RESEARCH ARTICLE

Evolution of increased larval competitive ability in *Drosophila melanogaster* without increased larval feeding rate

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Abstract

Multiple experimental evolution studies on *Drosophila melanogaster* in the 1980s and 1990s indicated that enhanced competitive ability evolved primarily through increased larval tolerance to nitrogenous wastes and increased larval feeding and foraging rate, at the cost of efficiency of food conversion to biomass, and this became the widely accepted view of how adaptation to larval crowding evolves in fruitflies. We recently showed that populations of *D. ananassae* and *D. n. nasuta* subjected to extreme larval crowding evolved greater competitive ability without evolving higher feeding rates, primarily through a combination of reduced larval duration, faster attainment of minimum critical size for pupation, greater efficiency of food conversion to biomass, increased pupation height and, perhaps, greater urea/ammonia tolerance. This was a very different suite of traits than that seen to evolve under similar selection in *D. melanogaster* and was closer to the expectations from the theory of *K*-selection. At that time, we suggested two possible reasons for the differences in the phenotypic correlates of greater competitive ability seen in the studies with *D. melanogaster* and the other two species. First, that *D. ananassae* and *D. n. nasuta* had a very different genetic architecture of traits affecting competitive ability compared to the long-term laboratory populations of *D. melanogaster* used in the earlier studies, either because the populations of the former two species were relatively recently wild-caught, or by virtue of being different species. Second, that the different evolutionary trajectories in *D. ananassae* and *D. n. nasuta* versus *D. melanogaster* were a reflection of differences in the manner in which larval crowding was imposed in the two sets of selection experiments. The *D. melanogaster* studies used a higher absolute density of eggs per unit volume of food, and a substantially larger total volume of food, than the studies on *D. ananassae* and *D. n. nasuta*. Here, we show that long-term laboratory populations of *D. melanogaster*, descended from some of the populations used in the earlier studies, evolve essentially the same set of traits as the *D. ananassae* and *D. n. nasuta* crowding-adapted populations when subjected to a similar larval density at low absolute volumes of food. As in the case of *D. ananassae* and *D. n. nasuta*, and in stark contrast to earlier studies with *D. melanogaster*, these crowding-adapted populations of *D. melanogaster* did not evolve greater larval feeding rates as a correlate of increased competitive ability. The present results clearly suggest that the suite of phenotypes through which the evolution of greater competitive ability is achieved in fruitflies depends critically not just on larval density per unit volume of food, but also on the total amount of food available in the culture vials. We discuss these results in the context of an hypothesis about how larval density and the height of the food column in culture vials might interact to alter the fitness costs and benefits of increased larval feeding rates, thus resulting in different routes to the evolution of greater competitive ability, depending on the details of exactly how the larval crowding was implemented.

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Introduction

The theory of density-dependent natural selection is perhaps the most successful bridge between population genetics and population ecology, being premised on the notions that genotypic fitnesses are functions of population density, and that often no single genotype will be the most fit at both low and high densities (Mueller 2009). Following the early development of simple models of density-independent (*r*-selection) and density-dependent (*K*-selection) selection by MacArthur (1962) and MacArthur and Wilson (1967), a loose verbal

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theory attempting to explain life history variants via the *r*- and *K*-selection dichotomy took form (Pianka 1970). Subsequently, formal population genetic models of density dependent selection were developed (Gadgil and Bossert 1970; Roughgarden 1971; Clarke 1972; Anderson and Arnold 1983; Asmussen 1983), and these models, *inter alia*, highlighted the importance of *r–K* tradeoffs in mediating the evolutionary response to differences in population density. The first rigorous attempt to study *r*- and *K*-selection empirically was through laboratory selection on *Drosophila melanogaster* populations maintained either at low density by culling (*r*-populations), or at high density by allowing populations to reach their carrying capacity (*K*-populations). This work revealed a trade-off between *r* and *K* for the first time (Mueller and Ayala 1981): the *K*-populations had a higher per capita rate of population growth than the *r*-populations when assayed at high densities, but had a lower per capita population growth rate than the *r*-populations when assayed at low densities. Further experiments showed the *K*-populations to have evolved a greater larval competitive ability (Mueller 1988), larval feeding rate (Joshi and Mueller 1988), foraging path length (Sokolowski *et al.* 1997), pupation height (Mueller and Sweet 1986; Joshi and Mueller 1993), but a reduced food to biomass conversion efficiency (Mueller 1990) such that larvae from the *K*-populations required more food to successfully complete development compared to the control *r*-populations. This finding, at odds with the classical expectation of the evolution of greater efficiency of food utilization under *K*-selection (MacArthur and Wilson 1967), along with the facts that the *K*-populations experienced higher densities than the *r*-populations both as larvae and adults, and that the *r*- and *K*-selection contrast was confounded with a discrete versus overlapping generation contrast, motivated a second selection study using *D. melanogaster* populations from a different geographic origin than the *r*- and *K*-populations, and selected for adaptation only to larval crowding (the CU populations, first described in Mueller *et al.* 1993).

Relative to the low larval density control populations (UU populations: Joshi and Mueller 1996), the CU populations evolved a set of traits very similar to that seen in the earlier study of the *K*-populations. The CU populations evolved greater egg to adult survivorship when assayed at high larval density (Mueller *et al.* 1993; Shiotsugu *et al.* 1997), largely through the evolution of increased larval feeding rate (Joshi and Mueller 1996), larval foraging path length (Sokolowski *et al.* 1997) and tolerance to nitrogenous waste like urea (Shiotsugu *et al.* 1997; Borash *et al.* 1998) and ammonia (Borash *et al.* 1998). The evolution of these traits was accompanied, as in the case of the *K*-populations, by a reduced food to biomass conversion efficiency (Joshi and Mueller 1996). CU egg to adult development time was similar to that of UU populations when assayed at low larval density, but lower than the UU populations when assayed at high larval density (Borash and Ho 2001). Urea and ammonia tolerance of the *r*- and *K*-populations were not assayed, while competitive ability of the CU and UU populations was not assayed. The results from these two sets of studies led to what might be termed the canonical view of adaptation to larval crowding in *Drosophila*: the evolution of greater competitive ability was mediated by the evolution of higher feeding rate and greater tolerance to nitrogenous waste, at the cost of efficiency of food utilization (Mueller 1997, 2009; Joshi *et al.* 2001; Prasad and Joshi 2003; Mueller and Cabral 2012).

A major element of this canonical view that developed was that increased larval feeding rate is a strong correlate of preadult competitive ability in *Drosophila*. Populations subjected to direct selection for increased larval feeding rate also evolved to be better competitors (Burnet *et al.* 1977), whereas populations selected for rapid egg to adult development evolved both reduced larval feeding rate and reduced larval competitive ability (Prasad *et al.* 2001; Shakarad *et al.* 2005; Rajamani *et al.* 2006). Moreover, populations selected for increased resistance to hymenopteran parasitoids also evolved both reduced larval feeding rate and reduced larval competitive ability (Fellowes *et al.* 1998, 1999). The suggestion that increased larval feeding rate has a fitness cost unless larval densities are high (Joshi and Mueller 1996) was supported by the observation that larval feeding rates rapidly reverted to control levels in CU populations that were maintained at moderate densities (Joshi *et al.* 2003). In addition, there also appeared to be a trade-off between urea/ammonia tolerance and larval feeding rate. Populations selected for greater urea and ammonia tolerance, respectively, exhibited the correlated evolution of reduced larval feeding rate (Borash *et al.* 2000) and larval foraging path length (Mueller *et al.* 2005), whereas populations selected for greater larval urea tolerance did not exhibit higher egg to adult survivorship than controls at high larval density (Shiotsugu *et al.* 1997). Overall, results from multiple studies suggested that larval feeding rate and foraging path length were positively correlated (Joshi and Mueller 1988, 1996; Sokolowski *et al.* 1997; Borash *et al.* 2000; Prasad *et al.* 2001; Mueller *et al.* 2005). Consequently, it appeared that the evolution of competitive ability in *Drosophila* depended upon the outcome of a balance between mutually antagonistic traits like increased larval feeding and forgaing behaviour, greater tolerance to nitrogenous wastes, and a reduced efficiency of conversion of food to biomass. Supporting this view, it was found that the CU populations evolved a development time based polymorphism for two of these traits: compared to controls, offspring of early eclosing flies in a crowded culture showed higher feeding rates, whereas offspring of late eclosing flies showed greater urea/ammonia tolerance (Borash *et al.* 1998).

Recent studies in our laboratory on species of *Drosophila* other than *D. melanogaster*, however, suggested that there were alternative routes to the evolution of adaptation to larval crowding that did not involve a concomitant increase in larval feeding rate. Populations of *D. ananassae* and *D. nasuta nasuta* subjected to larval crowding under a slightly different maintenance regime than the CU

populations evolved greater larval competitive ability and egg to adult survivorship at high density, relative to controls, but without the evolution of increased larval feeding rate, foraging path length and tolerance to nitrogenous wastes, contrary to what was seen earlier in the *K*- and CU populations (Nagarajan *et al*. 2016). The selected populations of both these species evolved a shorter egg to adult development time, when assayed at both low and high larval densities, and also showed a reduced minimum critical feeding time, the minimum time duration of larval feeding required to sustain successful pupation and eclosion subsequently (Nagarajan *et al.* 2016). Thus, results from the *D. ananassae* and *D. n. nasuta* crowding adapted populations clearly showed that the evolution of greater competitive ability does not necessarily involve the evolution of higher larval feeding rate or foraging path length or tolerance to increasing urea and ammonia in the food medium.

Nagarajan *et al.* (2016) discussed three possible reasons for the differing phenotypic correlates of increased competitive ability between the studies on *D. ananassae* and *D. n. nasuta* and the earlier studies on *D. melanogaster*. (i) Species-specific differences in the genetic architecture of traits correlated with competitive ability. (ii) Differences in genetic architecture between the relatively recently wild-caught populations of *D. ananassae* and *D. n. nasuta* and the long-term laboratory populations of *D. melanogaster* used in the earlier studies, due to very different durations of rearing under domestication. (iii) Differences in the details of the laboratory ecology of how larval crowding was implemented between the studies on *D. ananassae* and *D. n. nasuta* and the earlier studies on *D. melanogaster*. The main difference between the maintenance regime used in the *D. melanogaster* studies and that used in the studies on *D. ananassae* and *D. n. nasuta* was in the number of eggs per unit volume of food and the total amount of food used for larval rearing in the crowding-adapted populations (described in detail by Nagarajan *et al.* 2016). The rearing densities in the *D. ananassae* and *D. n. nasuta* crowded populations were 550–600 eggs in 1.5 mL food, and 350–400 eggs in 2 mL of food, respectively, per vial. In the *K*- and CU population of *D. melanogaster*, egg densities were not explicitly controlled but were generally higher than those used in the *D. ananassae* and *D. n. nasuta* studies, but with a larger total volume of food, too. It is, therefore, possible that the time course of food depletion and nitrogenous waste build-up in the *D. ananassae* and *D. n. nasuta* crowded cultures was different from that in the *K*- and CU populations. It has been shown theoretically that optimal feeding rates are likely to decline as the concentration of nitrogenous waste in the food increases (Mueller *et al.* 2005; Mueller and Barter 2015). Thus, at least in principle, it is possible that the optimal feeding rates in the *D. ananassae* and *D. n. nasuta* crowded populations were actually less than they were for the *K*- and CU populations, and that is why increased feeding rates did not evolve in the former (Nagarajan *et al.* 2016).

In this paper, we describe results from a study of adaptation to larval crowding in long-term laboratory *D. melanogaster* populations that are descendents of the UU control populations used in one of the earlier studies (Mueller *et al*. 1993; Joshi and Mueller 1996), but are maintained at a density of about 600 eggs in 1.5 mL of food per vial, similar to the maintenance regime of the *D. ananassae* and *D. n. nasuta* crowded populations of Nagarajan *et al.* (2016). We show that these *D. melanogaster* populations evolve a suite of traits very similar to that seen in the *D. ananassae* and *D. n. nasuta* populations of Nagarajan *et al.* (2016) and, unlike the earlier studies on *D. melanogaster*, increased competitive ability in these populations is not accompanied by greater larval feeding rate or a reduced efficiency of food utilization. Consequently, these results allow us to rule out genetic architecture differences as a cause for the discrepancies between the studies of Nagarajan *et al.* (2016) and the earlier *D. melanogaster* studies, especially on the CU populations. It seems clear, therefore, that differences in egg density and total volume of food used in the crowding selection regime can mediate the evolution of increased competitive ability through different suites of phenotypes.

Materials and methods

Study populations

We used eight laboratory populations of *D. melanogaster* for this selection experiment: four populations selected for adaptation to larval crowding (Melanogaster Crowded as larvae and Uncrowded as adults: MCU-1..4), and four ancestral control populations (Melanogaster Baseline: MB-1..4), whose derivation and ancestral history are summarized in figure 1. Both the MCU and MB populations were derived from the MGB populations, which were created by mixing the four JB populations (first described by Sheeba *et al.* 1998), themselves direct descendents of four of the UU populations (Mueller *et al.* 1993; Joshi and Mueller 1996), and then splitting the four-way hybrid population into five independent replicates, the MGB populations (figure 1).

The MB populations are maintained at 25 ± 1°C, ~90% relative humidity and constant light on a 21-day discrete generation cycle on cornmeal medium (table 1). The egg density is kept moderate by collecting 70 ± 10 eggs per vial (9.5 cm height, 2.4 cm inner diameter) in 6 mL of food. Forty such vials are set up per population. On the 11th day after egg collection, the eclosed flies (~75–80% flies eclose by this day) are transferred into Plexiglas cages ($25 \times 20 \times 15$ cm^3). Thus, each population consists of over 2000 breeding adults per generation. In the cage, food plates are changed on the 12th, 14th and 17th day after egg collection. On 18th day after egg collection, each cage is provided with a food plate containing excess supplementary live yeast–acetic acid paste. Three days later (i.e. on day 21 postegg collection), fresh food plates are provided and the flies are allowed to

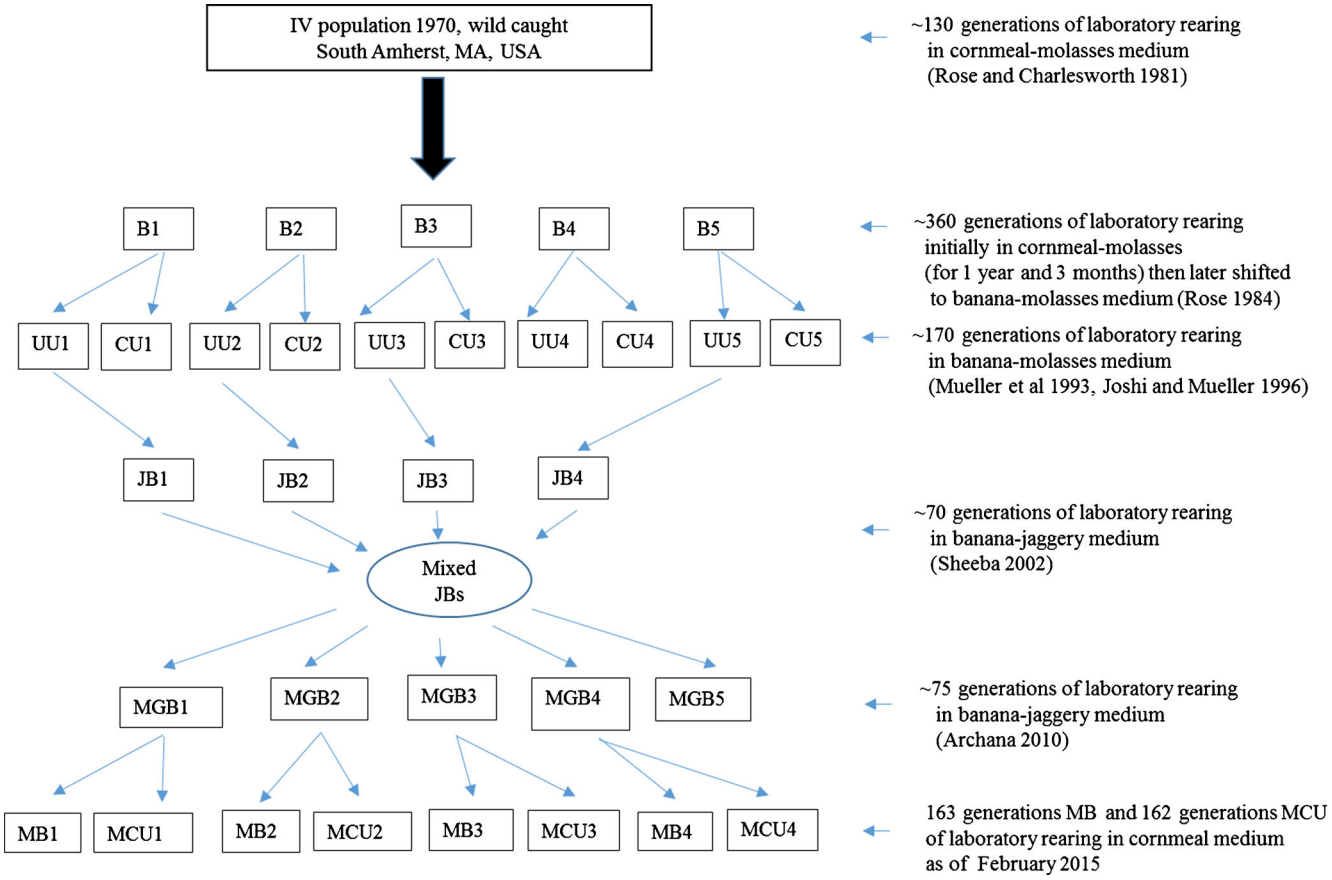

Figure 1. Schematic showing the ancestry of the populations discussed in this study. All populations were kept on a 21-day discrete generation cycle, except the IV and B populations that were on a 14-day discrete generation cycle.

oviposit for 18 h, and eggs from these plates are transferred into vials to initiate the next generation.

The MCU populations are maintained in a manner identical to the MB populations except that the rearing density is about 600 eggs in 1.5 mL of food, and that, once eclosions begin, eclosing flies are collected into cages from the rearing vials daily until day 18 postegg collection, a period of roughly 8 days. Initially, the MCU populations were reared at 800 eggs in 1.5 mL of food for a few generations; the density was subsequently reduced to avoid population size crashes. Initially, 24 vials used to be set up per replicate population, but after generation 135 of MCU selection, the number of vials was reduced to 15 per population due to increase in overall egg to adult survivorship over the course of selection. The number of breeding adults is over 2000 per population, as in the MB controls. Each MCU population was derived from one MB population and, hence, MB-*i* and MCU-*i* ($i = 1..4$) are related and were therefore treated as constituting the *i*th block, representing ancestry, in the statistical analyses. The salient features of MB and MCU maintenance together with that of the CU populations of Joshi and Mueller (1996) are summarized in table 2 for ease of comparison.

Table 1. Composition of cornmeal medium (1 L).

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

For a 1 L cornmeal medium cook, all the ingredients are weighed and added together to 1 L of water. The mixture is then allowed to homogenize on heat, with continuous stirring, after which 120 mL of extra water is added to maintain consistency. The mixture is then pressure cooked for 20 min. The prepared medium is then cooled to 60°C, after which preservatives (1 g methyl-p-hydroxybenzoate dissolved in 10 mL of ethanol and 1 mL propionic acid) are added.

We used two marked mutant populations of *D. melanogaster* as common competitors for the MCU and MB populations in assays of egg to adult survivorship in the presence of a competitor population. One was a white eye population (WE), obtained from spontaneous mutations in one of our wild-type JB populations. At the time of assay, the WE population had been maintained in the laboratory for about 90 generations on a 21-day discrete generation cycle on banana–jaggery medium, with all other rearing conditions identical to the MB populations. The second population was an orange eye (OE) mutant population obtained from spontaneous mutations which occurred in the WE population. At

Table 2. Similarities and differences in the maintenance regimes of the MB, MCU and CU populations.

Population:	MB	MCU	CU
Larval number	∼70	∼600	∼1200–1500
Food volume	6 mL	1.5 mL	∼5 mL
Type of food	Cornmeal	Cornmeal	Banana–molasses
Type of culture vial	8 dram (2.4 cm diameter × 9.5 cm height)	8 dram (2.4 cm diameter × 9.5 cm height)	6 dram (2.2 cm diameter × 8.4 cm height)
Fly collection method	Eclosing adults were collected into cages on day 11 from egg collection	Eclosing adults were collected everyday into cages once eclosions began till day 18 postegg collection	Eclosing adults were collected everyday into fresh food vials once eclosions began and kept at a low density of 60–80 adults per vial till day 18 postegg collection
Fly collection duration	1 day	Over 7 days	Over 9 days
Yeast supplement duration	3 days	3 days	3 days
Generation cycle	21 day	21 day	21 day

the time of assay, the OE population had been maintained for over 48 generations under conditions identical to the MB populations, on cornmeal medium.

Standardization of populations

Prior to any assay, both control and selected populations were subjected to common (control) rearing conditions for one full generation to eliminate any nongenetic parental effects. About 70 ± 10 eggs were collected per vial in 6 mL of food and 40 such vials were set up per population. On the 11th day after egg collection, the eclosing flies (henceforth referred to as standardized flies) were transferred to cages. Eggs for assays were obtained from these standardized flies over a 14 h period after three days of being provided with food along with a supplement of live yeast–acetic acid paste. All assays were conducted at 25 ± 1°C, under constant light and ∼90% relative humidity.

Egg to adult survivorship at low and high density

Egg to adult survivorship of the MB and MCU populations was assayed at low and high larval density, in the absence (monotypic culture) and presence (bitypic culture) of a marked competitor strain, respectively, at different time points in the course of MCU selection. Egg to adult survivorship at high larval density is the primary trait expected to be under direct selection in the MCU populations exposed to high larval crowding every generation.

Generation 30: After 30 generations of MCU selection, eggs laid by standardized flies were placed into vials containing 1.5 mL of food at a density of either 70 or 800 per vial. Eight such vials were set up for each replicate MB and MCU population at each density in monotypic cultures. Eight such vials for each MB and MCU population at each density were also set up in bitypic cultures, with the WE population as a common competitor. For the bitypic cultures, eggs laid by standardized MCU, MB or WE flies were collected and placed into vials containing 1.5 mL of food at a density of either 70 (35 MCU or MB eggs and 35 WE eggs) or 800 (400 MCU or MB eggs and 400 WE eggs) per vial. The number of wild type and mutant (for bitypic cultures) flies eclosing in each vial was recorded and used to calculate egg to adult survivorship in monotypic and bitypic cultures. Once eclosion began, vials were checked for eclosion twice a day until there were no eclosions for four consecutive days.

Generation 82: After 82 generations of MCU selection, eggs laid by standardized flies were placed into vials containing 1.5 mL of food at a density of either 60 (10 vials per population) or 600 (six vials per population) per vial. The number of flies eclosing in each vial was recorded and used to calculate egg to adult survivorship in monotypic cultures. Vials were checked for eclosion twice a day once eclosions began, until there were no eclosions for four consecutive days.

Generation 112: After 112 generations of MCU selection, eggs laid by standardized flies were placed into vials containing 1.5 mL of food at a density of either 70 (six vials per population) or 600 (five vials per population) per vial. Each vial had half the eggs from MB or MCU populations and other half from the mutant OE population. The number of wild type and mutant flies eclosing in each vial was recorded and used to calculate egg to adult survivorship in bitypic cultures. Vials were checked for eclosion twice a day once eclosions began, until there were no eclosions for four consecutive days.

Egg to adult development time at low and high density

After 82 generations of MCU selection, egg to adult development time was assayed at two densities: 60 or 600 eggs per vial with 1.5 mL of food. Eggs from standardized MB and MCU flies were dispensed into each vial using a moistened paintbrush. Eight such vials were set up per replicate population. After the pupae darkened, vials were checked for every 6 h and the number of eclosing flies recorded. These checks continued till there were no eclosions for four consecutive days.

Minimum critical feeding time and dry weight after minimum feeding

In this study, conducted at generation 106 of MCU selection, the minimum duration of feeding on yeast required for larvae to successfully complete development was assayed. Eggs for the experiment were collected from the standardized flies which were provided with yeast paste for three days prior to egg collection. Eggs were collected in a narrow time window to synchronize the age of eggs. Eggs from each population were separated into 20 batches of 100–110 eggs each. Each batch was spread on a nonnutritive agar Petri dish (9.5 cm diameter) for hatching. Freshly hatched first instar larvae were subsequently transferred to Petri dishes containing a thin layer of nonnutritive agar overlaid with 37.5% suspension of yeast. Twenty such plates were set up per population, each containing ∼80 larvae. Larvae were removed from the yeast plates at 3 h intervals between 62 to 80 h from egg collection. At each time point, 90 larvae were removed randomly from these plates, washed, dried on a soft tissue, and then moved to nonnutritive agar vials and their survivorship till eclosion were recorded. At each time point, two batches of 15 larvae each per population were also collected, dried in a hot air oven at 70°C for 36 h, and then weighed using a Sartorius (CP 225D) fine balance.

Dry weight of freshly eclosed adults

After 106 generations of MCU selection, eggs of approximately identical age were harvested over a 1 h period from the standardized MB and MCU flies and dispensed onto Petri dishes containing nonnutritive agar at a density of 110 eggs per Petri dish. Eighteen such Petri dishes were set up per population. Twenty-two hours later, the freshly hatched first instar larvae from each Petri dish were transferred using a moistened paintbrush, to a fresh Petri dish containing agar with a thin layer of 37.5% yeast suspension overlaid. Ten such plates with ∼100 first instar larvae were set up per population and left undisturbed to allow pupation to occur. After 196 h had elapsed since egg laying, the pupae were transferred to vials with nonnutritive agar, and eventually eclosing adults were harvested from these vials, frozen and subsequently dried in a hot air oven at 64°C for 36 h and weighed in 10 batches each of either five males or five females per population using a Sartorius (CP 225D) fine balance.

Larval feeding rate

After 37 generations of MCU selection, the feeding rates of MB and MCU third instar larvae were measured at physiologically equalized ages, based on the difference in MB and MCU development time. This was done by collecting eggs from the standardized MCU flies 5 h later than the MB flies. Thus, at the time of assay, MCU larvae were ∼68 h from egg lay, whereas MB larvae were ∼73 h from egg lay. Following Joshi and Mueller (1996), about a hundred eggs over a 4 h period were collected from standardized flies and placed into two Petri dishes with nonnutritive agar each for the MB and MCU populations. Twenty-four hours later, 25 newly hatched larvae were transferred to a Petri dish containing a thin layer of nonnutritive agar overlaid with 1.5 mL of 37.5% yeast suspension. Four such Petri dishes were set up per population. The larvae were then allowed to feed till they were in the early third instar. At this point, 20 larvae from each population were assayed for feeding rate by placing them individually in a Petri dish containing a thin layer of agar overlaid with a thin layer of 10% yeast suspension. After allowing for a 15 s acclimation period, feeding rate was measured under a stereo zoom microscope as the number of cephalopharyngeal sclerite retractions in a 1 min period. Selected and control populations, matched by the subscripted indices, were assayed together, with one larva from the selected population and one from the control population being assayed alternately.

Larval foraging path length

After 37 generations of MCU selection, the foraging path length of early third instar larvae was measured. The harvesting of larvae was done as described for the larval feeding rate assay. Due to the development time difference between MB and MCU populations, at the time of assay, MCU larvae were ∼68 h from egg lay, whereas MB larvae were ∼73 h from egg lay. For the assay, 20 larvae from each population were placed individually in a Petri dish containing a thin layer of agar overlaid with a thin layer of 10% yeast suspension. After allowing for a 15 s acclimation period, the larvae were allowed to move on the Petri plate surface for 1 min. The paths that the larvae traversed in 1 min interval were then traced onto a transparency sheet and later measured to the nearest millimetre with thread and ruler. Selected and control populations, matched by subscripted indices, were assayed together.

Pupation height

Pupation height was measured after 34 generations of MCU selection for blocks 1 and 2, and after 37 generations of MCU selection for blocks 3 and 4. Eggs from standardized MCU and MB flies were collected off Petri dishes and exactly 30 eggs were placed in a vial with 6 mL of cornmeal medium. Five such vials were set up per population. Once all individuals had pupated, the pupation heights were measured following Mueller and Sweet (1986), the distance from the surface of the medium to the point between the anterior spiracles of the pupae. Any pupae on or touching the surface of the food were given a pupation height of zero.

Larval nitrogenous waste tolerance

After 30 generations of MCU selection, larval tolerance to urea and ammonia in the MB and MCU populations was assayed. Three different concentrations of urea (0, 14 and 18 g/L), and three different concentrations of ammonia (0, 15

and 25 g/L) were used in this assay. Eggs were collected from standardized flies and exactly 30 eggs per 6 mL of food were placed in each vial with different concentrations of urea or ammonia. Ten such vials were set up for each of these three concentrations per population. The number of flies eclosing in each vial was recorded. Nitrogenous waste tolerance was reflected in the mean egg to adult survivorship at different concentrations of urea or ammonia.

Statistical analysis

Mixed model analyses of variance (ANOVA) were carried out for all the traits. MCU and MB populations with a common numerical subscript were treated as random blocks which were crossed with the fixed factor selection regime. Depending upon the assay, other fixed factors were larval density, type of culture (monotypic or biypic) for egg to adult survivorship, preadult life-stage for instar and pupal duration, urea or ammonia concentration in the food for urea and ammonia tolerance, and larval feeding duration or survivorship for the assays on minimum critical feeding time and dry weight at eclosion after feeding for different time durations. The egg to adult survivorship data were arcsine-square root transformed before ANOVA. Population means were used for testing significance of fixed factors and interactions in all analyses, which were implemented using Statistica for Windows rel. 5.0 B, (StatSoft 1995). Multiple comparisons were done using Tukey's honest significant difference (HSD) test.

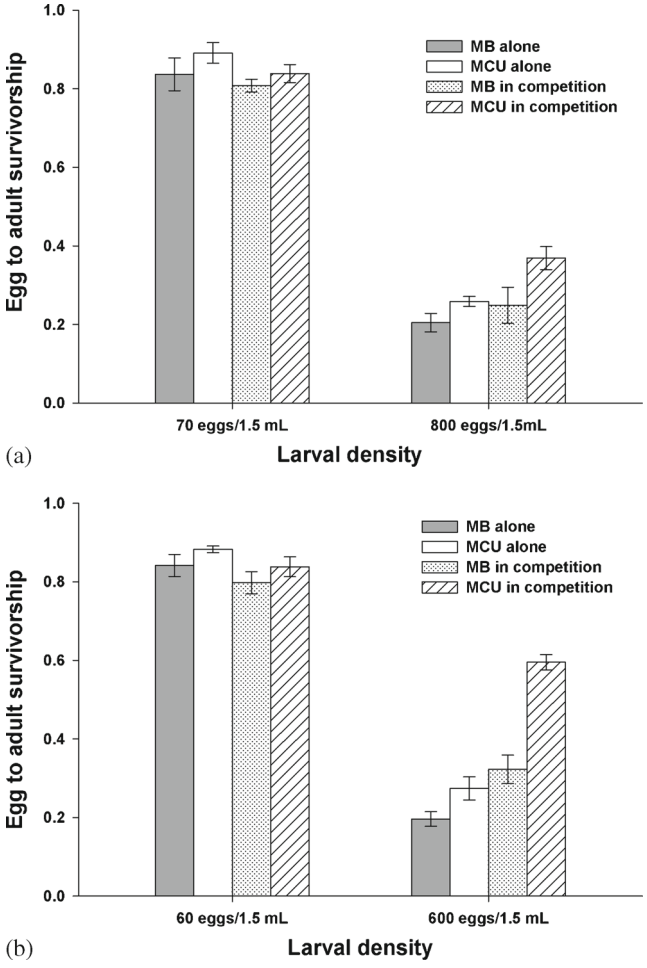

Figure 2. Larval competitive ability after generation (a) 30 of selection (b) 82 and 112 of selection. The graphs represent mean egg to adult survivorship of MB and MCU populations in the competition assay (either in a monotypic culture or in bitypic culture with a common competitor) at moderate and high larval densities. Error bars are the standard errors around the means of the four replicate populations.

Results

Egg to adult survivorship at low and high density

In the set of assays performed on monotypic and bitypic cultures at low and high density at different times during ongoing MCU selection, the overall trend was for egg to adult survivorship to be higher at low compared to high density, and for MCU populations to have higher egg to adult survivorship than MB controls, with the differences being more pronounced in bitypic cultures with a marked mutant competitor strain (figure 2; table 3). At generation 30 of MCU selection, egg to adult survivorship of the MCU populations was significantly higher than MB populations only in bitypic cultures, whereas the effect of density was significant in both monotypic and bitypic cultures (table 3). Later in the course of selection, egg to adult survivorship was significantly higher than controls in MCU populations, even in monotypic cultures at generation 82 (table 3). At generation 112, in bitypic cultures, there was clear evidence for greater competitive ability of the MCU populations, as there was a significant selection regime × density interaction, in addition to significant main effects of selection regime and density (table 3). At high density, the superiority of MCU populations over MB

Table 3. Results of ANOVA on arcsin-squareroot transformed mean egg to adult survivorship of MB and MCU populations when competed in monotypic culture and when competed against a common competitor (bitypic culture) at generations 30, 82 and 112 of selection. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

	Monotypic culture		Bitypic culture	
Effect	*F*	*P*	*F*	*P*
Generation 30				
Selection regime	2.877	0.188	35.819	0.009
Larval density	875.348	<0.001	169.459	<0.001
Selection regime × larval density	0.0704	0.807	3.252	0.169
Generation 82 and 112				
Selection regime	12.586	0.038	22.121	0.018
Larval density	1522.1	<0.001	133.124	0.001
Selection regime × larval density	0.621	0.488	31.923	0.010

controls was far greater in the bitypic cultures, in competition with the mutant OE strain than in the montypic cultures (figure 2b). A similar trend could also be seen in the generation 30 high density survivorship data (figure 2a), but the interaction between selection regime and density was not significant (table 3). There was no suggestion of an *r–K* trade-off manifesting in reduced survivorship of MCU populations at low density compared to the MB controls.

Egg to adult development time at low and high density

The ANOVA revealed a significant main effect of selection regime (table 4) with the MCU populations developing significantly faster than the MB controls at both low and high density (figure 3). The magnitude of the development time difference was about 10 h in males and 15 h in females at both low and high densities (figure 3), driving a significant selection regime × sex interaction (table 4). The other significant ANOVA effects were those of density (slower development at high density) and the sex × density interaction (table 4). Overall, females developed significantly faster than males at low density whereas the male–female difference was not significant at high density (Tukey's HSD test at $P = 0.05$ level of significance). In the MCU populations, females developed faster than males at both densities, whereas in the MB populations, females had similar development time as males at low density, but took about 3.5 h longer than males to complete development at high density (figure 3). None of these differences, however, were significant by Tukey's HSD test.

Table 4. Results of ANOVA on mean egg to adult development time assayed at low and high larval density, after 82 generations of MCU selection, with selection regime, larval density and sex as fixed factors, and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	*df*	MS	*F*	*P*
Selection regime	1	1294.25	35.020	0.009
Larval density	1	18197.1	100.63	0.002
Sex	1	8.442	3.345	0.164
Selection regime × larval density	1	0.130	0.005	0.947
Selection regime × sex	1	53.209	21.659	0.018
Larval density × sex	1	14.329	726.269	<0.001
Selection regime × larval density × sex	1	2.892	7.860	0.067

Minimum critical feeding time and dry weight after minimum feeding

The survivorship to adulthood of larvae allowed to feed for different durations of time after egg collection (62, 65, 68, 71, 74, 77 or 80 h) tended to increase with feeding duration for both MB and MCU populations (figure 4a; table 5: significant main effect of feeding duration). At each time point after 65 h of egg collection, however, MCU larvae showed significantly greater survivorship to adulthood than the MB controls (figure 4a; table 5: significant main effect of selection regime). There was no significant selection regime × feeding duration interaction (table 5).

The dry weight of larvae allowed to feed for different durations of time after egg collection (62, 65, 68, 71, 74, 77 or 80 h) also tended to increase with feeding duration for both MB and MCU populations (figure 4b; table 6: significant main effect of feeding duration). At each time point, the

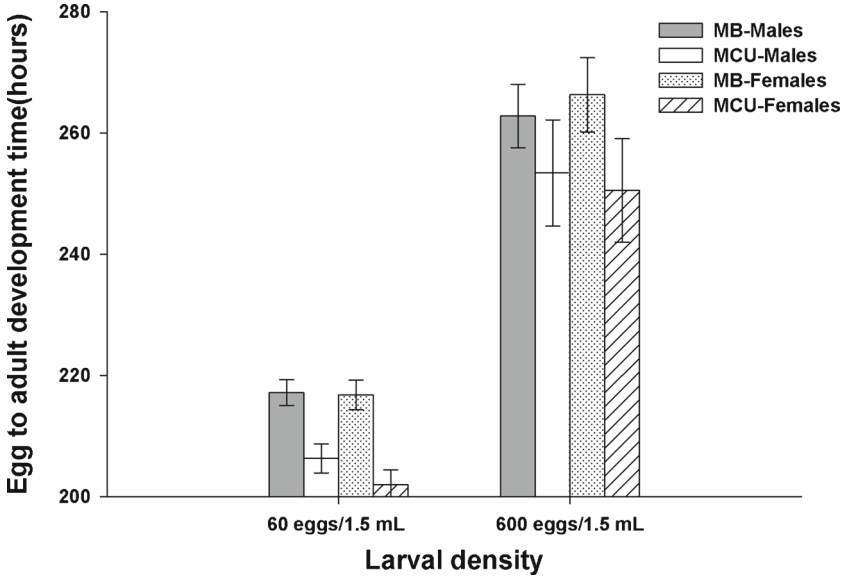

Figure 3. Egg to adult development time of MB and MCU populations after 82 generations of selection at moderate and high larval density. Error bars are the standard errors around the means of the four replicate populations.

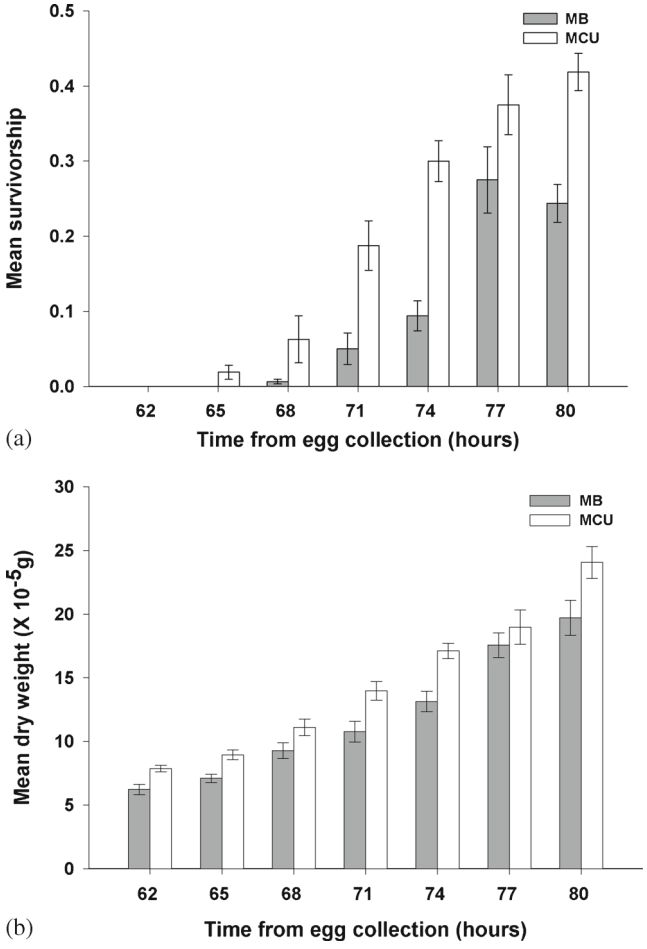

Figure 4. (a) Mean survivorship to eclosion of larvae when removed from food at different time points from egg lay and transferred to nonnutritive agar plates. (b) Mean dry weight of individual larvae when measured at different time points from egg collection. Error bars are the standard errors around the means of the four replicate populations.

MCU larvae were significantly heavier (except at 77 h) than the MB controls (figure 4b; table 6: significant main effect of selection regime). There was no significant selection regime × feeding duration interaction (table 6). The time points covered (62–80 h from egg collection) correspond roughly to mid–late second instar through approximately middle third instar.

Table 5. Results of ANOVA on mean survivorship to eclosion of larvae from the MB and MCU populations on nonnutritive agar, after having been allowed to feed for different durations of time. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted.

Effect	*df*	MS	*F*	*P*
Selection regime	1	0.315	297.848	<0.001
Feeding duration	6	0.538	29.609	<0.001
Selection regime × feeding duration	6	0.024	2.365	0.073

Table 6. Results of ANOVA on mean dry weight of larvae at different stages of larval development, in a span of 62 to 80 h from egg collection in MB and MCU populations, with selection and time as fixed factors and block as a random factor. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

Effect	*df*	MS	*F*	*P*
Selection regime	1	95.298	8832.264	<0.001
Feeding duration	6	238.881	58.033	<0.001
Selection regime × feeding duration	6	2.944	58.033	<0.430

Dry weight of freshly eclosed adults

The ANOVA on dry weight of freshly eclosed adults revealed significant main effects of selection regime and sex (table 7). Females were significantly heavier than males, and both MCU males (mean ± s.e. = $30.39 \times 10^{-5} \pm 0.40 \times 10^{-5}$ g) and females (mean ± s.e. = $40.82 \times 10^{-5} \pm 0.40 \times 10^{-5}$ g) were significantly lighter than MB males (mean ± s.e. = $30.96 \times 10^{-5} \pm 0.42 \times 10^{-5}$ g) and females (mean ± s.e. = $42.07 \times 10^{-5} \pm 0.95 \times 10^{-5}$ g), respectively.

Larval behaviours

The MB and MCU populations did not differ in any of the three larval behaviours assayed. Larval feeding rates in the MCU (mean ± s.e. = 108.8 ± 1.23 bites per min) and MB (mean ± s.e. = 108.2 ± 1.36 bites per min) populations did not differ significantly ($F_{1,3} = 0.51$; $P = 0.53$). Larval foraging path length also did not differ significantly ($F_{1,3} = 1.93$; $P = 0.26$) between the MCU (mean ± s.e. = 3.65 ± 0.036 cm) and MB (mean ± s.e. = 3.83 ± 0.033 cm) populations. Pupation heights, too, did not differ significantly ($F_{1,3} = 1.44$; $P = 0.32$) between the MCU (mean ± s.e. = 1.18 ± 0.11 cm) and MB (mean ± s.e. = 1.36 ± 0.088 cm) populations.

Larval nitrogenous waste tolerance

There was no evidence for any difference between the MCU and MB populations in tolerance to either ammonia (figure 5a) or urea (figure 5b). The only significant ANOVA effect on survivorship on both ammonia and urea was that of

Table 7. Results of ANOVA on mean dry weight at eclosion of MB and MCU populations, with selection and sex as fixed factors and block as a random factor for both analyses. In this design, the random factor (block) plus any random interactions are not tested for significance and are therefore omitted from the table.

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

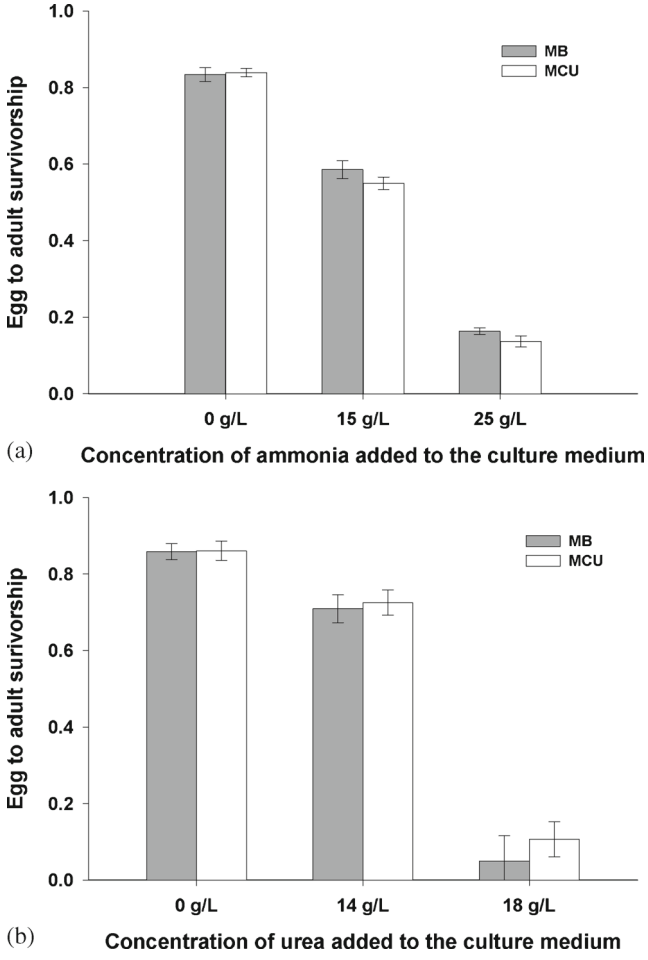

Figure 5. Mean egg-to-adult survivorship of MB and MCU populations in three different concentrations of (a) ammonia, and (b) urea, after 30 generations of selection. Error bars represent the standard errors around the means of the four replicate populations.

waste concentration (table 8), and in the case of both ammonia and urea, survivorship of both MCU and MB populations declined with increasing concentration (figure 5).

Table 8. Results of three-way ANOVA on egg to adult survivorship of MB and MCU populations in food containing different concentrations of ammonia and urea after 30 generations of selection. Selection and concentrations were fixed factors and block was a random factor.

Effect	*df*	MS	*F*	*P*
Ammonia				
Selection	1	0.003	3.82	0.145
Concentration	2	1.17	627.73	<0.001
Selection × concentration	2	0.0013	1.64	0.27
Urea				
Selection	1	0.012	4.83	0.115
Concentration	2	1.99	89.45	<0.001
Selection × concentration	2	0.007	2.69	0.146

Discussion

The present study was motivated by the earlier observation that crowding adapted populations of *D. ananassae* and *D. n. nasuta* evolved greater competitive ability and egg to adult survivorship at high density primarily through a combination of reduced duration of the larval stage, faster attainment of minimum critical size for pupation and greater efficiency of food conversion to biomass (Nagarajan *et al.* 2016). This was in contrast to the generally accepted view that the evolution of greater competitive ability in fruitflies was primarily mediated by the evolution of increased larval feeding rate, foraging path length and tolerance to nitrogenous wastes, at the cost of reduced efficiency of food conversion to biomass based on earlier studies with *D. melanogaster* (Mueller 1997, 2009; Prasad-and-Joshi 2003). We decided to ascertain whether the differences in the phenotypic correlates of greater competitive ability between these two sets of studies were due to either the different species used, or differences in the manner in which larval crowding was imposed, by examining the direct and correlated responses to selection for adaptation to larval crowding in populations of *D. melanogaster* related to those used in the earlier studies (UU populations, described by Joshi and Mueller 1996) but maintained under a rearing protocol similar to that used in the studies on *D. ananassae* and *D. n. nasuta* by Nagarajan *et al.* (2016).

Our results clearly show that the MCU populations of *D. melanogaster* have adapted to crowded larval conditions by evolving traits very similar to those seen in the *D. ananassae* and *D. n. nasuta* crowding adapted populations by Nagarajan *et al.* (2016), and very different from those seen in the CU populations of *D. melanogaster* (Mueller 1997, 2009; Prasad and Joshi 2003). Specifically, like the crowding adapted *D. ananassae* and *D. n. nasuta* populations (Nagarajan *et al.* 2016), the MCU populations appear to have evolved greater competitive ability (figure 2b) largely through reduced development time (figure 3), and a reduction in the minimum larval feeding duration needed to be able to successfully complete preadult development (figure 4a). Moreover, unlike the CU populations of *D. melanogaster* studied earlier (Mueller 1997, 2009; Prasad and Joshi 2003), the MCU populations do not differ from MB controls in larval feeding rate, foraging path length, pupation height or nitrogenous waste tolerance (figure 5).

Despite the broad similarities in correlated responses to selection for adapting to high larval density, however, there are some differences between the results reported here and the results on similar selected populations of *D. ananassae* and *D. n. nasuta* reported by Nagarajan et al. (2016). Our MCU populations did not show any evidence of an *r–K* trade-off (MCU survivorship was higher than controls at both low and high larval densities: figure 2), whereas the crowding adapted *D. ananassae* and *D. n. nasuta* populations showed a trend of lower survivorship than controls when assayed at low larval densities (Nagarajan *et al.* 2016). Moreover, a population

dynamics study on the *D. ananassae* populations suggested that *r* was actually lower in the crowding adapted populations, as compared to their controls (Dey *et al.* 2012). In the MCU populations, the development time reduction, relative to controls, was not magnified at high larval density (figure 3), whereas the development time difference between the crowding adapted and control *D. ananassae* and *D. n. nasuta* populations almost doubled when assayed at high larval density (Nagarajan *et al.* 2016). The crowding adapted *D. ananassae* and *D. n. nasuta* populations evolved greater pupation height than their respective controls (Nagarajan *et al.* 2016), but the MCU pupation height did not differ from controls. At this point, we do not have clear explanations for these differences in some of the correlated responses to selection across the three sets of populations.

In the crowding adapted *D. ananassae* populations, there was evidence for the evolution of reduced minimum critical time for feeding without a reduction in the minimum critical size for successfully completing subsequent development to adulthood (Archana 2010; Nagarajan *et al.* 2016). Although survival in *D. ananassae* selected populations was greater than controls after different durations of feeding, the dry weights of eclosing flies that had fed as larvae for different durations of time did not differ between selected and control populations. Similarly, larvae from the MCU populations exhibited a time advantage (head-start) over their controls of about 6 h in terms of survivorship after feeding for different durations (figure 4a), and a similar advantage of about 3 h for larval dry weight after feeding for different durations (figure 4b), suggesting no substantial change in minimum critical size in the MCU populations, relative to controls. Interestingly, til about mid third instar, MCU larvae were heavier than their control counterparts (figure 4b) but they are subsequently lighter than controls during the wandering larva stage (Sarangi 2013) and eventually eclose as lighter adults than their control counterparts (table 7). This pattern suggests that MCU populations have evolved a greater larval growth rate during second and early-to-mid third instar, followed by a reduction in the late third instar duration, leading them to stop feeding correspondingly earlier than controls, and life-stage-specific development time data support this view (Sarangi 2013).

Despite a few differences between correlated responses to selection shown by the MCU populations on one hand, and the *D. ananassae* and *D. n. nasuta* crowding adapted populations on the other, as discussed above, the overall similarity in the traits through which all three sets of crowding adapted populations in our laboratory evolved greater larval competitive ability clearly rule out two of the three hypotheses put forward by Nagarajan *et al.* (2016). The observation that the MCU populations, which share common ancestry with the CU populations of Joshi and Mueller (1996), nevertheless evolve a suite of traits similar to that seen in the crowding adapted *D. ananassae* and *D. n. nasuta* populations rule out differences between species, or between relatively recently wild-caught versus long-term laboratory adapted populations, in genetic architecture of traits relevant to fitness under larval crowding as being the cause for the different responses to selection seen in the earlier studies with *D. melanogaster* (Joshi and Mueller 1996) and the studies of Nagarajan *et al.* (2016). The remaining possibility, thus, becomes the most likely explanation: that differences in the details of the laboratory ecology of how larval crowding was implemented between the studies on the MCU populations and the crowding adapted populations of *D. ananassae* and *D. n. nasuta* on one hand, and the earlier studies on *D. melanogaster* on the other, led to the evolution of very different suites of traits conferring increased competitive ability in the two sets of studies.

As noted earlier, the main difference between the maintenance regime used in the earlier *D. melanogaster* studies and that used in this study, and the studies on *D. ananassae* and *D. n. nasuta*, was in the number of eggs per unit volume of food and the total amount of food used for larval rearing in the crowding-adapted populations (described in detail by Nagarajan *et al.* 2016). Compared to the three sets of crowding adapted populations used in our laboratory, the CU cultures had both a larger number of eggs and a greater total volume of food. It is, therefore, likely that the time course of food depletion and nitrogenous waste build-up in the MCU, *D. ananassae* and *D. n. nasuta* crowded cultures is different from that in the earlier studied *D. melanogaster* populations. Specifically, in vials with high larval density but only 1.5–2 mL of food (as in the MCU, *D. ananassae* and *D. n. nasuta* crowded cultures), the food gets used up very fast and probably also accumulates toxic levels of nitrogenous waste quite quickly as the total amount of food available for waste to diffuse into is small even early on in the culture. *Drosophila* larvae typically feed within a 1 cm depth from the surface of the food, and in vials with 1.5 mL of food, the height of the food column is less than 1 cm. If the food levels are closer to 5–6 mL (as in the CU populations), there is greater amount of food for waste to diffuse into and thus the effective waste concentration experienced by feeding larvae in the 1 cm depth feeding band is likely to be lower than that experienced by larvae in vials with only 1.5 mL of food, especially in the early stages of the crowded culture. At the same time, since feeding is restricted to a 1 cm deep band, effective competition for access to food, especially in early stages of a crowded culture, will be higher in vials with a greater absolute number of larvae (as in the CU populations), because the volume of food in the 1 cm deep feeding band is constant, but the number of larvae feeding in that zone is higher in the CU cultures compared to the MCU, *D. ananassae* and *D. n. nasuta* crowded cultures. As it has been shown theoretically that optimal feeding rates are likely to decline as the concentration of nitrogenous waste in the food increases (Mueller *et al.* 2005; Mueller and Barter 2015), it is likely that the optimal feeding rates in the 1.5 mL crowded cultures are lower than if there is 5–6 mL food per vial. Additionally, since food runs out very fast over time in crowded vials with only 1.5 mL of food, it might not permit better survival of

individuals with a high waste tolerance, unlike the case in crowded vials with 5–6 mL of food.

If the speculation above is correct, then the nature of selection acting on traits affecting competitive ability in *Drosophila* cultures is likely to depend critically both on egg density and total amount of food, specifically the height of the food column. This also suggests that although measures like egg density are often of heuristic value when thinking about adaptation to crowding, ultimately the ecological details of different types of crowded cultures can affect the form of the fitness functions acting on different traits related to competitive ability, thereby mediating the evolution of greater competitive ability via different sets of trait changes. In principle, it should be possible to directly test these predictions both by doing selection experiments at different combinations of egg density and total food volume, as well as by directly examining the effects of different combinations of egg density and total food volume on the phenotypic distributions of traits relevant to fitness under larval crowding in fruitflies.

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