 1 shows a summarised diagram of the maintenance regime for each population set.

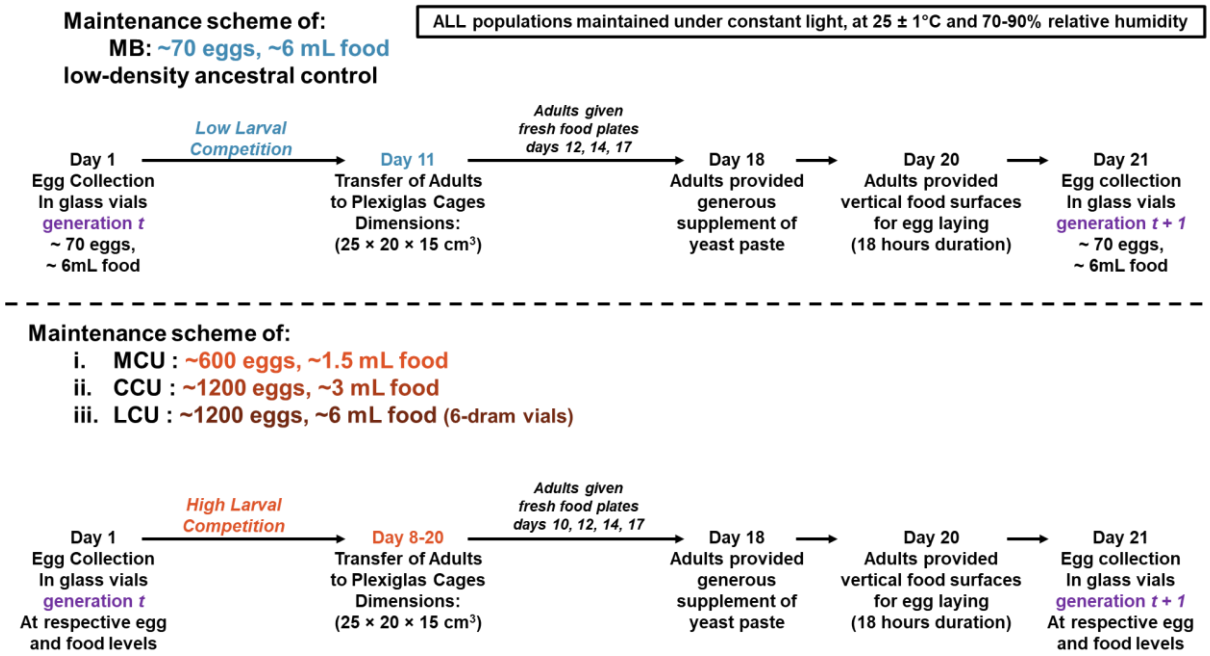

Figure 1: Maintenance schematic for all the populations used in the current study. In cultures with larval crowding, there is increased variation in pre-adult development time. Thus, the transfer of adults in MCU, CCU and LCU happens daily over multiple days during which adults are eclosing.

Two generations of common background rearing

Prior to assaying pre-adult competitive ability, we standardized each population to remove non-genetic parental effects. This was done by rearing all populations in a low-density environment for two generations. At both standardization generations, we collected ~70 eggs in ~6 mL of food for each population, in MB-type vial dimensions. We kept a total of 40 such vials per population.

On day 11 from egg collection, we transferred the eclosed adults to Plexiglas cages. In these cages, we provided the adults with food plates smeared generously with a paste of yeast with water and a few drops of glacial acetic acid.

In the first generation of standardization, we provided a food plate for egg laying on the 13th day from egg collection. On day 14, we collected eggs for the second standardisation generation.

We maintained the same protocol for generation 2 of standardisation as in generation 1 until day 13. On day 13, we first gave a regular egg-laying food plate to the adults for 1 hour, and discarded any eggs laid on it. This ensured that eggs laid from that point on would be relatively synchronized. After this step, we started the competition assay. Replicate populations 1, 3 of each set followed this schedule. For replicate populations 2 and 4 of each set, we started the experiment on day 16 due to logistical reasons, with the timing of yeast plate introduction also appropriately delayed.

Assay protocol

After the 1 h egg laying window described above, we gave another set of food plates to the adults for egg laying. These were harder agar plates, made of 2.4 g/L bacteriological agar along with yeast and sugar. The harder food surface facilitated easy removal of eggs. Once removed

from the plate, we placed the eggs onto a 1 g/L transparent agar plate. We counted these eggs using soft synthetic brushes and placed them in respective vials for the assay.

The assay involved imposing larval competition in high density duo-typic cultures, i.e., focal population (one of MB, MCU, CCU or LCU) + OE, with various larval age disadvantages to the focal populations (MB, MCU, CCU or LCU). This was achieved by giving larvae of the common competitor (OE) various durations of head start (figure 2). Briefly, from the standardized populations, 200 eggs of OE were added to 2 mL cornmeal food. These eggs were added 0, 3, 5, or 7 hours before 200 eggs of a focal population (MB, MCU, LCU or CCU). Each culture thus amounted to 400 eggs in 2 mL of food. For each combination of replicate population and head start duration, we used 5 replicate vials.

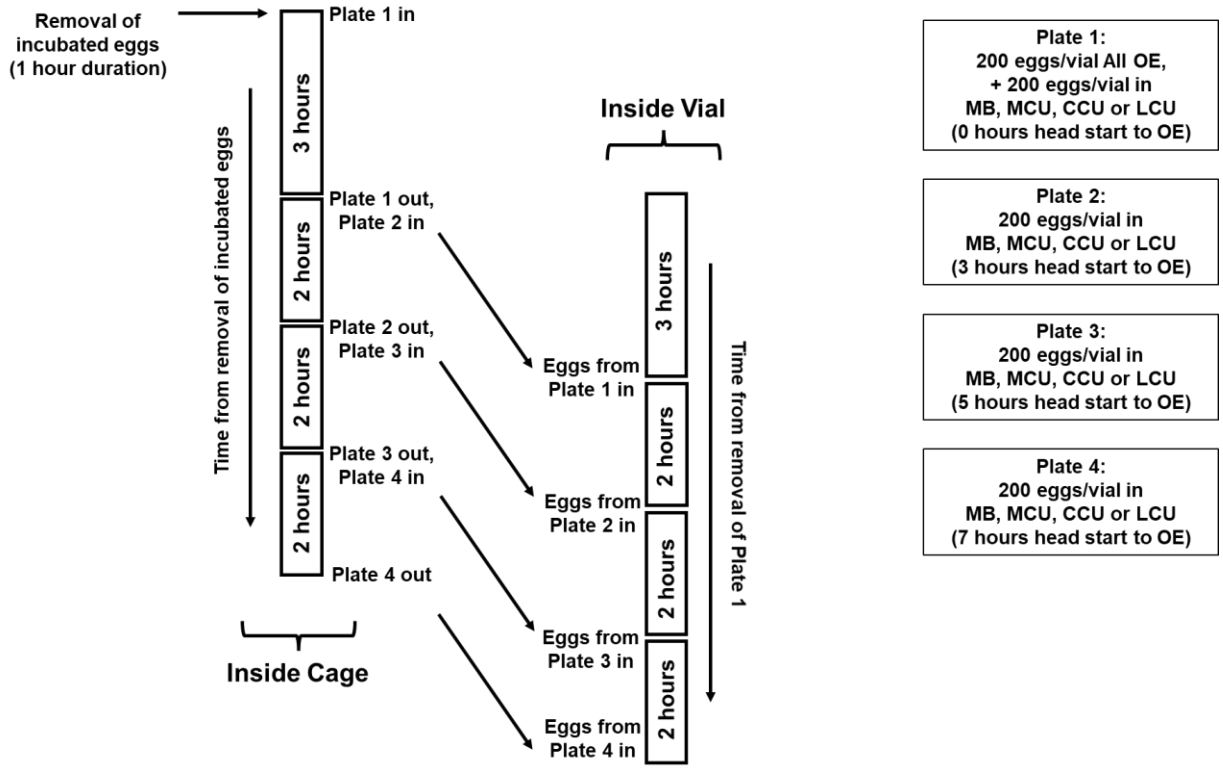

Figure 2: Schematic of the protocol for the pre-adult competition assay. Each plate represents a different head start duration given to OE. Plate 1 has either all the OE eggs, or eggs of the 0-hours head start treatment for focal populations. The subsequent plates contain eggs of the focal population for 3-, 5-, and 7-hours head start treatments, respectively.

We measured both pre-adult survivorship and pre-adult development time. We anaesthetised any adults eclosing from these vials (using CO_2) and counted them. We collected and counted eclosing flies twice a day at ~12-hour intervals for the first 3 days. This period was around day 8-10 from egg collection. Afterwards, the collection and counting frequency was relaxed to once a day. We continued this until eclosion stopped. Most flies eclosed by day 15-16 from egg collection, although a few stragglers could occasionally continue to emerge up to day 20-22. Due to logistical difficulties, eclosion checks for replicate 2 had to be stopped on the 21st day from egg collection, although by this time eclosion had ceased in most vials. The development time was calculated from time of egg plate introduction until mid-point of time window of eclosion.

Competitive performance

We wanted to test for changes in the outcome of competition, as reflected in pre-adult development time or pre-adult survivorship, due to the head start duration provided to OE. For this, we subtracted the competition outcome at 0-hours head start duration from the 3-, 5-, and 7-hour head start duration respectively. We did this for each focal population as well as for the OE competing against them.

This was calculated as follows, for both pre-adult development time and survivorship:

a) **Effect of competitive disadvantage on outcome of focal population** =

Outcome with given (3, 5 or 7 h) disadvantage – Outcome without disadvantage (0 h)

b) **Effect of focal population on outcome of OE with head start** =

Outcome with given (3, 5 or 7 h) head start to OE – Outcome without head start (0 h)

Statistical Analyses

We tested the difference in competitive outcome from the 0-hour head start condition, for each population. For this, we used a mixed-model ANOVA (model III) in a fully factorial design. The blocks representing ancestry comprised a random factor. Selection and age disadvantage duration (or head start duration for OE) were treated as fixed factors. There were 3 levels of head start to OE (3, 5, 7 hours), 4 levels of selection (MB, MCU, CCU, LCU), and 4 blocks (1-4). All ANOVA were done using STATISTICA 5 (Statsoft, 1995). Tukey's HSD was used for post-hoc pairwise comparisons. All results were plotted in R using the ggplot2 and tidyverse packages (R Core Team 2022; Wickham, 2016; Wickham *et al.*, 2019).

Results

Effects of competitive disadvantages on focal populations (pre-adult development time)

All focal populations faced, on average, a significant increase in pre-adult development time at every level of disadvantage. This can be seen in Figure 3, where y = 0 denotes no change compared to 0 hours head start. A 3 hour age disadvantage led to a 4.6 hour increase in development time, averaged across all populations. At 5 hours of disadvantage, this value was 8.2 hours. At 7 hours, it was 10.6 hours (Figure 3). The ANOVA also revealed a significant main effect of both selection and age disadvantage for the change in development time (Table 1).

All crowding adapted populations (MCU, CCU, LCU) faced a similar increase in development time compared to the 0 hours scenario, averaged over all levels of disadvantage provided to them (Figure 4). On average, MCU development time suffered by 5.6 hours, CCU by 4.9 hours, and LCU by almost 7 hours. Each of them fared better than the MB populations, which saw an

average increase of around 14 hours (Figure 4). The interaction of selection and age disadvantage was not significant (Table 1).

Effect	*df*	MS	*F*	*P*
Selection	3	251.602	34.262	<0.001***
Age Disadvantage	2	114.947	5.200	0.049*
Selection × Age Disadvantage	6	15.589	1.273	0.318

Table 1: Mixed-model ANOVA for the effect of competitive age disadvantage on pre-adult development time of focal populations. Three factors are used – selection (fixed, four levels), age disadvantage duration (fixed, three levels), block (random, four levels). Statistically significant effects are marked. Analysis was performed on population means, therefore main effect of block and its interactions were not tested for significance.

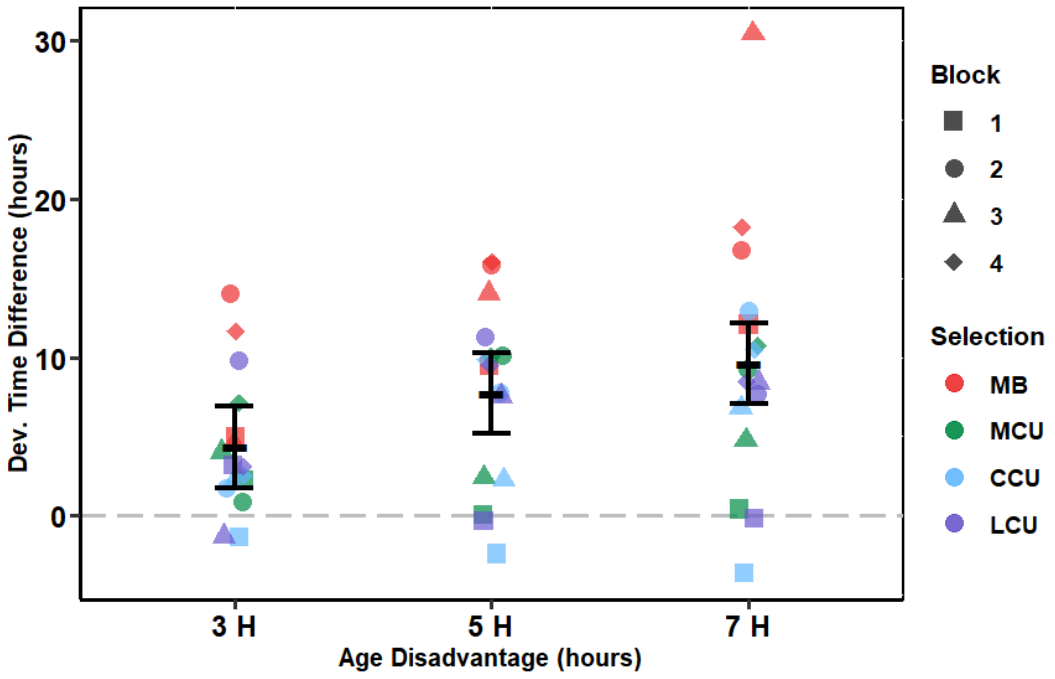

Figure 3: Effect of competitive age disadvantage on pre-adult development time of focal population. This is shown for the three levels of the age disadvantage given, averaged over all focal populations and blocks. The error bars show 95% confidence intervals around the mean, calculated from post-hoc Tukey's HSD, and thus can be used for visual hypothesis testing. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

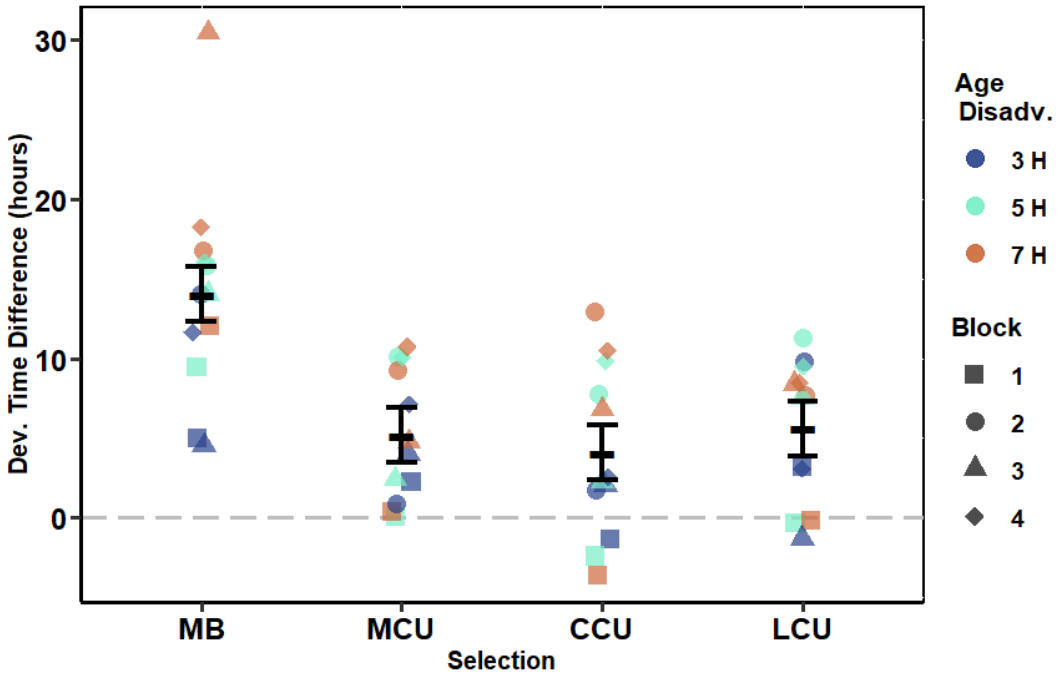

Figure 4: Effect of competitive disadvantage on pre-adult development time of focal population. This is shown for the four levels of the factor selection, averaged over all age disadvantage durations and blocks. The error bars show 95% confidence intervals around the population means, calculated from post-hoc Tukey's HSD, and thus can be used for visual hypothesis testing. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Effects of head start duration on the common competitor against each focal population (pre-adult development time)

As seen in Table 2, the ANOVA revealed a significant main effect for head start duration. Moreover, the interaction effect between selection and head start duration was also significant. Thus, we can analyze the effect of each focal population on the competitive performance of OE at every head start duration (see Figure 5 for the following results):

- 3 hours vs. 0 hours of head start to OE: No focal population differed statistically from another in its effect on OE development time. Compared to 0 hours of head start, OE with 3 hours of head start had significantly decreased development time against only CCU (~12 hours). On average, OE development time decreased by 7.8 and 5.5 hours when given a head start of 3 hours against MCU and LCU populations, respectively. Against MB, mean OE development time decreased by 1.4 hours.
- 5 hours vs. 0 hours of head start to OE: With 5 hours of head start, OE had significantly decreased development time in competition against every focal population, as compared to the scenario without any head start. No focal populations differed from each other in their effect on OE development time, but the pattern was that –OE development time decreased by around 24 hours in competition against both LCU and CCU, while this value was lower at 17.4 hours vs. MCU, and only 8.2 hours vs. MB.
- 7 hours vs. 0 hours head start to OE: Compared to the 0 hour head start scenario, OE had significantly shorter development time with 7 hours of head start in competition against each respective focal population. The development time decrease of OE against both CCU (35.3 hours) and LCU (38 hours) was significantly different from the development time decrease of OE against MB (10.4 hours). Moreover, the decrease of OE development time against LCU was also significantly more than the development time decrease against MCU (20.7 hours). A similar, but non-significant, pattern was seen for decrease in development time in competition vs. CCU and vs. MCU.

Effect	*df*	MS	*F*	*P*
Selection	3	745.391	1.714	0.233
Head Start Duration	2	1527.034	36.937	<0.001***
Selection × Head Start	6	113.931	2.990	0.033*

Table 2: Mixed-model ANOVA for the effect of focal populations on pre-adult development time of OE with head start. Three factors are used – selection (fixed, four levels), head start duration (fixed, three levels), block (random, four levels). Statistically significant effects are marked. Analysis was performed on population means, therefore main effect of block and its interactions were not tested for significance.

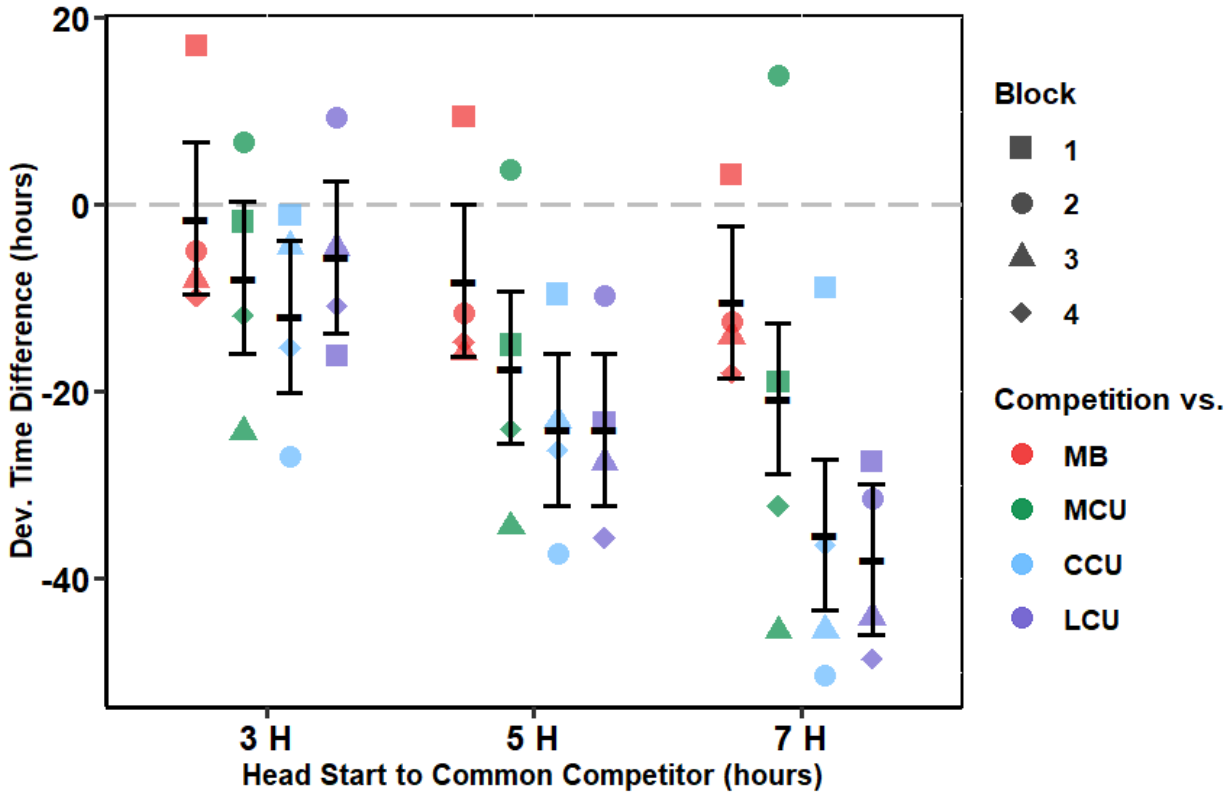

Figure 5: effect of focal populations on pre-adult development time of OE (common competitor) with head start. This is shown for the three levels of the age disadvantage and the four levels of focal populations, averaged over all four blocks. The error bars show 95% confidence intervals around the population means, calculated from post-hoc Tukey's HSD, and thus can be used for visual hypothesis testing. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

Pre-adult survivorship

Providing an age disadvantage did not impose any significant changes on the mean pre-adult survivorship of any focal population (Data not shown). Additionally, the common competitor did not experience any statistically detectable change in its pre-adult survivorship due to any of the head start durations provided (Data not shown).

Discussion

Overall patterns

We started the study with four predictions, as mentioned in the Introduction. These predictions have fared thus –

1. *All crowding adapted populations would be less affected by a competitive disadvantage than the low-density controls (MB).*

   This was observed. MB development time increased significantly more with age disadvantage than MCU, CCU or LCU (Figure 4).

2. *The common competitor would also gain the most competitive performance when given a head start against MB.*

   From Figure 5, this was rejected. MB induced the least decrease in OE development time when the latter received head starts (i.e., MB was most detrimental to OE competition) compared to MCU, CCU or LCU.

3. *The populations which evolved the largest eggs and the fastest hatching time (LCU) would be least affected by the age disadvantage.*

   This was rejected. No difference in the development time change was observed between any crowding-adapted population (Figure 4).

4. *The common competitor would gain the least competitive performance when given a head start against LCU.*

   This was rejected. The common competitor gained the **most** performance (decreased development time) against LCU, followed closely by CCU (Figure 5).

All populations selected for adaptation to larval crowding were affected to similar degrees by their competitive disadvantage (Figure 4). Moreover, the crowding-adapted population which evolved the smallest increase in head start mechanisms (MCU) reduced the competitive performance of the common competitor to the greatest extent (Figure 5).

We know from previous experiments that the larval competitive ability of OE, the common marked competitor, is lower than that of MCU, LCU and CCU. It is similar to that of MB, when tested across a range of egg and food combinations (Sarangi *et al.*, 2016; Sarangi, 2018; Chapter 2). The current study supports this and shows that MB larvae were most badly affected by the head start to OE (Prediction 1, Figure 4).

Predictions 2, 3 and 4, however, do not hold up to empirical tests. This unexpected pattern of results might have an underlying explanation in the effectiveness and tolerance of the focal populations.

Effectiveness and tolerance

We define *effectiveness* as the amount of growth rate inhibition a larva imposes upon its competitors. Conversely, a larva can also show some amount of *tolerance* to this growth inhibition imposed by other competitors on itself (usage inspired by Joshi and Thompson, 1995). These terms have been alternatively defined and formulated as aggressiveness and sensitivity (Breese and Hill, 1973), pressure and response (Mather and Caligari, 1983) or

aggression and response (Eggleston, 1985; Hemmat and Eggleston, 1988, 1990), although in those formulations response/sensitivity is inversely related with tolerance.

We performed a subsequent experiment to measure effectiveness and tolerance of the focal populations at the same density as the current study i.e., 400 eggs in 2 mL food (Chapter 2). The measurements were derived from the formulae for 'intrinsic competitive ability' used by Joshi and Thompson (1995). For pre-adult development time, the patterns for both effectiveness and tolerance of each set of focal populations were as follows:

MCU > CCU > LCU > MB

The MCU populations showed the highest effectiveness and tolerance against OE. The MB populations showed almost no change in effectiveness or tolerance compared to OE (i.e., they had the same competitive effect on OE larvae as the OE themselves).

We can consider the increase in development time due to age disadvantage to the focal populations as a decline in tolerance. Similarly, decreasing OE development time as the duration of head start to OE increases can be considered as a decline in the effectiveness of the respective focal population that competes against OE.

For seeing change in tolerance, Figure 6 shows the least square regression of focal population development time on age disadvantage duration. Exact values of intercepts and slopes are given in Table 3. The positive slope of development time vs. age disadvantage represents decline in tolerance. For all populations, there is a general decline in tolerance with increasing age disadvantage (Figure 6). MB has the most severe decrease in tolerance as OE is given a head start against it. This also follows from prediction 1 in the introduction (i.e., MB is expected to suffer the most from an age disadvantage). As MB has the lowest tolerance even without any head start to OE (Chapter 2), and also suffers the greatest decline in tolerance with age disadvantage, we can assume that the steepness of the decline in tolerance with age

disadvantage is proportional to the tolerance measured without any age disadvantage. With this assumption, based on the measurements made on tolerance without any age disadvantage (Chapter 2), MB should suffer the greatest decline, followed by LCU, followed by CCU, and finally by MCU, with the latter showing the lowest decline with head start to OE. However, all three crowding-adapted population sets show similar slopes (Figure 6). Thus, they suffer similar levels of decline in tolerance against OE that are given head starts against them. Based on measured tolerance, MCU should show a lower decline than CCU, with LCU showing the greatest decline among the three. The lack of a difference likely means that the greater egg size and faster hatching time of LCU and CCU help them offset a greater decline in tolerance. This provides some validation for prediction 3. However, the current argument is not consonant with the inference drawn from results in figure 4. Those seem to suggest that greater head start mechanisms (egg size and hatching time) of LCU and CCU do not help offset competitive age disadvantages any better than MCU. From the perspective of tolerance decline, the lack of a difference between the crowding-adapted populations highlights a possibly nuanced rather than straightforward role larger eggs or faster hatching may be playing in the process of competition.

Figure 7 shows the least squares regression of OE development time on head start duration. Exact values of intercepts and slopes are given in Table 3. We have plotted this for competition of OE vs. each focal population. The decrease in slope of development time vs. head start shows the decline in effectiveness of the focal populations. For the crowding-adapted populations, increasing head start durations lead to different declines in effectiveness (Figure 7). The rates of decline largely follow the patterns seen for effectiveness measurements without any head start to OE (Chapter 2). MCU, which has the greatest effectiveness, shows the lowest decline on increasing head start duration to OE. This is followed by CCU and LCU, with the latter having a marginally lower slope, i.e., greater decline in effectiveness. Even though CCU and LCU suffer more than MCU in effectiveness decline, we cannot preclude the possibility that

greater egg size and faster hatching time in these populations may be preventing an otherwise even steeper decline against increasing age disadvantage. Thus, prediction 4 is left unresolved.

The major exceptions to the patterns seen for effectiveness measurements are the MB. These low-density controls have the lowest measured effectiveness in competition with OE without any head start (Chapter 2) and yet display the least decline in effectiveness with head starts given to OE (Figure 7). This is also in contrast to the observed patterns of tolerance decline (Figure 6). We have provided a possible explanation for this in the extrapolations section below.

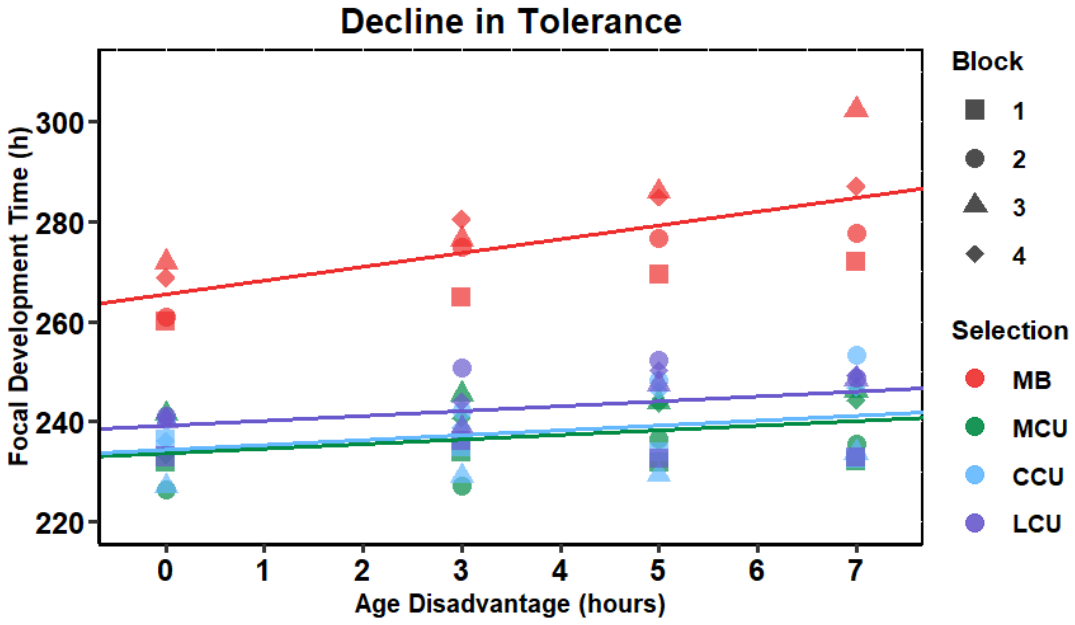

Figure 6: Pre-adult development time of focal populations vs. age disadvantage durations to the focal populations (by providing head start to OE). The positive slope of the least squares regression line denotes a decline in tolerance of focal populations. Exact values of slopes and intercept are given in Table 3. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

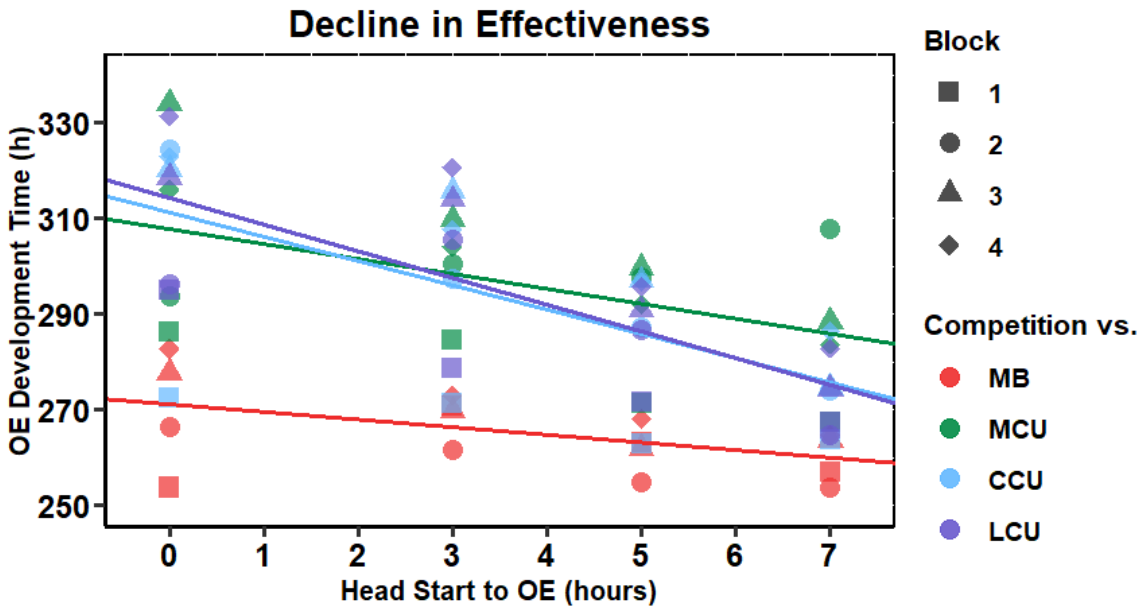

Figure 7: Pre-adult development time of OE against each focal population vs. head start duration to OE. The negative slope of OE development time with increasing head start duration denotes a decline in the effectiveness of focal populations. Exact values of slopes and intercepts are given in Table 3. Rearing details: MB = 70 eggs, 6 mL food; MCU = 600 eggs, 1.5 mL food; CCU = 1200 eggs, 3 mL food; LCU = 1200 eggs, 6 mL food.

	Fig. 6: Tolerance Decline		**Fig. 7: Effectiveness Decline**	
	Intercepts	Slopes	Intercepts	Slopes
MB	265.543	2.759	271.195	-1.601
MCU	233.620	0.931	307.770	-3.113
CCU	234.389	0.978	311.261	-5.081
LCU	239.266	0.967	314.348	-5.581

Table 3: Intercepts and slopes for the least squares regression lines in figures 6 and 7.

Extrapolations

Based on the trends of decline in effectiveness (Figure 7), we can calculate the head start duration to OE at which each crowding-adapted population should have zero effectiveness, on average. At this extrapolated head start duration, mean OE pre-adult development time in competition against 200 MCU, CCU, or LCU larvae, respectively, would approximate competition against 200 OE larvae. For this purpose, we assume that there remains a similar slope of decline in effectiveness of each crowding-adapted population (Table 3, Figure 7), as head start to OE increases beyond the bounds of the current study. From measurements of the subsequent experiment, we have calculated the effectiveness of MB as nearly zero against OE (Chapter 2). In the current study, we can simplify and assume the MB condition with no age disadvantage as the reference point for zero effectiveness. This zero point yields a development time of 271.2 hours for OE (Figure 7). With this, we extrapolate, from figure 7, the following head start durations to OE required to get zero effectiveness of the respective focal population:

vs. MB: 0 hours

vs. MCU: 11.75 hours

vs. CCU: 7.89 hours

vs. LCU: 7.73 hours

Both CCU and LCU have durations to reach zero effectiveness that nearly fall within the bounds of the current experiment. Their effectiveness is almost indistinguishable from that of OE (i.e., zero) at almost 8 hours of head start. MCU populations, owing to their high effectiveness and low slope of decline, are extrapolated to suffer nearly 12 hours of age disadvantage in order to get the same effectiveness as OE. We also note that at this zero effectiveness point, the mean pre-adult development time of each crowding-adapted population set would still be faster than OE by about 25-30 hours.

Further head starts to OE from this extrapolated duration may yield further loss in effectiveness, going below zero. This is observed in the MB populations, as their effectiveness declines to negative values with increasing age disadvantage (Figure 7). However, the MB populations already possess very nearly zero effectiveness in the absence of any imposed handicap. Given further age disadvantage, it is likely that the change in development time with head start duration may dampen somewhat as effectiveness becomes negative. At values below zero, MB would probably offer very mild competitive effects to OE. With greater head starts, there may not be much scope for effectiveness to reduce further. This may explain the somewhat strange results seen for Prediction 2 (Figure 5) and the minor decline of MB in effectiveness (Figure 7). The empirical verification of this argument will require future experiments with a larger range of head start durations to OE to test the limits of decline possible for effectiveness. Additionally, providing head starts to MB against OE should give the former positive effectiveness. This would allow the observation of changes in decline of effectiveness from a positive to negative range in MB populations.

Other outcomes of competition

At the density tested, we saw no differences in mean pre-adult survivorship. Conducting this experiment at a higher level of crowding may cause differences in survivorship. However, this may obscure trends in pre-adult development time due to very few surviving adults in some cases (Chapter 2). As seen in the current study, development time is sensitive to minor changes in the dynamics of competition.

Another sensitive output of competition is adult dry weight at eclosion (Sang, 1949a; Bakker, 1961, 1969; Ohba 1961 as cited by González-Candelas *et al.*; 1990; González-Candelas *et al.*, 1990; Chapter 2). Bakker (1961, 1969) saw changes in dry weight upon implementing multiple durations of head starts on two competing *D. melanogaster* populations. Nicholson (1948, as

cited in Nicholson, 1955) found that upon crowding the larvae of blow flies *Lucilia cuprina*, the variation in size of eclosing adults was accentuated due to batches of larvae getting various durations of head starts. Bryant (1971) also saw changes in dry weight of emerging house flies *Musca domestica* on conferring different durations of head starts to larvae of two strains. Due to logistical limitations, our study did not involve the measurement of adult dry weight. It does, however, have potential to show differences in future studies. We know that LCU-like crowded cultures show a large amount of variation in dry weight of eclosing flies (Sarangi, 2018). Adults eclosing earlier are larger. Later eclosing flies tend to be smaller, although the last eclosions are of medium-sized flies (Sarangi, 2018). Several other studies have also mentioned or shown patterns for earlier eclosing flies being larger than the later ones (Sang, 1949a; Bakker, 1969; Hughes, 1980; Chapter 2). Since there are large development time changes in our study, we might see differences in dry weight variation of both focal populations and OE at different head start durations.

Mechanistic explorations

The mechanism underlying the patterns seen in our experiment may be related to the dynamics of metabolic waste build-up in a crowded *Drosophila* culture . Metabolic waste products such as urea, ammonia and uric acid are known to cause mortality or increase development time in *Drosophila* larvae (Botella *et al.*, 1985). Food 'conditioned' by larvae of different strains can also cause differential mortality in larvae introduced later in the culture of *D. melanogaster* (Weisbrot, 1966; Dawood and Strickberger, 1969; but see Budnik and Brncic, 1975 for an exception) and related species (Budnik and Brncic, 1974, 1975). Moreover, the CU populations, which are ancestrally related to the populations used in our study, evolved different traits along the eclosion time axis (Borash *et al.*, 1998). From the CU-like crowded culture, offspring of earlier eclosing flies (first 72 h of eclosion) showed greater feeding rate. Those of later eclosing flies (post 500 h of eclosion, 48-72 h window) showed greater urea and

ammonia tolerance (Borash *et al.*, 1998). The same study also showed that waste (ammonia) accumulation occurred steadily over 20 days from egg collection.

Thus, waste dynamics likely play a larger role in the later stages of a crowded larval competition culture. The more time a larva spends in a culture, the greater the waste it likely is exposed to, and the longer it may take to develop. This means that larvae given age disadvantages may take a longer time to develop, as seen in our results (Figures 3, 4). Larvae given a head start likely face less waste and therefore may be able to develop faster (Figure 5). The delayed role of waste may also explain why LCU and CCU populations, with greater head start mechanisms, don't perform better than MCU. Even if the LCU or CCU larvae gain advantages early on compared to MCU in competition, the larvae of the latter could close the development time gap with potentially greater larval growth rates. Characterization of larval waste dynamics in future competition experiments with varying head starts could be used to test these predictions.

Additionally, as can be seen in figures 4 and 5, there exists large variation among blocks with respect to development time, under each selection and head start duration. This could occur due to a multitude of reasons, perhaps in combination:

i. Our observation is that whenever there are large delays to mean development time in crowded *Drosophila* cultures, there is typically also an increase in variance in development time within the crowded cultures (Chapter 6, compare figure 3Cii and figure s6a). Such large variances, coupled with the relatively small sample sizes of 4-5 vials that can be achieved within logistical possibilities, means that the standard error for each combination of block × selection × head start duration is likely to be high as well.

ii. There may be among-block variation in the response to crowding with respect to development time.

iii. There may also exist development time differences among blocks regardless of density.

On the importance of head starts in competition

We can potentially use results from the current study to ask another kind of question: how important are head starts in determining the outcome of competition?

The literature on plant competition is rife with studies attempting to address this question (reviewed in Ross and Harper, 1972; Wilson, 1988). Initial advantages to a plant in competition could lead to cumulative benefits, either through larger seed size (Black, 1958) or faster seedling emergence time (Ross and Harper, 1972). Ultimately, a small head start to a plant against somewhat equal competitors could provide an overwhelming supremacy to it. Newman (1973) termed this a "snowball" effect. In contrast, some studies showed that such cumulative snowballing may be limited to competition for light – for nutrient based, root competition, there was little evidence that initial advantages were supremely important (Newman, 1973; Newbery and Newman, 1978; Wilson, 1988).

Due to large differences in the biology of the model systems, such arguments from plant competition cannot directly be applied to the *Drosophila* larval competition context. However, the concept of a cumulative "snowball" effect in competition should be general enough to be explored in most model systems. Indeed, Bakker (1961) has reviewed evidence for and commented on the paramount importance of head starts in larval competition in nature.

Potentially cumulative advantages have been discussed in several studies which have looked at the ecological implications of head starts in larval competition. Populations have been seen

to switch larval competitive superiority after a few hours of head start in *Drosophila melanogaster* (Bakker, 1961, 1969), and house flies *Musca domestica* (Bryant, 1971). Several experimenters have studied competition by providing larval batches different durations of head start in *Drosophila* (Sang, 1949b; Seaton and Antonovics, 1967; Gilpin, 1974). Mueller and Bitner (2015) suggested that *Drosophila sechellia* larvae on the *Morinda* fruit may obtain a survival advantage from a large head start against otherwise competitively superior larvae from other *Drosophila* species. This could occur due to the former's significantly faster hatching time and *Morinda* toxin tolerance compared to other *Drosophila* species. Initial advantages of older larvae have been shown to be important in Tephritid fruit flies *Rhagoletis pomonella* (Averill and Prokopy, 1987), as well as different species of Ichneumonid parasitoid wasps (Fisher, 1961; Jørgensen, 2009). Based on several empirical observations, the importance of head starts through early larval hatching has been suggested for three species of Scolytid bark beetles (Beaver, 1974). Except for Bakker's (1969) study, none of the above-mentioned studies considered the effects of head starts on populations that had evolved to become competitively superior through laboratory selection.

In our results, there does seem to be some cumulative effect along the development time axis. This is evidenced by the large decrease in development time that OE experiences after gaining only a few hours of head start (Figure 5). This is additionally supported by the relatively large increases in development time of focal populations given a small age disadvantage (Figures 3, 4). However, development time of crowding-adapted populations was not affected as badly as the MB (Figure 4). Additionally, survivorship did not change significantly across any head start duration. In the case of a "snowball" effect, we would also expect survivorship to be affected by an age disadvantage.

An additional complication arises in such arguments when effectiveness and tolerance are taken into account. We consider the simplest case of OE getting a head start against an almost equal

competitor, MB. The latter's tolerance declines steeply with increasing head start to OE (Figure 7). This indicates the existence of a cumulative snowballing effect against MB. However, a similar pattern is not seen in the case of effectiveness. As seen in figure 6, OE gets the least advantage against MB, likely due to a dampened relationship between rate of decline for negative effectiveness and head start duration. This means that OE does not get nearly as much cumulative benefit against an equal competitor as it can get against potentially superior competitors such as LCU (Figure 6). Clearly, in *D. melanogaster* larval competition, cumulative effects from initial advantages are not obvious – they are dampened or exacerbated by the overall competitive ability, as well as relative effectiveness and tolerance, of the competitors. Further tests with different populations, varied densities and head start durations may help uncover these nuances in the process of larval competition.

Conclusions

We have shown that increased larval competitive ability evolved through three different chronic larval crowding regimes can help larvae of the respective populations fare better against age disadvantages in competition. However, among superior competitors, those that have evolved the greatest head start mechanisms may not show better performance against age disadvantage. Studying the effectiveness and tolerance of the competitors can highlight the subtle effects head start mechanisms may nevertheless have on the outcomes of competition. Even the effectiveness relationship with head start duration may change depending on the value of effectiveness of the population. We have discussed the possibility of waste dynamics being crucial to the underlying mechanisms of the patterns seen in the current study. Such competition experiments with artificially provided head starts can also be used to explore cumulative gains from initial advantages. From the current study, we have seen that a "snowball" effect may or may not occur depending on the considerations of effectiveness and tolerance. We end with the observation, in agreement with J.H. Sang (1949b), that such competition experiments with head

starts can give more nuanced understanding over and above simple larval crowding experiments.

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Chapter V

More than just density:

the role of egg number, food volume and container dimensions in mediating larval competition in *Drosophila melanogaster*

NOTE

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Introduction

Competition can potentially play a large role in shaping the ecology of populations and communities (Elton and Miller 1954; Nicholson 1955), and be a major force driving their evolutionary trajectories (Miller 1967; Wilson 2014). The importance of competition as an impetus for natural selection has been highlighted at least since the time of Darwin (Darwin 1859). Considerable research, both theoretical and experimental, has been done to define and understand competition, as well as its role in ecology and evolution (Sang 1949; Birch 1957; de Wit 1960; Bakker 1961; Miller 1967).

Over the last century, flies of the genus *Drosophila* have become some of the most popular laboratory model systems to experimentally study both intra- and inter-specific competition (Pearl and Parker 1922; Moore 1952; Miller 1964; Ayala 1969; Mueller 1985, 1988; Joshi and Thompson 1995). The holometabolous life cycle of *Drosophila* flies has allowed researchers to partition intra-specific competition for growth-related resources in the larval stage, from the competition for mates in the adult stage (Prasad and Joshi 2003). While studies of adult competition through induced adult crowding have also been conducted (Robertson and Sang 1944; Ohnishi 1976b; Joshi et al. 1998), the present study is focused on the larval phase of competition.

Larval competition in *Drosophila* has had a very rich history of study, and has contributed greatly to the understanding of density-dependent effects, density-dependent selection and the consequent population dynamics. The earliest studies showed that larval competition could be inferred from crowding eggs/larvae at high densities and observing the effects on fitness related traits such as pre-adult survivorship, pre-adult development time and size of eclosing adults (Sang 1949; Bakker 1961; Ohnishi 1976*a*). Several studies additionally investigated the differences in larval competitive ability of various strains of *D. melanogaster* (Bakker 1961,

1969; Gale 1964; Kearsey 1965; Mather and Caligari 1981; de Miranda et al. 1991). Models were also created in an attempt to capture the mechanisms of larval competition (Bakker 1961; De Jong 1976; Nunney 1983; Jansen and Sevenster 1997). Subsequently, selection experiments were carried out to adapt populations to chronic larval crowding, resulting in the evolution of increased larval competitive ability (Mueller 1988; Nagarajan et al. 2016; Sarangi et al. 2016; Chapter 2).

Researchers have also used the effects of larval crowding as proxies for increased stress at the juvenile phase. They have consequently studied the effects of crowding-adaptation on the correlated evolution of thermal stress resistance (Kapila et al. 2021*a*), as well as the immune system (Kapila et al. 2021*b*). Effects of crowding have also been used to explore yeast availability (Klepsatel et al. 2018), thermal stress (Sørensen and Loeschcke 2001; Henry et al. 2018), and larval crowding has enabled the manipulation of adult body size for sexual selection experiments (Mital et al. 2021). A recent study on transcriptomic consequences of larval crowding was also carried out (Morimoto et al. 2023).

Most studies exploring larval competition or otherwise implementing larval crowding have usually employed a single, or a gradient of, 'high density' larval cultures as the experimental regime(s), and compared it to a control 'low density' regime. While the low-density cultures were relatively consistent – they involved rearing larvae in high food volumes (5-8 mL, depending on the study) with low starting egg numbers (30-80 eggs, depending on the study), the high-density cultures employed in experiments varied a great deal (reviewed in Henry et al. 2018; Morimoto and Pietras 2020). These cultures varied widely in the number of eggs, the food volumes and the container dimensions used – with the focus being on studying the 'crowded' phenotype at an arbitrary high larval density, compared to the 'uncrowded' phenotype.

A series of long-term selection experiments over the last four decades, studying density-dependent selection using *Drosophila* populations adapted to larval crowding, ultimately challenged the notion that the consequences of crowding could be predicted based on larval density alone (reviewed in Chapter 1, Chapter 3). Initial studies from the 1980-1990s saw a canonical view emerge for adaptation to larval crowding (Mueller 1997; Joshi et al. 2001; Prasad and Joshi 2003; Mueller et al. 2005*b*; Mueller 2009; Mueller and Barter 2015). This view held that, based on empirical observation, a number of traits would consistently evolve in *Drosophila* populations adapted to larval crowding – such as increased larval competitive ability (Mueller 1988), primarily via increased larval feeding rate (Joshi and Mueller 1988, 1996) or increased metabolic waste tolerance (Shiotsugu et al. 1997; Borash et al. 1998), with a trade off in the form of a reduction in food to biomass conversion efficiency (Mueller 1990; Joshi and Mueller 1996), as compared to low-density control populations. Subsequent studies also carried out such larval crowding adaptation experiments – albeit at a different combination of eggs and food than used earlier (Nagarajan et al. 2016; Sarangi et al. 2016). While the older studies crowded larvae in starting cultures of roughly 1500 eggs in 6 mL food in 6 dram vials, the more recent studies employed cultures with approx. 600 eggs in 1.5 mL food, in wider 8 dram vials. These more recent studies resulted in the evolution of traits very different from what the canonical view predicted. While greater larval competitive ability did evolve, there was no change in feeding rate or waste tolerance. Instead, the crowding-adapted populations evolved shorter development time and greater efficiency of biomass accumulation with respect to time (Nagarajan et al. 2016; Sarangi et al. 2016).

Thus, after three decades of selection studies using larval crowding, it was evident that, while most crowding-adaptation experiments would lead to the evolution of increased larval competitive ability compared to the low-density adapted populations, the specific traits that

evolved in different populations could vary widely depending on the details of how exactly the larval crowding was imposed (Sarangi et al. 2016; Sarangi 2018).

These effects of the details of how crowding was imposed were further explored in recent years. One study examined if crowded cultures with the same overall density achieved through different egg numbers and food volumes would differ in their density-specific fitness functions (Sarangi 2018). This was done by crowding larvae of low-density reared populations in two cultures at the same overall density: in either 600 eggs in 1.5 mL food or 1200 eggs in 3 mL of food, in the same kind of vial. Quite surprisingly, the two types of cultures with the same density led to very different patterns of pre-adult survivorship and development time (Sarangi 2018), indicating that density-specific fitness functions of two cultures with the same density achieved via different combinations of egg numbers and food volume could be quite different. A subsequent evolutionary experiment also yielded concordant results. Two sets of populations evolved at the exact same density through different egg and food combinations (600 eggs in 1.5 mL food vs. 1200 eggs in 3 mL food) led to the evolution of differences in terms of feeding rate, pre-adult development time (Sarangi 2018), egg size and egg hatching time (Chapter 3). These populations also showed differences in the effectiveness and tolerance components of larval competitive ability across a gradient of high-density cultures (Chapter 2).

The significance of these findings can be understood by applying them to most of the earlier studies carried out on the effects of larval competition and comparisons of competitive ability. How could the results across several studies applying a range of different high-density conditions be comparable, when the same density in closely related populations resulted in large differences in density-specific fitness-functions? Clearly, the commonly used parameter of total density of eggs per unit volume of food itself is not enough to predict the ecology or evolution of traits in larval crowding. There are likely other factors that also influence the

ecological and evolutionary outcomes of larval competition beyond the total density of a crowded culture.

The current study aims to explore the aspects of larval crowding beyond the total eggs/food density that may be important in determining the density-specific fitness functions of crowded cultures. For this purpose, we studied two fitness related traits that have been shown to change under the influence of larval crowding – pre-adult survivorship and pre-adult development time (Sang 1949; Bakker 1961; Ohnishi 1976a) – in order to ask a fundamental question: how do these fitness-related traits change with density, when the density change is accomplished by varying different parameters like egg number, food column height and food column diameter?

Some clues to a better understanding of the different facets of larval density are provided by the extensive studies by J. H. Sang in the 1940s. His initial studies showed that keeping all else equal, food volume and egg number could both be varied to change the degrees of larval crowding. While this is now well understood, and has remained the basis of work on larval crowding ever since, Sang also studied two different implementations of changing food volume – firstly, keeping the same cylindrical container and changing the height of the food column (which changes the food volume) and secondly, changing the width (i.e. cross-section surface area) of the cylindrical container itself, while keeping the same food column height. He found that these two ways of implementing crowding led to different outcomes in terms of survivorship, development time and weight of adult flies (Sang 1949). He further calculated that the same food volume cast in different dimensions could have different effects of surface area and food column height (Sang 1949). It should additionally be noted that most subsequent studies altered food volume primarily via changing food column height in an unchanging container type. Unfortunately, these secondary results from Sang's studies appear to have been ignored due to the importance of the primary result from the research article, which was one of

the first demonstrations of the general effects of larval crowding in *D. melanogaster* (Sang 1949).

In the current study, we performed a rigorous follow-up of Sang's finding on the effects of different ways of implementing crowding. We conducted a fully factorial study of the three factors that can influence larval crowding – 1) egg number, 2) food volume via changing food column height, and 3) food volume via changing the surface area of food exposed to air.

A fully factorial design allowed us to set up several comparisons which within themselves had the same combination of egg number and food volume, but were cast in different cylindrical container dimensions. We asked if there are any differences to be found between any of these cultures. We further explored why there might be reason to find any differences between cultures that essentially appear to be the same implementation of crowding in terms of egg number and food volume, ignoring the container.

Methods

Populations used

We used four replicate large (1500+ adults), discrete generation, laboratory *D. melanogaster* populations (designated MB_{1-4}) whose ancestry and maintenance have previously been described in detail (Sarangi et al. 2016). These populations have also served as ancestral controls to three sets of larval crowding-adapted populations (see Chapter 3), and are thus maintained at a relatively low larval density. We started each generation of maintenance by collecting eggs from adults of the previous generation in vials at a low density of 60-80 eggs in ~6 mL of cornmeal-sugar-yeast medium food (see Sarangi et al. 2016) in cylindrical glass vials approx. 95 mm tall, with an inner diameter of around 22.5 mm^2, resulting in a cross-

section surface area of approx. 400 mm^2 (the food column height was approx. 15-20 mm). After egg collection, we waited until 11 days for the metamorphosis to complete for most of the individuals, and transferred the eclosed adults of each population to a respective Plexiglas cage (25 x 20 x 15 cm^3). We provided fresh cornmeal medium food plates to the adults in the cages on days 11, 12, 14 and 17 from the day of egg collection (day 1), as well as a moist cotton ball to maintain high relative humidity (>70%). On day 18 from egg collection, we replaced the food plate with a fresh one covered in a yeast-water paste mixed with a drop of glacial acetic acid. On day 20, we removed the yeast-covered plate and provided a food plate cut into two parts for 18 hours. This provided a vertical surface to the *Drosophila* for easier egg laying. Finally, on day 21, we removed the two food plate sections, now heavily laden with eggs. The collection of eggs from these plates served as day 1 for the next generation. This completed a single generation of maintenance. All populations were maintained in constant light (LL), at around 25°C, and a relative humidity of 70-90%.

Collection of eggs for experiment

For the start of the assay, we singled out one replicate population per generation and collected an additional 80 vials (each with 60-80 eggs, ~6 mL food, approx. 400 mm^2 cross section surface area per vial). On day 11 from egg collection, we transferred the eclosed adults from the extra vials to two new cages – adults from 40 vials were transferred to each cage, at random. These cages were each provided a food plate covered with yeast-water paste (containing a drop of glacial acetic acid). On day 14, we provided the cages with fresh cornmeal plates for 1 hour with vertical edges for egg laying. These were discarded after removal from the cages, as they served to collect the eggs that were previously incubated in the females. This would ensure that the subsequent eggs laid by the females would be freshly fertilised, and no inadvertent head starts due to early hatching would be provided to the competing larvae in the experiment. Following plate removal, we provided another egg collection plate to each cage for 4 hours.

These were harder plates, containing twice the usual amount of agar (2%), with only yeast and sugar added, and facilitated easier removal of eggs for counting (see Sarangi 2018; Chapter 3). After 4 hours, we removed the egg collection plates, and used the eggs laid thereon for the experiment. We added another one of the harder yeast-sugar-agar plate to each cage for 4 hours, in case the first batch did not yield the required number of eggs. The eggs were removed from plates of both cages and were transferred to a non-nutritive 1% agar plate (see Chapter 3), and thereafter thoroughly mixed prior to starting the egg counting.

After removal of the second harder agar plate, we provided another food plate covered with yeast paste to each cage. On day 15 from egg collection, we removed the yeast plate and repeated the egg collection process of day 14. Eggs taken on day 14 were assigned the Day 1 factor level, and those taken on day 15 were assigned the Day 2 factor level (see below).

The experimental set up

In the introduction, we listed three ways in which the degree of larval crowding in cylindrical culture vials may be affected, assuming all else is kept unchanged (fig. 1B):

1) Changing starting egg number.
2) Changing food volume via food column height, keeping the same container dimensions.
3) Changing food volume via changing the diameter of the cylindrical vial, thereby changing the surface area of food in contact with air.

We used the collected eggs on 1% agar to conduct an experiment incorporating all three crowding-inducing variables in a three-way, fully factorial design.

1) The eggs were counted exactly for three levels of starting egg numbers: 200, 400 and 600.
2) We used three levels of food column height (~5 mm, ~10 mm and ~15 mm): hereafter referred to as column height.

3) We used three levels of vial cross section surface area (~200 mm^2, ~400 mm^2 and ~600 mm^2): hereafter referred to as surface area.

The complete experimental design is detailed in figure 1. Using three levels of column height and three levels of surface area in a factorial design gave rise to a total of 9 combinations of culture types (fig. 1C). Among these 9 combinations, the food volumes of 2, 3 and 6 mL were present in two types of culture each (fig. 1C, colour matched). The first type had lower surface area and greater column height; the second type had greater surface area and lower food column height. The remaining 3 food volumes – 1, 4 and 9 mL – were unique.

To each of the 9 combinations of surface area and column height, we added 200, 400 or 600 eggs. There were thus 27 different combinations of egg number × surface area × column height. Subsets of these 27 treatments were used to draw relevant comparisons (see Results).

Each of the 27 treatments had four replicate vials for each of the four replicate MB populations, split across two days of egg collection. Thus, we collected two replicate vials per treatment combination on day 14, as well as two replicate vials on day 15.

In total, each replicate population had 108 vials, for a grand total of 432 vials across replicates. A total of 172,800 eggs were used for the entire experiment.

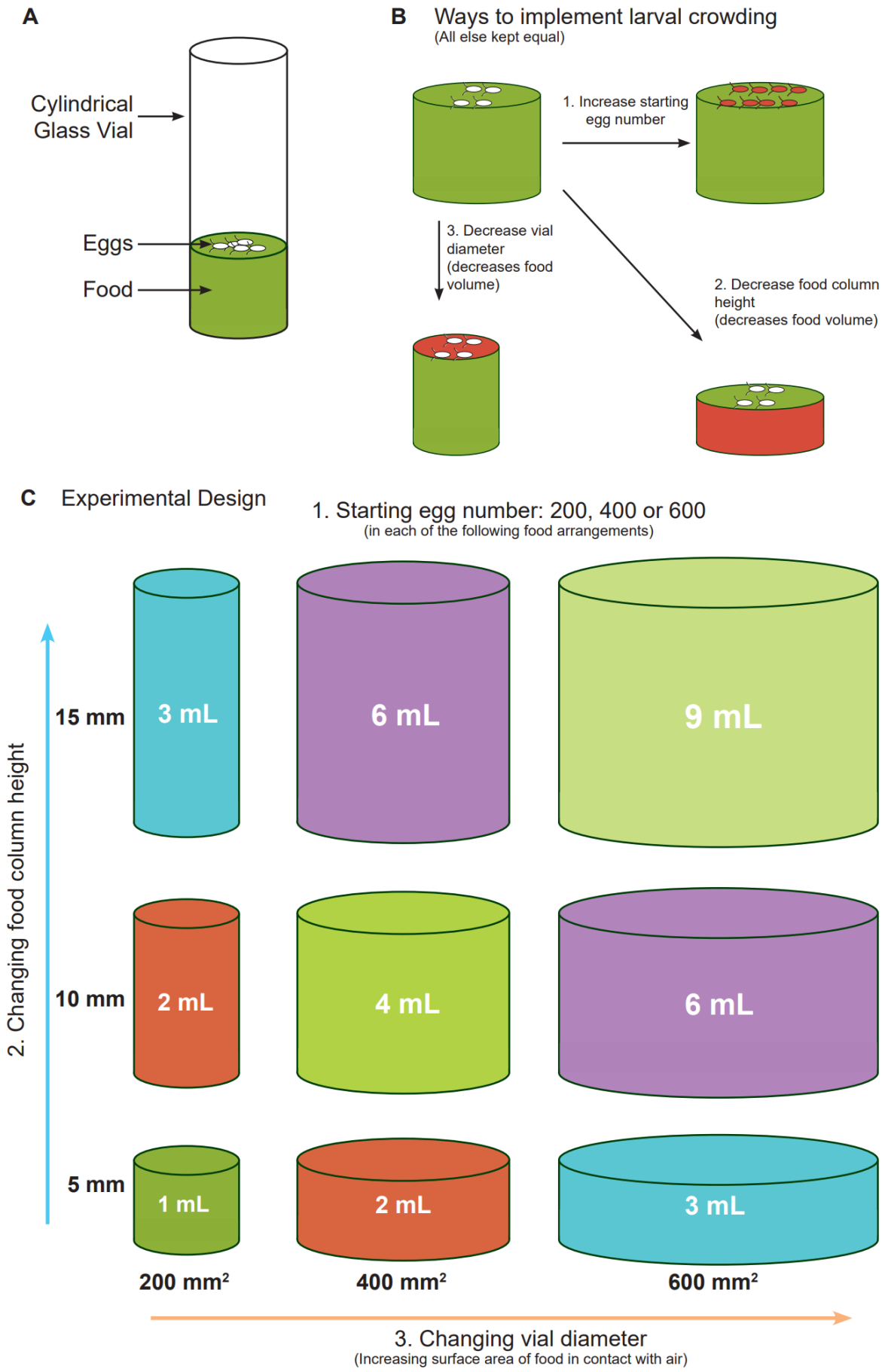
A
Cylindrical Glass Vial
Eggs
Food
B
Ways to implement larval crowding
(All else kept equal)
1. Increase starting egg number
3. Decrease vial diameter (decreases food volume)
2. Decrease food column height (decreases food volume)
C
Experimental Design
1. Starting egg number: 200, 400 or 600
(in each of the following food arrangements)
2. Changing food column height
15 mm
10 mm
5 mm
3 mL
6 mL
9 mL
2 mL
4 mL
6 mL
1 mL
2 mL
3 mL
200 mm²
400 mm²
600 mm²
3. Changing vial diameter
(Increasing surface area of food in contact with air)

Figure 1. The complete experimental design. A) diagrammatic representation of a cylindrical culture vial. B) The three ways of imposing crowding, assuming all other conditions are constant. C) The design of the experiment, with three levels each of three factors used 1. Egg number; 2. Food column height; 3. Vial cross-section surface area (affected by vial diameter).

Collection of eclosing flies

We collected all eclosing flies from each of the 432 experimental vials, split across four replicate populations, in order to measure pre-adult survivorship, as well as pre-adult development time. For this, we checked the assay vials every twelve hours once the first pupae started darkening. These checks were done starting on the 8^{th} day from egg collection, 12 hours apart. Once the first flies started eclosing (usually on the morning of the 9^{th} day), we transferred the flies to an empty vial (each of the 108 vials per replicate population had a corresponding transfer vial of similar dimensions). The flies were sacrificed by dousing the transfer vials in liquid nitrogen. Thereafter, they were removed to a clean white surface and counted. The checks were held 12 hours apart until day 12 from egg collection, following which they were instead conducted 24 hours apart. The vials were checked daily until all eclosion ceased.

The total number of adults eclosing from a vial, divided by the number of eggs added to the starting culture, was the measure of pre-adult survivorship per vial. The development time information of each fly was obtained by the total time taken from the mid-point of the parental egg laying interval to the mid-point of the time-window within which that individual eclosed.

Statistics

We carried out fully factorial mixed model Analysis of Variance (ANOVA) on each of pre-adult survivorship per vial and pre-adult development time (both mean and variance per vial).

Survivorship was also arcsine square root transformed and analysed to verify the results seen with non-transformed values.

All ANOVA were carried out on Statistica for Windows (Statsoft 1995). Egg number, food column height and vial cross-section surface area were treated as fixed factors with three levels each in the analysis, respectively. Additionally, day of experiment set up (day 14 or day 15 from egg collection) was also a fixed factor with two levels. Finally, population replicate number was treated as a random block factor with four levels. Pairwise post-hoc comparisons were done using Tukey's HSD.

We also carried out linear regression of the pooled data on pre-adult survivorship, mean pre-adult development time, variance of pre-adult development time and coefficient of variation of pre-adult development time, respectively. These were each treated as response variables against either total density or effective density as predictor variables (see results section).

The plotting of data was done on R release 4.2.2 (R Core Team 2022), using ggplot2 and tidyverse packages (Wickham 2016; Wickham et al. 2019).

Results

Main effects

Each of the three factors of interest – egg number, column height and surface area had a significant main effect on pre-adult survivorship, as revealed by the ANOVA (table s1). On an average, increasing the number of eggs, as well as decreasing the volume of food through reduction in either surface area or column height decreased pre-adult survivorship (fig. s1a-c). Although all three effects were significant for survivorship (table s1), the magnitude of change of each outcome with the factors was different. While large changes in survivorship were seen

across the three levels employed for egg number (fig. s1a) and surface area (fig. s1b), column height induced relatively smaller changes in survivorship with changing levels (fig. s1c).

The pattern for mean pre-adult development time differed based on the factor tested, although the ANOVA revealed a significant main effect for each factor (table s2). In case of egg number, mean pre-adult development time decreased with decreasing egg number (fig. s1d). For surface area, decreasing levels resulted in an increase in mean development time (fig. s1e). However, for column height, decreasing levels saw a small decrease in mean development time (fig. s1f). Thus, averaged over all other factors, an increase in crowding through reduced column height resulted in a slightly shorter development time. It should, however, be noted that the extent of differences in mean development time induced by the changing column height were relatively minor as compared to those induced by changes in both egg number and surface area. Variance of development time showed similar patterns as the mean (fig. s1g-i); however, there was no significant main effect of food column height (fig. s1i)

Comparisons of cultures with the same egg number and food volume in containers of different diameters

Different patterns of development time via increasing surface area vs. column height indicated some fundamental difference in how larval crowding was experienced as a result of reduced food volume achieved by these two modes. This was further explored by looking at subsets of comparisons for the three-way interaction of egg number × column height × surface area. These interaction effects were statistically significant for all three outcomes of crowding investigated (tables s1, s2, s3).

Subsets of comparisons were made among cultures with the same food volume and egg number, but across different vial diameters. As seen in figure 1C, the food volumes of 2, 3 and 6 mL are present in two culture types – one with larger surface area and reduced column height, and the

other with smaller surface area and a greater column height. Thus, for each of the three egg numbers used – 200, 400 and 600 – there are two cultures at 2 mL, 3 mL and 6 mL respectively, with not just the same overall density, but the exact same combination of egg number and food volume, achieved via different combinations of surface area and column height.

Figure 2 shows the mean pre-adult survivorship and mean pre-adult development time in the contrasting cultures of both 2 mL (fig. 2A and 2C) and 3 mL (fig. 2B and 2D) across increasing egg numbers. In case of the 2 mL cultures, when increasing the starting egg numbers, the culture with the narrow surface area and greater height (coloured blue) showed a greater loss in survivorship (fig. 2A) and a greater increase in mean development time (fig. 2C), compared to the culture with the greater surface area and shorter column height (coloured orange). These differences between the 'blue' and 'orange' cultures were further exacerbated at the 3 mL food volume (fig. 2B and 2D).

Similar results were also seen in the case of 6 mL food, although the differences were relatively smaller in magnitude, and often non-significant (fig. s2a, s2c). The variance in development time also showed differences, very similar in pattern to the average development time, in most cases (fig. s3a, s3b, s3c). Additionally, we have also plotted the data for 1 mL, 4 mL and 9 mL cultures for survivorship (fig. s2b), as well as the mean and variance in development time (fig. s2d, s3d).

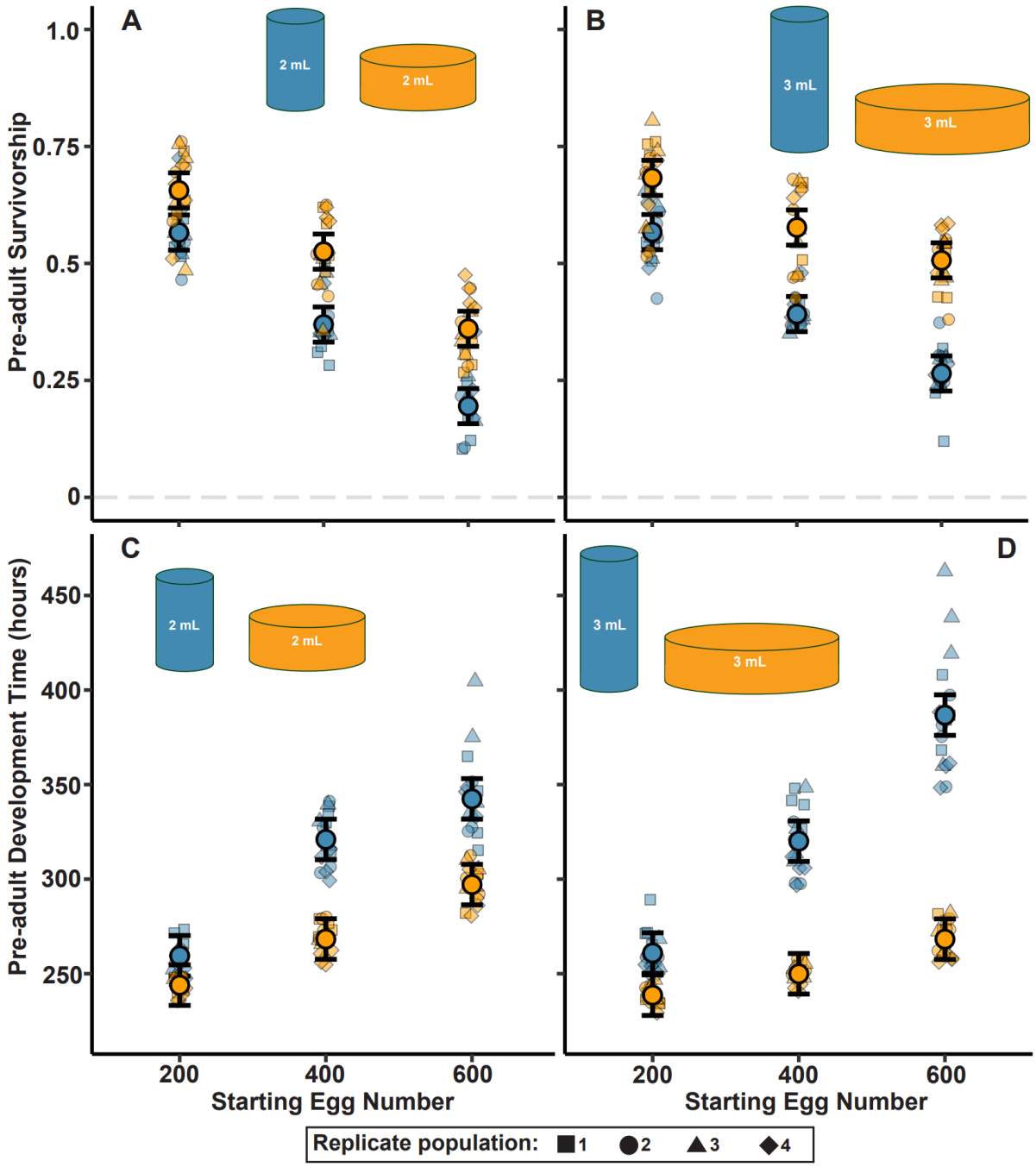

Figure 2. Mean pre-adult survivorship and mean development time at the same food volume. The food volumes and shapes into which they are cast are subsets of the experiment design showed in fig. 1C. In the current figure, A, C denote 2 mL; B, D denote 3 mL starting food volume. The error bars denote 95% C.I. around the overall means for the respective traits at each egg number, for the Tukey's post-hoc test.

Larval feeding band, total density and effective density

Our results quite clearly showed a large difference in survivorship and development time – and hence the underlying fitness functions – of two cultures that had the same egg number and food volume, with the main differentiating factor likely to be the surface area and column height that the food was cast into. To further investigate the possible role of surface area and column height, we focused on the larval 'feeding band' in highly crowded cultures. We define the feeding band as the volume of food close to the surface, with access to air, to which larval feeding is usually restricted (fig. 3A), implying that the maximal depth of food that a larva can access is roughly equal to the length of the larva. Consequently, relatively shallow food columns should allow the larvae to access most of the food (fig. 3Aiii), whereas, high food columns that extend beyond the feeding band are likely to have some food at the bottom that would not be accessible by the larvae until the top layer of starting food is sufficiently depleted (fig. 3Ai). Thus, two cultures with the same food volume that differ in surface area and food column height, may have very different volumes of initially accessible vs. inaccessible food, assuming similar feeding band depths. This is likely what we see in our experimental design, with food volumes of the 2, 3 and 6 mL combinations (fig. 2, s2). For example, in the 3 mL culture contained within the relatively narrow vial (~200 mm^2 surface area), the food column was relatively high (~15 mm) (fig. 2B, 2D; 'blue' cultures). In contrast, for the 3 mL culture contained within the wider vial (~600 mm^2 surface area), the food column was much shorter (~5 mm) (fig. 2B, 2D; 'orange' cultures). Given the shallow depth of food in the wider vial, larvae could possibly access a lot more of the food at any given time, compared to larvae in the narrow vial which might not be able to penetrate to the depths of 15 mm.

For the following distinction between total vs. effective density, we assumed the feeding band depth to be a constant 6 mm, with the caveat that the actual feeding band depth is likely to be a more complex parameter (see Discussion). In addition to the total density (eggs per unit food

volume in the vial), we define a new term – effective density – calculated as the number of eggs per unit volume of the feeding band. The effective density better reflects the actual crowding experienced by feeding larvae in the upper 6 mm of the food column.

We then calculated the total and effective density per culture combination for each of the 27 cultures, as shown in fig. s4. Cultures with short food column height, which have the feeding band volume equal to the total food volume end up having the same total and effective densities. Cultures with longer food column heights have feeding band volume as a subsection of the total food (up to 6 mm deep), and thus have higher effective density than total density. For the 3 mL food example, the wider vial has a feeding band that provides access to the entirety of the food (fig. 3Bv), whereas the 3 mL food in the narrow vial has only ~1.2 mL food accessible in the feeding band (fig. 3Biv), thereby leading to a far higher effective density for each given egg number (fig. s4).

Additionally, the calculated total and effective density for each treatment combination yielded multiple cultures which had the same total density, but different effective densities. Three levels of total density had at least 5 different representatives each. These were: 200 eggs/mL food; 100 eggs/mL food and 66.67 eggs/mL food (fig. 3B, s5A, s5B, colour matched). For example, the first case, a total density of 200 eggs/mL food, could be obtained in the following scenarios:

1) 200 eggs in 1 mL food (fig. 3Bi).
2) 400 eggs in 2 mL food in ~200 mm^2 surface area, 10 mm column height (fig. 3Bii).
3) 400 eggs in 2 mL food in ~400 mm^2 surface area, 5 mm column height (fig. 3Biii).
4) 600 eggs in 3 mL food in ~200 mm^2 surface area, 15 mm column height (fig. 3Biv).
5) 600 eggs in 3 mL food in ~600 mm^2 surface area, 5 mm column height (fig. 3Bv).

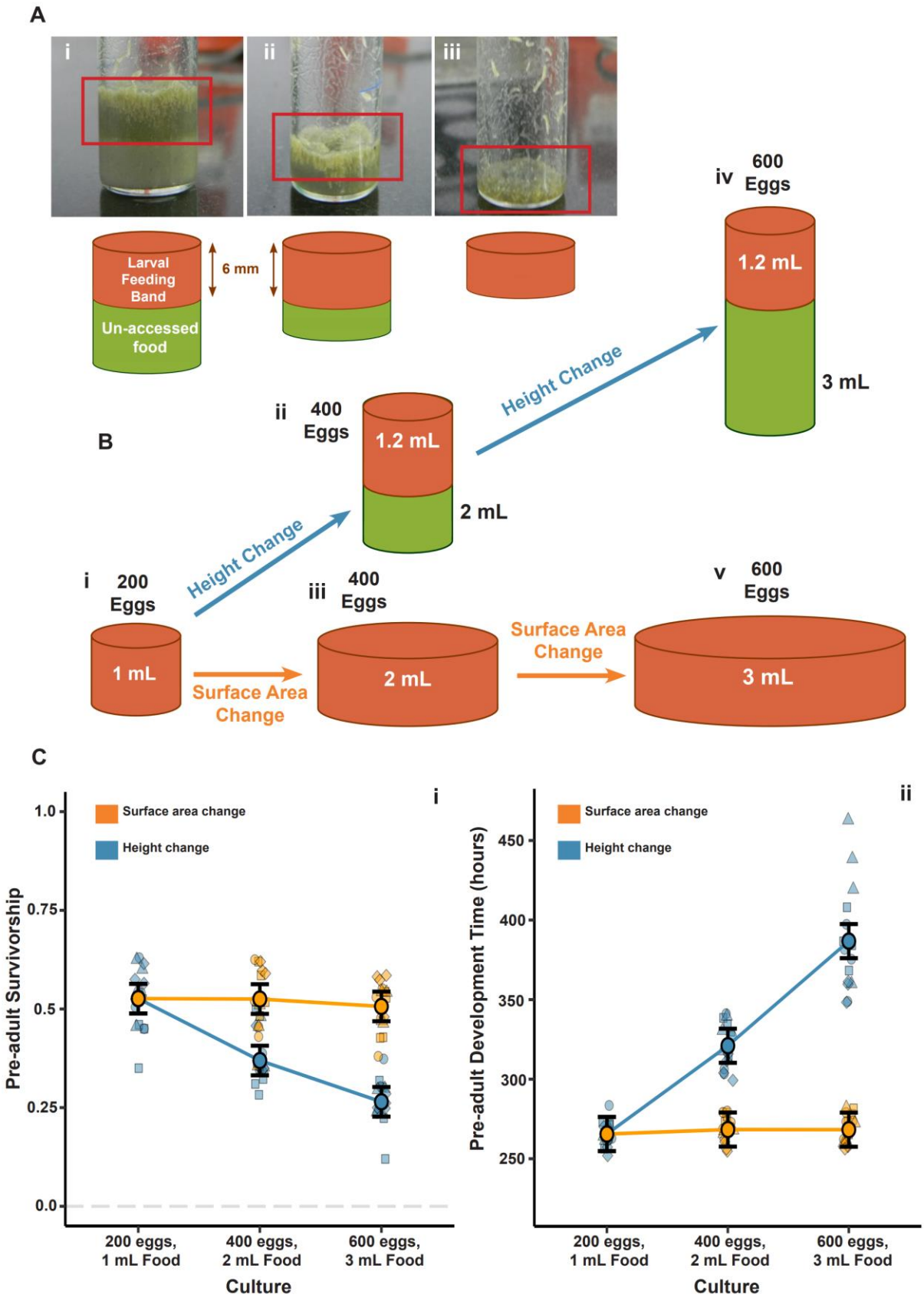

Figure 3. A. The presence of the feeding band and its diagrammatic representation across three food column heights. In case of tall food columns (i and ii), the food at the bottom in not accessed by larvae

until the top layer of food is depleted. In shallow food columns (iii), the entire food volume comprises the feeding band. B. For a total eggs/food density of 200 eggs/mL, there are 5 ways to achieve it by altering egg and food quantities, in our experimental design (i-v), as shown in the diagram. If food column height is increased (ii, iv), the feeding band is restricted to 1.2 mL food, thus changing the effective density in the feeding band (fig. s4). In case surface area is increased (iii, v), keeping a shallow food column, the total density and effective density are the same (fig. s4). C. The outcomes of crowding at a total density of 200 eggs/mL in 5 different ways as listed in B. (i) shows pre-adult survivorship; (ii) shows pre-adult development time (hours). The orange line represents changing surface area, keeping total density and effective density the same. The blue line represents changing food column height, which results in effective density differing from total density. Error bars represent 95% C.I. around the overall mean for a given culture and vial type, for each respective trait, as calculated from Tukey's post-hoc test.

Similar comparisons for the cases with 100 eggs/mL and 66.67 eggs/mL can be found in supp. figure s5A, s5B, respectively.

The results show that cultures wherein the total and effective density were the same (1, 3, 5 from list above), had very similar pre-adult survivorship as well as both mean and variance of pre-adult development time (fig. 3C, s6). When effective density was different from total density, there were large differences in the pre-adult survivorship, and also in the mean and variance of pre-adult development time (fig. 3C, s6). Similar results were seen for the other total densities as well (fig s5A, s5B).

Linear regressions: total vs. effective density

To further examine the role of total vs. effective density in mediating competitive outcomes, we also performed linear least-squares regressions by pooling all treatments, with either total density or effective density as the predictor variable. The response variables were either pre-

adult survivorship, mean development time per vial, or variance/coefficient of variation in development time per vial. The results can be seen from the table in fig. 4, wherein the R^2 values for both regressions are shown for each response variable. The fitted lines are plotted in fig. s7.

For each of the traits examined, a linear model using effective density substantially better predicted the response variable than total density. This showed that simple linear models with effective density were better predictors of the outcomes of competition.

Least-squares Regression of Fitness-related trait (Response variable)	Total density as predictor variable *R*2 value	Effective density as predictor variable R^2 value
Pre-adult survivorship	**0.66**	**0.82**
Pre-adult development time	**0.31**	**0.74**
Variance in pre-adult development time	**0.33**	**0.64**
Coefficient of variation in pre-adult development time	**0.41**	**0.60**

Figure 4. Goodness-of-fit (R^2) values for linear regressions performed by pooling the entire experimental data for each of the outcomes of larval crowding studied as response variables. The predictor variables are either total eggs/food density (left column), or effective (larval feeding band) density.

Discussion

We conducted a three-way, fully-factorial experiment on implementing larval crowding in different ways and found differences in density-specific fitness functions of cultures with the same egg and food combination, in different cylindrical dimensions. Across nine pairwise comparisons of the same combination of eggs and food volume, 7 out of 9 showed differences in pre-adult survivorship (fig. 2A, 2B, s2a), and 5 out of 9 comparisons showed differences in mean pre-adult development time (fig. 2C, 2D, s2c). In each case where the comparison yielded a significant difference (and some in which there was a non-significant difference), the culture which had a greater surface area of food in contact with air, with a reduced food column height, had greater survivorship than the same culture cast in a relatively lesser surface area and a greater food column height (fig. 2A, 2B, s2a). Similarly, the cultures with greater surface area and reduced column height also had shorter mean development time, where significant, than their respective counterparts with lower surface area and greater column height (fig 2C, 2D, s2c). These results are also in agreement with the study by Sang (1949). Variance in development time largely followed the same pattern as mean development time (fig. s3a-c).

In order to find out why there were such stark differences simply by property of culture dimensions, we looked at the larval feeding band, assuming a depth of 6 mm as a simplified representative of the depth to which larvae could potentially feed at all times. We thus calculated effective density (initial number of eggs/feeding band volume) for each of the 27 culture types (fig. 1C, s4), in addition to the total density (number of eggs/total food volume), for each culture. We found that while up to five types of cultures could have the same total density by virtue of their eggs and food combinations, their survivorship and development time outcomes were only similar if the effective density was equal to the total density (fig. 3, s5, s6). These strikingly similar outcomes were achieved via an increase in surface area, which kept the total and effective density the same. Additionally, the linear regression of every

outcome of larval crowding examined yielded a higher R^2 value when effective density was the predictor variable, as opposed to total density (fig. 4). Overall, these results indicate that effective density plays a far more important role in shaping the outcome of larval competition than total density, despite the latter having been the benchmark for larval crowding till date.

Even in terms of the eclosion profiles, effective density seemed to be very important. The eclosion distribution increases in variance at high effective densities coupled with long food columns (fig. 5, s6). At shallow depth of food, eclosion distributions are generally less spread out (fig. 5).

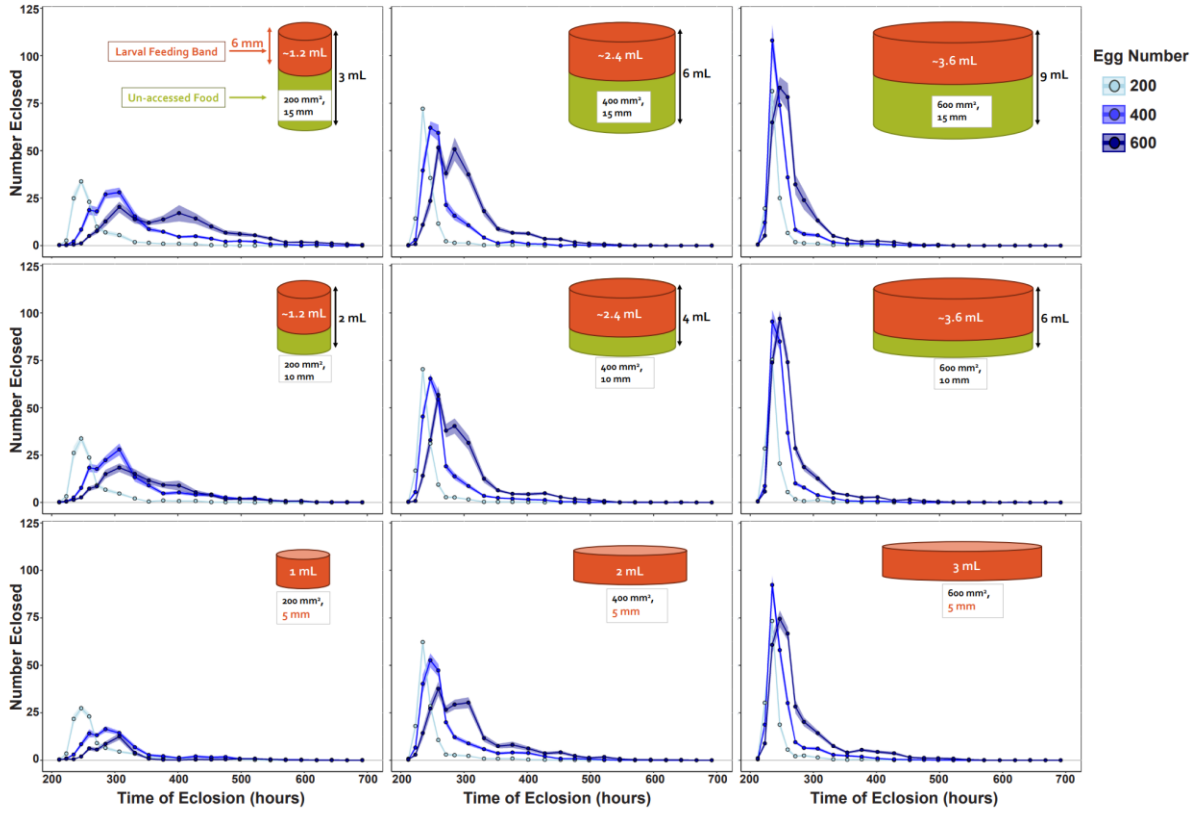

Figure 5. Distributions of time of eclosion of adult flies for each of the 27 treatments used in the current study. The representative culture diagrams are as shown in fig. 1C, with derived feeding band volumes as shown in fig. s5.

Our results also indicate that beyond effective density, the overall volume and depth of the food column may also help determine the ultimate fate of a crowded larval culture. This is apparent in the comparison of 600 eggs in 2 mL vs. 600 eggs in 3 mL food, cast into 10 mm and 15 mm column heights, respectively (200 mm^2 surface area each). While both cultures have the same calculated effective density of around 500 eggs per mL of feeding band (fig. s4), the same effective density in a 15 mm food column (3 mL total food) has significantly higher development time (fig. 2) and development time variance (fig s3a, s3b), and a higher, though non-significantly so, survivorship (fig. 2), than in a 10 mm food column (2 mL total food). This does not occur in any other scenario wherein the effective density is the same between two cultures. Thus, for two cultures having the same (high) effective densities, a greater volume of food – achieved via increased food column height – may lead to an average delay in development time along with an increase in variance, perhaps at the benefit of a slight advantage in survivorship. This needs to be explored further, with greater effective densities tested at several food levels beyond those used in the current study.

Due to logistical limitations, the measurement of a 6 mm deep feeding band is a necessary simplification of a complex phenomenon. An early study on larval length of *D. melanogaster* ascertained the maximum measurement to be around 4.5-4.6 mm, which is well within the 6 mm cut-off (Alpatov 1929). We also note that in his studies, Sang (1949) had discussed the existence of the feeding band as well, defining it at 5 mm deep. Given that 5-6 mm is the longest depth to which maximally grown larvae can extend (notwithstanding digging), we assumed 6 mm to be the liberal estimate of the feeding band depth. At the start of the culture, the freshly hatched larvae would be expected to feed in a much shallower feeding band than 6 mm. The feeding band would increase as the larvae grow and gain longer lengths and greater reach. Thus, assuming a depth of 6 mm as constant, any differences captured at this simple calculation are likely to be exacerbated with more precise measurements. Moreover, such precise

measurements may be difficult to carry out – realistically, the larval feeding band is likely to be complex over space and time. Spatially, the larvae tend to cluster themselves into feeding grooves near the surface, rather than spread themselves out uniformly over the feeding band (previously observed by Gilpin 1974, Chippindale et al. 1994, Mueller and Barter 2015, S. Venkitachalam, personal observation; also see Gregg 1990). Variable digging length may also be a possibility, although in overcrowded feeding clusters, the larvae could face the risk of drowning due to spatial constraints. The effective larval density of a culture would itself be dynamic and changing throughout the life of the culture, as the larvae grow, drown or leave the culture. This would make the prospect of exactly calculating the feeding band depth or the effective density very difficult.

Given that this study has formally added a new descriptor of larval crowding, it is important to apply this insight to any future studies that aim to implement any form of high density to larval cultures. It becomes extremely relevant to list out not just the number of starting individuals and the total food volume (as well as the type of food used), but also the details of the container – the best practice would be to list the effective density or the average food column height and the surface area of food with access to air. Even in case of studies in nature (Atkinson 1979; Grimaldi and Jaenike 1984; Morimoto and Pietras 2020), this finding is likely relevant. We cite the current study as a cautionary note against interpreting larval number in a whole fruit as a measure of density experienced by the larvae. The density of larvae in a fruit is likely to vary across its dimensions, creating local zones of high and low effective densities within a single fruit itself. Furthermore, unlike in controlled vial conditions in the laboratory, factors such as larval movement, head starts due to differential hatching time or oviposition timings, different hardness of rotting fruits across their respective volumes, to name a few among myriad factors, are likely to create nuances in the competition conditions experienced within local zones per given fruit. This would be in addition to other confounds such as resource overlap and

competition with other species, predation, parasitism, desiccation, disease etc. We could thus imagine a natural food source in the wild as a kaleidoscope of ever-changing effective densities and micro-environmental conditions.

Future studies on larval crowding in *D. melanogaster* across research labs worldwide should ultimately test the inferential reach of our findings, although we suspect that major exceptions to our results will be rare (and interesting). Besides J.H. Sang, who found similar results in very different strains, another study made note of the effects of surface area on larval competition (Scheiring et al. 1984). Several other studies discussed the possibility of the effects of surface area on the outcomes of larval crowding (Bakker 1961; Gilpin et al. 1976; Chippindale et al. 1994). The literature on larval crowding is also populated with references to un-accessed food at the bottom of high-density cultures with relatively high food volumes (Gilpin 1974; Bierbaum et al. 1989; Mueller 1990).

In earlier studies, *D. ananassae* and *D. nasuta* populations showed similar adaptations to larval crowding in a shallow depth of food with low food volume as *D. melanogaster* (Nagarajan et al. 2016, Sarangi et al. 2016). It remains to be seen whether they would follow similar patterns with respect to the predictability of crowding outcomes by effective density, as seen in the current study. Finally, there have also been findings of the effects of surface area in larval systems from other holometabolous insect clades, such as the moth species *Ephestia cautella* (Smith 1969), *E. kuehniella*, *E. elutella* and *PIodia interpunctella* (Bell 1976), as well as the flour beetles *Tribolium castaneum* (Wool 1969). These studies indicate that feeding-band-like features may exist across several insect groups, although the exact consequences of such a feature may depend on the biology of the species being crowded. However, this sets up the background for potentially interesting future studies that can explore the complex aspects of larval competition and crowding across species.

Mechanistically, it is likely that constrained space in the feeding band at high effective densities may be one major factor driving the outcome of *Drosophila* larval competition. In high food columns with small surface area, most larvae may be unable to access food beyond the depth of the feeding band. Moreover, a large number of larvae trying to feed in a constrained space likely leads to space limitation, despite potentially large volumes of food remaining uneaten at the bottom of the food column. On the other hand, high food columns may have the possible benefit of the diffusing of harmful metabolic waste products away from the feeding band and into the depths of the yet-inaccessible food (Sarangi 2018). This may explain the large variance in egg to adult development time in 600 eggs at 3 mL food at the 15 mm food column length (fig. 3Civ). Under such high effective densities, some space constrained larvae may survive on low amounts of food until most other competitors have left the food, drowned or been poisoned due to high waste levels. Thereafter, these surviving larvae could resume feeding (provided they can tolerate the elevated waste levels), leading to an extended developmental period. Alternatively, some larvae may feed faster and dig deeper into the un-accessed food, leading to a faster development time. Both may be viable strategies, but while the former scenario would delay developmental time, the latter scenario would select for faster digging, feeding and growth. This would ultimately lead to a culture with both increased mean and variance in development time. Our reasoning is also in agreement with some previous studies, which implemented larval crowding at approx. 1500 eggs in 6 mL food cast in high food columns with relatively narrow cross section surface area (Borash et al. 1998; Mueller et al. 2005*a*; Mueller and Barter 2015). Thus, these studies inadvertently used high effective density cultures with long food columns, achieving very large variance in development time. The authors found that early eclosing flies had offspring with increased feeding rate while late eclosing flies had high waste tolerance, as compared to the individuals of the non-selected control populations (Borash et al. 1998). In contrast, in high effective density cultures with shallow food column

heights, the food would run out much faster, due to a large number of larvae accessing all of a limited quantity of food. Space constraints might be secondary to food constraints, leading to a relatively truncated feeding period, thus reducing both the mean and variance of the pre-adult development time. This is likely what was observed in later selection studies on chronic larval crowding in *D. ananassae*, *D. nasuta* and *D. melanogaster* (Nagarajan et al. 2016, Sarangi et al. 2016). Most crowded cultures used in laboratories, and perhaps even in nature, likely lie between these two extreme conditions, often leading to a lack of consensus on the effects of crowding and competition.

Several avenues can be explored for future studies. With the experimental design used in the current study, we can also potentially obtain dry weight data for freshly eclosed adults, and map dry weight distribution over time for each culture type. Both mean and variance of dry weight per vial can be measured, as was done for pre-adult development time in the current study. The body weight of emerging flies is one of the most sensitive indicators of the existence of competition (Sang 1949; Bakker 1961; Santos et al. 1997). Moreover, studies have indicated that elongated development time distributions can give rise to differences in adult weight depending on the time of eclosion (Hughes 1980; Sarangi 2018; Chapter 2). This would be especially relevant for comparisons between cultures which have widely varying development time distributions (fig. 5).

We also know that populations adapted to high effective density cultures in shallow food column depths (total density similar to effective density) differ in evolutionary trajectory from populations adapted to high effective density in longer food columns (total density differing from the effective density) (Sarangi et al. 2016, Sarangi 2018, Chapter 3). Given the framework we have established, we can test whether these two kinds of population sets differ in their competitive ability depending on the type of culture used. A recent competitive ability experiment carried out by us tested each of the different crowding adapted population sets in

varying volumes of food (with shallow food column depths), and the results indicated the existence of culture-dependent competitive ability (Chapter 2).

Finally, starting new populations within this factorial framework would allow us to adapt populations to very different culture ecologies while keeping the same egg number and food volumes. While logistically intensive, this would be the best test for whether different crowding ecologies can lead to specialised competitive ability i.e., populations exhibiting higher competitive ability under the precise conditions in which they experienced crowding. It may be possible that greater digging distance may be important in competing in high effective density cultures with higher food columns. However, a previous study did not find differences in average digging length in populations adapted to crowding under relatively large volumes of food (Mueller 1990).

In conclusion, we have described effective density as the density of larvae in the feeding band – a volume of food close to the surface of food with access to air, where the larvae feed. The effective density is a far better predictor of three fitness-related outcomes of larval crowding compared to the total density, which is the total number of eggs in the total volume of food of a culture. This finding has important implications for the design of future studies on larval competition in *Drosophila* and perhaps other clades as well.

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Supplementary material for Chapter 5

More than just density: the role of egg number, food volume and container dimensions in mediating larval competition in *Drosophila melanogaster*

Effect	*df*	MS	*F*	*P*
Eggs	2	2.5593	400.881	< 0.0001***
Surface Area	2	2.5436	222.149	< 0.0001***
Food Height	2	0.2061	62.624	< 0.0001***
Day	1	0.3686	6.740	0.0806
Surface Area × Food Height	4	0.0357	25.669	< 0.0001***
Surface Area × Day	2	0.0878	16.405	0.0037**
Food Height × Day	2	0.0045	0.824	0.4828
Surface Area × Eggs	4	0.1565	32.952	< 0.0001***
Food Height × Eggs	4	0.0197	6.552	0.0049**
Day × Eggs	2	0.0682	23.124	0.0015**
Surface Area × Food Height × Day	4	0.0004	0.166	0.9515
Surface Area × Food Height × Eggs	8	0.0090	3.425	0.0091**
Surface Area × Day × Eggs	4	0.0039	2.542	0.0944
Food Height × Day × Eggs	4	0.0029	1.264	0.3371
Surface Area × Food Height × Day × Eggs	8	0.0037	1.623	0.1704

Table s1. ANOVA results for mean pre-adult survivorship.

Effect	*df*	MS	*F*	*P*
Eggs	2	101652	351.39	<0.0001***
Surface Area	2	125726	468.79	<0.0001***
Food Height	2	2356	6.79	0.0288*
Day	1	1	0.00	0.9488
Surface Area × Food Height	4	4716	19.56	<0.0001***
Surface Area × Day	2	3	0.01	0.9868
Food Height × Day	2	0	0.00	0.9983
Surface Area × Eggs	4	11565	48.32	<0.0001***
Food Height × Eggs	4	2284	16.48	<0.0001***
Day × Eggs	2	70	0.90	0.4553
Surface Area × Food Height × Day	4	81	0.83	0.5330
Surface Area × Food Height × Eggs	8	2831	13.27	<0.0001***
Surface Area × Day × Eggs	4	74	0.37	0.8276
Food Height × Day × Eggs	4	137	1.23	0.3488
Surface Area × Food Height × Day × Eggs	8	165	1.34	0.2741

Table s2. ANOVA results for mean pre-adult development time.

Effect	*df*	MS	*F*	*P*
Eggs	2	282326582.8	367.422	<0.0001***
Surface Area	2	404285765.8	245.276	<0.0001***
Food Height	2	2363303.6	1.703	0.2595
Day	1	1116630.7	0.792	0.4389
Surface Area × Food Height	4	15152499.2	8.261	0.0019**
Surface Area × Day	2	87741.1	0.058	0.9441
Food Height × Day	2	1652457.8	1.041	0.4091
Surface Area × Eggs	4	42424753.5	25.181	<0.0001***
Food Height × Eggs	4	3231437.7	2.653	0.0852
Day × Eggs	2	2205768.4	4.350	0.068
Surface Area × Food Height × Day	4	850900.5	0.296	0.8751
Surface Area × Food Height × Eggs	8	10830484.9	4.206	0.0029**
Surface Area × Day × Eggs	4	684030.1	0.828	0.5326
Food Height × Day × Eggs	4	2843090.7	1.294	0.3266
Surface Area × Food Height × Day × Eggs	8	1979722.5	1.202	0.339

Table s3. ANOVA results for variance in pre-adult development time.

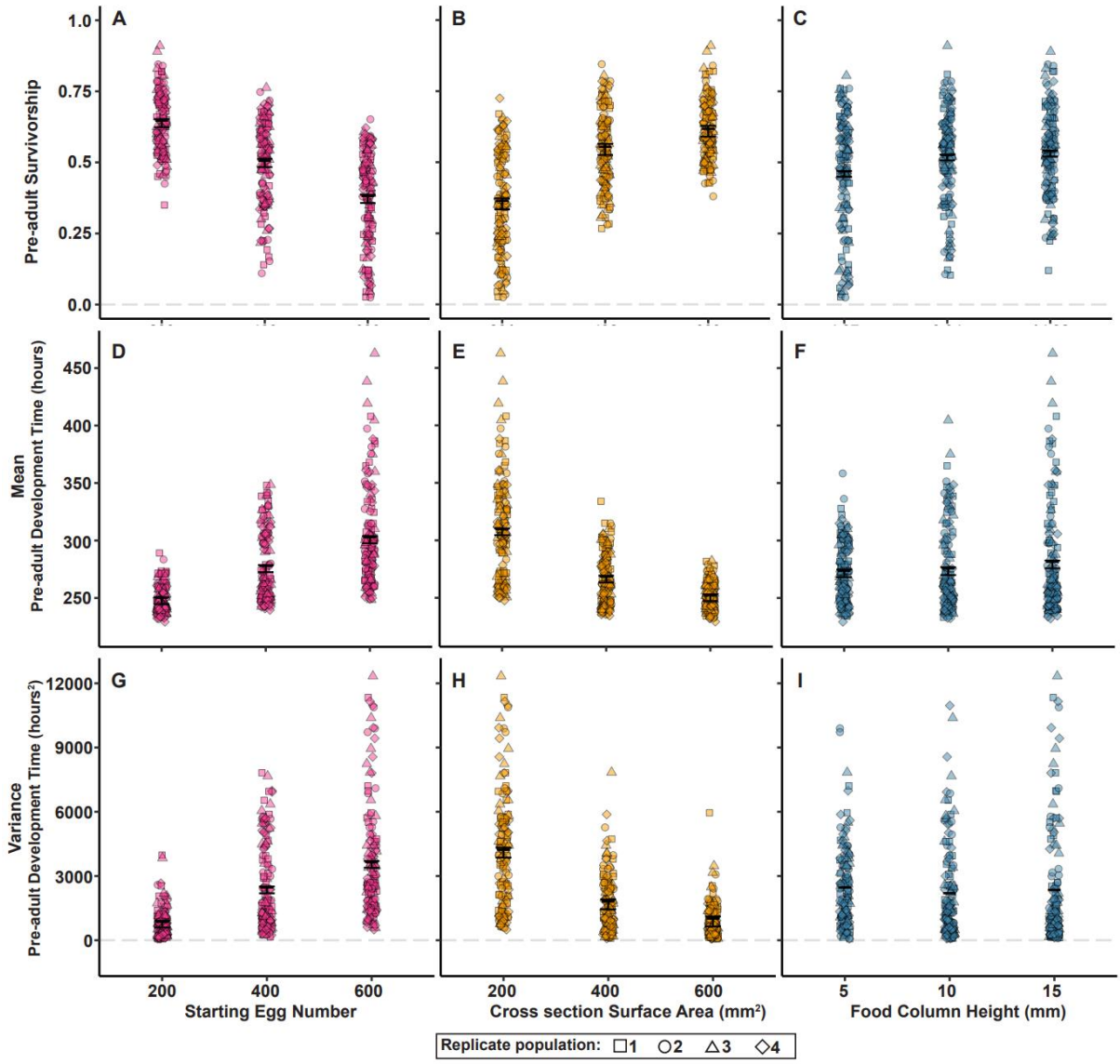


Fig. s1. Main effects of the three crowding-inducing factors (egg number, surface area and column height) on mean pre-adult survivorship (A, B, C); mean pre-adult development time (D, E, F); and variance in pre-adult development time (G, H, I), respectively. Error bars represent 95% CI around the means as calculated from Tukey's post-hoc test. No CI are shown in (I), as there was no significant main effect of column height on variance in development time.

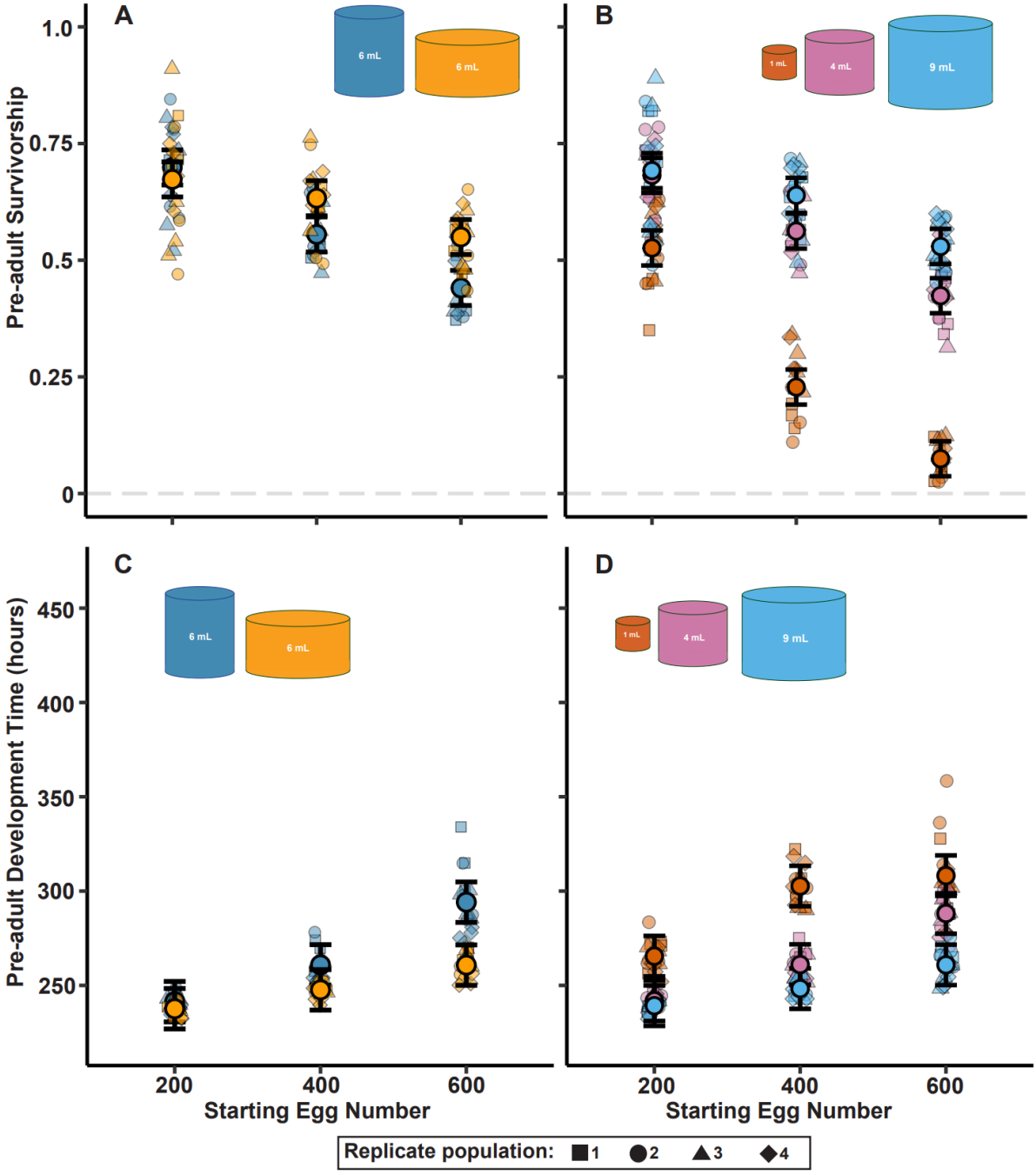


Figure s2. Mean pre-adult survivorship and mean pre-adult development time for (A, C) two variants of the 6 mL cultures; (B, D) Unique cultures of 1, 4 and 9 mL food volumes, for each starting egg number. Error bars represent 95% CI around the means as calculated from Tukey's post-hoc test.

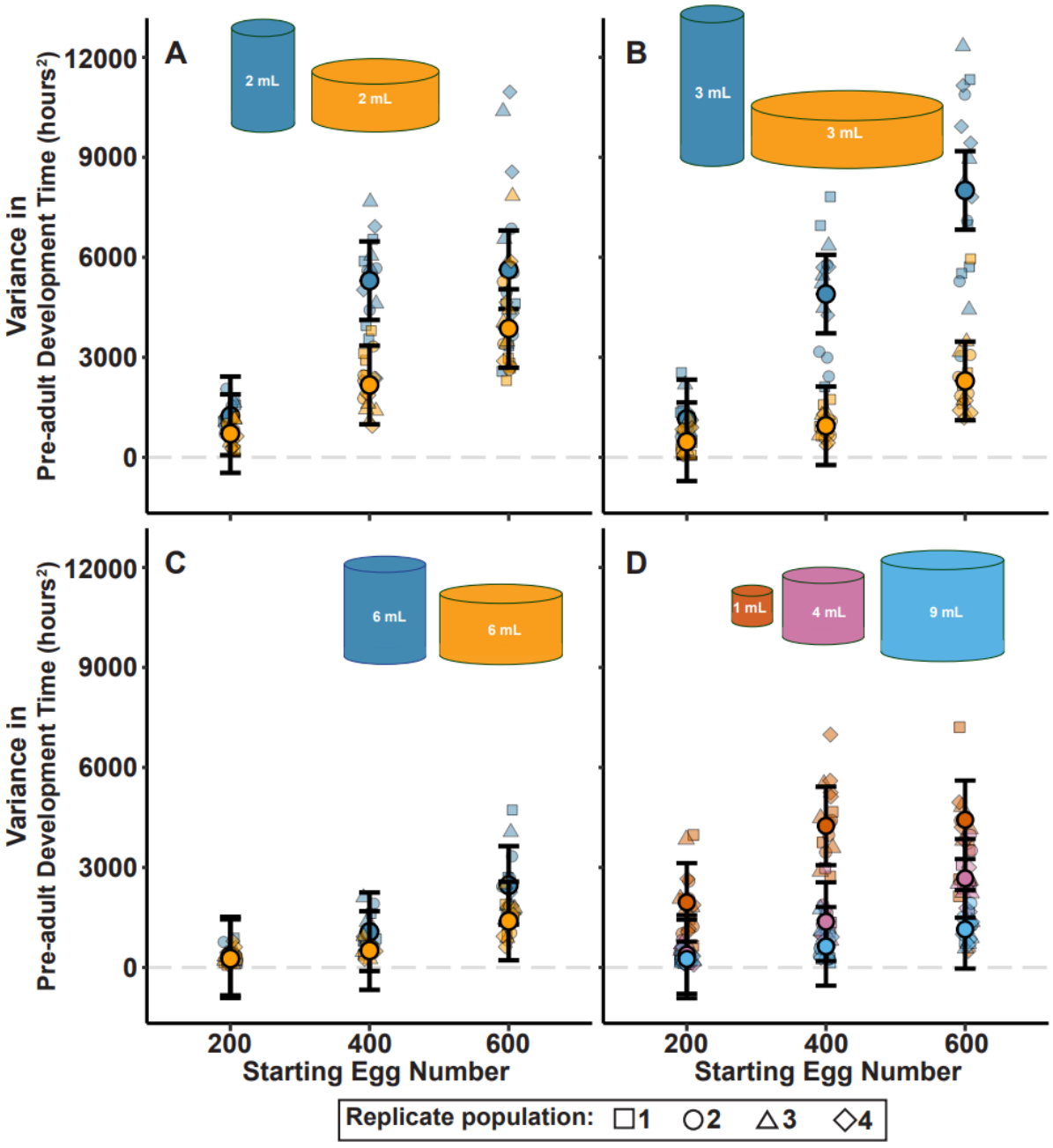
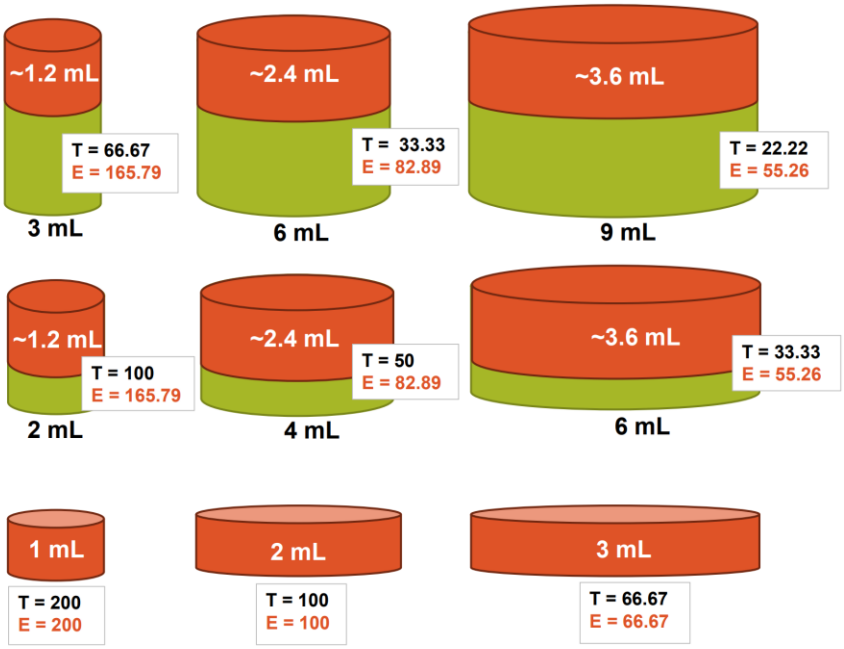


Figure s3. Variance in development time across 200, 400 and 600 eggs seeded in: A. 2 mL food cast in different combinations of surface area and column height; B. 3 mL food cast in different combinations of surface area and column height; C. 6 mL food cast in different combinations of surface area and column height; D. 1, 4 and 9 mL food, respectively. Error bars indicate 95% C.I. around the means, as calculated from Tukey's post hoc test.

200 eggs

T = Total Density (eggs/mL total food)

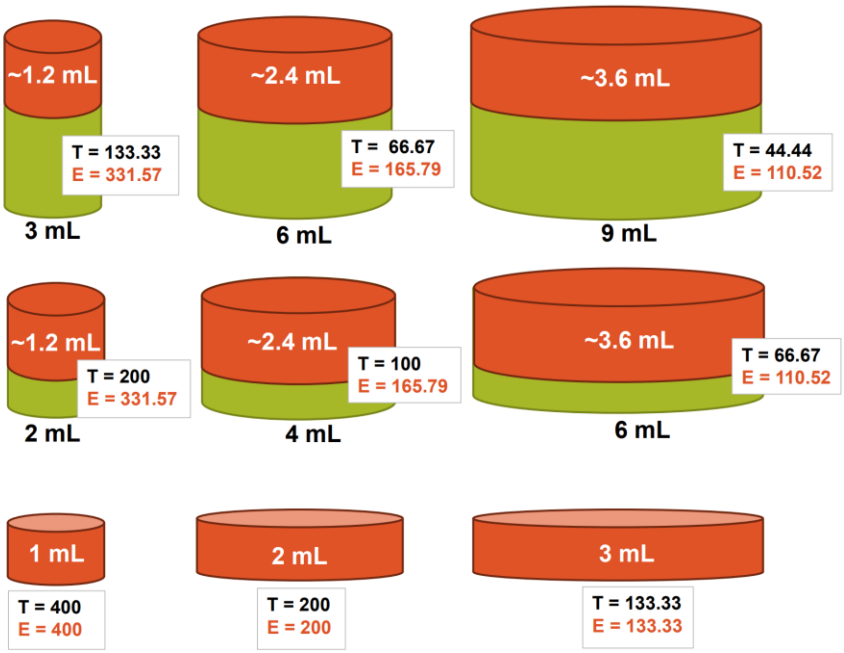
E = Effective Density (eggs/mL food in feeding band)



400 eggs

T = Total Density (eggs/mL total food)

E = Effective Density (eggs/mL food in feeding band)



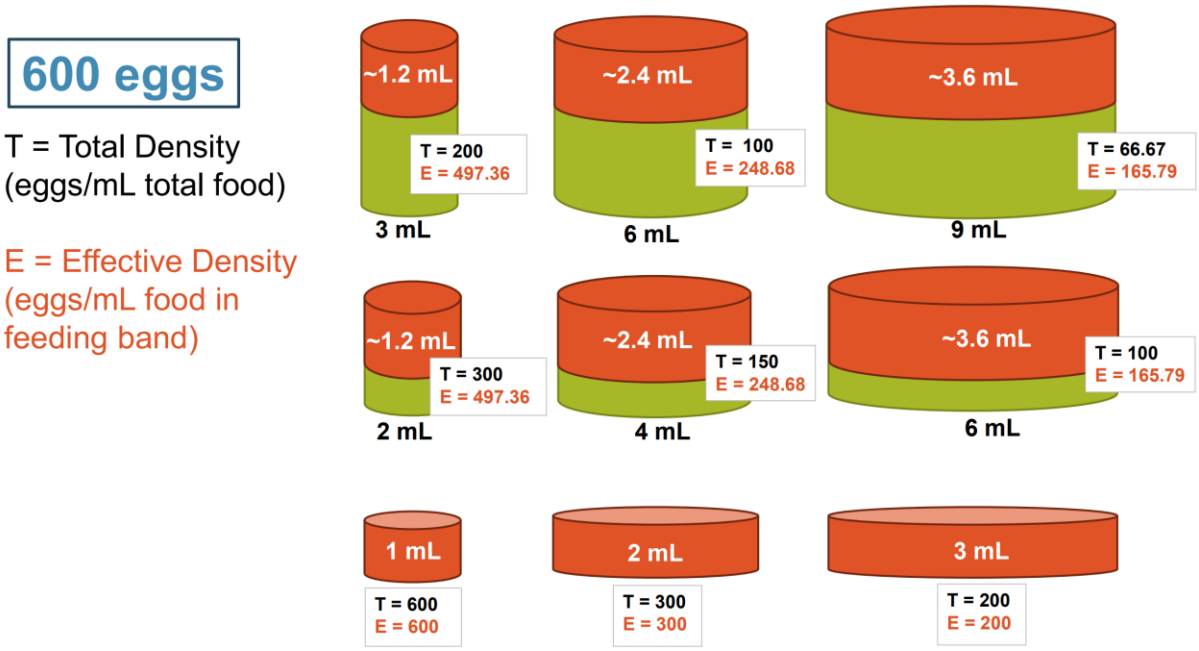


Figure s4. Total density and effective density for each of the 27 treatments used in the experiment.

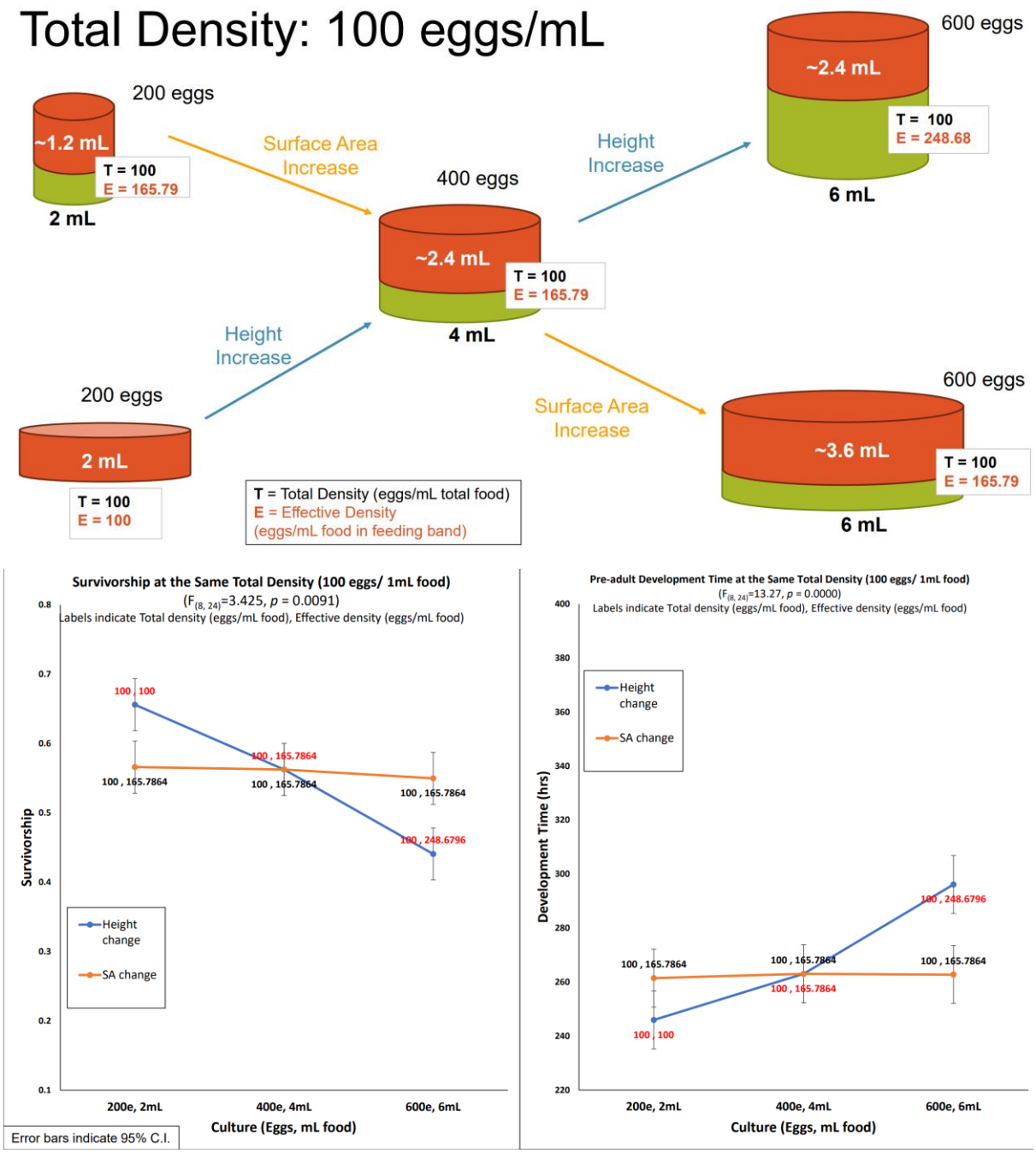


Figure s5A. Mean pre-adult survivorship and mean pre-adult development time for each culture with total density of 100 eggs/mL.

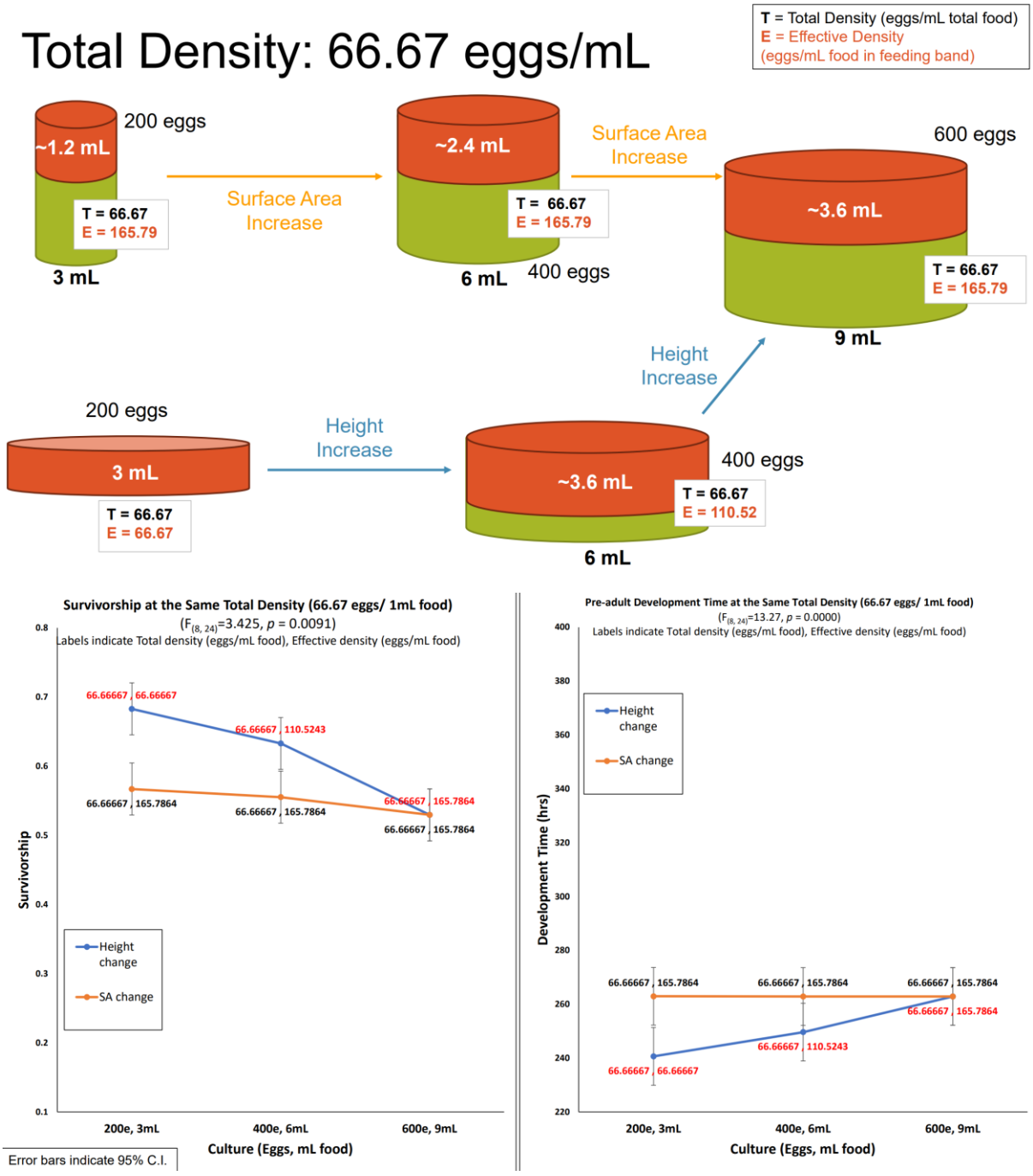


Figure s5B. Mean pre-adult survivorship and mean pre-adult development time for each culture with total density of 66.67 eggs/mL.

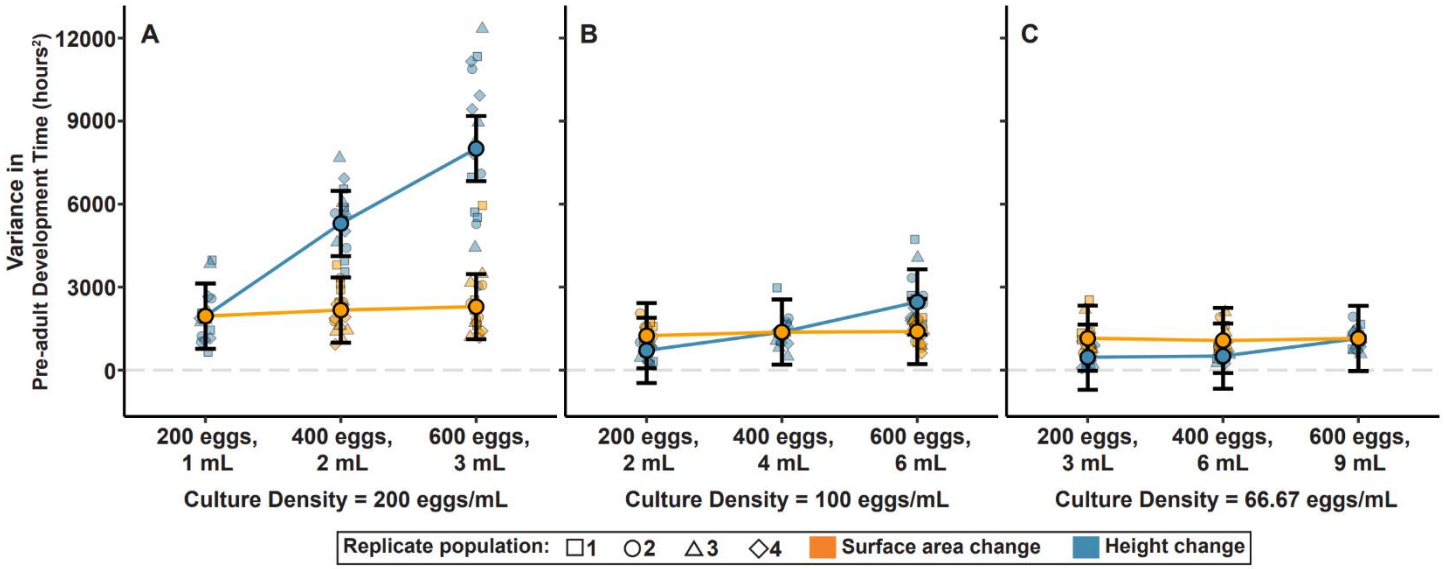
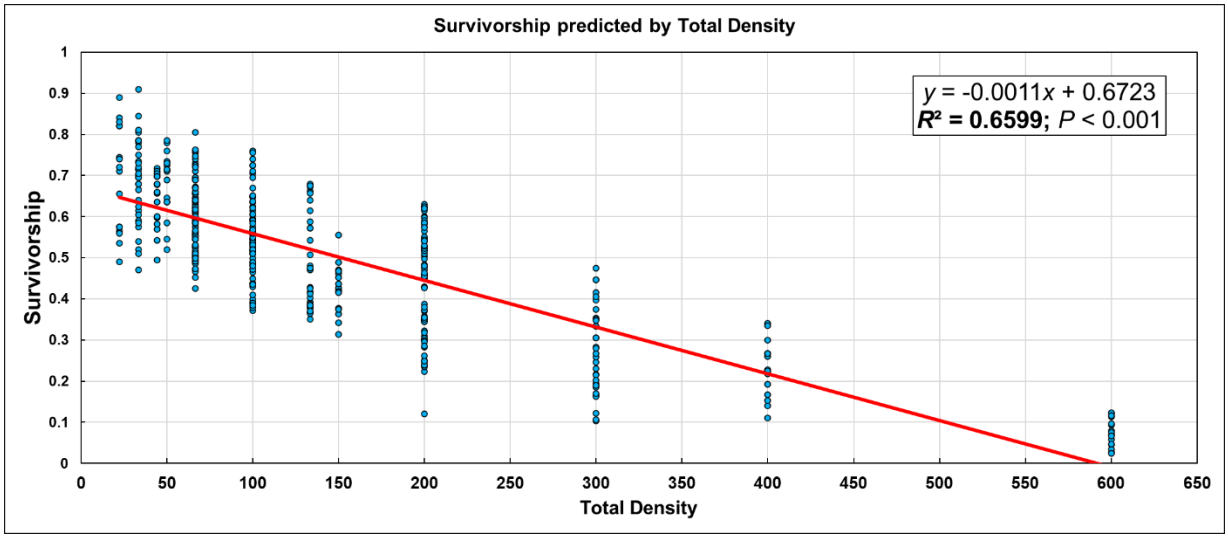
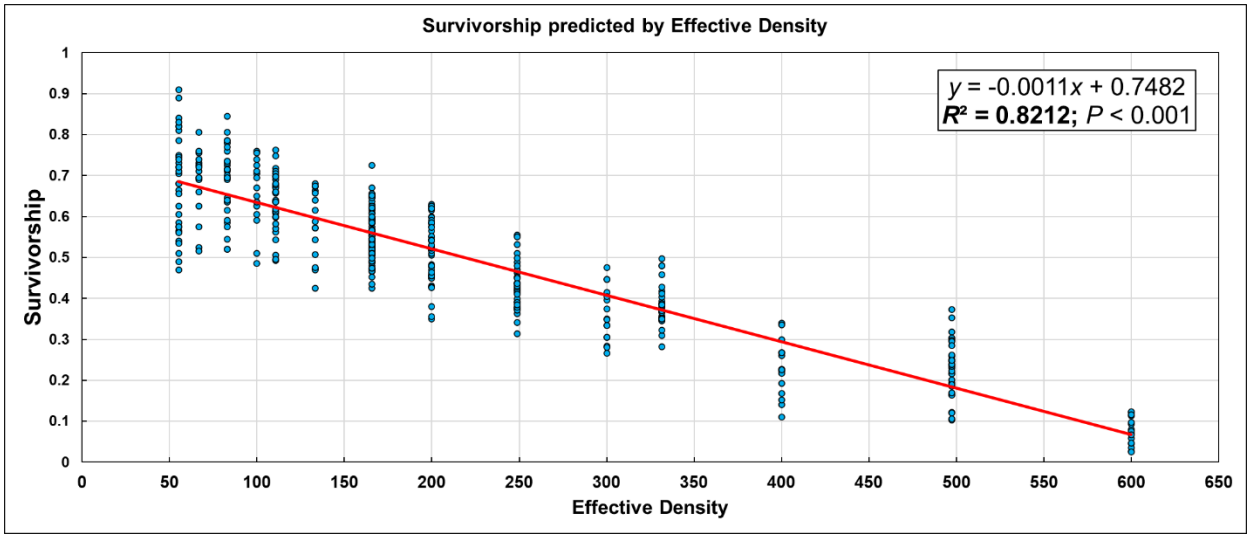
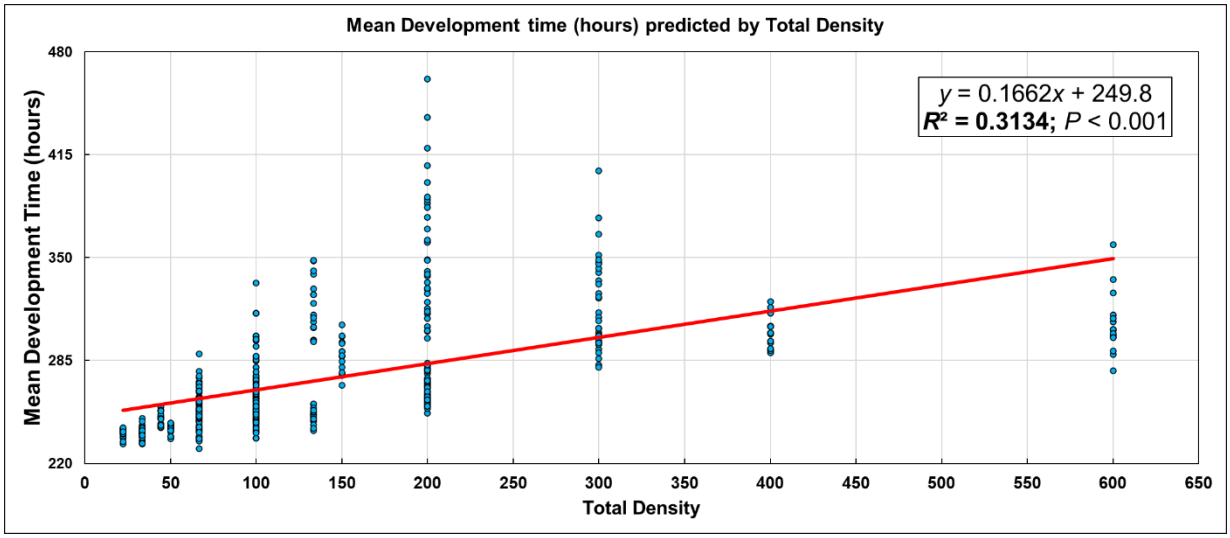
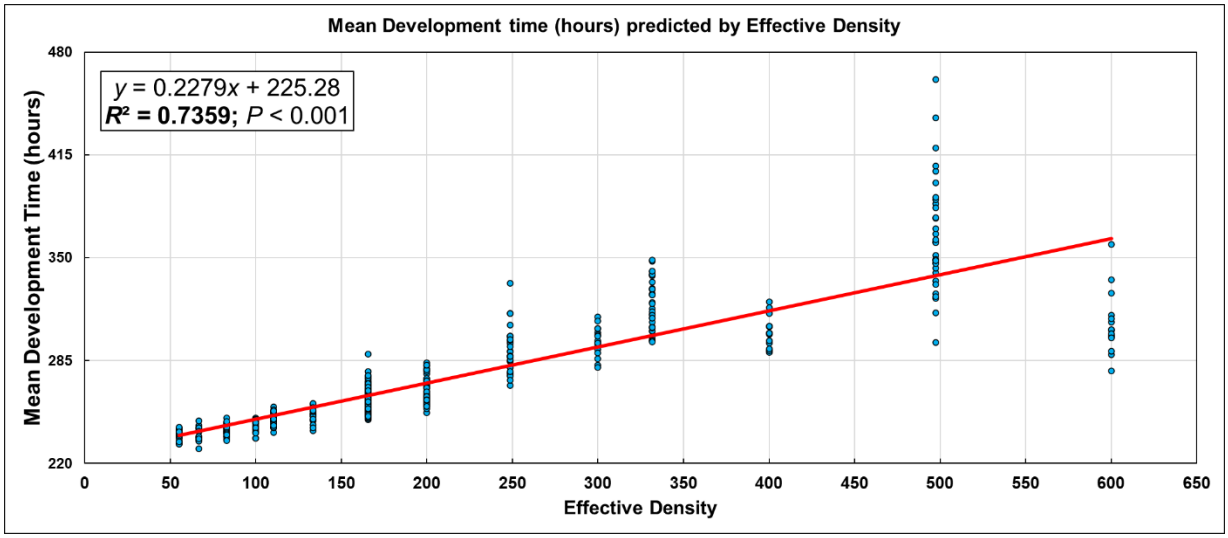


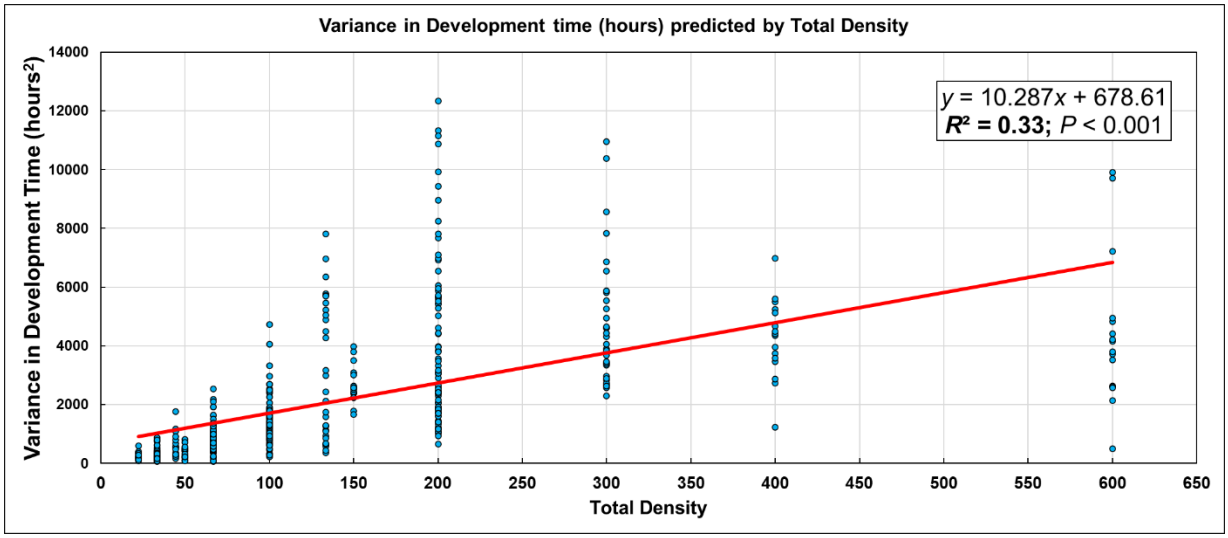
Figure 6. Variance in pre-adult development time for various total density scenarios (see fig. s4): A. Total density of 200 eggs/mL (see fig. 3C); B. Total density of 100 eggs/mL (see fig. s5A); C. Total density of 66.67 eggs/mL (see fig. s5B). Error bars indicate 95% CI around the means, as calculated from Tukey's post hoc test.

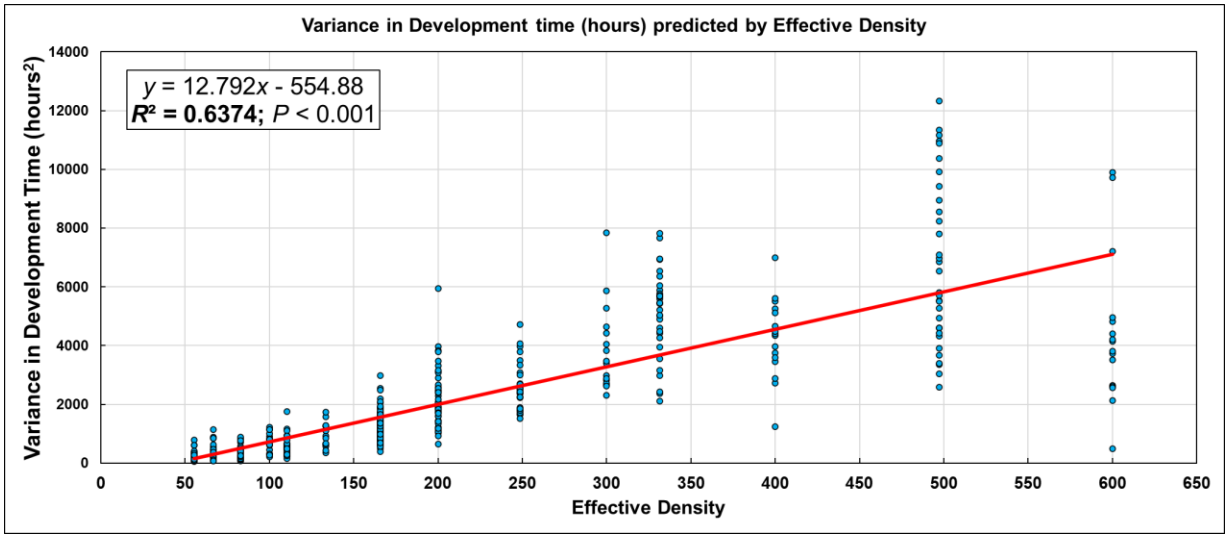

Survivorship predicted by Total Density
y = -0.0011x + 0.6723
R² = 0.6599; P < 0.001
Survivorship
Total Density
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
50
100
150
200
250
300
350
400
450
500
550
600
650


Survivorship predicted by Effective Density
y = -0.0011x + 0.7482
R² = 0.8212; P < 0.001
Survivorship
Effective Density
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
50
100
150
200
250
300
350
400
450
500
550
600
650


Mean Development time (hours) predicted by Total Density
y = 0.1662x + 249.8
R² = 0.3134; P < 0.001
Mean Development Time (hours)
Total Density
480
415
350
285
220
0
50
100
150
200
250
300
350
400
450
500
550
600
650


Mean Development time (hours) predicted by Effective Density
y = 0.2279x + 225.28
R² = 0.7359; P < 0.001
Mean Development Time (hours)
Effective Density
480
415
350
285
220
0
50
100
150
200
250
300
350
400
450
500
550
600
650


Variance in Development time (hours) predicted by Total Density
Variance in Development Time (hours²)
Total Density
y = 10.287x + 678.61
R² = 0.33; P < 0.001
0
2000
4000
6000
8000
10000
12000
14000
0
50
100
150
200
250
300
350
400
450
500
550
600
650


Variance in Development time (hours) predicted by Effective Density
Variance in Development Time (hours²)
Effective Density
y = 12.792x - 554.88
R² = 0.6374; P < 0.001
0
2000
4000
6000
8000
10000
12000
14000
0
50
100
150
200
250
300
350
400
450
500
550
600
650

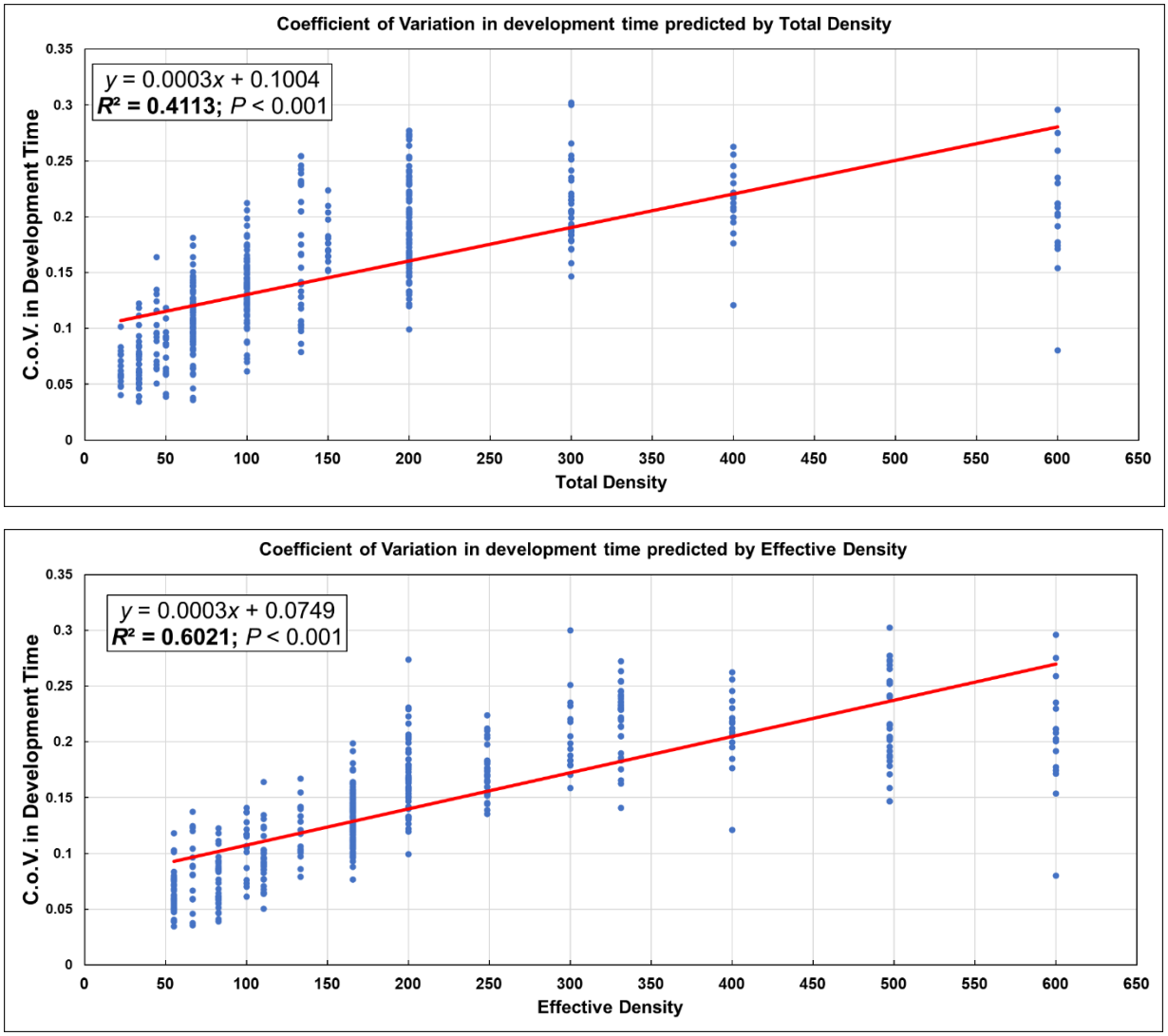


Figure s7. Least-square linear regressions of pre-adult survivorship, mean pre-adult development time, variance of pre-adult development time or coefficient of variation of pre-adult development time as response variables, respectively, as predicted by total density and effective density.

Chapter VI

An individual-based simulation framework exploring the ecology and mechanistic underpinnings of larval crowding in laboratory populations of *Drosophila*

NOTE

This chapter is a reproduction of a preprint on bioRxiv with the following citation:

Venkitachalam, S. and Joshi, A. 2023. An individual-based simulation framework exploring the ecology and mechanistic underpinnings of larval crowding in laboratory populations of *Drosophila*. bioRxiv. https://doi.org/10.1101/2023.07.30.551144

Introduction

The theory of density-dependent selection, first formally described by MacArthur (1962) and MacArthur and Wilson (1967), is a vital interface between the evolutionary and ecological approaches to competition (reviewed in Mueller, 1997, 2009; Joshi et al., 2001). At its core, it posits that fitness landscapes associated with various traits can change across population densities – i.e., the relationship of a trait variant with Darwinian fitness could depend on the population density it experiences.

Over the last few decades, many theoretical and experimental studies have aimed to examine the evolution of traits under different population density conditions (reviewed in Mueller, 1997; Joshi et al., 2001). On the experimental side, some of the most rigorous and detailed work has been carried out through selection experiments using outbred laboratory *Drosophila* populations (reviewed in Sarangi et al., 2016; Chapter 3). Adaptation to chronically crowded conditions has been used to explore the evolution of traits under high-density selection compared to respective low-density reared control populations.

The earliest studies focused on crowding populations at all life-stages through serial transfers (Mueller and Ayala, 1981). While this approach yielded some insights into the evolution of traits under high density conditions, crowding experienced at both larval and adult stages was a confounding factor when drawing inferences (Mueller et al., 1993). Thus, later studies focused largely on adaptation to larval crowding (but see also studies on adaptation to adult crowding by Joshi and Mueller 1997; Joshi et al., 1998). These larval crowding studies have previously been described in detail (reviewed in Prasad and Joshi, 2003; Sarangi et al., 2016; Chapter 3). The overall results from these studies were the following –

- Larval competitive ability evolves to be greater in crowding-adapted populations as compared to the low-density reared controls, under most selection studies carried out

so far (Mueller, 1988a; Nagarajan et al., 2016; Sarangi et al., 2016; Sarangi, 2018; Chapter 2; see Joshi and Mueller (1996) for an indirect inference of the same).

- The exact details of initial egg number, food volume and container dimensions (usually a cylindrical glass vial) determine which competitive ability linked traits evolve, and to what extent, in a given crowded scenario (Sarangi, 2018; Chapter 3, 2023a).
- Even under the same high-density conditions implemented via different combinations of initial egg numbers and food volume, the outcome of crowding with respect to fitness-related traits can be different, and this can also lead to the evolution of different traits under each condition (Sarangi, 2018).

Thus, it became apparent that a better understanding of the ecology of larval competition under different kinds of crowded scenarios would be vital to better understanding the evolution of larval competitive ability.

In parallel with, and also preceding the studies on evolution of adaptations to crowding, there was a long tradition of experimental and theoretical studies concerned with the ecology of larval competition in *Drosophila*. These studies ranged from describing the general outcome of larval crowding in *Drosophila* (Sang, 1949; Chiang and Hodson, 1950; Bakker, 1961; Ohnishi, 1976), to the role of waste in crowded conditions (Weisbrot, 1966; Botella et al., 1985), the cessation of developmental activity (Ménsua and Moya, 1983; González-Candelas et al., 1990),and the measurement of larval competitive ability in competition experiments between various strains (Bakker, 1961, 1969; Gale, 1964; Kearsey, 1965; Seaton and Antonovics, 1967; Mather and Caligari, 1981; de Miranda et al., 1991; Santos et al., 1992). Among these, studies by K. Bakker were particularly detailed, and inspired several models to understand and predict the results of larval competition, in addition to a verbal model described by Bakker himself (Bakker, 1961). These models, which at least in part covered the outcomes of larval competition, ranged from verbal (Bentvelzen, 1964) to mathematical (De Jong, 1976;

Nunney, 1983; Mueller, 1988b; Jansen and Sevenster, 1997; Tung et al., 2019). In each of these models, which were based directly on Bakker's data or from results of similar experiments, the primary end point of a culture experiencing competition under extreme crowding was the running out of food – and the primary outcomes were the differences in survivorship and body size of various types. These models largely considered different numbers of 'larvae' competing for some limited quantity of food, thereby varying larval density. Moreover, most of these models were concerned with describing or predicting the long-term consequences of the overall results of larval crowding as a whole. The understanding that the details of crowding experienced at similar overall densities, but at differing combinations of egg number and food volume, could result in different outcomes of fitness-related traits was largely absent. Also missing from these earlier models were the effects of metabolic waste products, which could impact the outcomes of competition along the axis of pre-adult development time in addition to survivorship (but see an independently-developed model by Moya and Castro (1986) for some consideration of the effects of metabolic waste on larval competition).

Adding to these unconsidered details, a recent study conducted by us decomposed the effects of larval crowding along the axes of starting egg number, height of the food column and the cross-section surface area of the cylindrical container (Chapter 5). This was largely inspired by the studies conducted by J.H. Sang (Sang, 1949). We found that the same combination of egg number and food volume cast into cylinders of different dimensions, via different food column height and surface area combinations, could greatly affect the egg to adult survivorship and the distribution of development time in a culture. Furthermore, we found that the density of larvae at the 'feeding band' – a shallow volume of food in contact with air, to which larval feeding is restricted – predicts the outcome of larval crowding better than the total density, as described by the starting number of eggs divided by the total starting volume of food.

In light of the recent advances in our understanding of the nuances of larval competition and of the adaptation to chronic larval crowding, there is a need to mechanistically explore how the differences in food surface area and food column height may be leading to differences in vital fitness-related traits such as survivorship and development time. Exploring this whole range of possible combinatorial scenarios experimentally is a daunting task from a logistical perspective. Therefore, we have developed an individual-based simulation framework that explores the mechanisms by which the outcomes of larval crowding are ultimately mediated. Such a model that predicts the results of larval competition under realistic scenarios can also be used to explore the consequences of long-term selection for adaptation to larval crowding under different conditions, further refining our understanding of density-dependent selection. This aspect has been elaborated in the discussion section (section 4.4).

The simulation framework described in the current study attempts to capture the processes of larval growth and the varied ways in which competition may actually occur in a crowded culture. This work is a follow up and expansion of a simple novel model developed by us and published as a Master's thesis by one of the authors (Venkitachalam, 2017). The current study limits the simulation to a single generation of a *Drosophila* larval culture, studying the outcome in terms of the distributions of fitness-related traits from various kinds of crowded cultures drawn from our recent empirical study (Chapter 5). The simulation framework is useful inasmuch as it may be helpful in generating predictions, confronting hitherto unconsidered mechanisms and directing precise experiments in situations wherein general exploratory experiments may be logistically daunting.

1. Traits involved in larval competition

Before setting up an individual-based simulation on larval competition, we list the traits being imparted to every competing individual. Each of these traits has been, or can potentially be, implicated in some way to be important to larval competitive ability – either in empirical studies involving the evolution of increased larval competitive ability, or in some form of logical argument. For this listing of traits, we describe a heuristic model for understanding the importance of various traits to larval competition, across three distinct time-periods of a culture.

1.1. *A heuristic model of larval competition in a crowded culture*

We imagine a crowded culture wherein we single out one individual (fig. 1.a). Over the timespan of the culture, this individual starts in the culture as an egg, which hatches into a larva. This larva then feeds on the available food and grows (through three instars), in competition with the myriad other larvae competing for the same limited resources. Ultimately, the crowded culture starts running out of food and/or building up toxic concentrations of metabolic wastes. In order to survive, the focal larva must achieve at least its minimum critical mass for pupation by feeding on a minimum amount of food (Beadle et al., 1938; Chiang and Hodson, 1950; Bakker, 1959, 1961; Robertson, 1963) before the food runs out and/or the ambient waste concentration becomes lethal for further food consumption.

From the above description, we can imagine three (more or less) distinct time stages in the culture in which different traits can affect the focal larva's competitive ability (fig. 1.a).

1.1.1. *Time stage 1: Initial stage of a culture*

Assuming simultaneous placement of all eggs into the culture (see Jansen and Sevenster (1997) for a model without this assumption), all else being equal, the focal individual can have greater competitive ability by having a faster egg hatching time than other competitors, or by having a larger mass at hatching (fig. 1.b, discussed in Bakker, 1969). Both of these aspects increase the

effective head start that the larva can have in its growth period (reviewed in Chapter 4). While a faster hatching time obviously confers greater head start, a larger initial larval mass reduces the amount of growth required by the larva to achieve its minimum critical mass (all else being equal), assuming larger eggs hatch into larger individuals. The evolution of both these traits has indeed been observed in multiple population sets adapted to respectively different details of larval crowding – each crowding adapted population, regardless of the exact conditions of larval crowding imposed, evolved greater egg length and egg width, as compared to the low-density control populations (Chapter 3). Out of the three population sets studied, one set also evolved significantly reduced egg hatching time compared to controls, while the other two sets showed non-significant reductions (Chapter 3).

1.1.2. *Time stage 2: larval growth period*

In case of larvae feeding at the initial time window of a crowded culture, faster growth rate (i.e., rate of weight gain) should be paramount for survival. All else being equal, the focal larva would be competitively superior to other larvae by growing faster than them (fig. 1.c, discussed in Bakker, 1961). There are two primary ways by which growth rate could be affected:

1.1.2.1. *Modification of overall feeding rate*

An increased rate of food ingestion, all else being equal, could drive faster larval growth rate. This could happen via either increased bite rate, measured as the number of bites taken per time step or increased bite size, leading to more food ingested per bite (Bakker, 1961; Robertson, 1963).

Bite rate has usually been termed ‘(larval) feeding rate’ in empirical studies, and has a rich history of study across laboratories and populations. It is measured as the total number of sclerite retractions per minute while feeding (Sewell et al., 1974; Joshi and Mueller, 1988). Initial studies on adaptation to larval crowding showed a positive relationship of feeding rate

with larval competitive ability (Joshi and Mueller, 1988, 1996). Moreover, a selection study on increased feeding rate also resulted in the evolution of increased larval competitive ability (Burnet et al., 1977). This led to the general assumption that the evolution of increased larval feeding rate would also be associated with increased larval competitive ability (Joshi and Mueller, 1996; Borash et al., 2000). However, in later selection studies, crowding-adapted populations of *D. ananassae* and *D. nasuta nasuta* evolved increased larval competitive ability without evolving increased feeding rate (Nagarajan et al., 2016). The underlying explanation for this contrasting set of results was revealed to be dependent on the exact conditions of larval crowding imposed. *D. melanogaster* populations crowded at the same conditions as the *D. ananassae* and *D. nasuta nasuta* populations (different from the earlier *D. melanogaster* studies) also evolved greater larval competitive ability without increased feeding rate (Sarangi et al., 2016). Further complicating the relationship of feeding rate with larval crowding was a recent study carried out in our research laboratory by M. Sarangi (2018). The results from that study showed that crowding-adapted populations of *D. melanogaster* which had unchanged feeding rate compared to controls when assayed singly or under low-density conditions, showed the highest feeding rate in their native crowded conditions in vials (Sarangi, 2018). Thus, the plasticity of larval feeding rate across the crowding gradient has emerged as another potential factor that contributes to larval competitive ability, although further experimentation is required to elucidate the details and extent by which such plasticity manifests itself under differently crowded conditions.

Little is known about the bite size of *Drosophila* larvae, and likely even less about the dynamics and variance of bite size across different stages of larval growth under crowded conditions. A study by Robertson (1963) showed that mouth part size increased along with larval size across the larval growth period. We can thus assume bite size to also increase with larval size.

1.1.2.2. *Modification of food to biomass conversion efficiency*

All else being equal, the focal larva could also enhance its growth rate by being more efficient in food utilisation than its competitors (Bakker, 1961). This efficiency could manifest itself in two possible ways – fraction of food converted to biomass, and time taken to convert the same quantity of food to biomass.

The importance of increased resource utilisation efficiency under high population density conditions has been stressed at least since the time density-dependent selection was formalised (MacArthur and Wilson, 1967; but see Joshi et al. (2001) for a contrasting view). However, results from experiments on larval crowding adaptation have yielded mixed results. Earlier selection studies indicated that crowding-adapted populations had evolved reduced efficiency with respect to minimum food required for pupation, indicating a reduction in the fraction of food converted to biomass (Mueller, 1990; Joshi and Mueller, 1996). Recent larval crowding studies on *Drosophila* populations, using a shallow food column, reported an increase in efficiency of crowding-adapted larvae, with respect to the time taken to convert the same volume of food to biomass (Nagarajan et al., 2016; Sarangi et al., 2016).

1.1.2.3. *Time stage 3: the end stage of larval crowding*

The end stage of crowding can be imagined as the time when the food runs out or the waste concentration becomes unbearably high for the larvae (fig. 1.a). At this stage, a different set of traits would help the focal larva survive, all else being equal. In particular, a greater waste tolerance would allow the focal larva to continue eating at a waste concentration intolerable to other larvae (fig. 1.di). Metabolic waste products such as ammonia, uric acid or urea have been shown to increase in crowded cultures in multiple studies (Botella et al., 1985; Borash et al., 1998; Sarangi, 2018). Furthermore, waste tolerance also evolved in populations adapted to crowding at high food volumes (Shiotsugu et al., 1997; Borash et al., 1998; but see Sarangi,

2018), but not in populations adapted to low food volumes (Nagarajan et al., 2016; Sarangi et al., 2016). In cultures with high food volumes, the food column length is greater (assuming similar container dimensions), and there is substantial food remaining even at the end stage of larval crowding (Bierbaum et al., 1989; Mueller, 1990; Chapter 5). Thus, it is likely that, when faced with long food columns, some larvae could feed slowly and survive late into the life of a culture, assuming they can tolerate the increased waste build-up expected due to the numerous larvae feeding and excreting in the vial (Borash et al., 1998; Mueller and Barter, 2015). One study showed that offspring of early eclosing adults from crowding adapted populations had higher larval feeding rate and low waste tolerance, whereas offspring of late eclosing adults had greater waste tolerance along with lower larval feeding rate (Borash et al., 1998). This point further reinforces our assumption that the growth stage and end stage of a crowded culture can be quite different ecologically, and have different underlying fitness functions at different times in the culture.

Finally, the end stage may also be tackled by larvae via a different approach – a reduction in the minimum critical mass required for pupation (Bakker, 1961). The focal larva could survive simply by reducing its requirements and commit to wandering at a smaller size (fig. 1.dii). While primarily useful in conditions where food quantities are fleeting, a reduced minimum critical mass could also potentially reduce some of the duration spent in the increasingly uninhabitable environment at the end stage of the culture. No crowding-adapted population till date has been known to show the evolution of reduced mean minimum critical mass to pupation, although some populations have evolved overall smaller sized adults under uncrowded conditions, which might indicate an evolution of smaller minimum critical mass as well (Sarangi, 2018; Chapter 2).

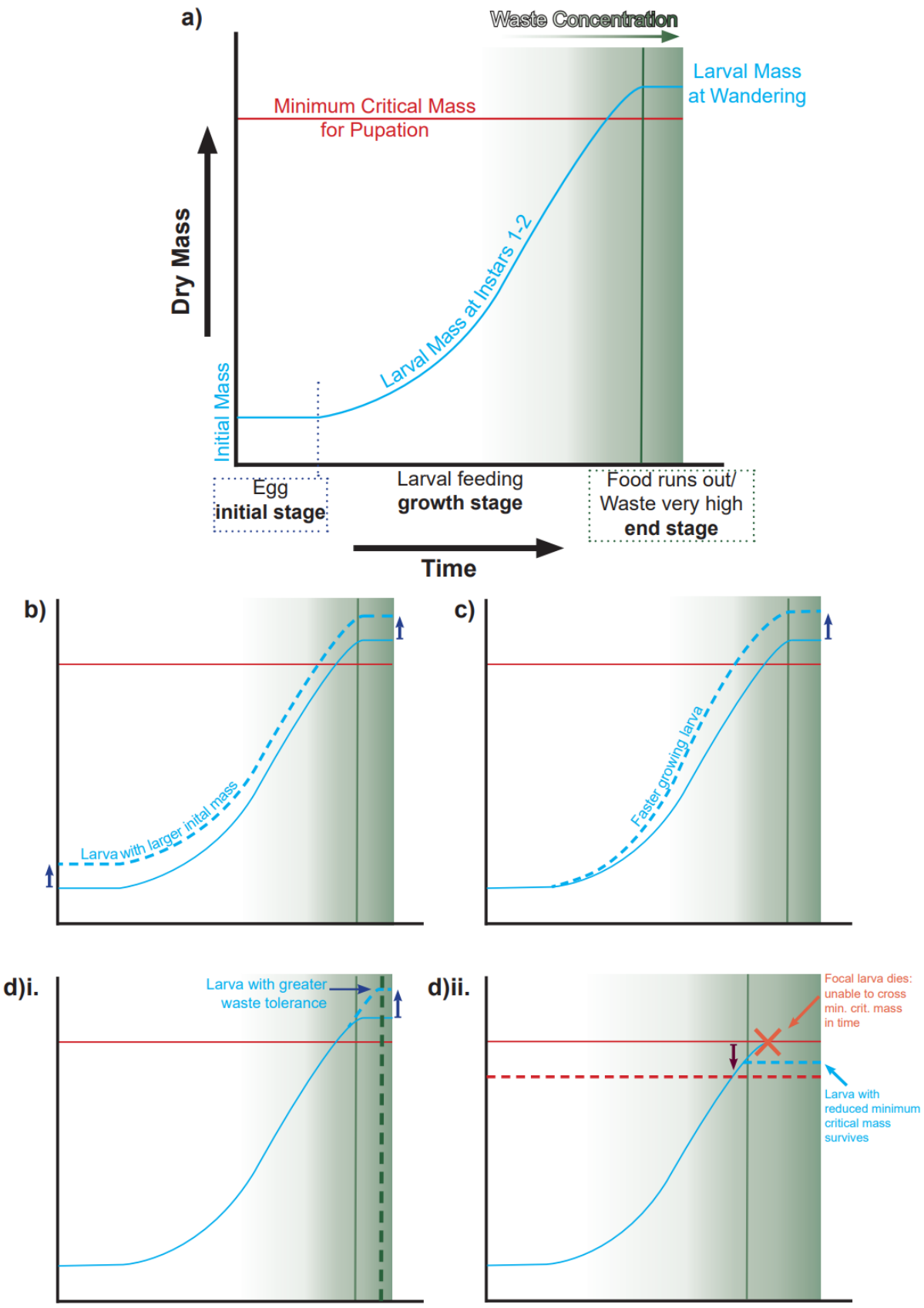


Figure 1. Heuristic model of larval competition (see introduction section 1.1 for more details).

a) This plots the growth profile of a single imagined individual from an imagined crowded *Drosophila* larval culture. The individual can go through three fairly distinct stages through the life of the crowded culture. It begins in the initial stage as an egg. The time of hatching determines the beginning of the growth stage of the larva. As multiple larvae feed in a crowded culture, food starts running out and/or metabolic waste concentration starts building up, as highlighted by a green background. This determines the end stage of the culture for the larva, when it must leave the system due to food running out or waste toxicity becoming too high, denoted by the solid green line. In order to commit to wandering stage and become an adult, the larva must cross its minimum critical mass required for pupation, else it dies. This threshold mass is drawn as a red line.

b) Traits that may improve competitive ability at the initial stage (see Table 1). Besides the focal individual (solid blue line), we imagine another competing individual (dashed blue line). This second individual has a larger initial larval mass, achieved via greater egg mass, compared to the focal individual. All else being equal, the individual with the larger egg mass will have a greater body mass than the focal larva by the end stage, giving it a potential competitive edge. An imagined individual with a faster egg hatching time would get a similar competitive edge, by feeding earlier and effectively having a larger mass by the time the focal individual hatches.

c) Traits that may improve competitive ability at the growth stage (see Table 2). We imagine another individual (dashed blue line) with a greater growth rate of larval mass than the focal larva (solid blue line). All else being equal, the individual with the greater growth rate will have a greater body mass than the focal larva by the end stage, giving it a potential competitive edge.

d) Traits that may improve competitive ability at the end stage (see Table 3). At this stage, a second larva (dashed blue line) can be more competitive than the focal larva (solid blue line), all else being equal, via two distinct ways:

i. Increased waste tolerance – the larva with the higher waste tolerance can continue feeding in greater concentrations of waste (dashed green line denotes the increased waste tolerance threshold), eclosing later but at a greater mass.
ii. Decreased minimum critical mass to pupation – the larva with lower minimum critical mass requirement, denoted by the dashed red line, can escape very high waste levels and become an adult, while a larva with a greater threshold requirement will succumb.

Methods

2.1. Recurrent 'short-hand' terms

Surface area: cross-sectional surface area of the cylindrical food column in a vial into which this food is cast in experiments.

Column height: starting food column height in a culture vial.

Food volume: starting food volume of a culture vial.

Eggs: starting egg number in a culture vial.

2.2. Units used

While our model is simple enough that any units used are at best vague abstractions of reality, the scale at which we have implemented the simulation attempts to work in units relevant to those used experimentally, and which can be followed intuitively. The units used are:

Dry mass (micrograms – μg): most of the reliable mass estimates on larvae and adult flies are found in units of dry mass (Bakker, 1961, 1969; Santos et al., 1997; Sarangi, 2018; Chapter 2).

Thus, the starting food culture is in units of μg dry mass. The larval body mass is also measured in units of dry mass (μg).

Time (hours): the measurement of development time (egg to wandering, pupal or adult stage) is typically done in hours (Nagarajan et al., 2016; Sarangi et al., 2016). Thus, time steps in the simulation framework are on the scale of hours. This is also logistically useful, as it requires much shorter computation time than implementing time in a per minute scale. The primary caveat is that feeding rate measurements, which are empirically measured at the scale of minutes (e.g., Sewell et al., 1974), have here been converted to the hourly format. This conversion may not be entirely accurate, due to the presence of inter-feeding bouts (Ruiz-Dubreuil et al., 1996) which may be prevalent over longer feeding periods. In our text, 'hour' and 'time step' are used interchangeably.

Space (mm): the culture set up in cylindrical dimensions, as well as some larval characteristics in the expanded model, such as digging depth and larval cross section surface area, depend on units of space.

2.3. A brief overview of the simulation framework

We simulated cultures of given cylindrical dimensions in which 'larvae' started as eggs, hatched and then fed until hitting a defined limit. If uncrowded, the larvae stopped feeding after achieving an optimum size and committed to the 'wandering' stage. Under crowded conditions, when faced with severe resource limitation or when facing a toxic environment due to high waste build up, the larvae needed to cross their respective minimum critical mass to pupation in order to commit to wandering. Larvae unable to cross this threshold when the environment was no longer habitable ended up 'dying'.

We started with a 'base' model (section 2.4), onto which we subsequently overlaid more nuanced details of larval competition under the 'expanded' model (section 2.5).

2.4. The 'Base' model of larval competition

2.4.1 Simulating uncrowded conditions

The initial model framework attempted to capture the overall patterns of time to reach wandering stage and dry mass achieved at wandering stage, seen in earlier empirical studies in uncrowded cultures at 25°C under constant light conditions and 60-80% relative humidity (Santos et al., 1997). We describe 'uncrowded' as a starting egg number of around 70, in a total food volume of 6 mL (400 mm^2 surface area, 15 mm food column height). We assumed any egg number below 70 in 6 mL of food would give the same culture outcomes in terms of % survivorship, average mass and average development time. Additionally, we did not incorporate any possibility of non-zero density-independent mortality.

2.4.2. Culture set up

Empirically, the food in a culture is usually measured in volume (mL, or mm^3), which are components of space rather than mass. Given that most laboratory culture media use a very specific ingredient list (e.g., Sarangi et al., 2016), it was relatively simple to convert a given volume of food into dry nutrient mass. We did not partition the food further into macro-nutrient mass. The dry nutrient density for the cornmeal-sugar-yeast medium used in our selection experiments was rounded to 160 μg/mm^3 for the simulations.

We started by setting up the culture in terms of the food column cross-section surface area and height, which were converted to the total food volume (E1). This food volume was subsequently converted to a corresponding dry mass value (E2).

E1.

$$food\ volume(mm^3) = height\ of\ food\ column(mm) \times surface\ area(mm^2)$$

E2.

$$dry\ mass\ of\ food(\mu g) = food\ volume(mm^3) \times nutrient\ density\left(\frac{\mu g}{mm^3}\right)$$

2.4.3. Adding eggs to the culture and setting up stochastic variability among individuals

A culture with a given food mass was then populated with a specified number of 'eggs'. The eggs were added simultaneously. These eggs 'hatched' into 'larvae' which proceeded to feed on the food. Each individual was endowed with a given set of traits (see section 1.1.) when it was first added to the culture. These could be uniform or variable across individuals. When variable, we assumed the traits to be complex enough for their variation to be described by a corresponding normal distribution (but see Borash and Shimada, 2001). Each individual's trait value was drawn randomly from a normal distribution with given mean and coefficient of variation (see Tables 1-5).

2.4.4. Setting up traits

2.4.4.1. Initial advantage related traits

The details of traits likely to contribute to competitive ability at the initial stage are listed in Table 1. We further calculated egg volume as a cylinder with egg length as height and egg width as diameter. Finally, this was converted to egg dry mass. For this, we assumed that an egg with volume calculated from a mean length of 0.5 mm and mean width of 0.18 mm would have a dry mass of 2.7 µg. This value of mass was obtained from Bakker's findings (1961), although the association of this mass value with the specific egg volume was arbitrary. However, once the egg volume and egg mass were calibrated in such a way, any combination of egg length and egg width would yield a corresponding egg mass. An individual's initial larval mass was equal to its egg mass (the mass of the egg shell was not considered – but see Bakker, 1961, 1969).

Trait	Significance	Notes	Mean	c.o.v.	Source(s)
i. Egg hatching time	The egg hatching time determined the time step at which an egg hatched into a larva.	Each individual's hatching time was implemented as a countdown from the start of the culture time.	20 hours	1%	Chapter 3
ii. Egg length	This translated into the initial larval length.	Egg shell dimensions were not considered.	0.5 mm	5%	Chapter 3
iii. Egg width	This translated into the initial larval cross-section diameter.	Egg shell dimensions were not considered; diameter was used to calculate initial cross-section surface area of larva.	0.18 mm	5%	Chapter 3

Table 1. Traits that may be important to competitive ability at the initial stage.

2.4.4.2. Growth rate related traits

The traits likely to contribute to competitive ability at the larval growth stage are listed in Table 2. Bite rate in uncrowded conditions was assumed to have a positive relationship with larval mass, for a given larva. The relationship was modelled as follows:

E3.

$$number\ of\ bites_{(t,i)} = number\ of\ bites_{(0,i)} \times \left(1 + \frac{larval\ dry\ mass_{(t,i)}(\mu g)}{maximum\ possible\ larval\ mass_{(i)}(\mu g)}\right)$$

Where t = time step (0 = initial time step); i = larval identity; and:

E4.

$$maximum\ possible\ larval\ mass_{(i)}(\mu g) = 3 \times minimum\ critical\ mass_{(i)}(\mu g)$$

At maximum mass (see Table 3.i), the larva in uncrowded conditions would, thus, have twice the initial bite rate (Santos et al., 1997).

For bite mass, estimates were roughly derived from available larval growth and feeding rate data (Santos et al., 1997), assuming an average food conversion efficiency of 30% (Table 2.iii). We further used extensive mouth part length measurements carried out by Alpatov (1929) to get a general idea of the possible order of magnitude for bite mass, calculated from assuming the bite size as a sphere using a fraction of the mouthpart as the diameter (data not shown). Mouth part size has also been shown to increase over the course of larval growth within the same instar (Robertson, 1963). The association bite mass with larval mass was calculated using a bite scaling factor which scaled bite mass of i^{th} larva to its body mass at time t. The calculations are as follows:

E5.

$$bite\ mass_{(t,i)}(\mu g) = bite\ scaling_{(t,i)} \times larval\ dry\ mass_{(t,i)}(\mu g)$$

E6.

$$bite\ scaling_{(t,i)} = bite\ scaling_{(0,i)} - \frac{larval\ dry\ mass_{(t,i)}(\mu g)}{maximum\ possible\ larval\ mass_{(i)}(\mu g)} \times \left(0.5 \times bite\ scaling_{(0,i)}\right)$$

The bite scaling factor had a negative correlation with larval mass. Simulated bite mass increased over the course of larval growth, but the rate of increase of bite mass itself reduced, capping at a minimum rate of zero. Most importantly, we did not consider any variation among larvae in bite scaling (Table 2.ii).

We relied on estimates by de Miranda and Eggleston (1988) to derive values of food to biomass conversion efficiency in *Drosophila* larvae. There were two relevant points in their study – the average efficiency values lay between 20-40% of food eaten depending on the strain, and there was a large amount of variance in estimating efficiency across the time of larval sampling (de Miranda and Eggleston, 1988). Another study by Bakker on multiple populations of *D. melanogaster* found the larval food conversion efficiency values to lie between 20-25%

(Bakker, 1969). We accounted for both the mean efficiency and the temporal variation in efficiency per larva (Table 2.iii, iv).

We were unable to find many data regarding the values of time taken to convert food to biomass. However, the study by de Miranda and Eggleston (1988) found the time window to lie within the bounds of 60 minutes. Furthermore, a study on crowding-adapted populations measured food passage time through the gut in the order of minutes (Joshi and Mueller, 1996). Thus, we did not incorporate this facet of larval competitive ability in the current study.

Trait	Significance	Notes	Mean	c.o.v.	Source(s)
i. Initial Bite Rate	For a given bite mass, the initial bite rate determined the rate of food intake.	The bite rate increased in value across the larval growth period (see text).	3000 bites per hour	5%	Joshi and Mueller, 1996; Santos et al., 1997; Joshi et al., 2003 (c.o.v.); Sarangi et al., 2016
ii. initial bite mass	For a given bite rate, the initial bite mass determined the rate of food intake.	The bite mass decreased in value across larval growth period (see text).	0.007 % of initial larval mass	None	Arbitrary
iii. food to biomass conversion efficiency mean	Determined the fraction of food eaten that was converted to growth in larval mass, each time step.	This determined the mean efficiency per larva across time steps.	30% of food eaten in a given time step	5%	(Only mean) Bakker, 1969; de Miranda and Eggleston, 1988
iv. Efficiency within larva c.o.v.	Determined the variation across time steps in food to biomass efficiency for a larva.	The efficiency of a larva per time step was randomly drawn from a normal distribution with mean as iii, and c.o.v. determined by iv.	5%	5% variation in c.o.v. across larvae	Inspired by de Miranda and Eggleston, 1988

Table 2. Traits that may be important to competitive ability at the growth stage.

2.4.4.3. End stage related traits

The traits likely contributing to competitive ability towards the end stage of a crowded culture are listed in Table 3.

The waste tolerance threshold per larva was calculated as follows:

E7.

$$waste\ tolerance\ limit_{(t,i)}(\mu g) = tolerance\ factor_{(i)} \times larval\ dry\ mass_{(t,i)}(\mu g)$$

Trait	Significance	Notes	Mean	c.o.v.	Source(s)
i. Minimum critical (dry) mass to pupation	Determined the dry body mass up to which a larva must grow in order to pupate.	Also determined maximum mass possible for a larva as: 3× min. crit. mass (largely based on patterns seen in previous data).	180 µg	5%	Bakker, 1961; Santos et al., 1997
ii. Waste tolerance factor	Determined the threshold of total dry mass of metabolic waste that could be non-lethally ingested by a larva.	The waste tolerance threshold of a larva at a given time was calculated as: the waste tolerance factor × current larval mass.	0.095	10%	Arbitrary

Table 3. Traits that may be important to competitive ability at the end stage.

2.4.5. Compiling traits and behaviours

The above factors constituted the larval characteristics in the 'base' model, which attempts to capture the understanding of *Drosophila* larval competition as it was around the end of the twentieth century. Each of the traits was incorporated in the process of larval feeding in uncrowded conditions, as well as in various degrees of crowding.

2.4.5.1. Egg hatching

Each added egg started with a counter for its hatching time (Table 1), which reduced by 1 per elapsed time step. After the counter reached 0, the larva hatched at the mass determined by the egg mass (Table 1), and began feeding.

2.4.5.2. Feeding and growth

Every time step *t*, each larva *i* fed as follows:

E8.

$$food\ eaten_{(t,i)}(\mu g) = bite\ mass_{(t,i)}(\mu g) \times no.of\ bites_{(t,i)} \times \left(\frac{total\ food_{(t)}(\mu g)}{total\ food_{(t)}(\mu g) + total\ waste_{(t)}(\mu g)}\right)$$

In the base model, growth was simply a property of multiplying food eaten by conversion efficiency, whose value was drawn randomly from a normal distribution every time step. This distribution of possible values had a mean equal to the larval mean efficiency value (Table 2.iii) and coefficient of variation equal to the mean value in Table 2.iv.

E9.

$$growth_{(t,i)}(\mu g) = food\ eaten_{(t,i)}(\mu g) \times food\ biomass\ efficiency_{(t,i)}$$

Once the i^{th} larva at time *t* crossed its minimum critical mass, it could feed for 36 hours or until it reached its maximum mass, depending on whichever was achieved first. The time limit was derived roughly from the post-critical feeding period seen by Santos et al. (1997), and the knowledge that larvae have a fixed feeding period after achieving their minimum critical mass to pupation (Robertson, 1963; also discussed in Burnet et al., 1977; Prasad and Joshi, 2003). A contrasting result which we did not consider in the current study, was the possible elongation of the third instar in the 'larval stop' phenomenon (Ménsua and Moya, 1983; González-Candelas et al., 1990).

Simulated larval growth profiles in an uncrowded condition are shown in figure 2.a, and the outcomes in terms of development time and dry mass at wandering can be found in figure 2.b, 2.c.

In the simulations, if food ran out before a larva completed feeding, there could be two possibilities. If the larva had crossed the minimum critical mass required for pupation, it committed to wandering at the moment of food cessation. However, if the larva had not reached its required minimum critical mass, it died at the moment of food cessation.

2.4.5.3. Waste generation, larval consequences and tolerance

Each feeding larva excreted 2.5% of the total food eaten at the previous time step as metabolic waste (the value used was arbitrary). This waste was representative of, but not limited to, known excretory materials such as uric acid, urea and ammonia, and assumed to be potentially toxic (Botella et al., 1985; Borash et al., 1998; Sarangi, 2018). The waste was excreted by the larva 1 hour after the corresponding food had been eaten (time lag approximated from de Miranda and Eggleston (1988)).

The waste ingested per larva per time step was calculated as follows:

E10.

$$waste\ eaten_{(t,i)}(\mu g) = bite\ mass_{(t,i)}(\mu g) \times no.of\ bites_{(t,i)} \times \left(\frac{total\ waste_{(t)}(\mu g)}{total\ food_{(t)}(\mu g) + total\ waste_{(t)}(\mu g)}\right)$$

This ingested waste was added to the total pool of waste previously ingested by the larva. If the total waste ingested by a larva crossed its current waste tolerance threshold, then the outcome was similar to food running out. If the larva had crossed its minimum critical mass to pupation, it committed to wandering; if not, it died.

Furthermore, below the waste tolerance threshold, as long as the total waste ingested by a larva was non-zero, it also reduced the bite rate of the larva according to the following non-linear formulation:

E11.

$$realised\ no.of\ bites_{(t,i)} = no.of\ bites_{(t,i)} \times bite\ rate\ modifier\ W_{(t,i)}$$

Where,

E12.

$$bite\ rate\ modifier\ W_{(t,i)} = \frac{1}{1 + e^{\left(15\times\left(\left(\frac{total\ waste\ ingested_{(t,i)}(\mu g)}{waste\ tolerance\ threshold_{(t,i)}(\mu g)}\right)-0.5\right)\right)}}$$

Note: we capped the lowest possible value of the waste ingestion modifier to 0.01, in order to prevent bite rate values from going to 0 and stalling the simulation.

Under the mechanism adopted in this simulation, larvae under uncrowded situations faced very little increase in waste across their growth period, and thus their waste ingested and the associated bite rate modifier values showed little change, leading to very similar uncrowded growth profiles (fig. S3) compared to the base model without waste (fig. 1). In crowded cultures, as food ran out and waste concentration increased, greater waste ingestion caused the bite rate modifier (Eq. 12) to reduce rapidly. Those larvae which had greater waste tolerance reached the inflection point of bite rate reduction at a greater waste concentration.

2.4.6. Base model – introducing larval crowding

Reducing the food volume and/or increasing egg number introduced larval crowding in the simple base model. The results for these with and without the incorporation of waste are given in section 3.1.2.

While our culture set-up method allowed us to vary food volumes in different cylindrical dimensions, the base model described above did not yield differences in cultures with the same egg number and food volume cast into different cylindrical dimensions (detailed in section 3.2 and fig. S4, S5). The base model was thus insufficient in capturing details of recent empirical findings on the effects of egg number, surface area and food column height (Chapter 5). For these, we expanded the base model to include mechanisms that could affect crowding along the axes of changing food column cross-section surface area or height, respectively.

2.5. The 'Expanded' model of larval competition

Recent findings on the importance of feeding band density, as well as the existence of some form of diffusion of metabolic waste into the food column, were incorporated to construct the 'expanded' model. We put the disclaimer that the mechanisms for these recently discovered phenomena are not known in detail, and we have put our best speculations into the model, based largely on personal observations, speculations from earlier papers, or logical assumptions about the overall process. These have been classified according to mechanisms and the larval traits involved in interfacing with those mechanisms.

2.5.1. Mechanism: how does food column cross-section surface area affect the outcomes of crowding?

In all likelihood, this is primarily a function of competition for limited space. One of the clearest lines of evidence for this lies in cultures with relatively small surface area and long food columns, wherein much of the food at the bottom is left untouched, presumably due to lack of larval access to food at the bottom of the vial. Moreover, our personal observation as well as those of some other authors (Gilpin, 1974; Botella et al., 1985; Moya and Castro, 1986; Gregg et al., 1990; Chippindale et al., 1994; Mueller and Barter, 2015) have been that crowded larvae

tend to feed in packed clusters rather than spread out over the complete surface of the food in contact with the air, even in cultures within very narrow vials. This is presumably done for more efficient feeding by breaking down the food, either physically through mouthparts (Burnet et al., 1977) or through enzymes such as amylase, resulting in easier consumption (Gregg et al., 1990; Sakaguchi and Suzuki, 2013). In conditions of food shortage, this could be particularly important, as easier consumption of food could possibly lead to faster growth.

Thus, the simulated mechanism for limiting surface area was implemented by considering a) difference between total surface area of a vial vs. the effectively available cross-section surface area of the food, as limited by the larval clustering and breaking down of food; and b) difference between effectively available surface area of the food and the total space taken up by the larvae. At the start, each culture had a small fraction of the total vial cross-section surface area as the effectively available surface area. This was meant to represent some initial points which the larvae would start feeding at, which could be chosen either because the food at those points was softer or more fractured, or just arbitrarily as an initial feeding point.

Moreover, we introduced another larval measurement – its cross-section surface area. The initial larval cross-section surface area translated directly from the cross-section surface area of the egg (Table 1.c). As a larva started feeding and growing, its own cross-section surface area also increased in proportion to its mass. This was calculated by assuming (arbitrarily) that a larva with a minimum critical mass of 180 μg would have a cross-section surface area of around 1.54 mm^2 when at its maximum mass. The larval surface area was calculated from a cross-section diameter of 1.4 mm at maximum mass, measured by Kaznowski et al. (1985). When the larvae, each with their own cross-section surface area, fed in a column of food, they occupied a proportion of the effectively available surface area. The occupancy of the effective surface area was calculated by dividing the total larval cross-section surface area in a culture, by the effective surface area of the vial. In case of a large number of larvae present in a limited

vial surface area, as was expected to occur in crowded scenarios, the effective surface area occupancy was likely to be >=1 (fig. 4.ci, cii). Meanwhile, larval feeding activity would also be expected to further loosen and liquefy the surrounding substrate over time, which we represented by the effective available surface area increasing every time step, the rate of which was dependent on whether or not the summed larval surface area completely filled up the available surface area.

We defined a 'crowded' situation as one where the effectively available surface area was at least completely occupied by feeding larvae. Besides the clustering feeding behaviour observed, a recent study revealed an additional plastic behaviour that exists for crowded vs. uncrowded situations in larvae. A set of crowding adapted populations showed greater larval feeding rate only under crowded conditions (Sarangi, 2018), but not when assayed singly (or in small groups) on a petri plate (Sarangi et al., 2016; Sarangi, 2018). We thus implemented a plastic increase in feeding rate in the simulation framework, triggered by crowding of the surface area. This was done through the incorporation of two additional traits in the larvae – crowding threshold and peak bite rate modifier (Table 4). We assumed that larvae could feed faster if they detected crowded conditions, as represented by each larva's crowding threshold. A larva with a lower threshold could potentially detect the presence of crowding at a lower surface occupancy than other larvae, and begin feeding faster a few hours before the other larvae detected crowding and increased their own bite rate. The degree to which the feeding could become faster was determined by the peak bite rate modifier (Table 4). The realised bite rate of a given larva per time step was its bite rate modifier (suffixed *C* i.e., under crowding) multiplied by its bite rate under uncrowded conditions. This bite rate modifier was calculated as follows for each larva *i* at time step *t*:

E13.

$$bite\ rate\ modifier\ C_{(t,i)} = 1 + \frac{peak\ bite\ rate\ modifier_{(i)}}{1 + e^{20\times(crowding\ threshold_{(i)} - surface\ area\ occupied_{(t)})}}$$

Moreover, there was a detrimental effect of crowding for limited space, represented by a loss of food eaten per time step. Our imagined mechanism was the following:

As the larvae crowded around limited cluster spots, they collided against and displaced one another (also speculated by Bakker, 1961; Gilpin, 1974), and not all bite attempts led to food being consumed. We further assumed that larger larvae were better able to embed themselves into the food than smaller larvae (also speculated by Gilpin, 1974), and were thus given less disadvantage in terms of loss of food eaten.

The realised food eaten by a larva per time step was the food eaten under uncrowded conditions multiplied by the food eaten modifier at that time step. The mass percentile of a given larva *i* at time step *t* was calculated for its larval mass as compared to all other larvae at that time step.

E14.

$$food\ eaten\ modifier_{(t,i)} = 1 - \frac{1}{1 + e^{20\times(mass\ dependence_{(t,i)} - surface\ area\ occupied_{(t)})}}$$

Where:

E15.

$$mass\ dependence_{(t,i)} = 0.9 + 0.1 \times \frac{mass\ percentile_{(t,i)}}{100}$$

The minimum value of food eaten modifier was set to 0.01 – food eaten per time step could not dip below a hundredth of the uncrowded value. This was done to prevent stalling the simulation.

Trait	Significance	Notes	Mean	c.o.v.	Source(s)
i. Crowding threshold	Determined the occupancy of the effective surface area at which the bite rate modifier peaked in value.	This was the threshold of space limitation at which a larva detected 'crowding'.	1.05	5%	Arbitrary
ii. Peak bite rate modifier	Determined the peak bite rate at the crowding threshold. See equation E13.	This represented the amelioration of detrimental effects of crowding for limited space.	1	5%	Sarangi, 2018

Table 4. Traits that may be important to competitive ability under crowding due to surface area limitation.

2.5.2. Mechanism: how does food column height affect the outcomes of crowding?

The presence of long food columns under conditions of crowding has a very consistent outcome – the presence of relatively unused food at the bottom of the vial. This has been discussed in previous crowding-adaptation studies (Bierbaum et al., 1989; Mueller, 1990). Our recent study also showed similar outcomes in conditions with a narrow surface area and a deep food column (Chapter 5). Likely due to their need for access to air, larvae tend to feed with their mouths facing downwards, and the posterior spiracles facing the food surface in contact with the air (Sang, 1949; Green et al., 1983; Ruiz-Dubreuil et al., 1996). The larvae can also dig to a certain length in order to procure food (Sang, 1949). When faced with intense crowding, smaller larvae may be unable to dig too deep, as they could subsequently fail to come back up for air and thus drown. Moreover, the larvae excrete the waste material (along with egestion) via the anus (reviewed in Kuraishi et al., 2015) at the top of the food when facing downwards. It has been speculated that under crowded conditions, larger larvae would be able to feed on relatively untainted food whilst polluting the food for smaller larvae closer to the surface of the food (Gilpin, 1974). An earlier study from our group has also given evidence for the presence of

larval metabolic waste at the lower half of long food columns after a period of 4-7 days, while the waste at the top saturates around the same period (Sarangi, 2018).

Consequently, we implemented a mechanism for the permeation of waste into the food column over time. The food column was divided into 0.01 mm thin uniform 'layers' – each layer having some uniform capacity to hold a certain dry mass of waste in it (arbitrarily, 4.5% waste concentration per layer). As the larvae fed and excreted, the waste first entered the topmost food layer. With waste build up, this layer reached its waste saturation limit at some time point. Subsequently excreted waste went down to the layer below the top layer. Once that got saturated, the process repeated and waste filled up the next layer, with layers continuing to get saturated with waste in a top-down manner (fig. 4a, b). If all the layers were saturated with waste, the new waste was deposited only on the top layer, with the assumption that the rest of the layers were unable to hold any more waste (fig. 4a).

In addition to dry mass and cross-section surface area, we also assigned a length (mouth to anus) characteristic to larvae. Larval length helped determine how deep a larva could dig and access food. The initial length of a larva translated directly from its egg length (Table 1b). As done for larval cross-section surface area, a larva having minimum critical mass of 180 μg was arbitrarily associated with a length of 4.6 mm at maximum mass. This maximum length measurement was obtained from Alpatov, (1929), as well as Kaznowski et al. (1985) (also verified by Bakker, 1959).

Furthermore, each simulated larva was assigned a trait, digging length multiplier, which could vary across individuals (Table 5). This value, multiplied by larval length at a given time step, determined the maximum digging distance for a given larva (fig. 4bi, bii). At any given time, an actively feeding larva could access certain food layers containing 0-4.5% waste concentration. It was assumed that a larva could access food layers from near its mouth

position, down to its maximum digging length. The furthest food layer accessible away from the top layer was rounded up from the digging length of the larva. The nearest food layer accessible from the top was rounded down from the larval length. A larva only faced the waste concentration available in accessible food layers. For example, if only layers 1-3 from the top were saturated with waste, while a given larva was feeding at the layers 4-6 from the top, it experienced no waste. If the total digging distance of a larva were to exceed the total number of food layers, it then experienced the entirety of waste in the totality of food. Empirically, this is likely to occur in scenarios where the food column length is shallow or the food gets consumed, resulting in a shallow food column length (fig. 4a).

Trait	Significance	Notes	Mean	c.o.v.	Source(s)
Digging length multiplier	Determined the depth to which a larva could dig, as a proportion of its own body length.	Digging depth = current larval length × digging multiplier	1.5	5%	Inspired by discussion in Sang, 1949

Table 5. Traits that may be important to competitive ability under crowding with long food columns.

2.6. Total treatments used – replication of experimental design

In order to capture the full scope of experimental results seen, we attempted to replicate the design used in our recent study (Chapter 5). This design consisted of a 3×3×3 factorial design with egg number, food column height and vial surface area as factors, respectively. The egg numbers used were 200, 400, 600; food column height levels were 5, 10 and 15 mm respectively; surface area levels were 200, 400 and 600 mm^2, respectively. The entire setup was run for 30 replicates per treatment combination. An expansion of this framework for predictive purposes is described in section 4.2.

2.7. Note on absence of Statistics

Following the reasoning used by White et al. (2014), we have refrained from performing statistical analyses on our simulation results, instead focusing more on the patterns of

differences seen among simulation runs with varying parameter values. In brief, given that we can replicate our simulations manifold for cheap, it is inappropriate to use statistical tests designed for comparisons in experiments which are resource intensive and involve limited replication. Most factors used in greatly replicated simulations would be statistically significant simply from having artificially inflated statistical power (White et al., 2014). Moreover, when trying to capture patterns seen in the experimental results with our simulations, we already knew that a subset of parameter combinations mimicking the treatment combinations in Chapter 5 would yield very different outcomes, based on prior limited simulation runs. Thus, as White et al. (2014) note, we knew *a priori* that the null hypothesis of no effect of different parameter combinations was false. Therefore, rather than do significance testing, we focused instead on the overall patterns observed in our simulated datasets, and compared them with corresponding patterns in empirical data. We further extrapolated our simulations to make gross predictions of patterns beyond the reference experimental set up used (section 4.2).

2.8. Simulation code

All code was written on Python 3 (Van Rossum and Drake, 2009), using the NumPy package (Harris et al., 2020). Plots were created using Matplotlib (Hunter, 2007) and Seaborn (Waskom et al., 2017) packages.

All the code for running the simulation can be found at the following GitHub link

(Note: this is a private repository until publication – please contact authors for access to code if required prior to publication):

https://github.com/SrikantVenkitachalam/Single-Generation-Simulation-Framework.git

Results

3.1.1. Base model – uncrowded conditions

The growth profiles over time for 70 individuals in 6 mL food (400 mm^2 surface area, 15 mm height) are shown in figure 2.a. The corresponding time to wandering and mass at wandering data are shown in figure 2.bi, bii. These closely corresponded to empirical values observed (Santos et al., 1997).

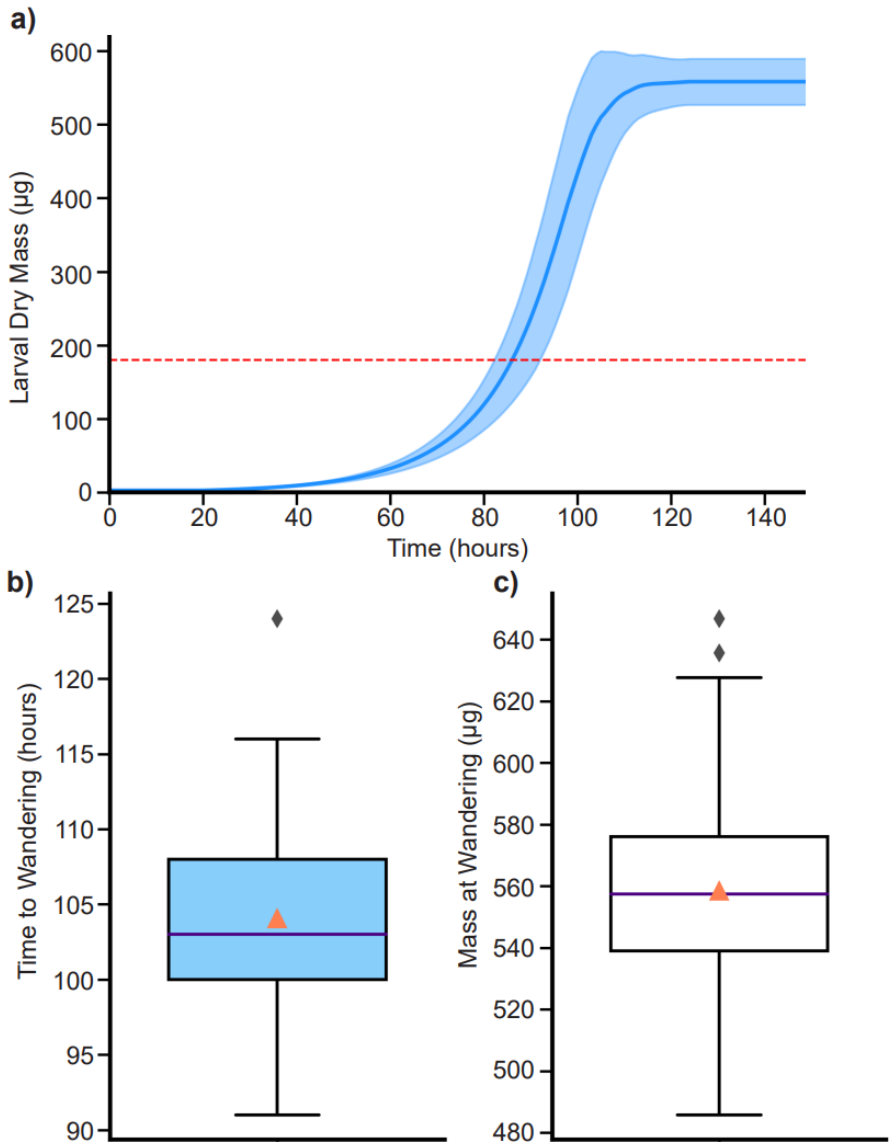


Figure 2. Base model (waste absent), uncrowded: 70 eggs, 6 mL food cast in 400 mm^2 surface area and 15 mm height.

a) Growth profile for 70 individuals. The solid blue line denotes the mean larval dry mass. The shaded region denotes standard deviation in larval dry mass. The dashed red line denotes the mean minimum critical mass to pupation (180 μg). Once the larvae commit to wandering, their mass remains unchanged.

b) Time to wandering distribution. The line within the box denotes the median. The orange triangle denotes the mean.

c) Mass at wandering distribution. The line within the box denotes the median. The orange triangle denotes the mean.

3.1.2. Base model – with larval crowding

The results of increasing egg number from 70-700 in 2 mL (400 mm^2 surface area, 5 mm height) food are given in figures S1, S2. Results shown in fig. S1 were generated using the base model without considering the effects of waste. Results in fig. S2 had the added effect of waste in the base model. These show the changes in egg to wandering survivorship (fig. S1.a, S2.a), as well as distributions of time to wandering (fig. S1.b, S2.b) and mass at wandering, respectively (fig. S1.c, S2.c). While patterns of survivorship and mass at wandering were largely similar with or without the addition of waste, it was in the distributions of time to wandering that the effects of waste came into play (fig. S1.b vs. S2.b). Without waste, the mean development time decreased across increasing egg number, and the variation collapsed (fig. S1.b). When the effects of waste were considered, mean development time increased, along with an increase in the variation around the mean (fig. S2.b).

3.2. A factorial framework for larval crowding

In addition to simpler consideration of changes only in egg number in fixed volumes of food (fig. S1, S2), we also tested the base model in the fully factorial framework of egg number, food column cross-section surface area and height.

This was a simulated 'replica' of the experiment reported in Chapter 5, examining 3 levels of egg numbers (200, 400 and 600 eggs), 3 levels of food column height (~5, ~10 and ~15 mm) and 3 levels of food column cross-section surface area (~200, ~400 and ~600 mm^2), in a fully factorial design. Within these 27 treatments lay several pairs of the same combination of egg and food volume, cast into different cylindrical dimensions (varying height and surface area). In total, there were 9 such pairs (200, 400, 600 eggs in two variants each of 2-, 3- and 6-mL food, respectively).

We implemented this experimental paradigm in our base simulation framework (with waste) for egg to wandering survivorship (fig. S4) and time to wandering (fig. S5). Cultures with the same food volume are highlighted using colour-matched text (e.g., fig. S4.i and S4.ix). In the base model, there were no differences observed for any pair of cultures containing the same volume of food, contrary to the experimental observations (Chapter 5).

3.3. The Expanded model

The 27-combination simulation of the experiment described by Chapter 5 was also run for the expanded model, incorporating waste diffusion, variable digging lengths and crowding for limited realised vial surface area (Methods section 2.5).

With the expanded model, there were major differences under various conditions of crowding compared to the base model (fig. 3 vs. fig. S4), while no qualitative differences were seen under

uncrowded conditions (fig. 2 vs. fig. S6). Figure 3 shows the egg to wandering survivorship obtained from the expanded model simulations for the 27 treatments described in section 3.2, with 30 randomly seeded replicates for each. Figure S7 shows the corresponding mean time to wandering data. Some of the salient observations are as follows:

1. On average, survivorship decreased as egg number increased under every food culture (fig. 3.d). This was also observed in empirical data (all empirical comparisons in this list are published in Chapter 5).
2. Survivorship increased with an increase in surface area (fig. 3.c) – also observed in empirical data.
3. Survivorship increased with increase in column height only for the transition from 5mm to 10mm (fig. 3.b). Even in that, there was a very large variance in data. In the base model, increasing height improved survivorship in the exact same way as increasing surface area did (fig. S4.b vs. S4.c). In empirical data, height increase also resulted in increasing survivorship, but to a very low degree compared to egg number and surface area. This result was thus close to what was seen in the expanded model. Furthermore, the more prominent change was from 5 mm to 10 mm height, similar to our model, although in the empirical data there was also a small but significant change observed from 10 mm to 15 mm, unlike in the simulation (fig. 3.b).
4. For each pair of cultures containing the same egg number and food volume, survivorship was higher in cultures with greater surface area and shallow depth of food, as compared to narrow surface area and long food column (fig. 3.a. i vs. ix, 3.a. iv vs. viii, 3.a. ii vs. vi). This was consistent with the empirical data. This pattern was not captured by the base model (fig. S4).

5. Mean time to wandering increased with increasing egg number, and decreased with increasing vial surface area (fig. S7.d, S7.c, respectively). This was consistent with empirical results.
6. There was a non-linear change in mean time to wandering along the food column height axis (fig. S7.b). Time to wandering increased from 5 mm to 10 mm height. However, there was a slight decrease in the overall time to wandering from 10 mm to 15 mm height. This was unlike the empirical results, wherein the development time increased almost monotonically, albeit to a very small degree, from 5 mm to 15 mm height. We speculate about possible explanations for this discrepancy in discussion section 4.3.1.
7. For pairwise simulated comparisons of the same combination of egg number and food volume (fig. S7.a. i vs. ix, S7.a. ii vs. vi, S7.a. iv vs. viii), mean time to wandering was higher across all egg numbers in the deeper food column with narrower surface area in 2 mL and 3 mL food. In case of 6 mL, the culture cast in the deeper column with the narrower surface area had similar time to wandering at 200 eggs and 400 eggs, but had lower time to wandering at 600 eggs than its culture counterpart cast into a shallower and wider cylinder. In the empirical results, the egg to adult development time was greater in the deeper versions of each food volume only at the higher egg numbers, and not at 200 eggs. This pattern was particularly exaggerated at 3 mL food.
8. In simulation results, within the deeper versions of 2 mL and 3 mL food, there was little difference in time to wandering across increasing egg numbers (fig. S7.a.iv and S7.a.i, respectively). In the experiment, there was a clear increase in development time across increasing egg numbers in these cultures – particularly in 3 mL food.

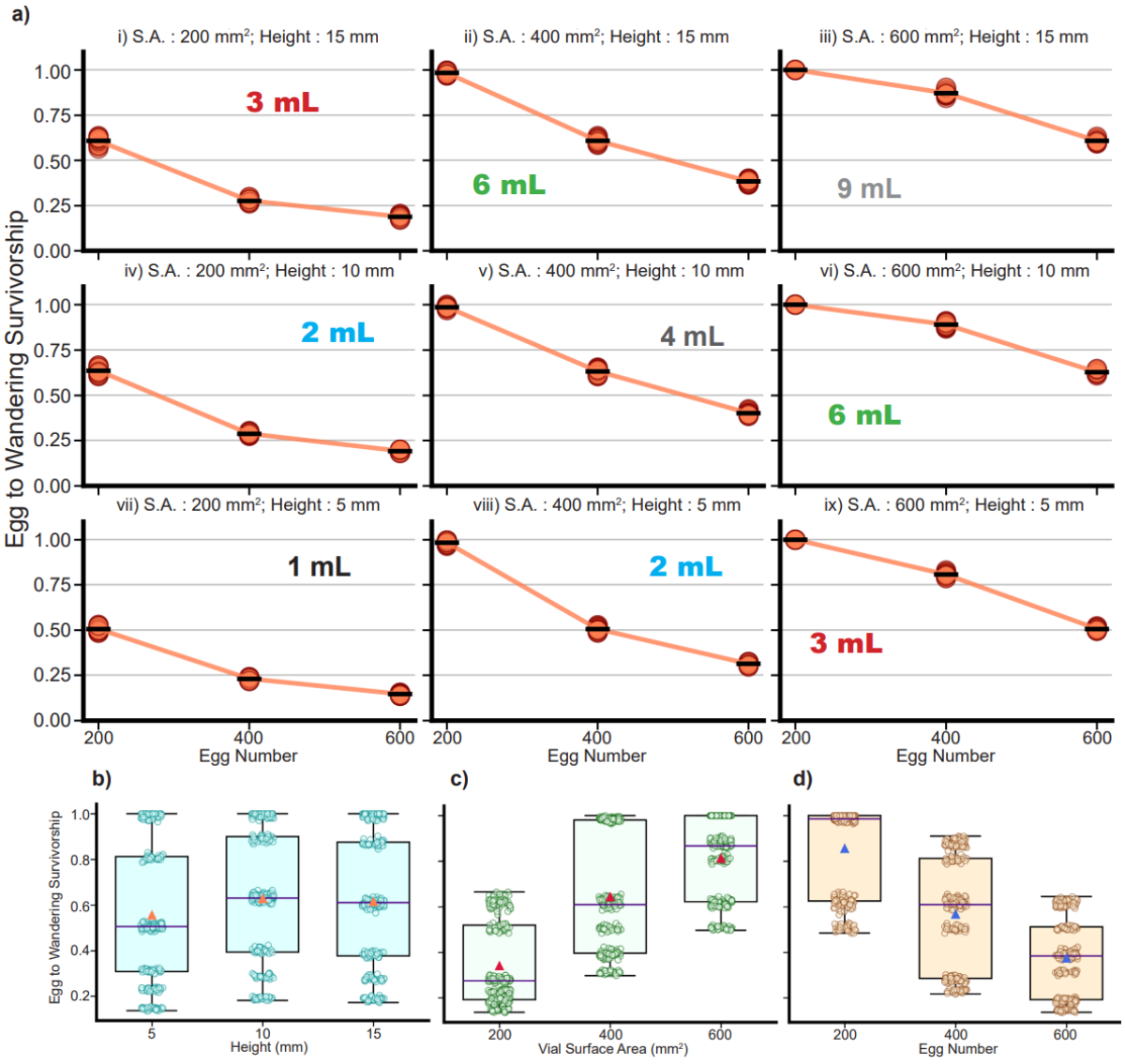


Figure 3. Egg to wandering survivorship in a large factorial simulated experiment (see Methods). S.A.: Cross-section surface area of the food column; Height: starting height of the food column.

a) Survivorship of 30 replicates for each of the 27 simulated treatments. The '-' symbol in black represents the overall mean for 30 replicates for each treatment. The food volume is also highlighted for each surface area and column height combination.

b) Survivorship values for the three levels of food column height pooling data across all other factors. The indigo line within the box represents median, the orange triangle represents the mean.

c) Survivorship values for the three levels of surface area of the food column, pooling data across all other factors. The indigo line within the box represents median, the red triangle represents the mean.

d) Survivorship values for the three levels of starting egg numbers, pooling data across all other factors. The indigo line within the box represents median, the blue triangle represents the mean.

As can be seen from the results, there were several differences in survivorship and time to wandering when the same number of eggs were added to the same volume of food cast into cylinders of different dimensions. We further elucidated the differences between the most extreme such pair of the crowded cultures – 600 eggs in 3 mL food – through differences seen in waste permeation, food depletion, digging depth distribution and crowding of realised vial surface area (fig. 4).

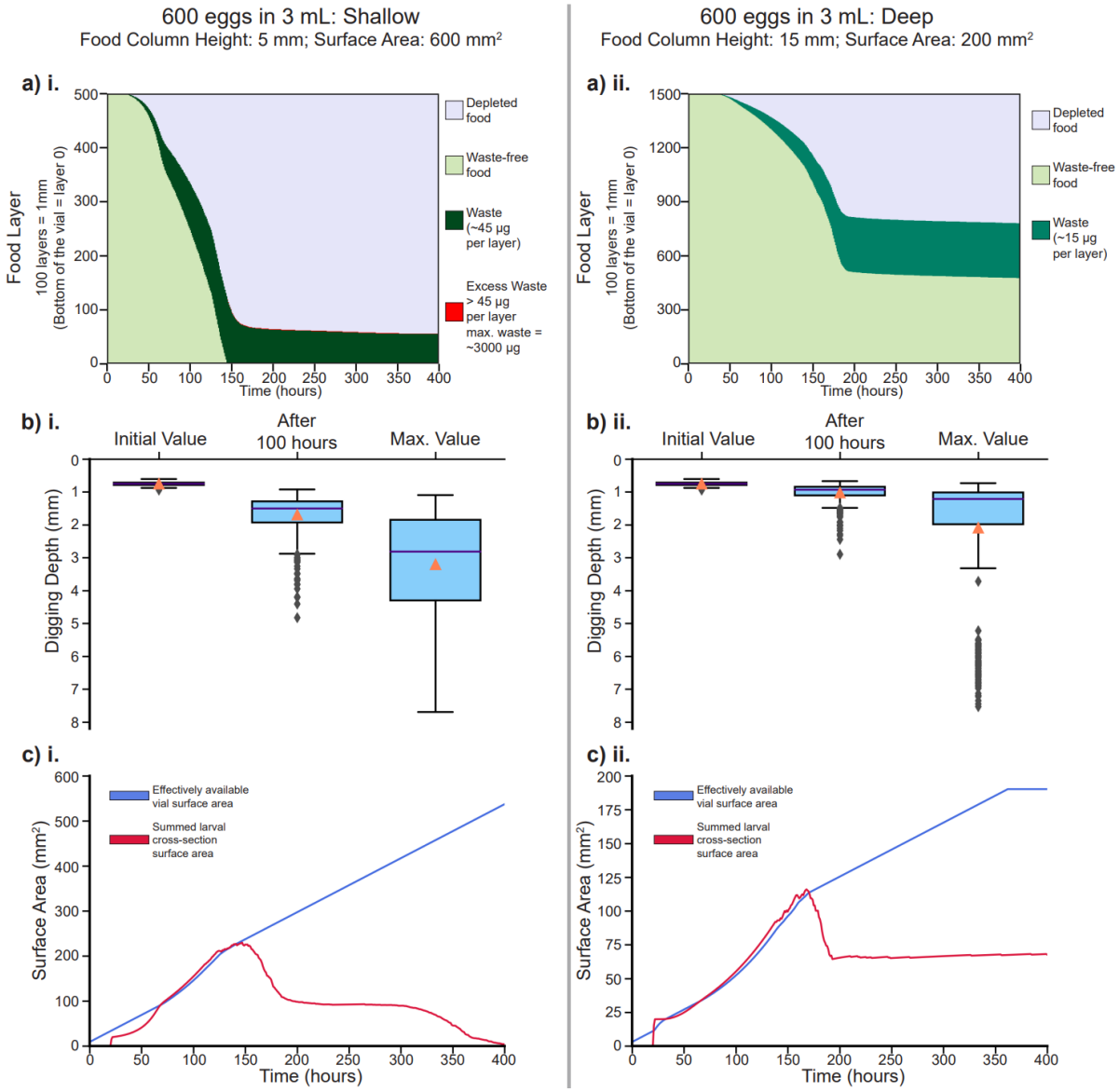


Figure 4. Properties of two simulated cultures with the same egg number and food volume, cast into two different cylindrical dimensions of food. The same individuals are initially seeded in both cultures. The left column denotes properties of the 'Shallow' type culture (see figure title); the right column denotes properties of the 'Deep' type of culture.

a) Waste permeation and food depletion over time in both cultures. Each point on the X-axis denotes a temporal snapshot of the status of the food layers presented in the Y-axis (see key).

   i. In the shallow type culture, the food depletes rapidly, until less than 1 mm deep food remains after 150 hours. At this point, all layers of food are saturated with waste, and

freshly excreted waste pools at the top food layer. This excess waste can be seen as a thin red line at the interface of depleted food layers and waste saturated food.

ii. In the deep type culture, food depletes until about 175 hours, after which the depletion starts plateauing. Almost half the food layers remain untouched by waste. Over half the food remains in the culture even after 400 hours, and little changes afterwards (data not shown).

b) Digging depth of larvae – showing the initial digging depth of freshly hatched larvae, at 100 hours from simulation start, and the maximum digging depth achieved by each larva, just before the point of wandering or death. While the initial distributions are the same in both cultures, the mean, median and quartile range of the shallow culture (b.i.) exceeds that of the deep culture (b.ii.) from 100 hours onwards, until the maximum. In case of the deep culture, only a few larvae achieve high values of digging depth, exceeding 5 mm, signified by outliers in b.ii., while most larvae remain small and dig to depths within 3 mm. In the shallow type culture, the mean digging depth at the max. point is itself at 3 mm, and many more larvae feed upto depths >3 mm.

c) The crowding dynamics of both types of cultures, signified by the value of summed larval cross section surface area (red) in relation to the expanding values of the effectively available vial surface area (blue). In both cultures, the summed larval cross section is initially zero when larvae are in their egg stage, and the value becomes >0 after hatching. We define crowding as the time when the summed larval cross-section surface area exceeds the effectively available vial surface area. The shallow type of culture (c.i.) has a much smaller window of time (from 75 hours to 150 hours) when it undergoes crowding, compared to the deep type culture (c.ii.), wherein crowding starts right from the moment of hatching at around 20 hours, and lasts until around 175 hours.

In our empirical study, major insight was gained by considering the outcome of crowding in terms of larval feeding band density (or effective density) instead of the total (eggs/food) density (Chapter 5). We defined the feeding band as the shallow volume of food in contact with air, to which larval feeding remains restricted. We had considered the top food volume of up to 6 mm depth as the feeding band (this value was reduced to the total volume of food if starting column height was lower than 6 mm).

We carried out the same exercise with our simulation results replicating the experimental design (fig. 3, fig. S7). We tested for similarities in the overall results with respect to cultures having the same total density across different effective densities. We considered the most crowded case of 200 eggs/mL total density. There were 5 cultures in total – 200 eggs in 1 mL, 400 eggs in 2 mL cast into deep or shallow cylinders respectively, and 600 eggs in 3 mL cast into deep or shallow cylinders respectively (both 2 mL and 3 mL cultures also had corresponding changes in cross-section surface area along with changes in height).

The survivorship and mean time to wandering of these simulated cultures are plotted in fig. 5. Similar to empirical results, there was no discernible change in both fitness related traits when total and effective density were the same (fig. 5.a.i., fig. 5.a.ii), accomplished by keeping the height constant and changing food volume via surface area, regardless of the egg and food combination used. In case the effective density exceeded the total density, accomplished by changing food column height while keeping vial surface area constant, survivorship decreased monotonically with increasing egg number (fig. 5.a.i). This was seen in empirical results as well (Chapter 5). While the increase in mean development time with increase in egg number was monotonic in case of experimental results, our simulations differed at least partly. While there was a relatively large increase in average time to wandering from 200 eggs 1 mL to 400 eggs 2 mL, the overall mean time to wandering fell somewhat from 400 eggs 2 mL to 600 eggs 3 mL (fig. 5.a.ii). We address this discrepancy in section 4.3.1.

Finally, we pooled the entire dataset with respect to survivorship and carried out a simple linear regression using either total density or effective density (i.e., feeding band density) as predictor variables. The survivorship data were log transformed for linearisation, as the drop in survivorship from low to high density was non-linear. This was unlike the empirical results wherein there was non-zero mortality even at the lowest densities used, leading to an overall linear relationship between total density and survivorship (Chapter 5). In the experimental paradigm used, the decreased survivorship at the relatively lower (but not uncrowded) densities was likely a combined effect of density-dependent and density-independent mortality, the latter not having been considered in our simulation framework.

Linear regression for survivorship as the dependent variable in the simulated data showed patterns very similar to the empirical results – effective density could explain far more variation for log survivorship than total density could. Total density as a predictor variable had an R^2 of 0.68 (fig. 5.b.i), while effective density as a predictor variable had an R^2 of 0.96 (fig. 5.c.i)

For time to wandering, however, a simple linear regression could not be carried out. Unlike in the experimental data, the simulated development time data were very clearly showing two different clusters changing differently with total or effective density (fig. 5.b.ii and 5.c.ii). The part of the data that had different total and effective densities, in case of survivorship, came to lie more closely on a line predicted from data that had the same total and effective densities. This was largely not the case for time to wandering.

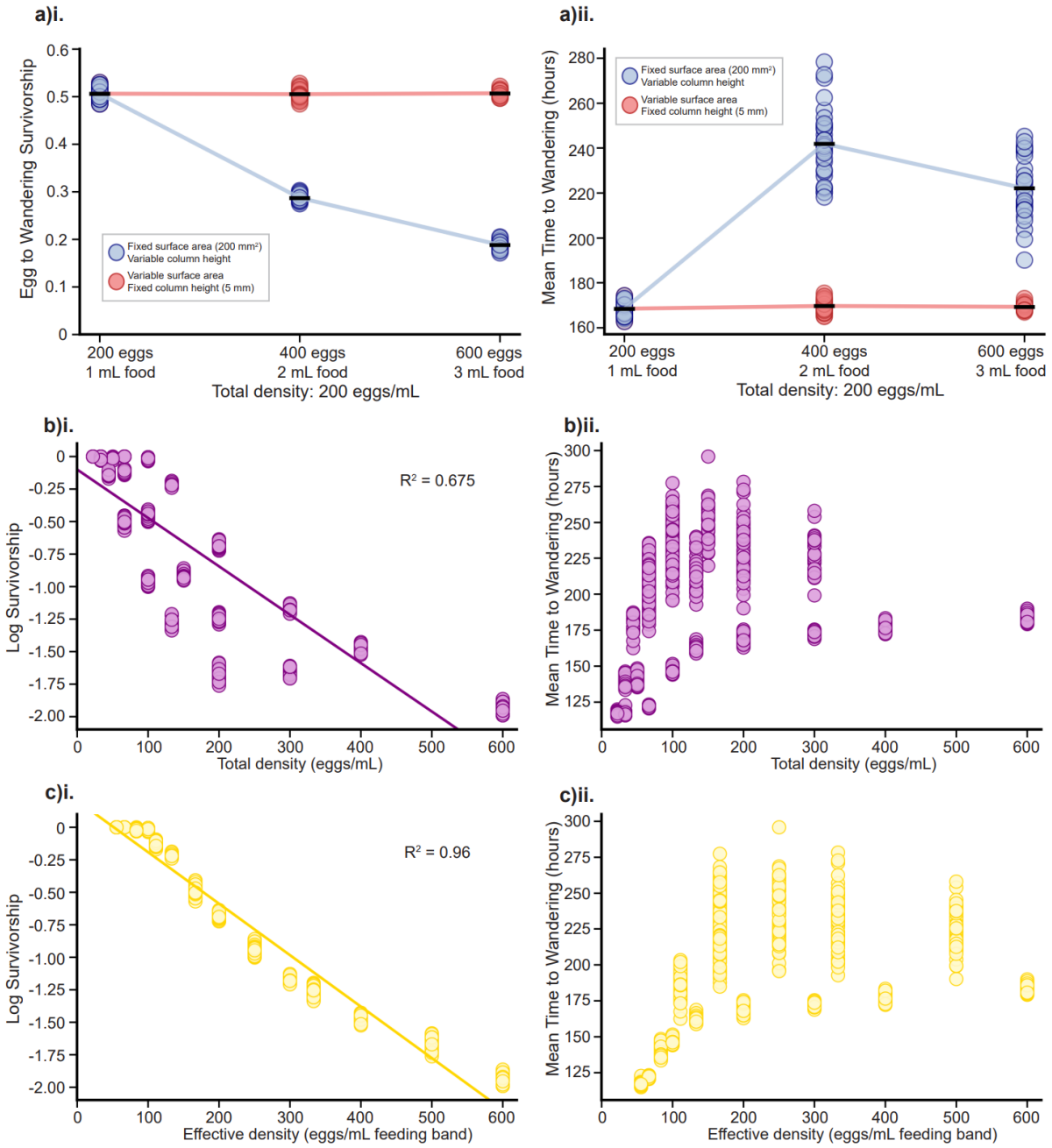


Figure 5. a) i) Survivorship and ii) Mean time to wandering for each simulated culture having 200 eggs/mL total density. The '-' symbol in black represents the overall mean for 30 replicates for each culture type. Plots b.i. and c.i. show the linear regression of log survivorship (pooled data from simulated experiment seen in fig. 3), as predicted by total density and effective density, respectively. b.ii. and c.ii. show the scatter of mean time to wandering of the pooled data, vs. total density and effective density, respectively.

Discussion

4.1. Findings from overall results

Similar to earlier models (e.g., de Jong, 1976; Nunney, 1983; see Introduction), the base model in our simulation framework captured Bakker's (1961) data quite well (fig. S1.a, S1.b). However, we also found that the base model did not account for changes in development time (fig. S1.c), which is an important fitness-related trait affected by larval competition (reviewed in Chapter 2). To account for development time variation, we introduced waste into the system, whose ingestion slowed the overall development time of our model larvae, in addition to killing the ones which ingested too much waste without having achieved their respective minimum critical mass (section 2.4.5.3). The resultant simulation better captured patterns seen in experiments done until the end of the twentieth century, in terms of survivorship, development time and dry mass (fig. S2).

We note that Bakker's (1961) data, quite uniquely in hindsight (given the many exceptions seen in subsequent studies), represented a purely "exploitation" mode of competition among larvae (*sensu* Park, 1954; reviewed in Birch, 1957), wherein differences in feeding rates were enough to predict the competitive outcomes of various strains of larvae. The crowded cultures in that study were implemented by seeding larvae over a thin layer of yeast covering a thick layer of non-nutritive agar (Bakker, 1961). This may have dispersed a majority of the waste away from the top food-containing layer where the larvae had to feed. Consequently, the near-absence of waste would make competition almost purely exploitative.

This reasoning may explain the success of several models, including our base model, in capturing patterns of data seen by Bakker in his studies. The exploitation type of competition was confounded by Bakker with the term "scramble" (Bakker, 1969), which was described originally to mean incomplete success in competition (Nicholson, 1954). In her model, de Jong

(1976) described *Drosophila* competition as scramble + exploitative, i.e., the outcome of larval competition would lead to incomplete success for the survivors as a majority would not feed optimally, and even dying larvae would consume non-zero food. This incomplete success would be expected to come about through purely exploitative action – the larvae would not affect directly or indirectly the feeding process of other larvae (de Jong, 1976).

However, once larvae face increasing concentrations of metabolic waste, as is expected under crowded conditions in homogenous food media, competition may no longer be purely exploitative. Excreted larval waste, which potentially affects the survival and/or development of all competing individuals (Botella et al., 1985), adds elements of indirect interference (Park, 1954; Birch, 1957; de Jong, 1976) into the process of competition (also see Weisbrot, 1966; Dawood and Strickberger, 1969). This could differentially affect individuals, and thus add a new dimension to larval competitive ability (Shiotsugu et al., 1997; Borash et al., 1998). We suspect that *Drosophila* larval competition in most laboratory scenarios would be scramble type with components of both exploitation as well as indirect interference.

A reduction in bite rate would be expected to delay overall development time of the larva under any condition where waste is high. This reasoning follows from discussion by Botella et al. (1985), whose study demonstrated higher waste conditions due to crowding leading to elongated egg to adult development times. We also know from previous larval crowding work that average development time tends to increase under crowded conditions (Sang, 1949; González-Candelas et al., 1990; Sarangi, 2018; Chapter 2,b). Mechanistically, this would make sense if a larva has to slow down its bite rate in order to optimally detoxify the accumulating waste within its body. To affirm this mechanism, we would need to test the feeding rate of larvae in food laced with waste products. In the current study, we did not include an actual reduction in waste levels within larvae due to detoxification, although this can certainly be implemented in the future. Moreover, this relationship between waste levels and bite rate could

work independently of the knowledge that late-eclosing adults in some crowding-adapted populations evolved reduced feeding rate alongside increased waste tolerance (Borash et al., 1998), or that populations adapted to tolerate high levels of metabolic waste products such as urea or ammonia showed the evolution of lower feeding rates compared to unselected control populations (Borash et al., 2000; Bitner et al., 2021).

While introduction of waste in the simulation framework does replicate patterns seen in the experimental data to a certain extent, there is a fundamental aspect missing. Recent studies conducted by us showed that similar densities cast into different dimensions yield very different outcomes of fitness related traits (Sarangi, 2018; Chapter 5). This vital but nuanced aspect of larval competition was not captured by the base model even with the implementation of waste (fig. S4, S5).

To address this, we introduced the expanded model, which includes features that could work specifically with vial cross-section surface area and food column height characteristics (section 2.5). Under this updated framework, simulated larvae could dig into the food column while excreting waste which would saturate the top layers of food first, affecting the smaller larvae which are unable to dig too deep. These larvae would also have their own cross-section surface area. Too many larvae in a constricted cylindrical container would see their cumulative cross-section surface area exceed the relatively little accessible vial surface area, leading to competition for limited space. Ultimately, such crowded conditions could cause larvae to 'miss out' on food eaten, while also plastically increasing their bite rate as an ameliorative measure.

These incorporations led to the expanded model capturing several facets of empirical data (see Results 3.3.). In particular, simulated patterns of egg to wandering survivorship mimicked the experimental data quite well (fig. 3). Overall patterns seen in survivorship from simulated cultures having the same combination of eggs and food but cast into different cylindrical

dimensions were largely preserved (fig. 3). Similar to empirical observations, there was a lot of unused food left at the end of a simulated culture in a deeper, more constricted food column than when the same egg and food combination was present in a shallow but wider cylinder (fig. 4.a.i, 4.a.ii). The simulated food also depleted faster in wider and shallower cultures than in deeper, constricted cultures – this phenomenon yet remains to be studied experimentally. There was also an overall increase in realised bite rate seen in simulated crowded cultures (data not shown), similar to empirical data (Sarangi, 2018). Perhaps more importantly, regression patterns of survivorship as predicted by total density and effective density (fig. 5.b.i, 5.c.i) were quite consistent with empirical observations (Chapter 5). For these regressions, any contradictions of simulated data with those drawn from experiments were in the realm of low total densities, likely due to the absence of density-independent mortality in our model. Effective density in the simulations, like in the experiment, predicted egg to wandering survivorship far better than the total density (fig. 5.b.i, 5.c.i).

While some aspects of development time, represented here by time to wandering, were captured by our simulated data, exceptions were also seen (see section 3.3.). Changes in mean time to wandering along the egg number and vial surface area axes were largely similar to experimental data. However, there was a lack of congruence on the food column height axis. Mean time to wandering increased along with an increase from a very shallow food column (same total and effective density) to a moderate food column (total and effective density not similar). This pattern was consistent with experimental data. However, on increasing food column height further (greater dissimilarity between total and effective density) there was a decrease in mean time to wandering in the simulations, unlike the consistent increase seen in experimental data. This pattern of observations has been explored further in the 'Predictions' section below. Along with greater food column height increase, there was also a lack of change in survivorship (fig. 3), whereas in experimental systems it has been well established that

survivorship improves when culturing the same number of eggs along an increasing food column height (Sarangi, 2018). However, this improvement is far less than the change in survivorship when vial surface area is increased instead of food column height (Sang, 1949; Chapter 5).

4.2. Predictions

One of the primary goals of simulating a biological system, such as our current undertaking, is to make predictions beyond the domain of the existing empirical work, ultimately guiding the development of better experimental designs.

After having re-created an existing experimental design within our simulation framework, and achieving reasonable (albeit varying) congruence with empirical results, we simulated two major expansions to the existing experimental paradigm.

The first of these is straightforward. There are three easily observable outcomes of implementing larval competition – reduced egg to adult survivorship, increased mean egg to adult development time and reduced mean adult body mass at eclosion, as compared to the uncrowded conditions (Sang, 1949; Ohba, 1961 – as cited by González-Candelas et al., 1990; Sarangi, 2018; Chapter 2). Of these, only the first two were studied experimentally in the factorial experimental framework in Chapter 5, and subsequently explored via simulations (fig. 3, fig. S7). Thus, we make broad predictions with respect to body mass distributions in various crowded cultures in the experimental design replicated in this study. The simulated mean and variance in dry body mass at wandering have been shown in fig. 6, fig. S9, respectively. At high surface areas, body mass decreases across increasing egg numbers (fig. 6.a.iii, vi, ix), along with an increase in variance (fig. S9.a.iii, vi, ix). At constricted surface areas there is little change in the mean or variance across egg numbers (fig. 6.a.i, iv, vii; fig. S9.a.i, iv, vii). In

deeper and constricted food columns, a similar lack of change was seen for simulated mean and variance in time to wandering (fig. S7.a.i, iv, vii; fig. S8.a.i, iv, vii), but not in empirical data (Chapter 5). For the same egg number and food volume, a simulated culture cast into a deeper and more constricted column will have, on average, greater mean and variance in dry body mass (fig. 6.a.i vs. fig. 6.a.ix; fig. S9.a.i. vs. fig. S9.a.ix).

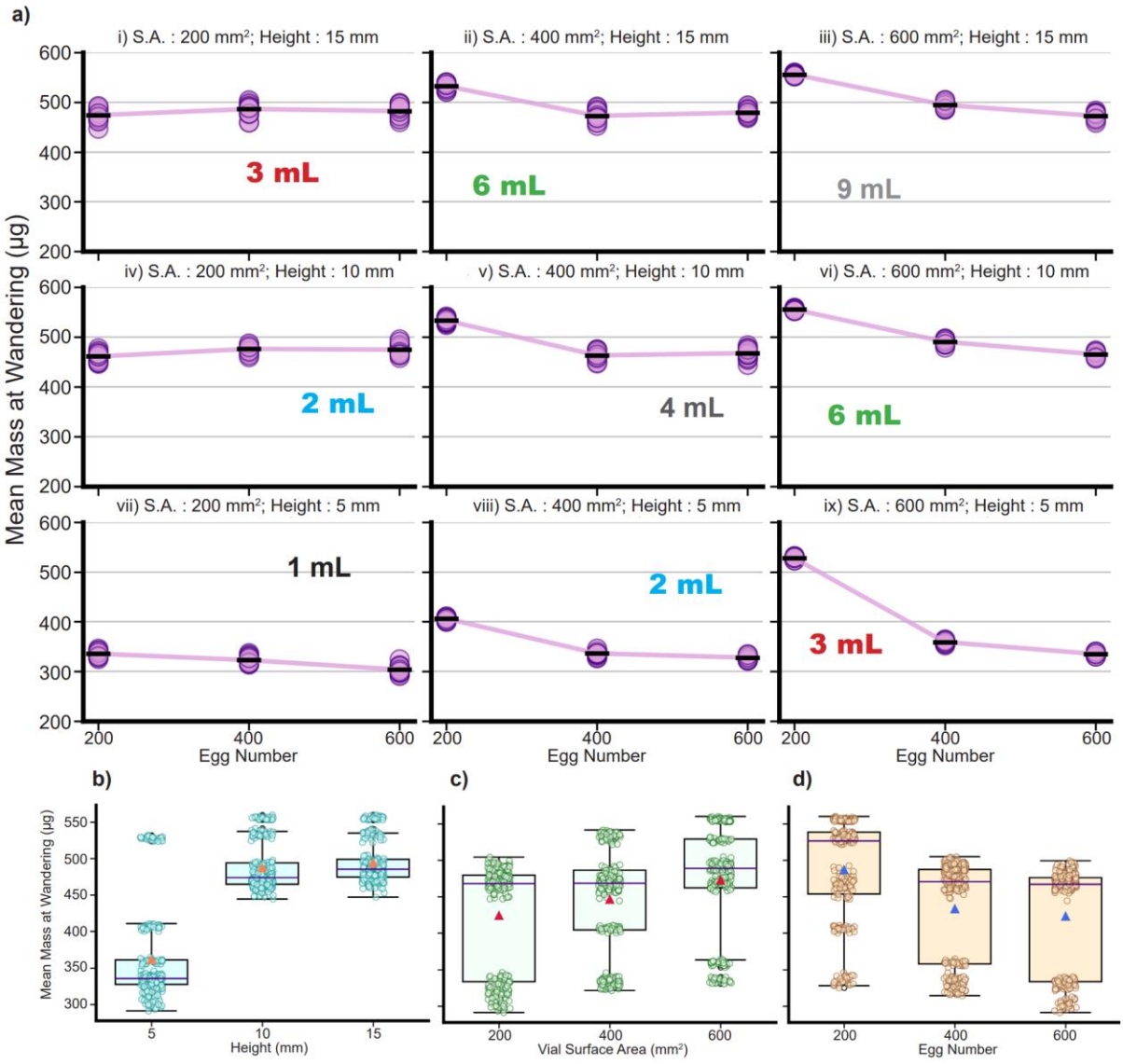


Figure 6. Mean mass at wandering (µg) in a large factorial simulated experiment (see Methods). S.A.: Cross-section surface area of the food column; Height: starting height of the food column.

a) Mean mass at wandering of 30 replicates for each of the 27 simulated treatments. The '-' symbol in black represents the overall mean for 30 replicates for each treatment. The food volume is also highlighted for each surface area and column height combination.
b) Mean mass at wandering values for the three levels of food column height pooling data across all other factors. The indigo line within the box represents median, the orange triangle represents the mean.
c) Mean mass at wandering values for the three levels of surface area of the food column, pooling data across all other factors. The indigo line within the box represents median, the red triangle represents the mean.
d) Mean mass at wandering values for the three levels of starting egg numbers, pooling data across all other factors. The indigo line within the box represents median, the blue triangle represents the mean.

The simulations perhaps have a unique situation wherein crowded cultures with lower surface areas have relatively unchanged development time and body mass of survivors across increasing egg numbers. This occurs along with a large reduction in survivorship. In empirical data, this pattern was observed at lower heights, but not at the 15 mm height (Chapter 5).

The second major prediction is with respect to the treatment levels used for the same total density. We implemented a total density of 200 eggs/mL across various combinations of eggs and food volume, resulting in a deeper and constricted food column, or a shallow and wide column (as seen in figure. 5.a). The simulated scenarios ranged from 100 eggs in 0.5 mL food, up to 1000 eggs in 5 mL food (fig. 7). We avoided predicting beyond these limits, as other factors such as humidity, or the lack thereof, are likely to become important at very constricted or very wide and shallow food columns. Within the limits seen, there is no change predicted in egg to wandering survivorship or mean time to wandering for increasing surface area (i.e.,

keeping total density and effective density the same) (fig. 7). When the change in food volume is via column height, egg to wandering survivorship is predicted to decrease consistently with increasing egg number and food volume (fig. 7.a). There is an interesting pattern seen in mean time to wandering – as the food volume increases, the mean time to wandering increases, peaks at 400 eggs in 2 mL food, and reduces to a middle value at higher egg numbers and food volumes, with a slight increase seen with increasing egg and food volumes (fig. 7.b). This is also observed in fig. 5.a.ii, wherein the time to wandering at 400 eggs in 2 mL was greater than in 600 eggs in 3 mL food.

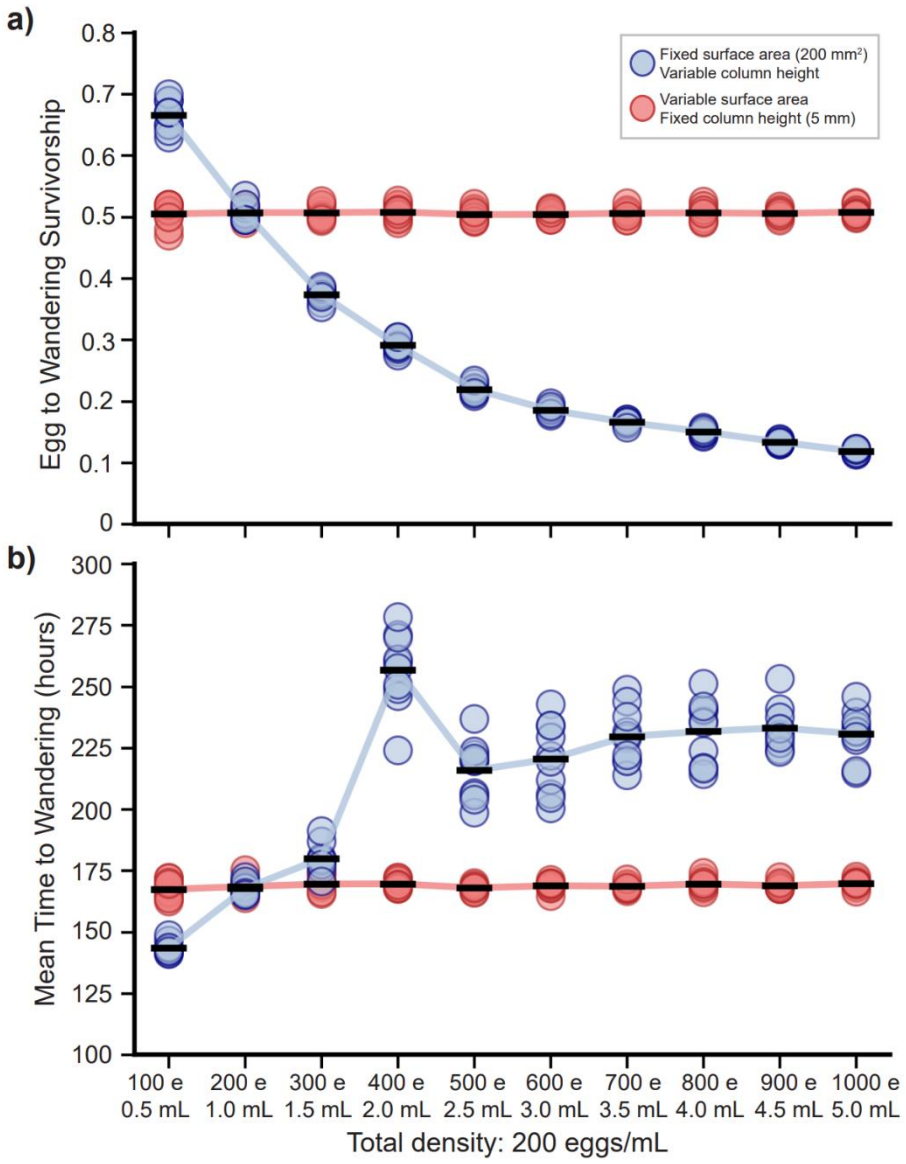


Figure 7. Simulations predicting multiple treatment combinations achieving a total density of 200 eggs/mL. This is an extrapolation of the cultures considered in figure 5.a. The figure shows a) egg to wandering survivorship and b) mean time to wandering for the extended design. The X-axis labels show egg number (shorthand 'e') and food volume (mL) combination used for every treatment pair (see legend). The '-' symbol in black represents the overall mean for 10 replicates per culture type.

This pattern likely occurs in the simulation because in a culture with 400 eggs constricted into a narrow column with 2 mL food, there is not enough waste accumulated to kill all the larvae as the waste permeates downwards, leading to several waste resistant larvae surviving much later into the life of the culture. Due to the relatively high numbers and lower surface area, there is also enough initial space crowding present such that there would be a further delay in development time. Upon increasing the egg numbers more than 400, the resultant increase in waste levels would kill a majority of the larvae which could have otherwise survived later into the culture if waste concentrations were lower, leading to an overall shorter mean time to wandering (also see Discussion 4.3.1.).

These traditional predictions from our simulations are fairly conservative, taking a cautious approach in expanding upon known empirical regimes, instead of making more general claims e.g., on the ecology and evolution of larval competitive ability in *Drosophila* as a whole, or on interspecific competition, or on natural systems.

The reason for our conservative outlook on ecological predictions is due to our novel predictions for mechanisms. This, we feel, is the most important test for the model – designing experiments to test the mechanisms predicted in this model would validate more general predictions that we may make in the future. Elucidation of the underlying mechanisms of larval competition under different contexts with respect to the effective density of cultures would then

further inform the simulation framework, and be used to make more robust ecological and evolutionary predictions.

The formulation of this simulation framework compelled us to peer inside the black box of larval competition, and we found the knowledge of mechanisms wanting. While some traits such as bite rates, egg mass, hatching time and minimum critical mass were fairly well studied and defined, most of our 'self-defined' traits (Tables 2-4) were deigned to be so because we did not find any relevant studies that had explored the exact mechanisms or measurements of these traits. These are particularly true for traits, as well as phenomena, used in the expanded model (section 2.5; Tables 4, 5). The mechanisms assumed in our simulations should be good starting points for exploration of these hitherto unexplored traits and characteristics relevant to crowded systems, as they yield a sufficient fit to empirical data. Of greatest import are understanding the following:

a) The complete content, and build-up of metabolic and other egested waste products, as well as their effects on larval feeding behaviour in various kinds of crowded cultures. Knowledge of the mean and variance of waste conversion from food would also be useful when comparing uncrowded and crowding-adapted populations.

b) The overall mechanism by which crowding for limited surface area may occur, as well as its effects on larval feeding behaviour.

c) The dynamics of larval digging – the mean and variance in larval length and digging distance achieved under various scenarios of crowding.

d) The overall phenomenon of waste permeation and/or diffusion – does waste predominantly saturate layer by layer, or does it travel down the food column and settle at the bottom? We did try to implement the latter process in our framework, but the

patterns in our simulation results (data not shown) did not capture those seen in empirical data.

So far, none of the points listed above have been pursued experimentally in larval crowding systems, to our knowledge.

We think that further additions and modifications to the mechanisms and traits of the current simulation framework would make more sense once some (if not all) of the implemented mechanisms are empirically better understood. We would, however, be remiss not to credit authors who, several decades prior to our work, made observations or speculations regarding most phenomena and behaviours explored in the current study (Sang, 1949; Bakker, 1961; Gilpin, 1974; Botella et al., 1985; Moya and Castro, 1986; Bierbaum et al., 1989; Mueller, 1990; Chippindale et al., 1994; Sokolowski et al., 1997; Sarangi et al., 2016; Sarangi, 2018).

4.3. Drawbacks and possible quirks of the simulation framework

4.3.1. Discrepancies with data

Currently, most of the major discrepancies of simulated results with respect to empirical data lie at high levels of crowding coupled with deep food columns. In simulations, there is no change in survivorship for the same egg number in increasing volumes of food beyond a certain height (fig. 3.a, 3.b). Meanwhile, the simulated time to wandering follows a non-linear pattern across increasing food column heights, both with the same egg number (fig. S7.a.i, iv, vii) as well as proportionately increasing egg numbers (fig. 5.a.ii, fig. 7b) – showing the lowest development time at the lowest food volume, then increasing at moderate volume, then coming to a middle value at a higher volume. In experiments, both survivorship and development time increase for the same number of eggs and increasing food column heights (Chapter 5).

There are two possibilities here – a) either we failed to capture the effect of food column height on a crowded culture, or b) we captured the overall pattern but could not capture the corresponding levels of height at which the pattern unfolds. These can be addressed through an expanded experimental design – increasing food volume through several levels of column height while keeping the number of eggs the same. If a) is correct, then both survivorship and development time will likely keep increasing with increasing height (or development time may not change beyond a certain height). However, if b) is correct, there should be no change in survivorship and/or a decrease in development time beyond a certain height. The latter would mean that our simulations captured the overall patterns of the effects of changing food column height, but did not match the dimensions of experimental culture vials correctly. For testing congruence of empirical development time with simulated data, an experiment using uniform total density across several levels of increasing egg numbers and food column heights may also suffice.

The lack of congruence with the outcomes of crowding on changing food column height also creates some discrepancies with data from other studies besides the experiment of Chapter 5. We know from previous studies (Sarangi, 2018) the survivorship, pre-adult development time and body mass patterns in cultures crowded at 600 eggs in 1.5 mL food (400 mm^2 vials), 1200 eggs in 3 mL food (400 mm^2 vials), and 1200 eggs in 6 mL food (350 mm^2 vials). While the survivorship data is well captured by the simulations at 600 eggs, 1.5 mL food, there is only a slight reduction in survivorship when doubling the egg number and food volume in our simulations (data not shown), whereas in experiments there is >10% reduction in survivorship (Sarangi 2018). More incongruent are the data from 1200 eggs in 6 mL food – while survivorship is the lowest amongst the three types of cultures in our simulations (data not shown), it is similar to survivorship in 600 eggs in 1.5 mL food, in experiments. This clearly shows the importance of the effect of food column height on survivorship, which does not seem

to be captured in our simulation framework beyond a point. However, mean time to wandering and mean mass at eclosion patterns in the respective simulated cultures are very much in line with experimental data (data not shown).

These discrepancies likely happen due to the mechanism of waste permeation considered by us. Given a certain number of eggs and a certain vial surface area, the pattern of waste permeation is likely to be very similar across all but the lowest food column heights. This once again highlights the need for future studies on waste permeation mechanisms and its effects on larval feeding.

An important aspect that has been seen in crowded cultures in 400 mm^2 vials containing food $\geq$ 2 mL is the distribution of mass at eclosion across the development time window. Adult mass is maximum for the earliest eclosing flies; those flies eclosing at the middle of the distribution have lower mass; and finally, the last eclosing flies have increased mass, similar to the early eclosing flies (Sarangi, 2018; Chapter 2). This pattern of experimental data is likely indicative of some detoxification mechanism in late feeding larvae, or some diffusion of waste away from the feeding band – both of which have not been incorporated in the simulations. Thus, in simulated crowded cultures, while the earliest wandering larvae (and hence eclosing adults) do have the maximum mass, all subsequent wanderers are much smaller, with no larva with delayed development time having increased mass (data not shown).

Finally, while there are several aspects of empirical data that have been successfully captured by our work despite the discrepancies, we do not yet know whether our 'best-guesses' employed here actually replicate the process of competition, or if they are simply approximating empirical consequences through non-biological causes. In either case, further experimentation using the leads from the current study are likely to yield greater understanding of the ecology and mechanism of larval competition.

4.3.2. Some additional features of the simulation that may have biological relevance

The following features (or quirks) of our simulation may be worth exploring, for they may represent important real-world relationships.

The first is the effect of waste on the outcome of crowding. We have observed that each time any waste is present in the base model, it becomes the predominant factor controlling the outcome of the culture (fig. S2). The larvae with the highest waste tolerance are the only ones that survive when faced with any amount of waste. No other larvae can survive regardless of any other trait advantage they may possess (data not shown). We know that a host of traits besides waste tolerance can evolve under experiments on adaptation to larval crowding (section 1.1). Thus, it is unlikely that waste is either this prominent or ever-present. As a possible answer to this, the incorporation of waste permeation in our simulations delayed the exposure of waste to most larvae, thus painting what might be a realistic picture for the role of waste in larval competition. It may also be possible that, in reality, the effects of waste on larvae are completely different from what was implemented in our simulations. Elucidating this possibility would require further experimentation.

Secondly, in order to get any realistic result, efficiency could not be plastically reduced due to high waste or high crowding, such as was done with bite rate or food eaten (section 2.4.5.3; 2.5.1). Any time efficiency was reduced, the larvae under crowded conditions even in relatively high volumes of food rapidly depleted all remaining food without converting most of it to growth. This led to almost no survivorship with completely depleted food from initially high food column lengths – a situation that has not yet been observed empirically. Thus, while greater efficiency may play a vital role in faster larval growth (section 1.1, fig. 1), and be selected for under certain situations of crowding, it may be unlikely to reduce plastically to large degrees under any crowding stress (assuming this feature of the simulation is realistic).

An additional feature is ‘crowding’ as defined in the expanded model (section 2.5.1), wherein we predicted the potential importance of the space occupied by larvae (measured in terms of cross-section surface area) and its changing dynamics with the overall accessible space in the feeding band. In the simulations, this feature was of central importance in the outcome of a crowded larval culture (fig. 4.ci, cii). A question that arises from this is whether high surface area occupancy is what represents ‘true’ larval crowding – which would mean that a crowded larval culture is only crowded for a period of time. True crowding may not be occurring initially and especially towards the end of a culture, due to increased availability of food surface area to the larvae (fig. 4.c; also discussed in Sokolowski et al., 1997). Depending on the vial surface area and the number of eggs seeded, the dynamics of crowding as defined here may be central to predicting the outcomes of competition among larvae.

4.4. Looking ahead

4.4.1. Factors left to incorporate

Before concluding, we wish to note several traits, characteristics and behaviours we omitted from our simulation framework, in order to keep it from becoming more complex than it already was. To start with the most well documented characteristic, we decided to remove larval instars from consideration. While earlier versions of our framework did have an implementation of instars (S. Venkitachalam and A. Joshi, *unpublished*), it was unclear if the simulations benefitted from the added feature. It is known that both feeding rate and bite mass can increase continuously over the period of larval growth (Santos et al., 1997; Robertson, 1963, respectively), rather than in discrete steps corresponding with instars. According to Bakker (1961), instar moulting episodes could possibly be relevant in terms of enforcing time

away from feeding that could be detrimental to a larva's competitive ability (see Green et al., 1983).

Another characteristic that we have not incorporated is the possibility of drowning of larvae under limited surface-area crowding in cultures with deep food columns. Given that there is limited space under such conditions, it is likely that there would be scope for larval collisions (Gilpin, 1974), and deep digging larvae may be blocked from access to air altogether, resulting in them drowning. This may be an important 'disoperative factor' (see Bakker, 1961) in reducing survival under high feeding-band density conditions, while drowning resistance could be a trait to counter it.

There is likely great room for exploration of the ecology and evolution of larval digging behaviour under crowded conditions. Recent studies have highlighted the possibility of 'cooperative digging' in *Drosophila* larvae (Dombrovski et al., 2017, 2019), and it remains to be seen if such phenomena are observed in our laboratory populations. Additional related questions also arise – whether such digging phenomena can show plasticity under different scenarios of crowding and whether the level of synchronicity in digging behaviour can evolve under selection for high-feeding band density coupled with deep food columns. The potency of enzymes such as salivary amylase secreted by digging larvae which can help break down the food substrate for easier consumption (Gregg et al., 1990; Sakaguchi and Suzuki, 2013), or rate of the physical breakdown of food by larval mouthparts (Burnet et al., 1977), are each potential candidate traits for the action of selection under crowded conditions.

Finally, in shallow food columns with high larval density, food can run out, leading to larval death (Sarangi et al., 2016; Chapter 2; see fig. 4a.i). When food runs out, we have caused the simulated larvae to instantly commit to wandering in case they have crossed the threshold minimum critical mass to pupation. However, real-world larvae may instead attempt to seek

out food through increased locomotion (Green et al., 1983; but see Ruiz-Dubreuil et al., 1996) and arrest their development for several hours if they run out of food. The latter is the mechanism described for the phenomenon of 'larval stop' observed in earlier studies (Ménsua and Moya, 1983), which has not yet been incorporated in our simulation framework. It is additionally worth pointing out that the presence of large amounts of waste (Botella et al., 1985), or high surface area crowding can also possibly slow down development time under crowded conditions and lead to 'larval stop' like phenomena.

4.4.2. Future expansions of the model

4.4.2.1. An evolutionary extension of the single generation framework

Understanding the importance of different traits under various scenarios of larval crowding would involve the extension of the current simulation framework into a multigenerational model. This would allow the study of evolutionary consequences of rearing larvae in given culture conditions. In this extended framework, the wandering larvae would end up as adults, mate and produce offspring with some rules of inheritance, perhaps a simple additive model for quantitative traits to begin with. For a given crowding regime, the offspring produced from multiple replicates at a single generation would be mixed and resampled into new cultures subjected to the same crowded regime, forming the next generation. This endeavour is currently underway (see Venkitachalam (2017) for a preliminary version of this model).

4.4.2.2. Traits likely to evolve across different types of cultures

As a first pass to predicting the traits that might be important in different kinds of crowded cultures, we have plotted the trait distributions of surviving and dying individuals (fig. S10) in two types of cultures – sharing the same number of eggs (600) in the same volume of food (3 mL) but cast into different types of cylinders ('shallow' or 'deep' – see fig. 4, S10). A brief overview of our findings is as follows:

Traits conferring initial advantage:

There were no discernible differences between surviving and dying individuals in terms of their hatching times, in either shallow or deep cultures (fig. S10.1.). Experimentally, reduced hatching time has evolved in some crowding adapted populations, but the extent of differences was less than an hour (Chapter 3). It is likely that this is not observed in simulations due to the 1-hour minimum resolution used in our simulations and low between-larva variation in hatching time (Table 1.a)

Large differences were observed between surviving and dying individuals in terms of both their egg lengths and egg widths, with survivors having larger eggs on average (fig. S10.2; S10.3). This difference was more exaggerated in case of the deep-type culture (fig. S10.2.aii, bii; S10.3.aii, bii), with most of the early surviving individuals having greater egg lengths and widths (fig. S10.2.cii; S10.3.cii). In the simulation, it is likely that an initial mass advantage is critical to surviving and feeding in the more intensely crowded 'deep' type of culture, wherein hatched eggs have to immediately face crowded conditions. Interestingly, a recent study by us showed that greater egg length and width evolved in three different sets of crowding-adapted populations compared to low density reared controls, with the largest eggs being found in the cultures adapted to high effective densities with deep food columns (Chapter 3).

Traits conferring growth rate advantage:

Initial bite rate was higher among survivors of both types of simulated cultures (fig. S10.4). However, the extent of difference between bite rate of surviving and dying individuals was greater in the shallow type culture (fig. S10.4.a.i). This may be due to the simulated shallow type cultures having a greater scope for larval growth in the initial period of absence of crowded conditions (fig. 4.c.i). In selection experiments, populations reared in crowded conditions similar to the shallow type cultures did not evolve greater feeding rate than controls when

assayed singly (Nagarajan et al., 2016; Sarangi et al., 2016). In contrast, survivors of the shallow simulated cultures would be expected to evolve higher bite rate, which would be visible regardless of culture conditions.

A similar pattern as initial bite rate was also seen for mean efficiency in simulations (fig. S10.7.). However, it is unknown if populations evolved in cultures resembling the shallow type have greater food to biomass conversion efficiency. Populations adapted to cultures similar to the deep type evolved lower efficiency than the controls, which may have been due to trade-offs between food acquisition and food utilization in the particular cultures studied (Mueller, 1990; Joshi and Mueller, 1996).

End stage related traits:

Surviving individuals in the shallow-type simulated culture had lower minimum critical mass than the dying ones (fig. S9.a.i), whereas no clear pattern emerged for the deep-type culture (fig. S9.a.ii). Till date, no crowding-adapted population from any selection experiment has been shown to evolve reduced minimum critical mass to pupation, but recent studies have indicated that some crowding adapted populations, particularly those reared in cultures similar to the shallow type, may have evolved smaller mass at uncrowded conditions (Sarangi, 2018; Chapter 2) – it is yet to be established whether these differences have a link to reduced minimum critical mass.

Both shallow and deep type simulated cultures showed slight differences between surviving and dying individuals in terms of their waste tolerance (fig. S10), with perhaps a greater difference in deep type culture (fig. S10.a). However, the more prominent results were visible in the time dependence of waste tolerance. While early survivors did not have any pattern in terms of their waste tolerance (fig. S10.c), there was a clear linear pattern for the same in dying individuals at later time stages. The early dying individuals had lower waste tolerance, with

late dying ones having greater waste tolerance (fig. S10.c). Among these deaths, a few survivors were also present, usually possessing slightly higher waste tolerance than dying larvae at that time step. In the simulations, the larval deaths were exclusively due to high waste concentration, and fig. S10c shows the time at which the waste got intolerable for many small larvae. Of note is also the observation that the larval death occurred earlier and in a smaller time window in the shallow type culture than in the deep type culture (fig. S10c). Experimentally, waste tolerance evolved primarily in populations adapted to cultures resembling the deep type of culture, and particularly in the late eclosing individuals of those populations (Borash et al., 1998; but see Nagarajan, 2009 and Sarangi, 2018 for some contrasting points).

Traits from the expanded model – crowding and digging:

The deep type simulated cultures had stark differences among survivors and dying individuals in terms of their crowding threshold (fig. S10.5.aii, bii), and slight differences in terms of peak crowding bite rate (fig. S10.6.aii, bii). The survivors appeared to detect crowding at lower thresholds and fed at higher bite rates under crowding compared to those individuals that perished. Smaller differences existed in the shallow type culture in terms of crowding threshold (fig. S10.5.ai, bi), and almost none in terms of peak crowding bite rate (fig. S10.6.ai, bi). This highlights the importance of amelioration of the detrimental effects of crowding in the deep type culture, which faces much higher feeding band densities and thus greater crowding for limited space (fig. 4.c.ii). Surprisingly, in selection experiments, the evolution of increased feeding rate visible as a plastic response to crowding occurred most drastically in populations reared in cultures similar to the shallow type (Sarangi, 2018). We speculate that this could be due to some limit to the plastic increase in feeding rate, which may be capped at lower effective densities, and further crowding would reduce this plastic increase. However, further

experimentation on feeding rates at different effective densities is critical to addressing this conundrum.

There was not much difference observable among surviving and dying individuals in terms of their digging length multiplier in the simulated shallow type culture (fig. S10.11.ai, bi). Survivors in the deep culture appeared to have slightly higher respective digging multipliers (fig. S10.11.aii, bii). This is plausible, as digging is likely to be more important in the deep food columns. This difference was probably not as drastic as was seen for other traits, perhaps because larval growth in simulations was a primary determinant of digging length – as long as a larva grew enough to avoid the upper waste-saturated layers, the digging length multiplier would likely only confer slight advantages on average.

4.4.2.3. Exploring population dynamics

We may additionally also carry out various population dynamics experiments using our simulation framework. Unlike in the abovementioned multi-generation extension, each population culture would have a given food volume in a given cylinder, but there would be no control over egg numbers laid each generation. For a given population, the number of eggs laid each generation would instead depend on the number of adults as well as their fecundity (for some such experiments, see Mueller et al., 2000; Dey et al., 2012; Pandey and Joshi, 2022a,b). Recently, it has been observed that populations reared under different combinations of food volumes, total densities and effective densities can evolve different degrees of constancy and persistence stability in their population dynamics (Pandey and Joshi, 2022a,b). Simulations would allow the investigation of dynamics induced in several different types of cultures, which may not be possible empirically due to logistical limitations.

A recent simulation study achieved significant congruence with an experimental population dynamics paradigm (Tung et al., 2019). Survivorship and mass distributions at low and high

food levels in the population dynamics experiment were successfully predicted by Tung et al. (2019), based on earlier studies following up on Bakker's findings (Bakker, 1961; Nunney, 1983; Mueller, 1988b). As their population dynamics experiment was run on set generation times, development time changes due to various egg numbers in limiting food may not be as important in predicting the larval stage outcomes every generation.

Future population dynamics experiments may be carried out to explore if predictions by Tung et al. (2019) can hold in different paradigms exploring various vial surface areas and food column lengths. Integration of some findings of our current study may be important with the earlier model in order to carry out further predictions in more nuanced setups.

In both multi-generation extensions as well as population dynamics simulations from our current study, it may be important to consider a potentially important caveat – even slight deviations from empirical reality in a causal model such as ours could snowball across multiple generations, leading to large deviations when making ultimate predictions. Despite this caveat, however, both the avenues of future studies could respectively direct important experiments for long term selection, or population dynamics, thus leading to a greater understanding of the ecology of competition and the evolution of competitive ability – ultimately rendering the theory of density-dependent selection both more robust and more nuanced.

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Supplementary material for chapter 6

An individual-based simulation framework exploring the ecology and mechanistic underpinnings of larval crowding in laboratory populations of *Drosophila*

Figure S1. Base model, with larval crowding – waste absent:
A single replicate is represented at each point. The X-axis denotes egg numbers. A) Egg to wandering survivorship across increasing egg numbers. B) The distribution of dry mass at wandering (µg) across increasing starting egg numbers. C) The distribution of time to wandering (hours) across increasing starting egg numbers. For the box plots in B) and C), the line within the box symbolises the median, the orange triangle symbolises the mean.

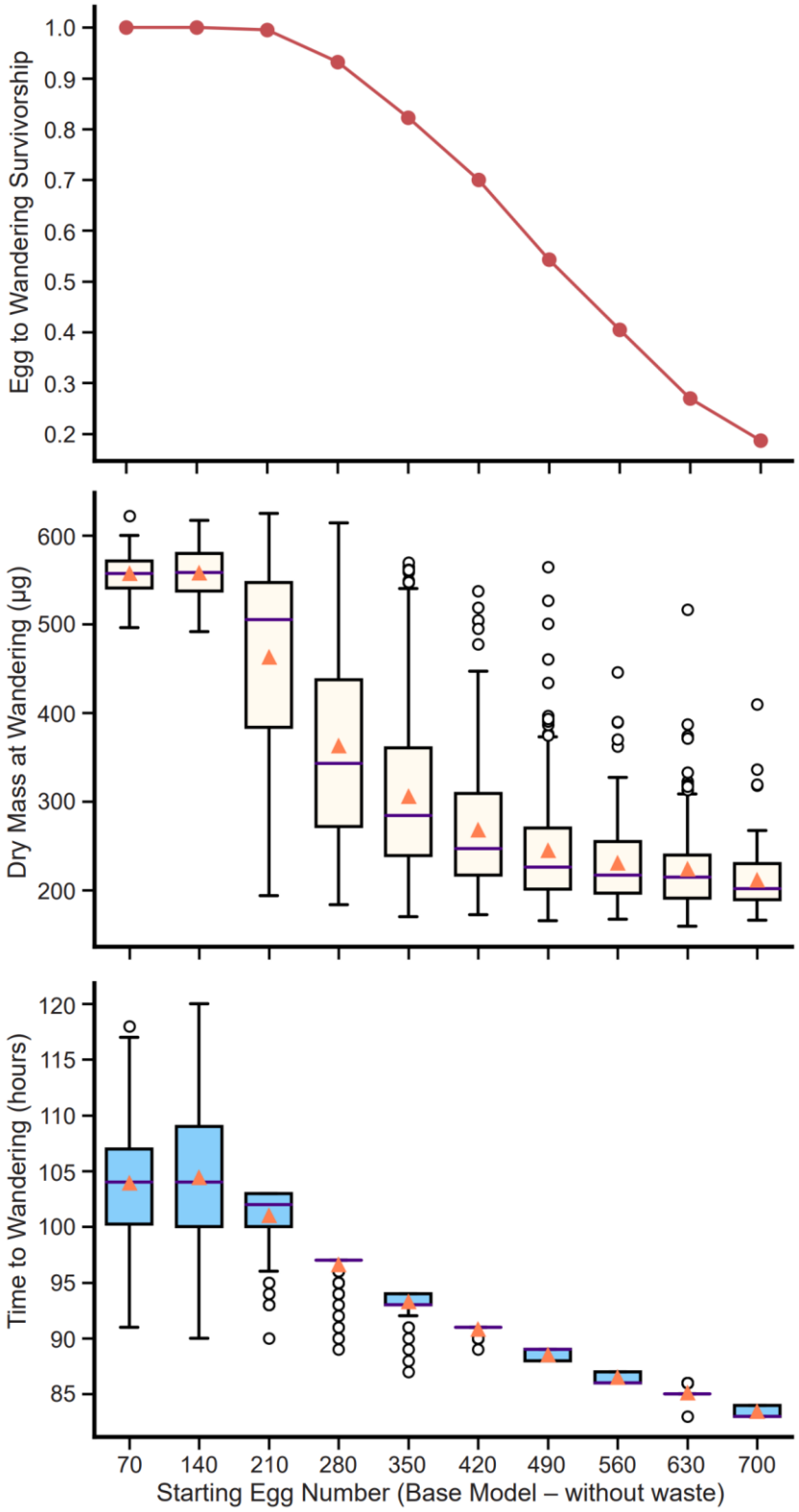


Figure S2. Base model, with larval crowding – waste present.
A single replicate is represented at each point. The X-axis denotes egg numbers. A) Egg to wandering survivorship across increasing egg numbers. B) The distribution of dry mass at wandering (µg) across increasing starting egg numbers. C) The distribution of time to wandering (hours) across increasing starting egg numbers. For the box plots in B) and C), the line within the box symbolises the median, the orange triangle symbolises the mean.

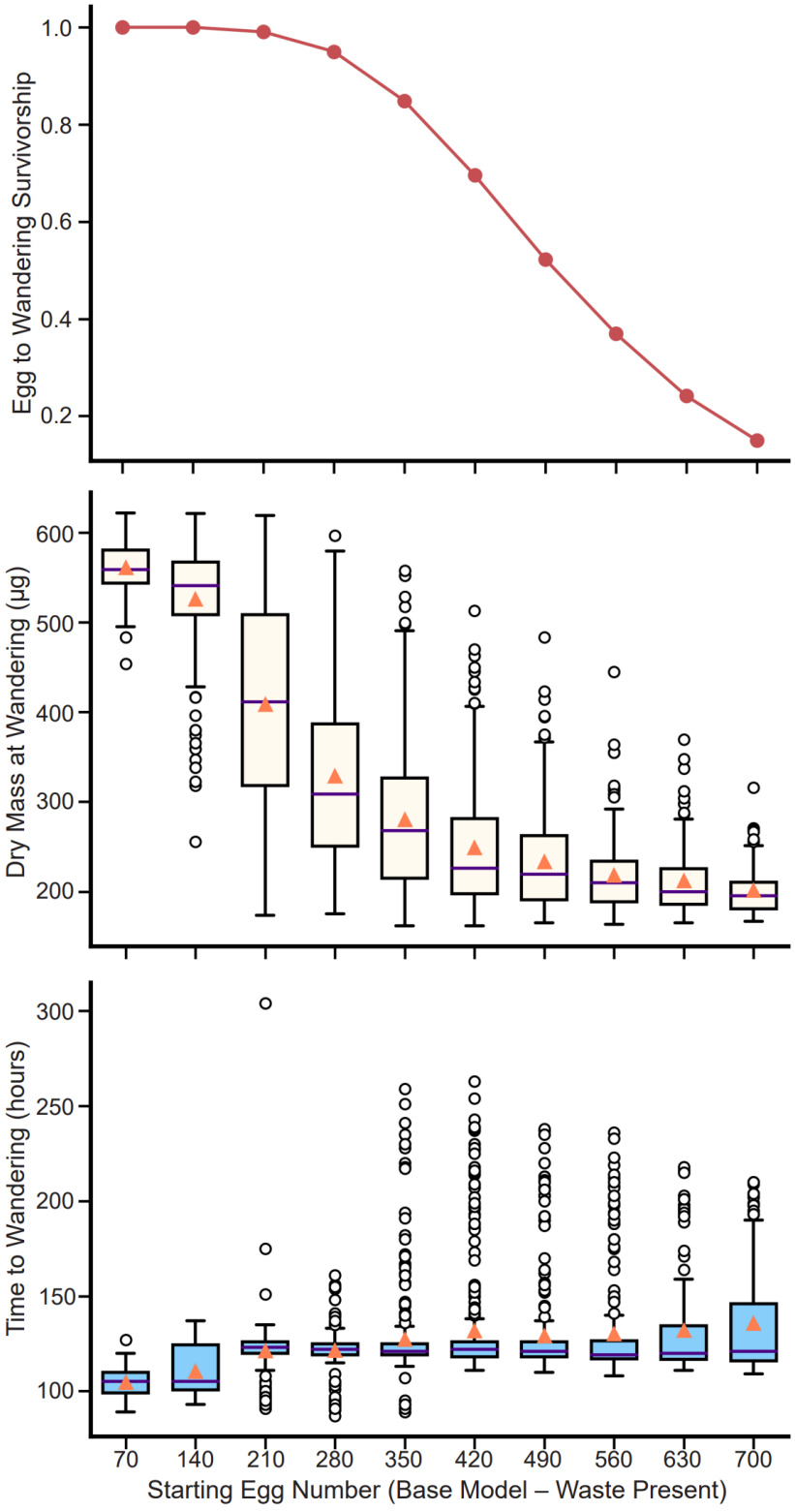


Figure S3. Base model – waste present:

Uncrowded (70 eggs in 6 mL food; 400 mm^2 surface area, 15 mm height).

a) Growth profile for 70 individuals. The solid blue line denotes the mean larval dry mass. The shaded region denotes standard deviation in larval dry mass. The dashed red line denotes the mean minimum critical mass to pupation (180 µg). Once the larvae commit to wandering, their mass remains unchanged.

b) Time to wandering distribution. The line within the box denotes the median. The orange triangle denotes the mean.

c) Mass at wandering distribution. The line within the box denotes the median. The orange triangle denotes the mean.

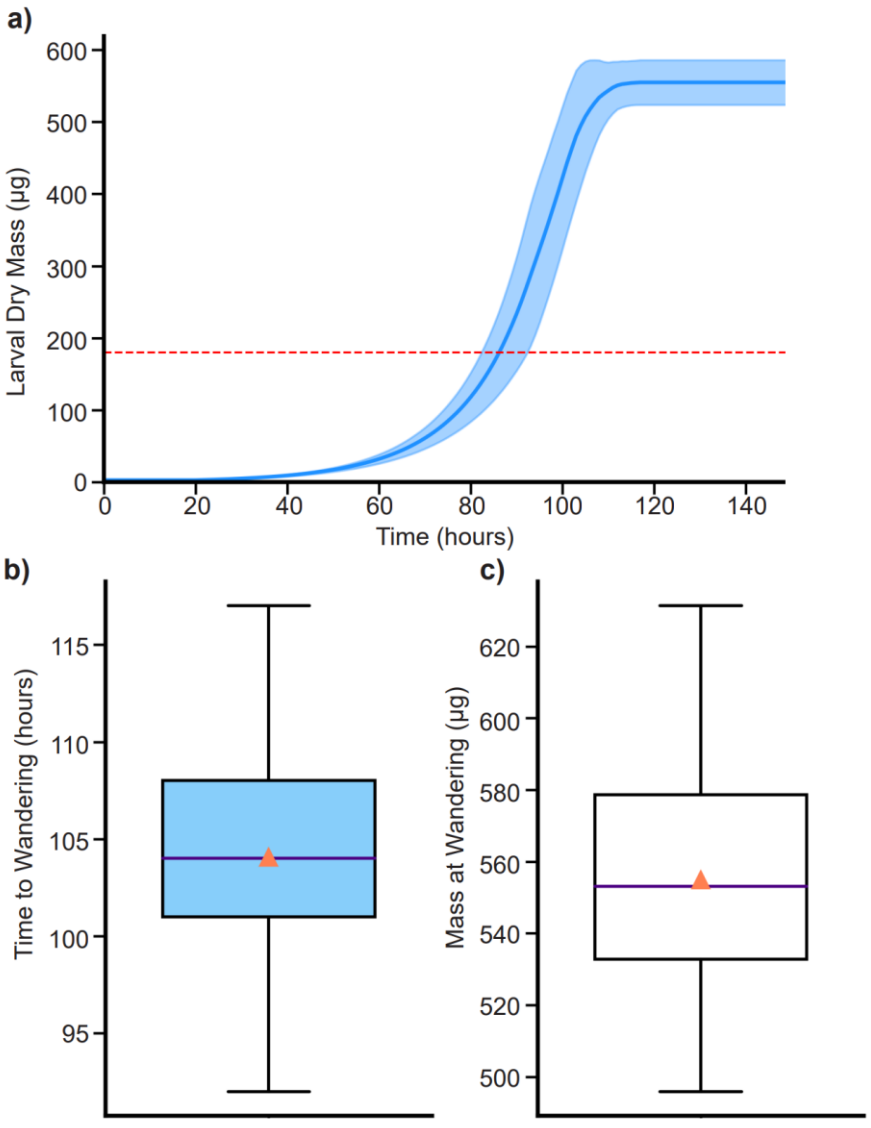


Figure S4. Base model – waste present:

Egg to wandering survivorship in a large factorial simulated experiment (see Methods).

S.A.: Cross-section surface area of the food column;

Height: starting height of the food column.

a) Survivorship of 30 replicates for each of the 27 simulated treatments. The '-' symbol in black represents the overall mean for 30 replicates for each treatment. The food volume is also highlighted for each surface area and column height combination.

b) Survivorship values for the three levels of food column height pooling data across all other factors. The indigo line within the box represents median, the orange triangle represents the mean.

c) Survivorship values for the three levels of surface area of the food column, pooling data across all other factors. The indigo line within the box represents median, the red triangle represents the mean.

d) Survivorship values for the three levels of starting egg numbers, pooling data across all other factors. The indigo line within the box represents median, the blue triangle represents the mean.

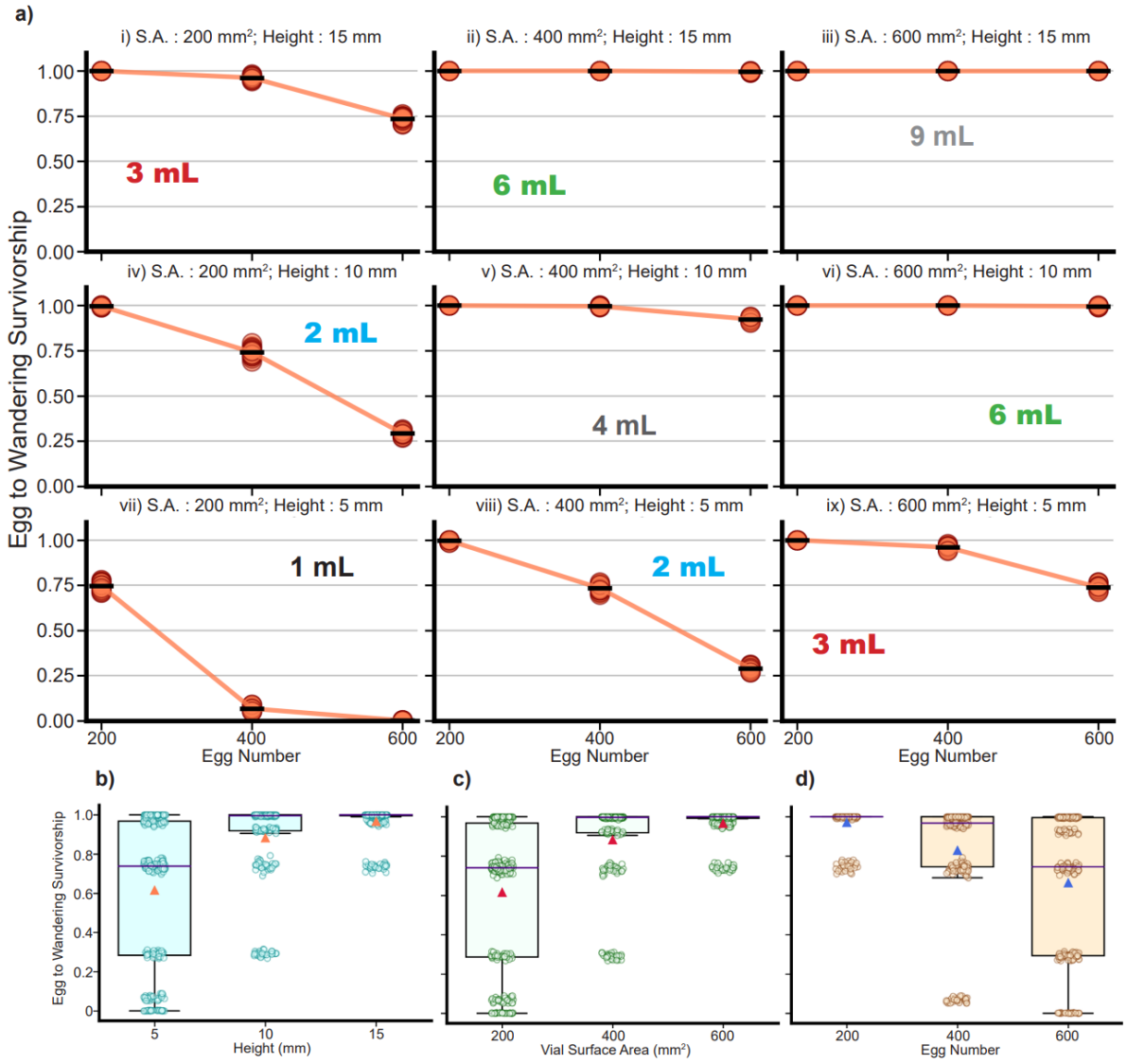


Figure S5. Base model – waste present:

Mean time to wandering (hours) in a large factorial simulated experiment (see Methods).
S.A.: Cross-section surface area of the food column;
Height: starting height of the food column.

a) Mean time to wandering of 30 replicates for each of the 27 simulated treatments. The '-' symbol in black represents the overall mean for 30 replicates for each treatment. The food volume is also highlighted for each surface area and column height combination.

b) Mean time to wandering values for the three levels of food column height pooling data across all other factors. The indigo line within the box represents median, the orange triangle represents the mean.

c) Mean time to wandering values for the three levels of surface area of the food column, pooling data across all other factors. The indigo line within the box represents median, the red triangle represents the mean.

d) Mean time to wandering values for the three levels of starting egg numbers, pooling data across all other factors. The indigo line within the box represents median, the blue triangle represents the mean.

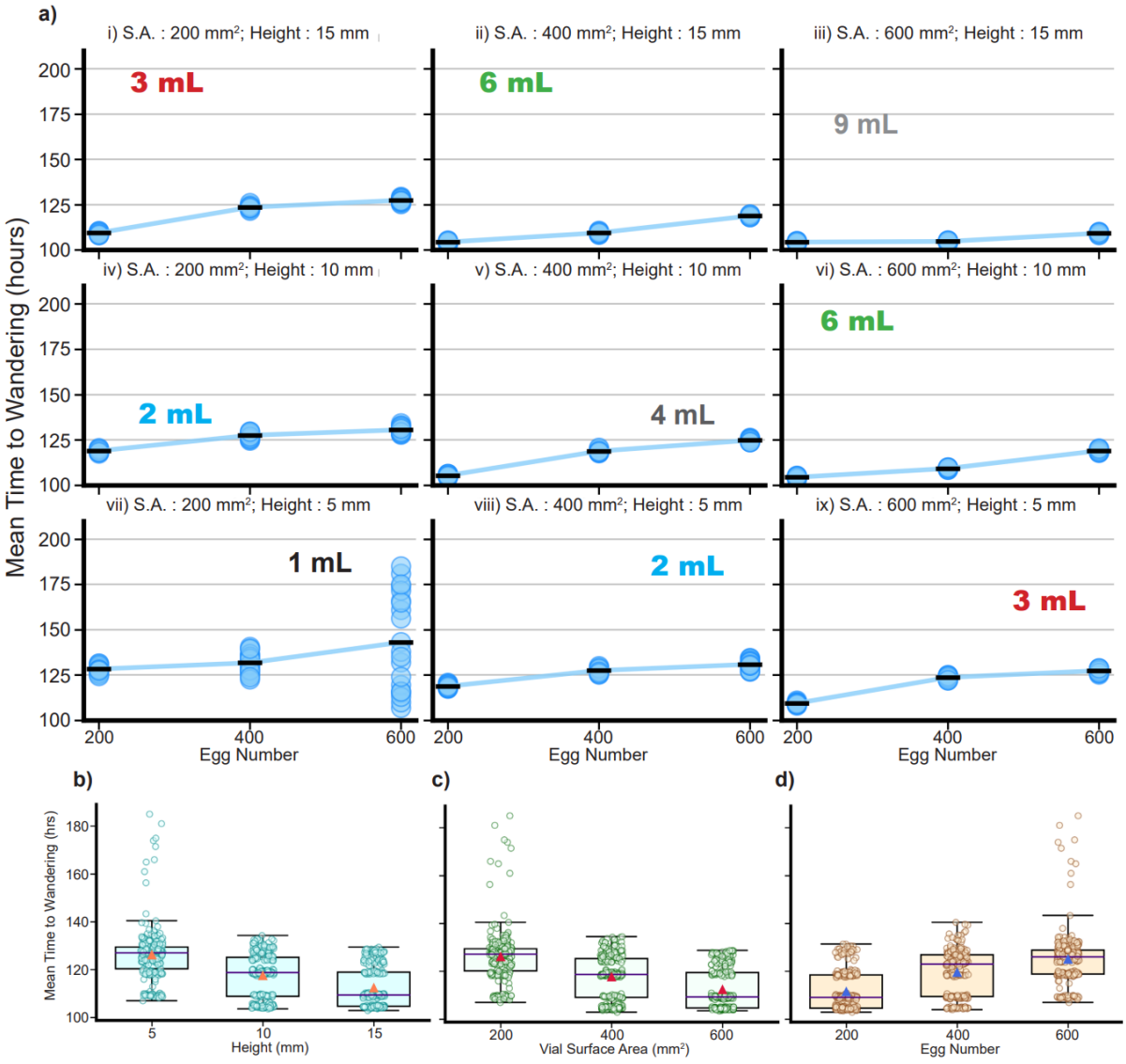


Figure S6. Expanded model:
Uncrowded (70 eggs in 6 mL food; 400 mm^2 surface area, 15 mm height).

a) Growth profile for 70 individuals. The solid blue line denotes the mean larval dry mass. The shaded region denotes standard deviation in larval dry mass. The dashed red line denotes the mean minimum critical mass to pupation (180 µg). Once the larvae commit to wandering, their mass remains unchanged.

b) Time to wandering distribution. The line within the box denotes the median. The orange triangle denotes the mean.

c) Mass at wandering distribution. The line within the box denotes the median. The orange triangle denotes the mean.

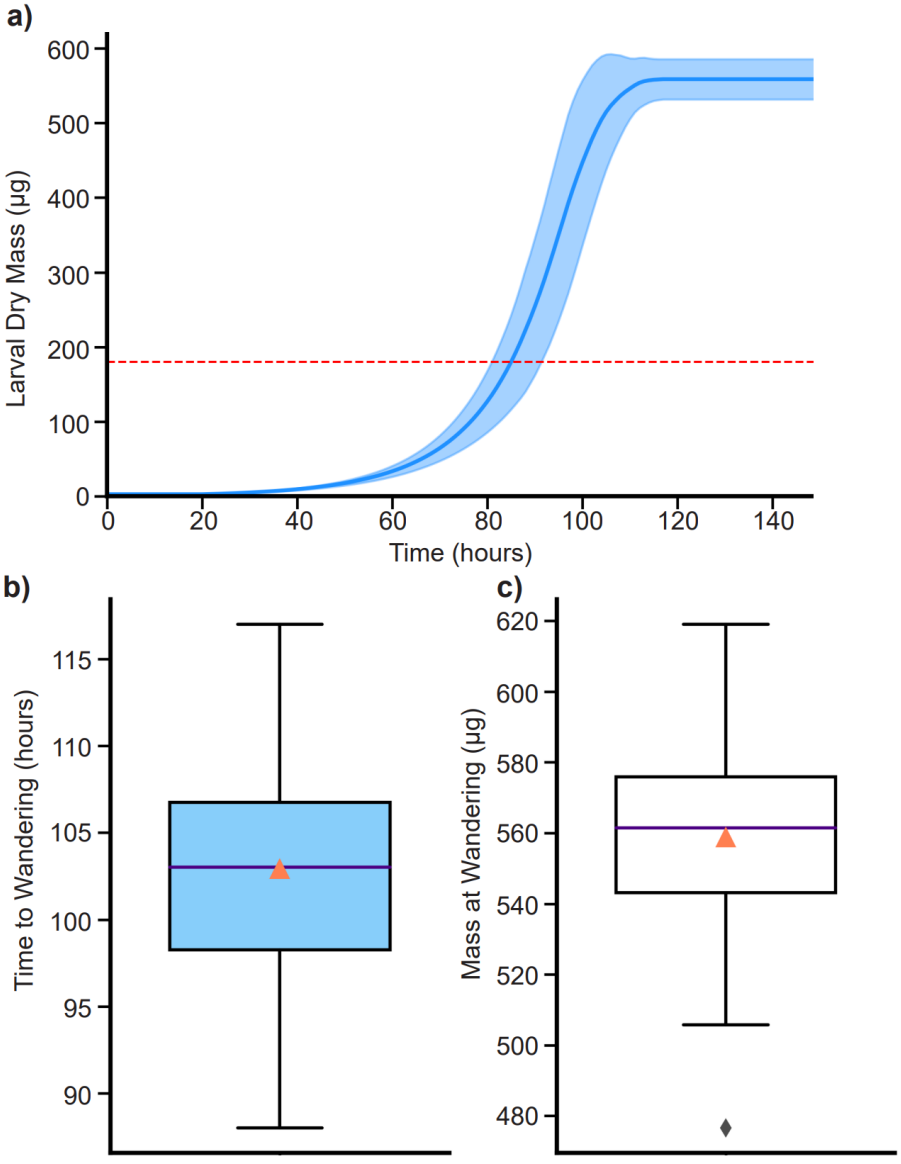


Figure S7. Expanded Model:

Mean time to wandering (hours) in a large factorial simulated experiment (see Methods).
S.A.: Cross-section surface area of the food column;
Height: starting height of the food column.

a) Mean time to wandering of 30 replicates for each of the 27 simulated treatments. The '-' symbol in black represents the overall mean for 30 replicates for each treatment. The food volume is also highlighted for each surface area and column height combination.

b) Mean time to wandering values for the three levels of food column height pooling data across all other factors. The indigo line within the box represents median, the orange triangle represents the mean.

c) Mean time to wandering values for the three levels of surface area of the food column, pooling data across all other factors. The indigo line within the box represents median, the red triangle represents the mean.

d) Mean time to wandering values for the three levels of starting egg numbers, pooling data across all other factors. The indigo line within the box represents median, the blue triangle represents the mean.

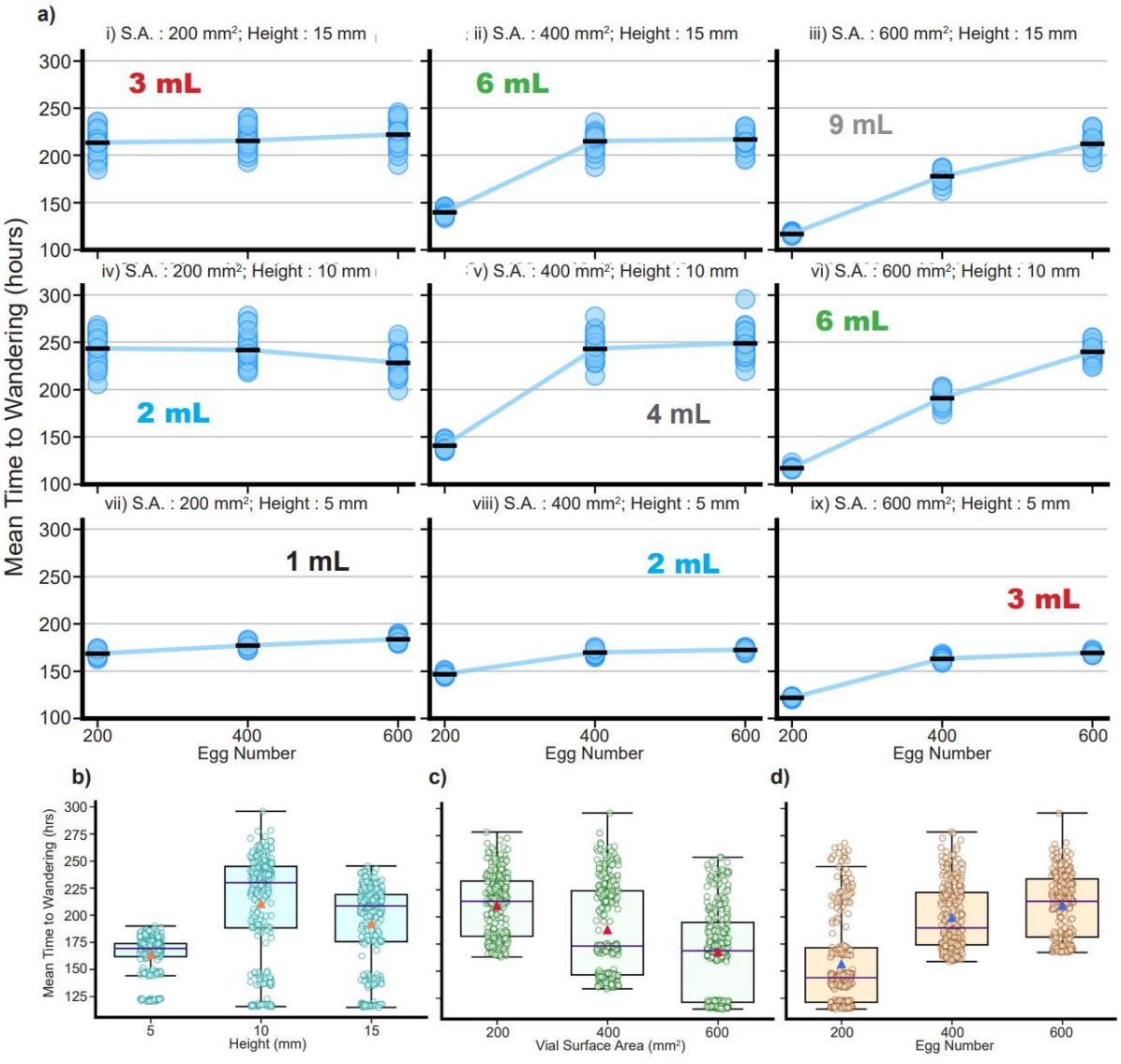


Figure S8. Expanded Model:

Variance in time to wandering (hrs) in a large factorial simulated experiment (see Methods).
S.A.: Cross-section surface area of the food column;
Height: starting height of the food column.

a) Variance in time to wandering of 30 replicates for each of the 27 simulated treatments. The '-' symbol in black represents the overall mean for 30 replicates for each treatment. The food volume is also highlighted for each surface area and column height combination.

b) Variance in time to wandering values for the three levels of food column height pooling data across all other factors. The indigo line within the box represents median, the orange triangle represents the mean.

c) Variance in time to wandering values for the three levels of surface area of the food column, pooling data across all other factors. The indigo line within the box represents median, the red triangle represents the mean.

d) Variance in time to wandering values for the three levels of starting egg numbers, pooling data across all other factors. The indigo line within the box represents median, the blue triangle represents the mean.

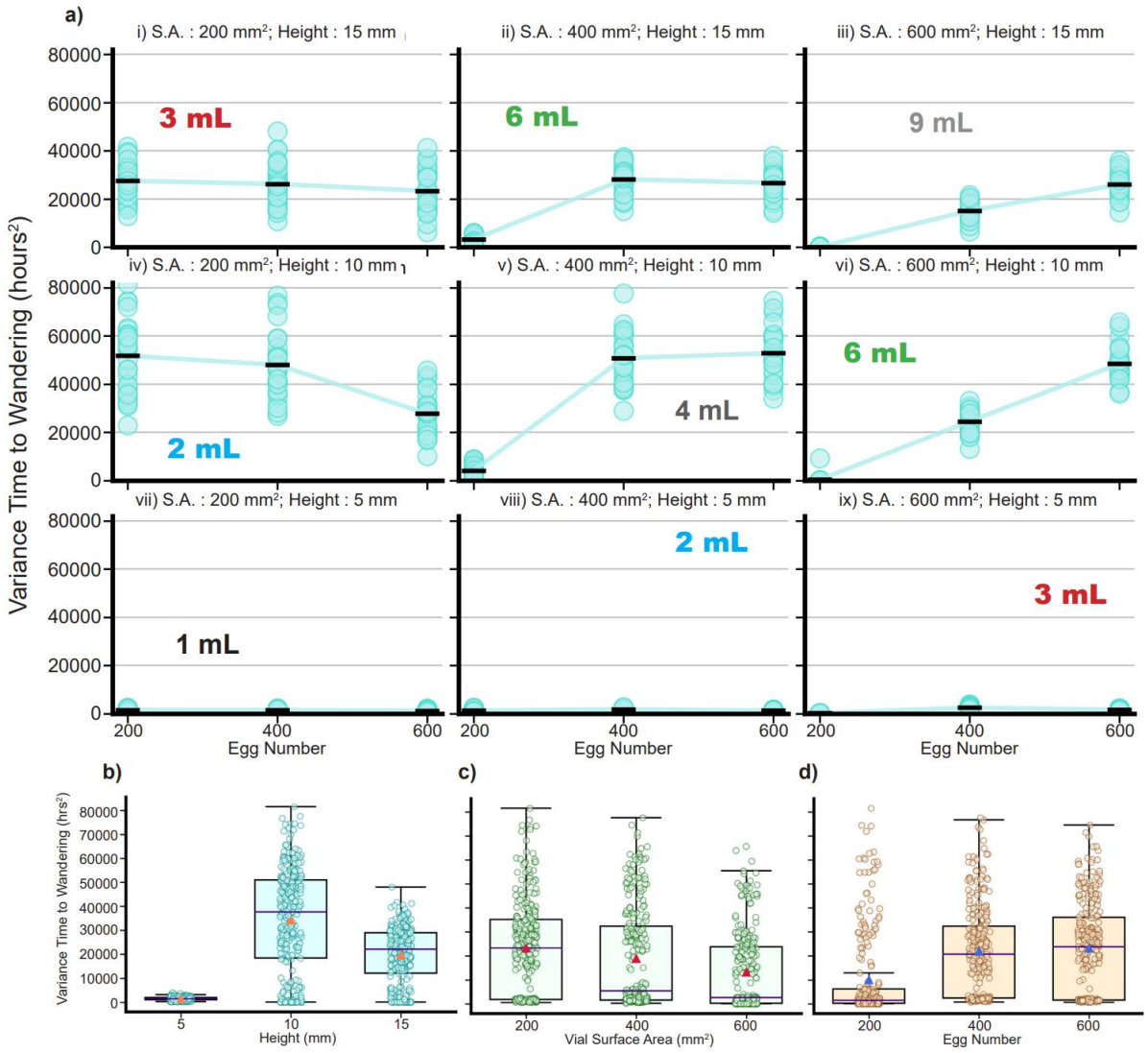


Figure S9. Expanded Model:

Variance in mass at wandering in a large factorial simulated experiment (see Methods).

S.A.: Cross-section surface area of the food column;

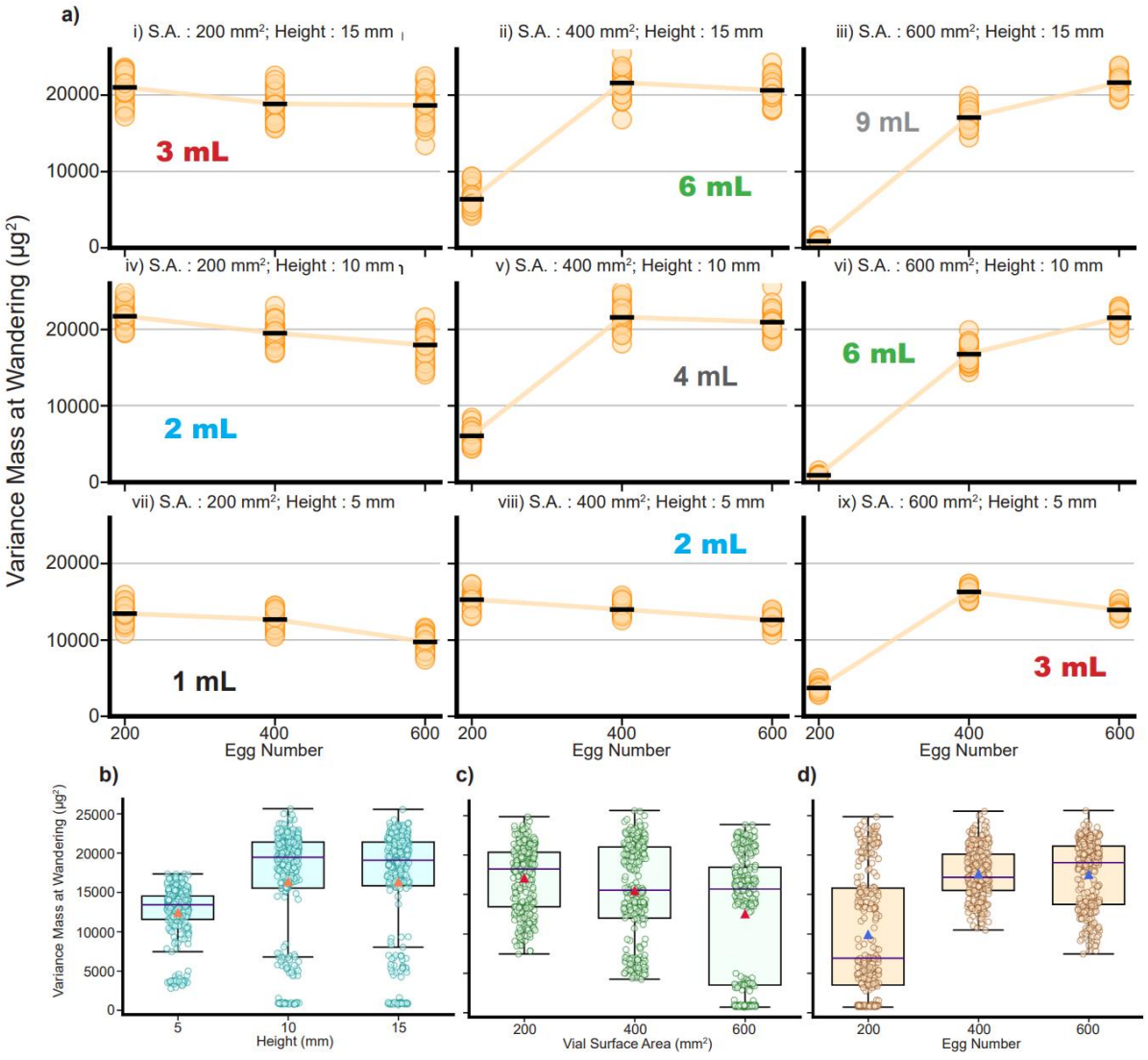
Height: starting height of the food column.

a) Variance in mass at wandering of 30 replicates for each of the 27 simulated treatments. The '-' symbol in black represents the overall mean for 30 replicates for each treatment. The food volume is also highlighted for each surface area and column height combination.

b) Variance in mass at wandering values for the three levels of food column height pooling data across all other factors. The indigo line within the box represents median, the orange triangle represents the mean.

c) Variance in mass at wandering values for the three levels of surface area of the food column, pooling data across all other factors. The indigo line within the box represents median, the red triangle represents the mean.

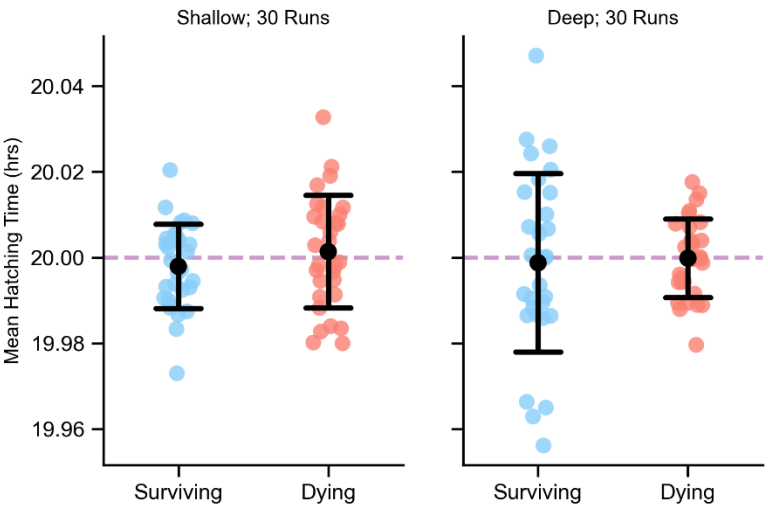
d) Variance in mass at wandering values for the three levels of starting egg numbers, pooling data across all other factors. The indigo line within the box represents median, the blue triangle represents the mean.

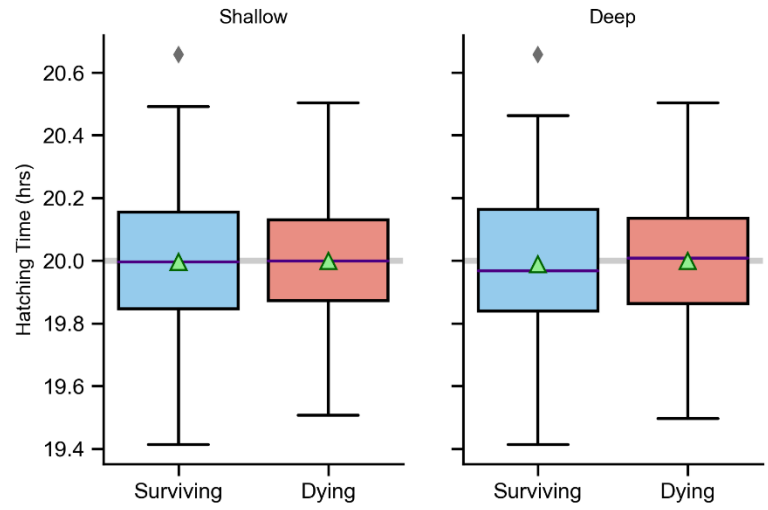


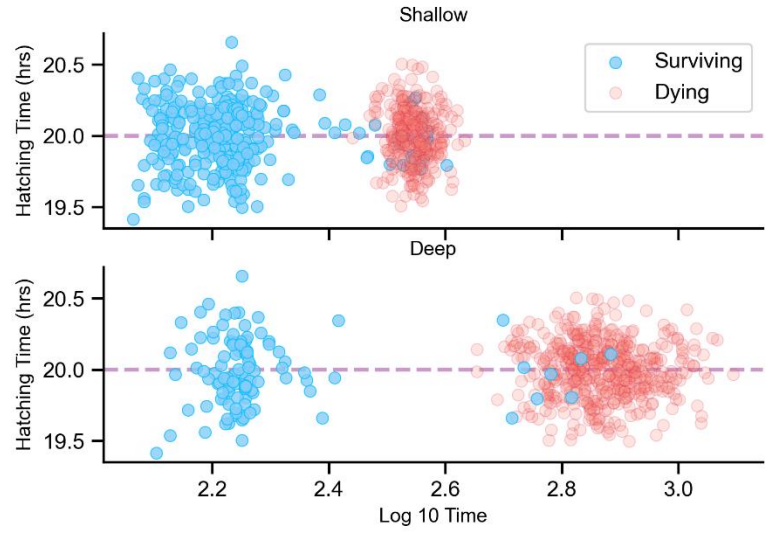
S10. Trait distributions of surviving (blue) and dying (red) larvae:

- Each numbered figure set represents a trait.
- The left column of plots represents trait distributions from shallow type cultures – 600 eggs in 3 mL food cast in cylinders of 600 mm2 cross-section surface area and 5 mm column height.
- The right column of plots represents trait distributions from shallow type cultures – 600 eggs in 3 mL food cast in cylinders of 200 mm^2 cross-section surface area and 15 mm column height.
- The first figure in each numbered set represents the mean trait distribution across 30 replicates for surviving and dying individuals, in shallow or deep type cultures. For each replicate, the starting individuals in both types of cultures are the same. Error bars represent standard deviation.
- The second figure represents the trait distribution of surviving and dying individuals within a single run. The line within the box plots represents the median for each distribution. The green triangle represents the mean for the respective distribution.
- The third figure represents the trait distribution of individuals across their respective times of wandering (blue) or dying (red).
- For each trait, the mean value of the distribution from which the traits were randomly drawn at the beginning of the simulation, is also plotted as a purple dashed line (first and third figure per numbered set), or as a grey solid line (second figure per numbered set).

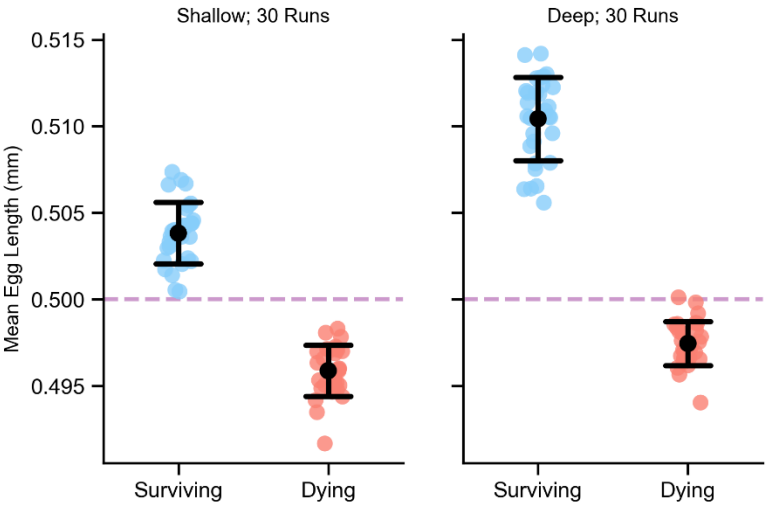
a) Hatching Time (hours)

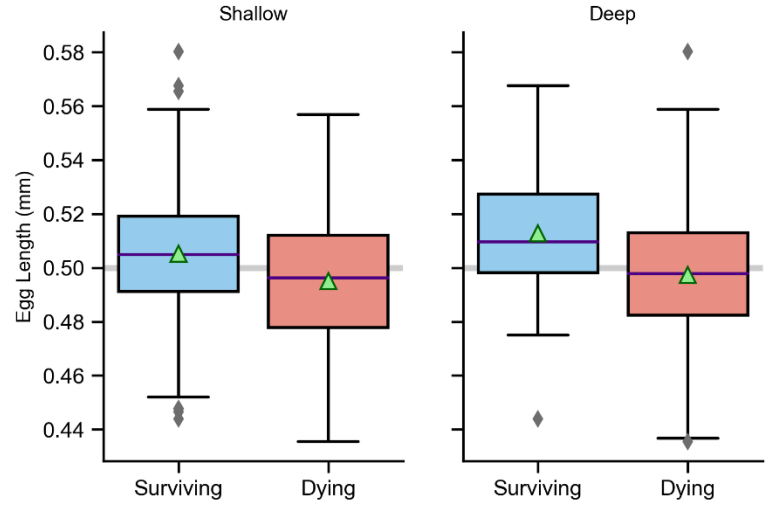


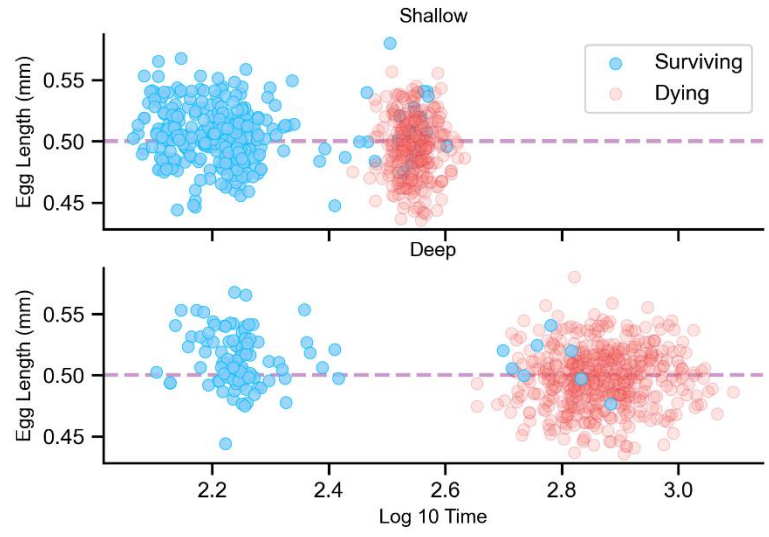




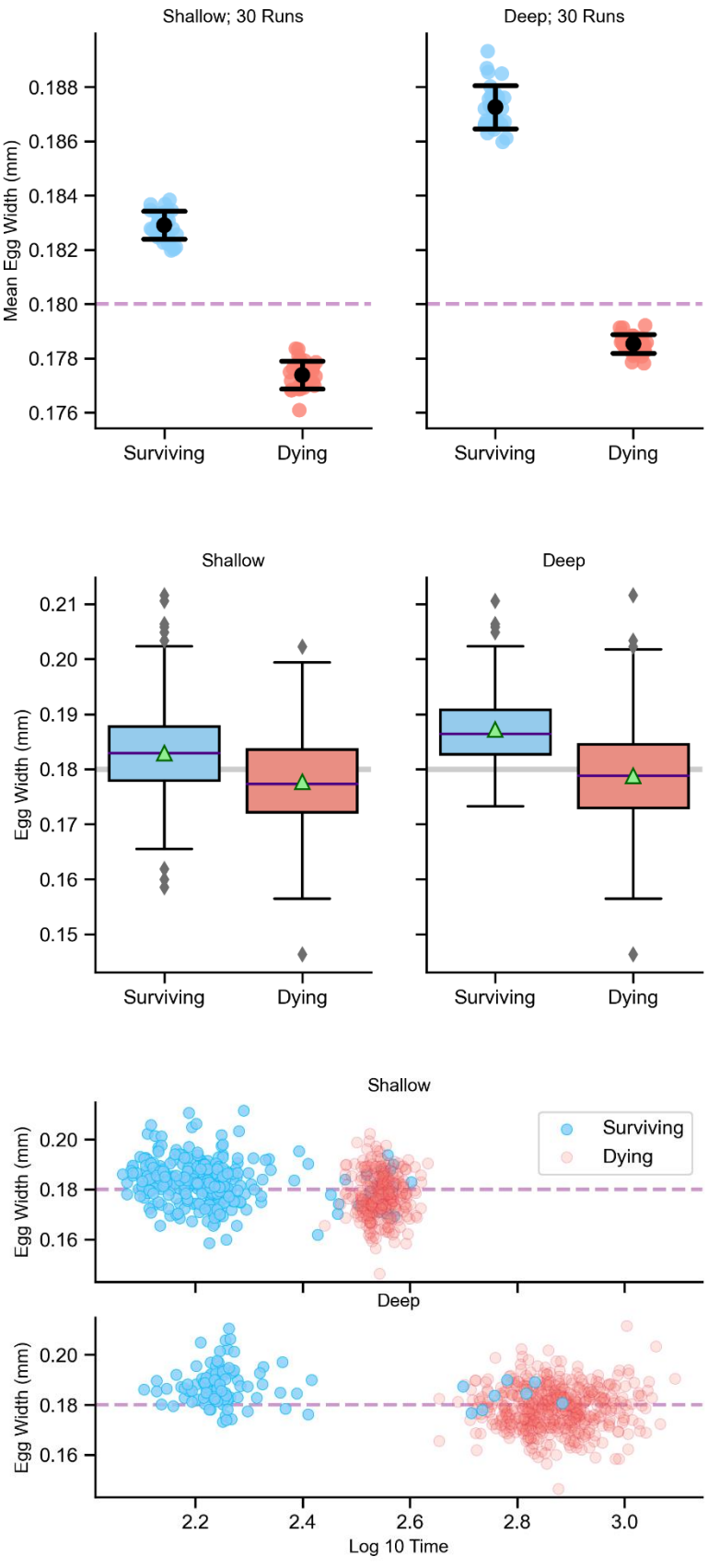
b) Egg Length (mm)



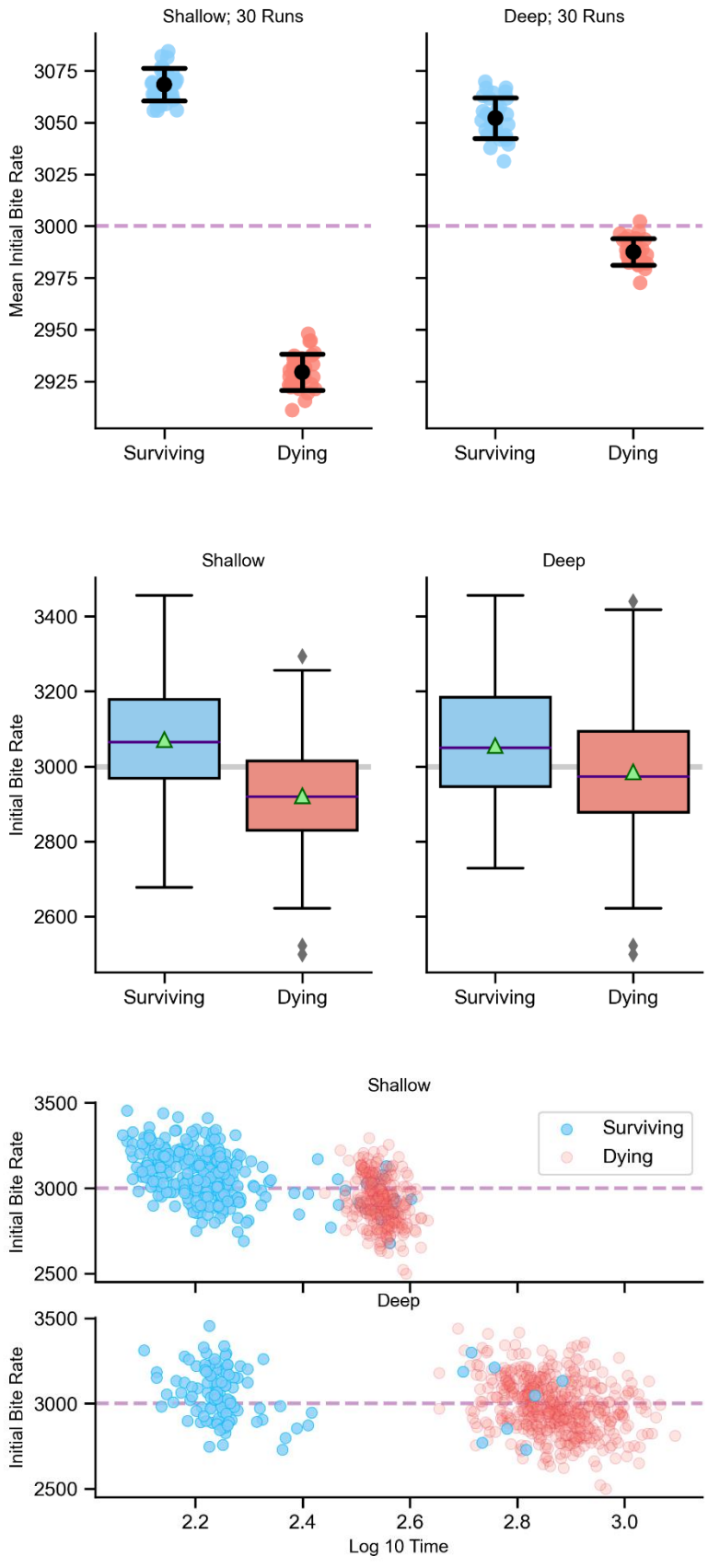




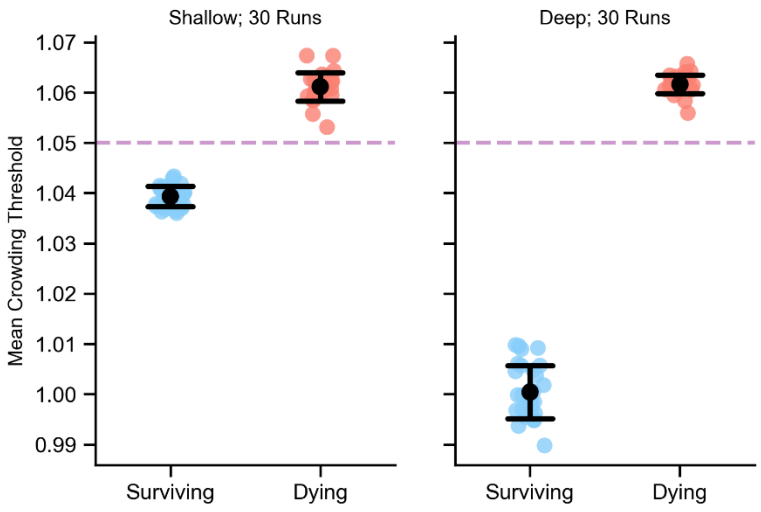
c) Egg Width (mm)

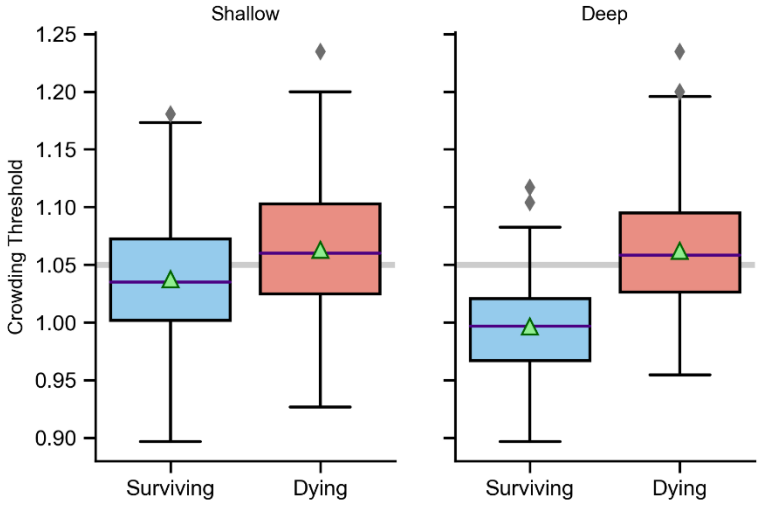


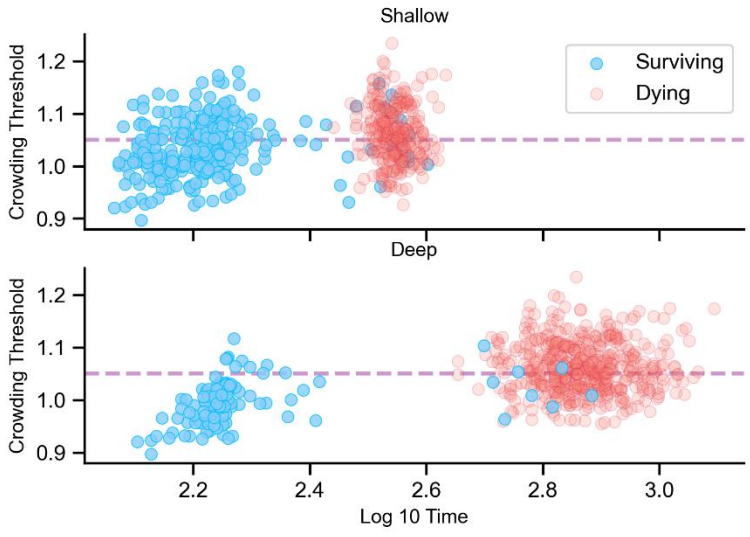
d) Initial Bite Rate



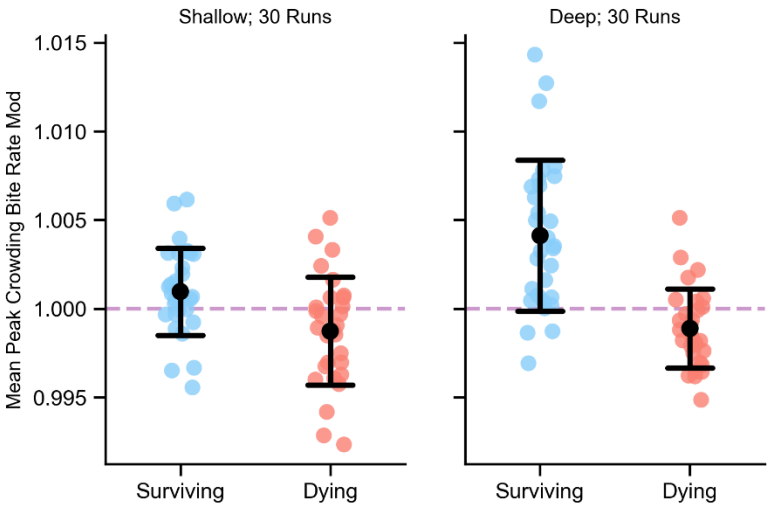
e) Crowding Threshold

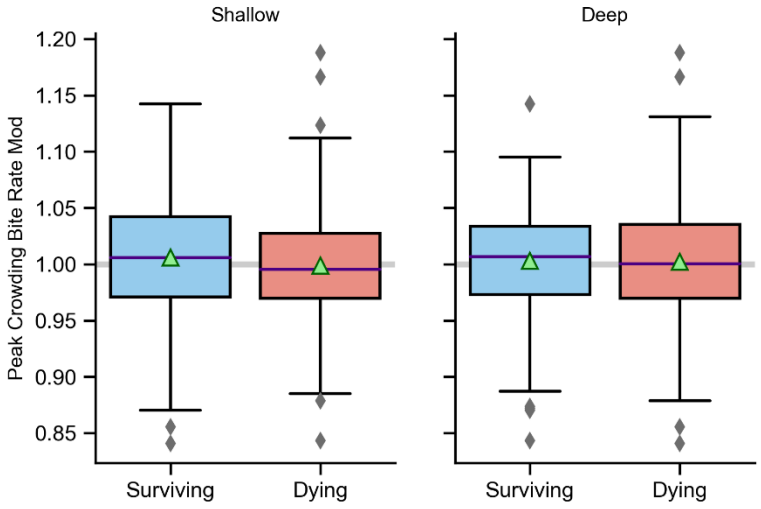


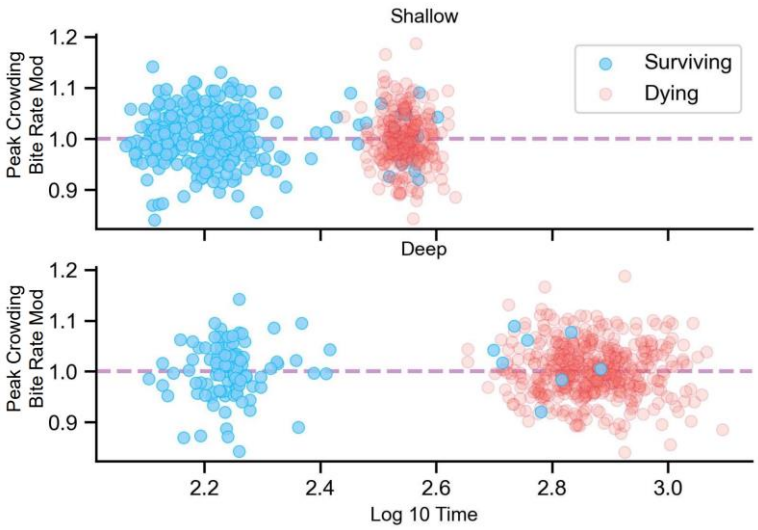




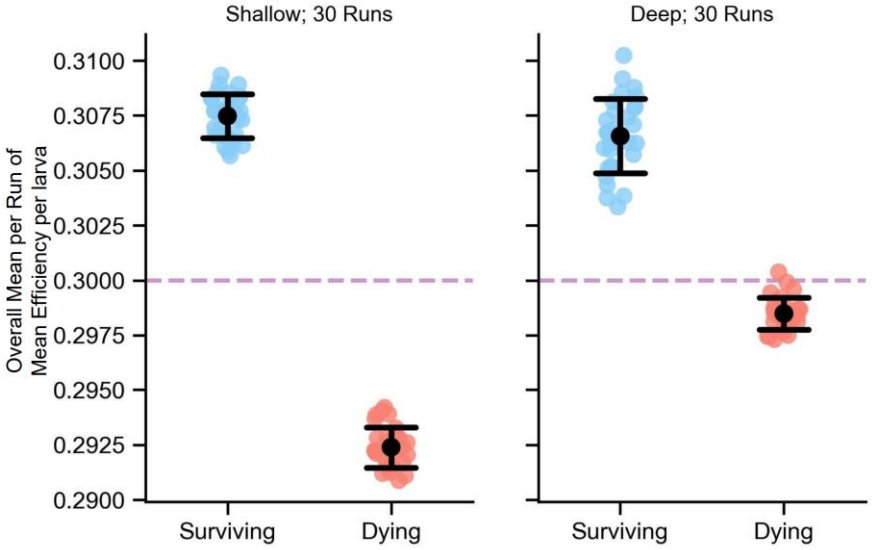
f) Peak Crowding Bite Rate Modifier

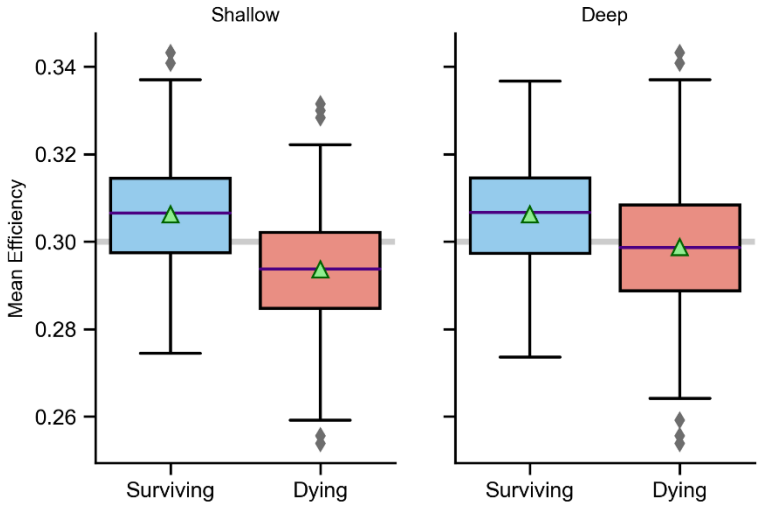


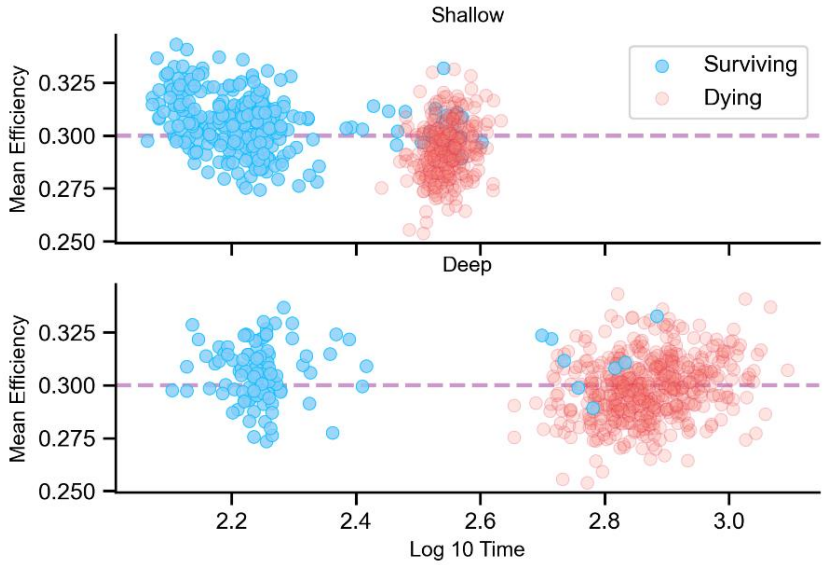




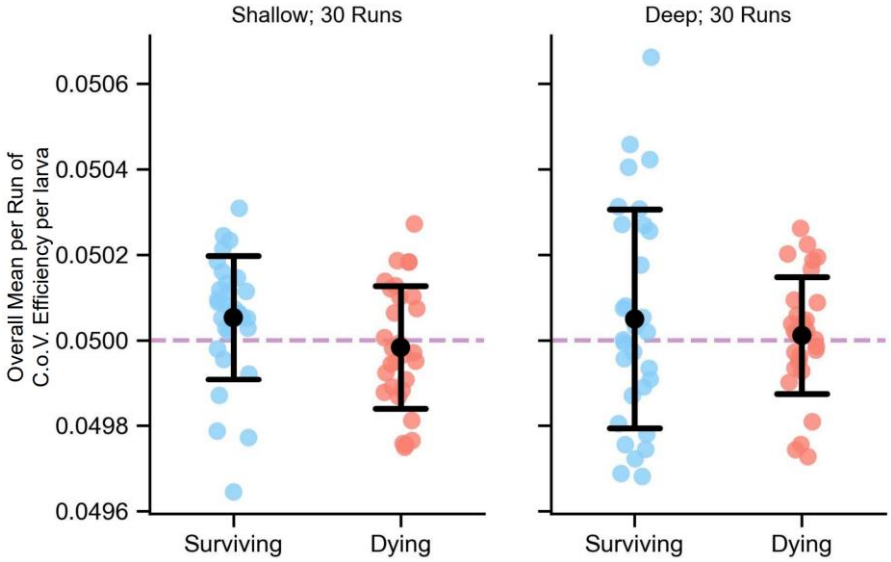
g) Mean Efficiency of food to biomass conversion, per larva

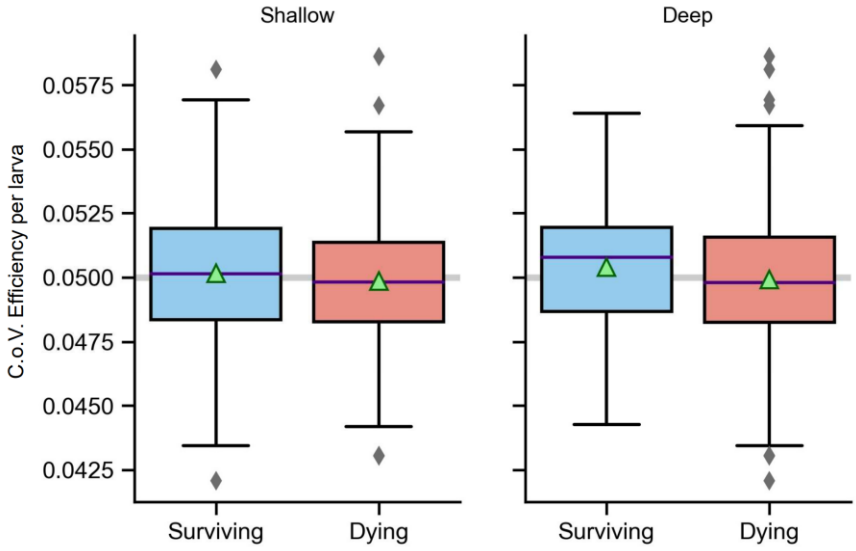


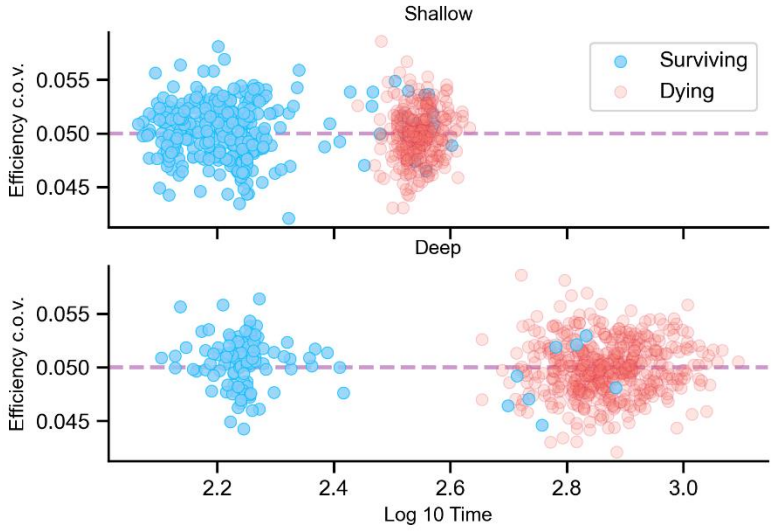




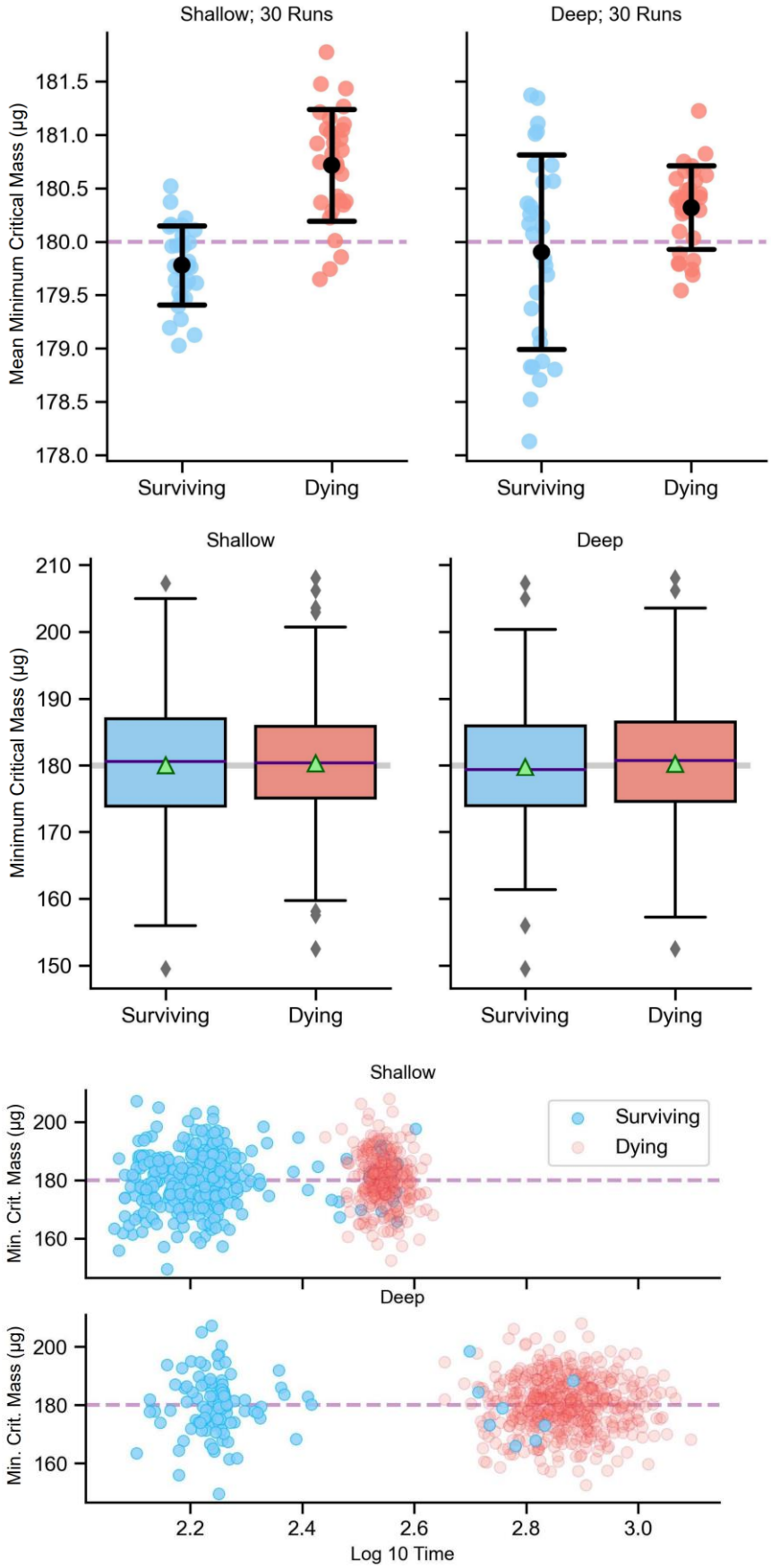
h) Efficiency c.o.v.



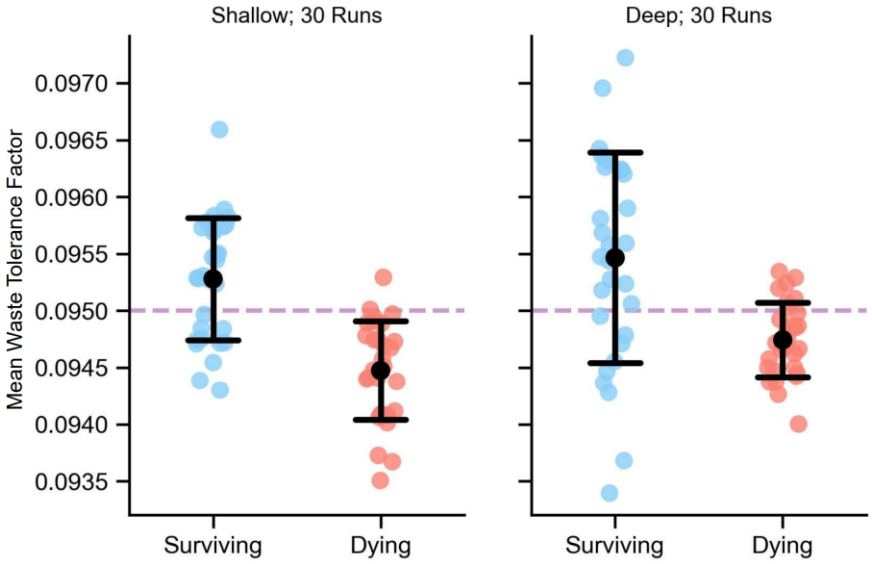


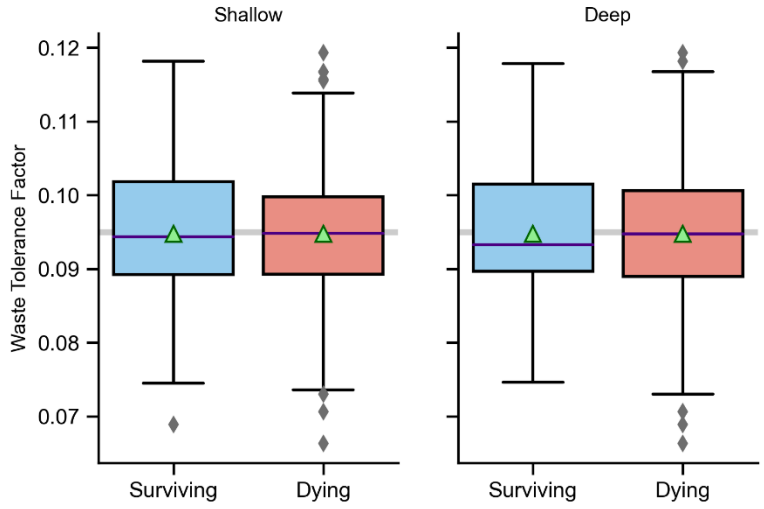


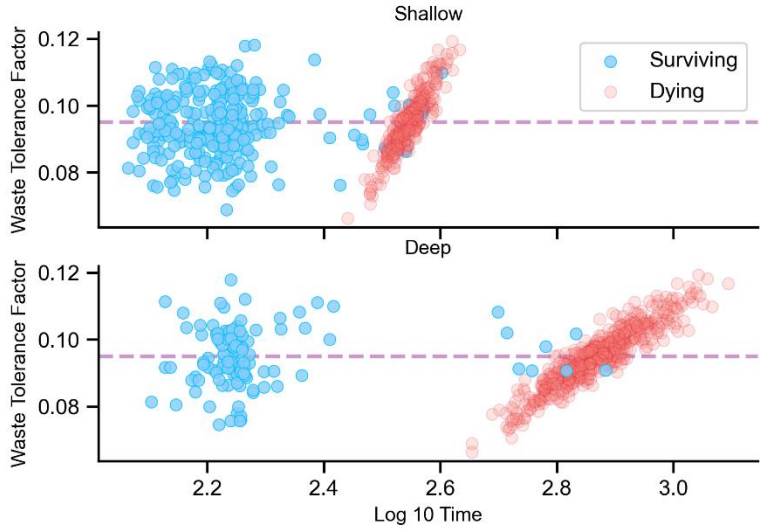
i) Minimum Critical Mass (µg)



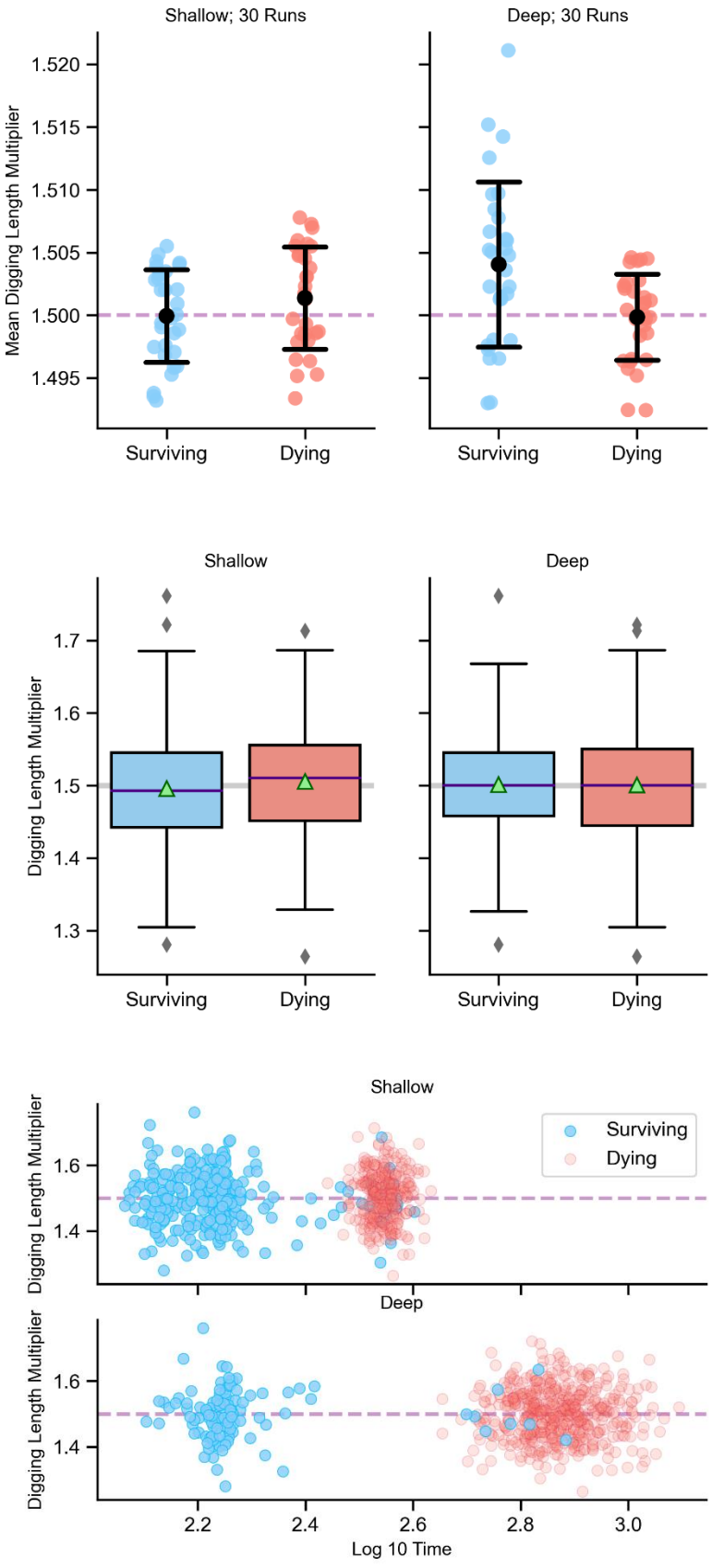
j) Waste Tolerance Factor







k) Digging Length Multiplier



Chapter VII

Conclusions and Potential Future Studies

Conclusions

The salient findings from chapters 2-6 in the thesis have been listed below:

Chapter 2

As seen across several long-term selection studies before (see Chapter 1), our results showed that laboratory *D. melanogaster* populations adapted to chronic conditions of larval crowding evolved increased larval competitive ability. Furthermore, larval competitive ability had increased to different degrees depending partly on the exact rearing conditions of each crowding-adapted population set. The competitive ability expressed by each population set was also seen to depend on the crowded conditions presented to the respective larvae with respect to the exact food volume used, the number and composition of individuals in a culture (mono-culture or duo-culture), and the outcome of competition measured.

To summarise, larval competitive ability, when split into components of effectiveness and tolerance, and measured for four different outcomes of competition, revealed several patterns in the data:

The crowding-adapted populations MCU, CCU and LCU exhibited greater competitive ability than MB, depending on the food level, and the outcome of competition examined.

The superior competitive ability of each crowding-adapted population was reflected in an increase in effectiveness alone, or an increase in tolerance, or both, for any given outcome of competition measured.

Among the crowding adapted population sets, the MCU appeared to have the most consistently high larval competitive ability across food levels and outcomes of competition.

LCU usually had lower competitive ability than MCU. At 1 mL food, the competitive ability of LCU was also lower than CCU.

At 1 mL of food, the larval competitive ability expressed by CCU was slightly greater than or equal to the larval competitive ability of MCU populations. However, CCU larval competitive ability was closer to that of LCU at 1.5 mL and 2 mL food volumes. From these results, we saw that CCU populations performed best at their rearing total density (eggs/food) i.e., 400 eggs in 1 mL food, which was the same total density as 1200 eggs, 3 mL food. However, the CCU populations had lower competitive ability at 400 eggs in 2 mL food, which was closer to their rearing food volume of 3 mL.

From the low-density assay cultures in addition to the crowded assay cultures studied, there was a strong indication that each crowding-adapted population had evolved reduced development time in uncrowded as well as crowded (400 eggs in 1, 1.5 and 2 mL) cultures.

From this work, we saw that competitive ability when measured across different axes of fitness-related traits can yield greater insights than just looking at pre-adult survivorship. Additionally, as seen in several previous studies, effectiveness and tolerance components could mediate the evolution of competitive ability to different degrees, depending on the focal population and the outcome of competition measured. We further established that both effectiveness and tolerance components may evolve to increase independently in the course of adaptation to larval crowding on varying combinations of egg numbers and food volumes.

From the eclosion distributions, dry biomass measurements across time, and dry weight measurements over time, we may infer that larval competition in *Drosophila* likely plays out in a very nuanced, time-dependent manner. This was seen in the extended development time of OE competing against the crowding-adapted populations, with some pattern of loss in dry mass per fly. Additionally, at the relatively higher food volume of 2 mL used in the assay, LCU larvae appeared to suppress the biomass of later eclosing OE better than CCU.

Results from this study indicate that there may be some culture- and density-specific components to larval competitive ability, which may have evolved to different degrees in the three sets of crowding-adapted populations.

Chapter 3

All crowding-adapted population sets (MCU, CCU, LCU) evolved some mechanism for gaining temporal head starts in larval competition, compared to their low-density reared ancestral control population set (MB).

With respect to egg hatching time, only the eggs from LCU populations were seen to hatch significantly faster than eggs from the MB populations. Although non-significant, we saw the following overall pattern of egg hatching time:

MB > MCU > CCU > LCU.

Moreover, all crowding-adapted populations evolved both greater egg length and egg width compared to MB. There were also differences among the crowding-adapted populations themselves – LCU evolved the greatest egg size metrics, while CCU egg size showed a pattern of being ≥ MCU egg size, although these results also varied depending on the rearing density conditions of the adults from which the eggs were collected. Thus, among the crowding-adapted populations, LCU had evolved the greatest head start parameters, followed by CCU and then MCU, indicating that head starts in competition may be the most important in crowded cultures resembling the rearing conditions of LCU.

Chapter 4

We provided head starts of varying durations to OE in larval competition against MB, MCU, CCU and LCU. Upon receiving an age disadvantage in competition, the crowding-adapted populations coped to similar extents, suffering a significant non-zero increase in development time. The MB populations fared worse than all crowding-adapted populations, with a significantly greater period of development when in competition against OE having head start.

In contrast, upon receiving a temporal head start against each crowding-adapted population, OE development time decreased the most against LCU and CCU, followed by MCU.

These results were better understood by considering an age disadvantage to the focal populations (MB, MCU, CCU, LCU) as inducing a decline in tolerance, and considering the head start given to OE as inducing a decline in effectiveness.

MCU likely had better effectiveness even with disadvantages compared to CCU and LCU populations, as OE benefitted less in terms of a decrease in mean pre-adult development time in competition against MCU, especially at 7 hours of head start, as compared to CCU (non-significant difference) and LCU (significant difference). Against MB, OE got the least development time benefit with head start, likely due to the low effectiveness of MB even without any disadvantage.

Ultimately, there was no clear benefit of evolving larger egg size and shorter hatching time to LCU, in terms of how well it coped against age disadvantages in competition, or in terms of how much it prevented OE from benefitting from a head start, as compared to MCU or CCU.

Chapter 5

We studied the consequences of larval crowding by changing egg number, or food volume via changing surface area in contact with air, or via changing food column height, implemented in a three-way, fully factorial design. Increasing crowding via increasing egg number or by decreasing food volume led to decreased survivorship, although to a lesser extent when the volume was changed via column height than via surface area. Furthermore, increasing food volume via surface area decreased pre-adult development time, whereas increasing food volume via column height increased development time, with the latter being prominent at higher egg numbers. Mechanistically, the height of the food column in the vial could determine the extent of diffusion of potentially harmful larval metabolic waste products such as urea and ammonia away from the feeding band, thus letting the larvae feed in relatively low concentrations of waste for longer periods of time. The surface area of food exposed to air in vial could be the determinant of the extent of space available for larvae to feed – a greater surface area would signify greater spatial access to the food, for the larvae. Both these mechanisms need to be studied independently and in greater detail in the future, in order to understand why column height and surface area affect the outcomes of crowding differently.

Moreover, we described a new term – "effective density", as the density of larvae in the feeding band – a volume of food close to the surface with access to air, where the larvae feed. In the results of our study, the effective density was a far better predictor of three fitness related outcomes of larval crowding (pre-adult survivorship, mean pre-adult development time and variance of pre-adult development time) compared to the total density, defined as the number of eggs per mL total food volume. Overall, competition for limited space may be driving the results of competition in high effective density cultures, as inferred from our study.

The findings from this study may be very relevant for any future study on larval competition in *Drosophila* and perhaps other clades as well.

Chapter 6

We constructed an individual-based simulation framework to better understand the evolutionary ecology and mechanistic underpinnings of larval competition in *Drosophila*. This involved the creation of a 'base' model, which re-created a simpler understanding of larval competition, in concordance with older models which had attempted to predict the results of larval competition. We also added dynamics of waste excretion and waste consumption in the 'larvae' of the simulation. With this base model, we were able to predict results as shown by the detailed studies of Bakker (1961), which was a benchmark of comparison for most previous models on *Drosophila* larval competition. We additionally recreated more realistic patterns of pre-adult development time distributions under crowded scenarios due to the inclusion of the effects of waste excretion and ingestion in the system. However, as with the previous models, the base models had no consideration of recent findings in larval competition, wherein the same total density, or even the same combination of eggs and food volume, cast into different container types, could lead to very different outcomes with respect to fitness related traits.

In order to make more robust predictions about the outcomes of larval crowding in different kinds of cultures, we implemented an 'expanded' model, which incorporated additional factors:

1. Space constraints resulting from the crowding of the limited food surface area at the denoted 'feeding band' by larvae in highly crowded cultures.
2. The resulting detrimental effects of space constraints on crowded larvae in terms of food eaten per time step, as well as the variable plastic increase in bite rate of larvae feeding in spatially crowded conditions.

3. The ‘permeation’ of waste down the food column from the top ‘layer’ of food wherein the larvae excrete. At a given time step, freshly generated waste would travel down the layers of food as the top layers got saturated with previously excreted waste.

4. The larvae, which would dig to variable lengths due to their own body length as well as digging ‘potential’, would also experience different waste concentrations depending on the ‘food layers’ the larvae had access to.

Results from the ‘expanded’ model predicted patterns of pre-adult survivorship from a simulated experiment of the assay design used in Chapter 5 very well. Some aspects of pre-adult development time were also captured, although there were some patterns of development time which the simulated experiment failed to mimic compared to the real-world assay. In addition, dry mass distributions from each type of culture used in Chapter 5 were also predicted using the simulations. Overall, the work in this chapter laid down a strong foundation to build further simulation expansions as well as predict experiments based on the mechanisms implemented in this study.

Potential future studies

The results of competitive ability measurements in Chapter 2, wherein population sets CCU and LCU showed patterns of having greater competitive ability at particular cultures but lower competitive ability in other types of crowded cultures, as well as the pattern of head start mechanisms evolved in crowding adapted populations in Chapter 3, wherein LCU populations clearly evolved the largest egg size and the fastest hatching time, add further evidence that larval cultures at different details of crowding could have very different underlying fitness landscapes as measured by larval competitive ability (also see feeding rate data on MB, MCU, CCU and LCU from (Sarangi, 2018). Such culture-specific components of increased larval

competitive ability may have evolved to different degrees in the three sets of crowding-adapted populations used in our studies (MCU, CCU and LCU).

Future studies may test this by measuring larval competitive ability in the focal populations across a larger array of culture types than were used in Chapter 2. Specifically, an experimental design such as the one used in Chapter 5 would allow us to explore the extent to which culture-specific differences in competitive ability have evolved in each crowding-adapted population set. These would be particularly interesting to test in two types of cultures kept at the same number of eggs and same food volume, through different combinations of food column height and surface area. This would allow us to ask whether MCU, CCU and LCU populations exhibit different larval competitive ability in cultures similar to their respective rearing densities. Would LCU show increased larval competitive ability compared to MCU in cultures having high food column lengths and high effective densities? Or would MCU competitive ability be largely superior across most types of cultures tested?

If logistically possible, future experiments on larval competitive ability may also be expanded to use multiple marked competitors (in addition to OE), and also employ more complex experimental designs to tease apart differences in competitive ability (e.g., De Wit 1960; Seaton and Antonovics, 1967; Mather and Caligari, 1981; de Miranda et al., 1991; Santos et al., 1992).

Regarding hatching time – would there be differences in hatching time of the crowding adapted populations, as seen in Chapter 3, if incubation times within the female body were not controlled for? For asking this question, a future assay could include eggs from females who could potentially have incubated their eggs for several hours. This would reveal whether there are any differences in the frequencies of incubated eggs of MCU, CCU and LCU as compared to MB.

As the egg length and egg width varied, though non-significantly, with adult rearing density, especially in CCU and LCU populations, future studies may be conducted on these populations to study the existence of plasticity in the size and shape of eggs depending on the rearing larval density of the adult females laying the eggs. Compared to the relatively modest densities used in chapter 3, more severe crowded conditions may also be used to rear adults of the various populations, which may yield some insight into the plasticity that could exist for egg size metrics across different crowding scenarios.

For the study investigating the effects of head starts in larval competition, such as the one used in Chapter 4, a richer experimental design might yield more insight, and thus future studies could incorporate the following:

- Keeping mono-culture control conditions for each focal population and OE.
- Testing the effect of head start to OE across different egg numbers and food levels, reflecting densities high enough that pre-adult survivorship might also be affected, in addition to just pre-adult development time.
- Providing additional magnitudes of head start durations to OE, as well as incorporating conditions with head-starts also provided to focal populations.
- Studying dry mass at eclosion and dry biomass across different head start durations to OE or to the focal populations.

Additionally, there are a large number of traits and other factors which have not been studied in crowded conditions in greater details, or in light of recent findings. Some pertinent questions could be:

- How do the waste dynamics play out across different effective densities and food column heights?

- What determines the delay in development time in late eclosing flies in crowded cultures at high food volumes?
- Is there variation in the plasticity in feeding rate as seen in Sarangi (2018) in different crowded cultures at the same combination of eggs and food volume but cast into cylindrical columns of different heights and surface areas?
- Has food to biomass conversion efficiency evolved in any of the MCU, CCU, or LCU populations?
- Are there differences in microbial build up and/or microbiome differences across different kinds of crowded cultures?
- How do microbial and/or microbiome build ups affect larval growth and survival in crowded cultures, and do crowding adapted populations get affected by microbial growth differently, or evolve different microbiomes, as compared to their low-density control populations?
- What are the consequences of different types of crowded cultures on adult mating behaviours and on variation in fecundity?

Finally, the simulation study in Chapter 6 could be expanded into a multi-generational design, in order to test the evolutionary consequences of different kinds of crowded cultures, with different combinations of egg number, food column height and surface area. Such an expansion could also be used to simulate population dynamics experiments across different kinds of column heights and surface areas.

Generalising the inferences of this thesis

While the studies of this thesis have made strides to uncover the ecology of larval competition through the classification of effective density (Chapter 5), explored the nuances in the evolution

of competitive ability (Chapters 2 and 4), and some traits that may contribute to it (Chapter 3), a lot more remains unknown, as described extensively through mechanistic conjectures in the simulation framework (Chapter 6). At its core, the take away message from the studies of this thesis is this: the process of competition in *Drosophila* larvae is very nuanced, and there are no general, context-free predictions to be made about the mechanisms, the ecology or the evolution of larval competitive ability.

How then could the results of this thesis be generalised to studies in the wild, or to other model systems?

In *Drosophila melanogaster*, competition clearly needs to be looked at from the context of the feeding band, and this can easily be tested in related laboratory *Drosophila* species, and other dipterans such as house flies *Musca sp.* (Bryant and Sokal 1967), and even various species of blow flies, which have been shown to be affected by larval crowding in similar fashion as *Drosophila* (Ullyett 1955). Beyond this, several studies exist in other species in both laboratory conditions and in the wild, which cite density as a measure of competition (e.g., Grimaldi and Jaenike 1984; Agashe and Bolnick 2010) – in such studies, it is important to consider if the total density is an appropriate measure of competition, and whether phenomena analogous to the feeding band exist in those systems.

Finally, this knowledge of context dependence in studying competition is likely to hold in most complex biological model systems across laboratories and field sites, even if the details of the context may vary.

Closing remarks

Studies on density-dependent selection have come a long way from the verbal speculations of Pianka (1970), or the initial simplified models (e.g., Roughgarden, 1971; Anderson, 1971). Decades of rigorous experimental studies have advanced the understanding of the consequences of selection at low vs. high densities tremendously – far beyond existing analytical models. We currently stand on the threshold of a new way of thinking about density-dependent selection, as shown by recent studies on population dynamics (Pandey and Joshi, 2022a, b), the evolution of competitive ability (Chapter 2), the importance of the feeding band (Chapter 5), and the role of various traits across various crowded conditions (Sarangi 2018, Chapter 3). These novel insights highlight the need to consider the consequences of the spatio-temporal unfolding of the process of competition before making generic long-term predictions. The potential importance of considering the seemingly insignificant nuances – from the surface area of the container, to the clustering behaviour of crowded larvae in the feeding band, to the un-accessed food at the bottom of long food columns, have been highlighted across various early studies (Sang, 1949; Bakker, 1961; Gilpin, 1974; Bierbaum et al., 1989; Mueller, 1990), and these factors have been systematically examined over the last decade in both ecological and evolutionary contexts. Our recent findings, however, are likely to have only scratched the surface – there is seemingly endless nuance and depth to the ecology of larval competition and the evolution of larval competitive ability in laboratory populations of *Drosophila*. All this, in one of the simplest systems for studying competition and density-dependent selection! Ultimately, intra-specific competition across different related and unrelated species will also have to be systematically compared with the results of the last decade of studies using *D. melanogaster*, in order to expand our understanding of density-dependent selection. Studies on inter-specific competition with different species combinations, across a variety of crowding

regimes, will also be required, and are likely to hold great promise in further elucidating the ecology of competition and the evolution of competitive ability.

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