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

Srikant Venkitachalam, Srijan Das¹, Auroi Deep² and Amitabh Joshi*

Evolutionary Biology Laboratory, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bengaluru 560 064, India.

¹*Present address: Molecular Genetics and Plant Biology Laboratory, Biology Division, Indian Institute of Science Education and Research Pune, Dr. Homi Bhabha Road, Pune 411 008, India.*

²*Department of Zoology, University of Delhi, North Campus, Chhatra Marg, Faculty of Science, University Enclave, Delhi 110 007, India.*

*: For correspondence, Email: ajoshi@jncasr.ac.in

Email addresses of all authors: srikantv@jncasr.ac.in, srijan.das@students.iiserpune.ac.in, auronideep777@gmail.com

Running title: Larger egg size evolves in crowded *Drosophila* cultures

Keywords: fruit-flies; egg hatchability; larval crowding; pre-adult competitive ability; head-starts in competition.

25Abstract

26Many different laboratory studies of adaptation to larval crowding in *Drosophila* spp. have all yielded the
 27evolution of pre-adult competitive ability, even though the ecological context in which crowding was
 28experienced varied across studies. However, the evolution of competitive ability was achieved through
 29different suites of traits in studies wherein crowding was imposed in slightly different ways. Earlier studies
 30showed the evolution of increased competitive ability via increased larval feeding rate and tolerance to
 31nitrogenous waste, at the cost of food to biomass conversion efficiency. However, more recent studies, with
 32crowding imposed at relatively low food levels, showed the evolution of competitive ability via decreased
 33larval development time and body size, and an increase in the time efficiency of conversion of food to
 34biomass, with no change in larval feeding rate or waste tolerance. Taken together, these studies have led to a
 35more nuanced understanding of how the specific details of larval numbers, food amounts etc. can affect
 36which traits evolve to confer increased competitive ability. Here, we report results from a study in which egg
 37size and hatching time were assayed on three sets of populations adapted to larval crowding experienced in
 38slightly different ways, as well as their low density ancestral control populations. Egg size and hatching time
 39are traits that may provide larvae with initial advantages under crowding through increased starting larval
 40size and a temporal head-start, respectively. In each set of populations adapted to some form of larval
 41crowding, the evolution of longer and wider eggs was seen, compared to controls, thus making egg size the
 42first consistent correlate of the evolution of increased larval competitive ability across *Drosophila*
 43populations experiencing crowding in slightly different ways. Among the crowding-adapted populations,
 44those crowded at the lowest overall eggs/food density, but the highest density of larvae in the feeding band,
 45showed the largest eggs, on an average. All three sets of crowding-adapted populations showed shorter
 46average egg hatching time than controls, but the difference was significant only in the case of populations
 47experiencing the highest feeding band density. Our results underscore the importance of considering factors
 48other than just eggs/food density when studying the evolution of competitive ability, as also the advantages
 49of having multiple selection regimes within one experimental set up, allowing for a more nuanced
 50understanding of the subtlety with which adaptive evolutionary trajectories can vary across even fairly
 51similar selection regimes.

52Introduction

53Populations adapted to high density conditions are expected to evolve greater competitive ability, a
 54prediction highlighted by the theory of density-dependent selection, first formulated by MacArthur (1962)
 55and MacArthur and Wilson (1967) (see Mueller 1997, 2009 for reviews on subsequent developments in this
 56area). Several rigorous long-term selection experiments on populations of *Drosophila* reared under high
 57larval density conditions subsequently validated this prediction, showing the evolution of increased pre-adult
 58competitive ability in the crowding adapted populations when compared to their low density controls
 59(Mueller 1988; Nagarajan *et al.* 2016; Sarangi *et al.* 2016). However, the traits that evolved as correlates of
 60the increased pre-adult competitive ability differed widely across the studies (Nagarajan *et al.* 2016; Sarangi
 61*et al.* 2016; Sarangi 2018).

62

63The first of these experiments was done using two sets of replicate populations: the *K*-populations,
 64maintained at high population density (larval and adult) by serial transfer, and the *r*-populations, maintained
 65at low population density by culling (Mueller and Ayala 1981). Compared to the *r*-populations, the *K*-
 66populations evolved greater larval competitive ability (Mueller 1988), increased larval feeding rate (Joshi
 67and Mueller 1988), greater pupation height (Mueller and Sweet 1986; Joshi and Mueller 1993), greater
 68larval foraging path length (Sokolowski *et al.* 1997), increased adult dry weight and pre-adult viability at
 69high density (Bierbaum *et al.* 1989), and increased minimum larval food requirement for completion of
 70development (Mueller 1990).

71

72The next selection study sought to validate the results from the *r*- and *K*-populations, as the earlier selection
 73regime confounded the effects of larval and adult crowding. Moreover, the *r*-populations were maintained
 74on discrete generations, whereas the *K*-populations were maintained on overlapping generations (Mueller *et*
 75*al.* 1993). Consequently, populations of *D. melanogaster*, originally derived from a different geographical
 76region than the ancestors of the *r*- and *K*-populations, were used in a selection experiment that differentiated
 77the effects of larval and adult crowding, and in which all selected populations and controls were maintained
 78on a three-week discrete generation cycle (Mueller *et al.* 1993). The populations reared at high larval, but
 79not adult, density were called the CU (Crowded as larvae, Uncrowded as adults), and the low density

80controls were called UU (Uncrowded as larvae, Uncrowded as adults) (Mueller *et al.* 1993). Similar to what
81was seen earlier in the *K*-populations, the CU populations evolved increased larval feeding rate and
82minimum larval food requirement for completion of development (Joshi and Mueller 1996), and larval
83foraging path length (Sokolowski *et al.* 1997). Moreover, the CU populations evolved increased pre-adult
84urea tolerance (Shiotsugu *et al.* 1997; Borash *et al.* 1998) and ammonia tolerance (Borash *et al.* 1998). The
85CU populations, however, did not evolve increased pupation height than controls (Joshi and Mueller 1996),
86unlike the *K*-populations; possible explanations are discussed by Joshi *et al.* (2003).

87
88The broadly consistent results from the *r*- and *K*-populations and the CU and UU populations, together with
89similar results from the *rK* and *r×rK* populations (Guo *et al.* 1991), resulted in the canonical model for
90adaptation to larval crowding in *D. melanogaster* populations: these populations would exhibit increased
91pre-adult competitive ability and larval feeding rate, foraging path length, and tolerance to ammonia and
92urea, but would show reduced food to biomass conversion efficiency as a trade-off (Mueller 1997; Joshi *et*
93*al.* 2001; Prasad and Joshi 2003; Mueller *et al.* 2005; Mueller 2009; Mueller and Cabral 2012; Mueller and
94Barter 2015; Bitner *et al.* 2021). The canonical model was further strengthened by observations in *D.*
95*melanogaster* of greater pre-adult competitive ability in populations selected for increased larval feeding rate
96(Burnet *et al.* 1977), and the evolution of reduced pre-adult competitive ability in populations that evolved
97reduced larval feeding rate due to selection for either rapid pre-adult development (Prasad *et al.* 2001;
98Shakarad *et al.* 2005; Rajamani *et al.* 2006) or for increased parasitoid resistance (Fellowes *et al.* 1998,
991999).

100

101The canonical model was, nevertheless, challenged later by three selection studies involving adaptation to
102larval crowding, in *D. ananassae*, *D. nasuta nasuta* and *D. melanogaster*, respectively (Nagarajan *et al.*
1032016; Sarangi *et al.* 2016). In all three studies, crowding adapted populations did evolve greater larval
104competitive ability compared to their respective low density controls, but did so through a suite of traits
105different from the canonical model. No evolution of increased feeding rate was seen, nor were there any
106changes in urea tolerance, compared to controls. Instead, the crowding-adapted populations seemed to
107evolve 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 was 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).

126

Interestingly, although the LCU and CCU populations did evolve greater pre-adult competitive ability compared to the MB controls (Sarangi 2018; S. Venkitachalam and A. Joshi, *unpubl. data*), 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

136time and a greater egg size than the MB controls. These traits may be important for larval competitive
137ability, as together they can effectively provide a temporal head-start and initial size advantage in
138competition (Sokolowski *et al.* 1997; Bakker 1961, 1969).

139 140 **Materials and methods**

141 ***Experimental populations***

142We used four sets of long-term laboratory populations of *D. melanogaster*, with each set consisting of four
143replicate populations, as briefly described below. The derivation and maintenance of all these populations
144have been discussed in detail by Sarangi (2018).

145**MB 1-4:** These are four low density reared populations that serve as ancestral controls to the three sets of
146crowding-adapted populations. They are maintained at a relatively low density of approx. 70 eggs in 6 mL
147of cornmeal-sugar-yeast medium, in cylindrical Borosilicate glass vials of 2.2-2.4 cm inner diameter and 9.5
148cm height.

149**MCU 1-4:** These populations experience larval crowding at ~600 eggs in ~1.5 mL of cornmeal medium, in
150the same type of vials as MBs. At the time of assaying, the MCUs had undergone at least 218 generations of
151selection (blocks (i.e. replicate populations) 1, 2 assayed at gen. 218; blocks 3, 4 assayed at gen. 219).

152**CCU 1-4:** These populations experience larval crowding at ~1200 eggs in ~3 mL of cornmeal medium, in
153the same type of vials as MBs. It should be noted that MCU and CCU have the exact same overall eggs/food
154density. At the time of assaying, the CCUs had undergone at least 97 generations of selection (blocks 1, 2
155assayed at gen. 97; blocks 3, 4 assayed at gen. 98).

156**LCU 1-4:** These populations experience larval crowding at ~1200 eggs in ~6 mL of cornmeal medium, in
157Borosilicate glass vials of ~2 cm inner diameter and ~9 cm height (approx. 6-dram volume, to mimic the CU
158populations of Mueller *et al.* (1993)). At the time of assaying, the LCUs had undergone at least 96
159generations of selection (blocks 1, 2 assayed at gen. 96; blocks 3, 4 assayed at gen. 97).

160
161While the pre-adult stages of each population are maintained in vials, the adults are transferred to Plexiglas
162cages ($25 \times 20 \times 15 \text{ cm}^3$) on the day of eclosion. Given the low larval density of MB populations, they are
163transferred to cages on the 11th day from egg collection. In the crowding-adapted populations, there is a large

164 amount of variation in eclosion time and thus, transfer of eclosing adults to cages is done daily from day 8 to
 165 day 21 from egg collection. Fresh cornmeal food plates are given (following a fresh plate given on initiation
 166 of transfers) on day 10, 12, 14 and 17 from egg collection. On day 18 from egg collection, the flies in the
 167 cages are provided a food plate with a generous smear of a paste of live yeast mixed with water and a few
 168 drops of glacial acetic acid. On day 20 from egg collection, the flies are provided cornmeal food plates with
 169 vertical edges present for egg laying, for 18 hours. Finally, eggs laid by the flies on these plates are used to
 170 initiate the next generation, with eggs being transferred to fresh vials containing the respective food volume
 171 assigned to each population. All populations are maintained under constant light, at $25 \pm 1^\circ\text{C}$ and 70-90%
 172 relative humidity.

173

174 ***Standardisation of populations***

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

183

184 ***Rearing environments***

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

187 ***Low density rearing:*** The first set was kept at a relatively low eggs/food density of ~70 eggs in 6 mL
 188 cornmeal medium per vial, with a total of 40 vials per replicate population. As in the standardisation, the
 189 adults eclosing in the vials were transferred to a cage on day 11 from egg collection. On day 17 from egg
 190 collection, the flies were provided a food plate with a generous smear of live yeast paste for ~48 hours.
 191 Following this, a “dummy” egg collection cornmeal plate was provided for an hour, which was for the

192 laying of any eggs previously incubating inside the females. Relatively synchronized eggs for the hatching
193 time and egg size assays were then obtained by providing a harder plate with double the usual agar and
194 different composition (only yeast, sugar added), for 45 minutes. This composition ensured easier egg
195 removal for counting.

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

202

203 *Egg hatching time*

204 The assay was carried out in plastic Petri plates (90 mm diameter × 14 mm height), in which a thin layer of
205 12 g/L agar solution (containing 2.4 g/L methyl 4-hydroxybenzoate, as preservative) was spread. A 6 × 6
206 square grid (36 square cells, each having 3 mm sides) was pasted on the bottom of each Petri plate, which
207 was visible through the transparent layer of agar. A total of 5 Petri plates were used per selection × rearing
208 density × block combination, with 36 eggs per Petri plate – one egg per cell of the grid (Figure 1). Checks
209 for egg hatching were done at 13, 15, 17, 18, 19, 20, 21, 22, 24, 26, 28 and 30 hours from egg laying,
210 respectively. At each check, eggs which hatched in the time interval between the current and previous check
211 were noted.

212

213 *Egg hatchability*

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

220

221 *Egg length and width*

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

228

229 *Statistical analyses*

230 Every replicate larval crowding adapted population shares ancestry with an MB population with the same
231 replicate subscript i.e. replicate population i in the MCU, CCU and LCU regimes is derived from replicate i
232 of MB ($i = 1..4$). This permits the use of a completely randomized block design in our statistical analysis,
233 with replicate populations bearing the same subscript treated as blocks. Assays were conducted concurrently
234 on all populations of a block. The data were subjected to a mixed model ANOVA (type III) in a fully
235 factorial design, with the block (4 levels) treated as a random factor. Selection (4 levels) and rearing density
236 (2 levels) were treated as fixed factors. For hatchability, the analysis was repeated after performing an
237 arcsine square root transformation on the data, to check for differences in the statistical significance of the
238 fixed factors. All ANOVAs were done using STATISTICA™ Windows release 5.0 (Statsoft 1995). Tukey's
239 HSD was used for post-hoc pairwise comparisons at $\alpha = 0.05$. The image measurements for egg size were
240 done using ImageJ (Rasband 1997-2018). Pearson's product-moment correlation coefficients were
241 calculated pairwise for population means of egg hatching time, egg length and egg width.

242

243

Results

244

245 *Egg hatching time*

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

248populations had shorter mean hatching times than the MB controls, the shortest being in the LCU
249populations, followed by CCU, and then by MCU (Figure 3). The ANOVA revealed a significant main
250effect of selection ($F_{3,9} = 4.142$, $P = 0.042$) on egg hatching time, but post-hoc pairwise comparisons showed
251a significant difference only between LCU and MB (Figure 3). There neither a significant main effect of
252rearing density ($F_{1,3} = 0.631$, $P = 0.485$), nor a significant selection $\times$ rearing density interaction ($F_{3,9} =$
2531.871, $P = 0.205$).

254

255Egg hatchability

256Mean egg hatchability ranged from about 75-90% across selection $\times$ rearing density combinations, with flies
257reared as larvae at high density (300 eggs in 2 mL food) tending to lay more viable eggs than those reared at
258low density (70 eggs in 6 mL food), most markedly so in the MCU populations (Figure 4). The ANOVA
259revealed no significant main effect of selection ($F_{3,9} = 1.067$, $P = 0.410$). There was, however, a significant
260main effect of rearing density ($F_{1,3} = 12.484$, $P = 0.039$), as well as a significant selection $\times$ rearing density
261interaction ($F_{3,9} = 4.236$, $P = 0.040$). Post-hoc comparisons revealed that only MCU showed significantly
262higher hatchability when flies were reared as larvae at high versus low density. Similar but non-significant
263differences were also seen in the MB and LCU, whereas mean hatchability of CCU reared at low versus
264high larval density was very similar (Figure 4). The pattern of significant ANOVA effects was unaffected by
265whether untransformed or arcsine transformed data were used.

266

267Egg length (μm)

268Mean egg length in MB populations was significantly less than any of the sets of populations selected for
269larval crowding (Figure 5), driving a significant ANOVA main effect of selection ($F_{3,9} = 22.104$, $P < 0.001$).
270Egg length, on an average, did not differ significantly between rearing densities (main effect of rearing
271density: $F_{1,3} = 8.109$, $P = 0.065$; Figure 5). LCU eggs were longer than those of MCU across both rearing
272densities, but longer than CCU eggs only at high rearing density. On the other hand, MCU eggs were shorter
273than CCU eggs at low density, but of similar length at high density (Figure 5), and these rearing density-
274specific differences among various crowding adapted sets of populations drove a significant ANOVA
275selection $\times$ rearing density interaction ($F_{3,9} = 4.830$, $P = 0.029$). This pattern of differences between CCU

276and the other crowding adapted populations was likely due to an average of 9 μm longer eggs laid by CCU
277females when reared at low as compared to high density, although this difference itself was not statistically
278significant (Figure 5).

279

280Egg width (μm)

281Overall, the egg width data were fairly similar to those for egg length (Figures 5,6), with egg width being
282considerably lower in MB populations compared to all crowding adapted populations (ANOVA main effect
283of selection: $F_{3,9} = 5.496$, $P = 0.020$), and not differing, on an average, between rearing densities (main
284effect of rearing density: $F_{1,3} = 0.584$, $P = 0.500$) (Figure 6). Eggs laid by MCU, CCU and LCU flies reared
285at low density did not differ much in mean width, whereas at high rearing density LCU females laid the
286widest eggs, and the MCU and CCU did not significantly differ in egg width (selection $\times$ rearing density
287interaction: $F_{3,9} = 7.971$, $P = 0.007$; Figure 6).

288

289Trait correlations

290There was a strong, positive correlation across population means between egg length and width ($r = +0.771$,
291 $P < 0.001$; Figure 7), indicating that populations with longer eggs also tended to have wider eggs, and vice
292versa. The correlations for mean hatching time and mean egg length ($r = -0.395$, $P = 0.025$), and for mean
293hatching time and mean egg width ($r = -0.548$, $P = 0.001$) were both negative, although the strength of the
294correlation was moderate in both cases, being stronger for hatching time with egg width (Figure 7). There
295were no discernible patterns for within population correlations between egg length and egg width, with the
296mean correlation coefficient being around 0.11, and no selection $\times$ rearing density combination exceeding a
297correlation coefficient of 0.3 (data not shown).

298

299

Discussion

300Despite the variation in which traits underlie the evolution of greater pre-adult competitive ability in
301*Drosophila* populations that experience larval crowding under slightly varying conditions (reviewed in
302Sarangi 2018), our results suggest one common adaptation across at least three such selection regimes
303covering a range of egg number and food amount combinations that more or less mimics the range of

304previous studies. Adults from the MCU, CCU and LCU populations laid eggs with greater length and width
305compared to the MB populations, when assayed at low (70 eggs in 6 mL food) or relatively high (300 eggs
306in 2 mL) density rearing conditions (Figures 5 and 6). Along with the strong positive correlation seen
307between the mean egg length and mean egg width across populations (Figure 7), these results indicate an
308increase in overall egg size of all these three sets of crowding adapted populations compared to the ancestral
309controls. Our results are also in agreement with earlier study from a different laboratory, which
310demonstrated an increase in egg size, relative to controls, in crowding adapted populations derived from our
311MCU populations and maintained on a similar regime (Kumar 2014).

312

313The eggs laid by LCU females were larger than those laid by MCU at both low and high density rearing
314conditions, with CCU eggs being intermediate in size (Figures 5 and 6). The differences among the MCU,
315CCU and LCU populations themselves are perhaps just as important as the consistent difference between the
316egg size of the crowding-adapted and MB populations. While previous comparisons of results from selection
317studies in differently crowded cultures have focused on the repeatability of qualitative differences found
318between a single set of crowding adapted populations against its controls (Joshi and Mueller 1996;
319Nagarajan *et al.* 2016; Sarangi *et al.* 2016), our study system permits more nuanced, quantitative
320comparisons between multiple types of high-density selection regimes.

321

322The importance of plasticity in egg size has been studied extensively from the perspective of non-genetic
323maternal effects, in the contexts of both competition and malnutrition (Kawecki 1995; Azevedo *et al.* 1997;
324Prasad *et al.* 2003; Vijendravarma *et al.* 2010; Yanagi *et al.* 2013). In our study, egg size did not show any
325statistically significant difference between parents reared at low or high larval density (Figures 5,6).
326However, the CCU populations did show a consistent trend for smaller eggs when crowded at the given
327density. It may be possible that either the relative scaling of egg size with female body size, or the sensitivity
328of female size to larval crowding, might be different across the MCU, CCU and LCU populations. This
329might be worth exploring in the future.

330

Moreover, since it is also known that crowding more severe than what we used can further decrease body size (Sang 1949; Bakker 1961; S. Venkitachalam and A. Joshi *unpubl. data*), observed effects of rearing density on egg traits in these populations may change under more extreme crowding, where size at eclosion and pre-adult survivorship are more severely impacted than they were in this study.

335

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.

353

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.

371

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 (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.

380

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,

387highlighting quantitative differences that may otherwise not be possible to see when comparing qualitative
388results between different long-term selection experiments.

389

390

Acknowledgments

391We thank Sajith V. S., Ramesh Kokile, Avani Mital, Medha Rao, Bhavya Pratap Singh, Rajanna N. and
392Muniraju P. for help with the experiments. S. Venkitachalam was supported by a doctoral fellowship from
393the Jawaharlal Nehru Centre for Advanced Scientific Research. S. Das and A. Deep had their stay supported
394by the Jawaharlal Nehru Centre for Advanced Scientific Research's Project Oriented Biological Education
395and Summer Research Fellowship Programme, respectively. This work was supported by a J. C. Bose
396National Fellowship from the Science and Engineering Research Board, Government of India, to A. Joshi
397and in part by A. Joshi's personal funds.

398References

- 399Azevedo R.B., Partridge L. and French V. 1997 Life-history consequences of egg size in *Drosophila*
400*melanogaster*. *Am. Nat.* **150**, 250–282.
- 401Bakker K. 1961 An analysis of factors which determine success in competition for food among larvae of
402*Drosophila melanogaster*. *Arch. Neerl. Zool.* **14**, 200–281.
- 403Bakker K. 1969 Selection for rate of growth and its influence on competitive ability of larvae of *Drosophila*
404*melanogaster*. *Neth. J. Zool.* **19**, 541–595.
- 405Bierbaum T.J., Mueller L.D. and Ayala F.J. 1989 Density-dependent evolution of life-history traits in
406*Drosophila melanogaster*. *Evolution* **43**, 382–392.
- 407Bitner K., Rutledge G.A., Kezos J.N. and Mueller L.D. 2021 The effects of adaptation to urea on feeding
408rates and growth in *Drosophila* larvae. *Ecol. Evol.* **11**, 9516–9529.
- 409Borash D.J., Gibbs A.G., Joshi A. and Mueller L.D. 1998 A genetic polymorphism maintained by natural
410selection in a temporally varying environment. *Am. Nat.* **151**, 148–156.
- 411Burnet B., Sewell D. and Bos M. 1977 Genetic analysis of larval feeding behaviour in *Drosophila*
412*melanogaster*. II. Growth relations and competition between selected lines. *Genet. Res.* **30**, 149–161.
- 413Chippindale A.K., Hoang D., Service P. and Rose M.R. 1994 The evolution of development in *Drosophila*
414selected for postponed senescence. *Evolution* **48**, 1880–1899.
- 415Chippindale A.K., Alipaz J.A., Chen H.-W. and Rose M.R. 1997 Experimental evolution of accelerated
416development in *Drosophila*. 1. Developmental speed and larval survival. *Evolution* **51**, 1536–1551.
- 417Fellowes M.D.E., Kraaijeveld A.R. and Godfray H.C.J. 1998 Trade-off associated with selection for
418increased ability to resist parasitoid attack in *Drosophila melanogaster*. *Proc. R. Soc. Lond. B* **265**, 1553–
4191558.
- 420Fellowes M.D.E., Kraaijeveld A.R. and Godfray H.C.J. 1999 Association between feeding rate and
421parasitoid resistance in *Drosophila melanogaster*. *Evolution* **53**, 1302–1305.
- 422Guo, P.Z., Mueller L.D. and Ayala F.J. 1991 Evolution of behavior by density-dependent natural selection.
423*Proc. Natl. Acad. Sci. USA* **88**, 10905–10906.
- 424Joshi A. and Mueller L.D. 1988 Evolution of higher feeding rate in *Drosophila* due to density-dependent
425natural selection. *Evolution* **42**, 1090–1092.

- 426Joshi A. and Mueller L.D. 1993 Directional and stabilizing density-dependent natural selection for pupation
427height in *Drosophila melanogaster*. *Evolution* **47**, 176–184.
- 428Joshi A. and Mueller L.D. 1996 Density-dependent natural selection in *Drosophila*: trade-offs between
429larval food acquisition and utilization. *Evol. Ecol.* **10**, 463–474.
- 430Joshi A., Prasad N.G. and Shakarad M. 2001 *K*-selection, α -selection, effectiveness, and tolerance in
431competition: density-dependent selection revisited. *J. Genet.* **80**, 63–75.
- 432Joshi A., Castillo R.H. and Mueller L.D. 2003. The contribution of ancestry, chance, and past and ongoing
433selection to adaptive evolution. *J. Genet.* **82**, 147–162.
- 434Kawecki T.J. 1995 Adaptive plasticity of egg size in response to competition in the cowpea weevil,
435*Callosobruchus maculatus* (Coleoptera: Bruchidae). *Oecologia* **102**, 81–85.
- 436Kumar L. 2014 *A study of female reproductive investment in populations of Drosophila melanogaster*
437*adapted to larval crowding*. M.S. Thesis, Indian Institute of Science Education and Research, Mohali, India.
- 438MacArthur R.H. 1962 Some generalized theorems of natural selection. *Proc. Natl. Acad. Sci. USA* **48**, 1893–
4391897.
- 440MacArthur R.H. and Wilson E.O. 1967 *The theory of island biogeography*. Princeton University Press,
441Princeton, NJ, USA.
- 442Mueller L.D. 1988 Evolution of competitive ability in *Drosophila* by density-dependent natural selection.
443*Proc. Natl. Acad. Sci. USA* **85**, 4383–4386.
- 444Mueller L.D. 1990 Density-dependent natural selection does not increase efficiency. *Evol. Ecol.* **4**, 290–297.
- 445Mueller L.D. 1997 Theoretical and empirical examination of density-dependent selection. *Annu. Rev. Ecol.*
446*Syst.* **28**, 269–288.
- 447Mueller L.D. 2009 Fitness, demography, and population dynamics in laboratory experiments. In:
448*Experimental evolution: concepts, methods and applications of selection experiments* (Eds. T. Garland Jr.
449and M.R. Rose) pp. 197–216. University of California Press, Berkeley, CA, USA.
- 450Mueller L.D. and Ayala F.J. 1981 Trade-off between *r*-selection and *K*-selection in *Drosophila* populations.
451*Proc. Natl. Acad. Sci. USA* **78**, 1303–1305.
- 452Mueller L.D. and Sweet V.F. 1986 Density-dependent natural selection in *Drosophila*: evolution of pupation
453height. *Evolution* **40**, 1354–1356.

454Mueller, L.D. and Cabral L.G. 2012 Does phenotypic plasticity for adult size versus food level in
455*Drosophila melanogaster* evolve in response to adaptation to different rearing densities? *Evolution* **66**, 263–
456271.

457Mueller L.D. and Barter T.T. 2015 A model of the evolution of larval feeding rate in *Drosophila* driven by
458conflicting energy demands. *Genetica* **143**, 93–100.

459Mueller L.D., Graves J. and Rose M.R. 1993 Interactions between density-dependent and age-specific
460selection in *Drosophila melanogaster*. *Func. Ecol.* **7**, 469–479.

461Mueller L.D., Rauser C.L. and Rose M.R. 2005 Population dynamics, life history, and demography: lessons
462from *Drosophila*. *Adv. Ecol. Res.* **37**, 77–99.

463Nagarajan A., Natarajan S.B., Jayaram M., Thammanna A., Chari S., Bose J., Jois S.V. and Joshi A. 2016
464Adaptation to larval crowding in *Drosophila ananassae* and *Drosophila nasuta nasuta*: increased larval
465competitive ability without increased larval feeding rate. *J. Genet.* **95**, 411–425.

466Prasad N.G. and Joshi A. 2003 What have two decades of laboratory life-history evolution studies on
467*Drosophila melanogaster* taught us? *J. Genet.* **82**, 45–76.

468Prasad N.G., Shakarad M., Rajamani M. and Joshi A. 2003 Interaction between the effects of maternal and
469larval levels of nutrition on pre-adult survival in *Drosophila melanogaster*. *Evol. Ecol. Res.* **5**, 903–911.

470Prasad N.G., Shakarad M., Anitha D., Rajamani M. and Joshi A. 2001 Correlated responses to selection for
471faster development and early reproduction in *Drosophila*: the evolution of larval traits. *Evolution* **55**, 1363–
4721372.

473Prasad N.G., Shakarad M., Gohil V.M., Sheeba V., Rajamani M. and Joshi A. 2000 Evolution of reduced
474pre-adult viability and larval growth rate in laboratory populations of *Drosophila melanogaster* selected for
475shorter development time. *Genet. Res.* **76**, 249–259.

476Rajamani M., Raghavendra N., Prasad N.G., Archana N., Joshi A. and Shakarad M. 2006 Reduced larval
477feeding rate is a strong evolutionary correlate of rapid development in *Drosophila melanogaster*. *J. Genet.*
478**85**, 209–212.

479Rasband W.S. 1997-2018 ImageJ. U.S. National Institutes of Health, Bethesda, MD, USA
480(<https://imagej.nih.gov/ij/>).

- 481Sang J.H. 1949 The ecological determinants of population growth in a *Drosophila* culture. III. Larval and
482pupal survival. *Physiol. Zool.* **22**, 183–202.
- 483Santos M., Borash D.J., Joshi A., Bounlutay N. and Mueller L.D. 1997 Density-dependent natural selection
484in *Drosophila*: evolution of growth rate and body size. *Evolution* **51**, 420–432.
- 485Sarangi M. 2018 *Ecological details mediate different paths to the evolution of larval competitive ability in*
486*Drosophila*. Ph.D. Thesis, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India.
- 487Sarangi M., Nagarajan A., Dey S., Bose J. and Joshi A. 2016 Evolution of increased larval competitive
488ability in *Drosophila melanogaster* without increased larval feeding rate. *J. Genet.* **95**, 491–503.
- 489Shakarad M., Prasad N.G., Gokhale K., Gadagkar V., Rajamani M. and Joshi A. 2005 Faster development
490does not lead to correlated evolution of greater competitive ability in *Drosophila melanogaster*. *Biol. Lett.* **1**,
49191–94.
- 492Shiotsugu J., Leroi A.M., Yashiro H., Rose M.R. and Mueller L.D. 1997 The symmetry of correlated
493selection responses in adaptive evolution: an experimental study using *Drosophila*. *Evolution* **51**, 163–172.
- 494Sokolowski M.B., Pereira H.S. and Hughes K. 1997 Evolution of foraging behavior in *Drosophila* by
495density-dependent selection. *Proc. Natl. Acad. Sci. USA* **94**, 7373–7377.
- 496StatSoft 1995 Statistica Vol. I: general conventions and statistics 1. StatSoft Inc., Tulsa, OK, USA.
- 497Vijendravarma R.K., Narasimha S. and Kawecki T.J. 2010 Effects of parental larval diet on egg size and
498offspring traits in *Drosophila*. *Biol. Lett.* **6**, 238–241.
- 499Yanagi S., Saeki Y. and Tuda M. 2013 Adaptive egg size plasticity for larval competition and its limits in
500the seed beetle *Callosobruchus chinensis*. *Entomol. Exp. Appl.* **148**, 182–187.

501Figure legends

502Figure 1: Apparatus for egg hatching time and hatchability measurements. The 6×6 grid is pasted on the
503bottom of a Petri plate containing a thin layer of agar solution. Each cell of the grid contains an egg, as can
504be seen in the image. The label denotes the selection $\times$ rearing density $\times$ block combination used, along with
505the replicate plate number ('1' in this case).

506Figure 2: Egg size measurement setup for a replicate containing 10 eggs. There were three such replicates
507for each selection $\times$ rearing density $\times$ block combination. Eggs were numbered from 1 through 10. The egg
508labelled '9' has two lines of measurement drawn for demonstration: the yellow line denotes egg length and
509the red line, egg width. The background is that of a Neubauer haemocytometer, which contains parallel lines
510set a known distance apart, and can thus be used to determine the scale in the image (parallel lines set either
511250 μm or 200 μm apart could be used, as marked in the figure).

512Figure 3: Mean egg hatching time in hours for the four levels of selection, averaged over all levels of rearing
513density and block. The error bars show 95% confidence intervals, calculated from post-hoc Tukey's HSD,
514and allow for visual hypothesis testing – identical superscript letters denote means that did not differ
515significantly, whereas different letters denote means that differed significantly.

516Figure 4: Mean hatchability (%), for all combinations of four levels of selection and two levels of rearing
517density, averaged across all blocks. The error bars show 95% confidence intervals, calculated from post-hoc
518Tukey's HSD, and allow for visual hypothesis testing – identical superscript letters denote means that did
519not differ significantly, whereas different letters denote means that differed significantly.

520Figure 5: Mean egg length (μm), for all combinations of four levels of selection and two levels of rearing
521density, averaged across all blocks. The error bars show 95% confidence intervals, calculated from post-hoc
522Tukey's HSD, and allow for visual hypothesis testing – identical superscript letters denote means that did
523not differ significantly, whereas different letters denote means that differed significantly.

524Figure 6: Mean egg width (μm), for all combinations of four levels of selection and two levels of rearing
525density, averaged across all blocks. The error bars show 95% confidence intervals, calculated from post-hoc

526 Tukey's HSD, and allow for visual hypothesis testing – identical superscript letters denote means that did
 527 not differ significantly, whereas different letters denote means that differed significantly.

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

531

532

533

534

535

536

537

538

539

540

541

542

543

544

545

546

547

548

549

550

551

552

553Figure 1

554

555
556
557
558

559

560Figure 2

561

Figure 3

Figure 4

590

591

592

593Figure 5

594

595

596

597

598Figure 6

599

Figure 7