Adaptation to larval crowding in *Drosophila ananassae* and *Drosophila nasuta nasuta*: increased larval competitive ability without increased larval feeding rate

Archana Nagarajan[1*], Sharmila Bharathi Natarajan[2*], Mohan Jayaram[3], Ananda Thammanna[4], Sudarshan Chari[5], Joy Bose[6] and Amitabh Joshi[†]

Evolutionary Biology Laboratory, Evolutionary & Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur P.O., Bangalore 560064, India.

*: equal contribution; †: Corresponding author

1. Present address: Centre for Organelle Research (CORE), Faculty of Science and Technology, University of Stavanger, N-4036 Stavanger, Norway.
2. Present address: Department of Pharmocology, School of Medicine, University of Pennsylvania, Philadelphia 19104, USA.
3. Present address: Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560012, India.
4. Present address: Jubilant Biosys Ltd., 96 Yeshwantpur Industrial Suburb IInd Stage, Tumkur Road, Bangalore 560 022, India.
5. Present address: Department of Zoology, Michigan State University, East Lansing, MI 48824, USA.
6. Present address: Department of Behavioural Biology, University of Osnabrueck, Osnabrueck D-49076, Germany.

Email addresses :

archana0681@gmail.com

sharmilanatarajan79@gmail.com

jmohan80@gmail.com

ananda_thammanna@jubilantbiosys.com

charisud@msu.edu

joy.bose@biologie.uni-osnabrueck.de

ajoshi@jncasr.ac.in

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ABSTRACT

The canonical view of adaptation to larval crowding in fruitflies, built on results from 25 years of experimental evolution studies on *D. melanogaster*, is that enhanced competitive ability evolves primarily through increased larval feeding and foraging rate, at the cost of efficiency of food conversion to biomass, and increased larval tolerance to nitrogenous wastes. We show that populations of *D. ananassae* and *D. n. nasuta* subjected to extreme larval crowding evolve greater competitive ability and pre-adult survivorship at high density 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 is a very different suite of traits than that seen to evolve under similar selection in *D. melanogaster* and seems to be closer to the expectations from the canonical theory of *K*-selection. We discuss possible reasons for these differences in results across the three species. Overall, the results reinforce the view that our understanding of the evolution of competitive ability in fruitflies needs to be more nuanced than before, with an appreciation that there may be multiple evolutionary routes through which higher competitive ability can be attained.

INTRODUCTION

Adaptation to crowding leading to greater competitive ability, first conceptualized as *r*- and *K*-selection theory (MacArthur and Wilson 1967), is an important phenomenon in ecology and evolution that has been extensively studied in *Drosophila melanogaster* over the past three decades, using experimental evolution approaches (reviewed in Joshi 1997; Mueller 1997; Prasad and Joshi 2003). With lifespan, fecundity, starvation resistance and dessication resistance, some of the other traits extensively examined via experimental evolution in *D. melanogaster*, there have been conflicting reports of differing patterns of correlated responses to selection between laboratories (Ackermann et al. 2001; Prasad & Joshi 2003; Rose et al. 1996), leading, in part, to a growing appreciation that evolution is often "local" (Rose et al. 2005). In the case of adaptation to crowding, however, the pattern of correlated responses to selection has been largely consistent over two separate selection experiments, involving *D. melanogaster* populations originating from different geographical sources (*r*- and *K*-populations: Mueller and Ayala 1981; UU and CU populations: Joshi and Mueller 1996), and the suite of traits through which selected populations evolved greater competitive ability was very different from the canonical version of *r*- and *K*-selection theory (Mueller 2009).

Results from the two selection studies on adaptation to crowding in *D. melanogaster* have been reviewed extensively (Joshi 1997; Mueller 1997; Joshi et al. 2001; Prasad and Joshi 2003; Dey et al. 2012) and, consequently, we will just summarize the major correlated responses of pre-adult traits to selection observed. Relative to the low density *r*-populations, the crowding adapted *K*-populations exhibited higher (i) pre-adult survivorship, when assayed at high larval density (Bierbaum et al. 1989), (ii) pre-adult

competitive ability (Mueller 1988), (iii) larval feeding rate (Joshi and Mueller 1988), (iv) larval foraging path length (Sokolowski et al. 1997), (v) pupation height (Mueller and Sweet 1986; Joshi and Mueller 1993), and (vi) minimum food requirement for pupation (Mueller 1990). Pre-adult survivorship of the *r*- and *K*-populations did not differ significantly when assayed at low larval density (Bierbaum et al. 1989). Pre-adult development time in the *K*-populations was lower than that of the *r*-populations at low (30 larvae per vial) and moderately high (160 larvae per vial) density, but greater at very high (320 larvae per vial) density (Bierbaum et al. 1989). The evolution of higher larval feeding rate and pupation height in crowding adapted populations was subsequently verified with a separate set of populations (*rK* and *r*×*rK* populations), by Guo et al (1991).

Relative to the control UU populations, the larval crowding adapted CU populations exhibited higher (i) pre-adult survivorship, when assayed at high larval density (Mueller et al. 1993; Shiotsugu et al. 1997), (ii) larval feeding rate (Joshi and Mueller 1996), (iii) larval foraging path length (Sokolowski et al. 1997), (iv) minimum food requirement for pupation (Joshi and Mueller 1996), and (v) tolerance to nitrogenous wastes like urea (Shiotsugu et al. 1997; Borash et al. 1998) and ammonia (Borash et al. 1998). Pre-adult survivorship of the CU and UU populations did not differ significantly when assayed at low larval density (Mueller et al. 1993; Shiotsugu et al. 1997). CU pre-adult development time was similar to the UU populations when assayed at low larval density, but lower than the UU populations when assayed at high larval density (Borash and Ho 2001). Pupation height of the CU populations was greater than the UU controls during early generations of CU selection (Mueller et al. 1993), but did not differ significantly from the controls after about 60 generations of selection (Joshi and Mueller

1996); possible reasons for this are extensively discussed by Joshi et al. (2003). Urea and ammonia tolerance were not assayed on the *r*- and *K*-populations, whereas competitive ability was not assayed on the CU and UU populations.

The *K*-populations differed from the *r*-populations in experiencing higher larval as well as adult density and, moreover, were kept on an overlapping generation regime whereas the *r*-populations were kept on discrete generations (Mueller and Ayala 1981). The CU populations, on the other hand, differed from the UU controls only in larval density (Joshi and Mueller 1996). Given the congruence in the pre-adult traits that evolved in the CU and *K*-populations, it is likely that these traits represented a response primarily to levels of larval crowding. Thus, the overall picture that emerged from these two studies was that long-term exposure to larval crowding in *Drosophila* selected for increased larval feeding and foraging activity, at the cost of efficiency at converting food to biomass, a greater tendency to pupate away from the food, and a higher tolerance to toxic levels of metabolic waste. It makes sense that these traits would contribute to greater pre-adult survivorship in a crowded larval culture characterized by diminishing food levels, greater chance of pupal drowning on the increasingly mushy food surface, and a rapid buildup of levels of nitrogenous waste. This has been the canonical view of adaptations to crowding in *Drosophila* for the past fifteen years or so (Mueller 1997; Joshi et al. 2001; Prasad and Joshi 2003; Mueller 2009; Mueller and Cabral 2012).

The notion that faster feeding is a strong correlate of pre-adult competitive ability in *Drosophila* has a lot of support. Populations selected for faster feeding rate are also better competitors (Burnet et al. 1977)), and populations selected for rapid pre-adult development evolve both reduced feeding rate and reduced competitive ability (Prasad et

al. 2001; Shakarad et al. 2005; Rajamani et al. 2006). Similarly, populations selected for increased parasitoid resistance evolve both reduced feeding rate and reduced competitive ability (Fellowes et al. 1998, 1999). The notion that there is a cost to faster feeding (Joshi and Mueller 1996) is supported by the rapid return of feeding rates to control levels when CU populations were maintained at moderate densities (Joshi et al. 2003). There is also evidence for a trade-off between urea/ammonia tolerance and larval feeding rate. Populations selected for greater urea and ammonia tolerance, respectively, also show reduced larval feeding rate (Borash et al. 2000) and larval foraging path length (Mueller et al. 2005), and populations selected for greater urea tolerance do not survive any better than controls at high larval density (Shiotsugu et al. 1997). In general, larval feeding rate and forgaing path length appear to be positively correlated (Joshi and Mueller 1988, 1996; Sokolowski et al. 1997; Borash et al. 2000; Prasad et al. 2001; Mueller et al. 2005). Thus, the evolution of competitive ability in *Drosophila* seems to be the outcome of a balance between mutually antagonistic traits like increased larval feeding and forgaing behaviour (and perhaps pupation height), greater tolerance to nitrogenous wastes, and a reduced efficiency of conversion of food to biomass. Indeed, the CU populations exhibit a temporal polymorphism for two of these traits: offspring of early eclosing flies in a crowded culture show higher feeding rates, whereas offspring of late eclosing flies show greater urea/ammonia tolerance than controls (Borash et al. 1998).

Given that the above view of adaptation to larval crowding in *Drosophila* is built around studies on a single species (*D. melanogaster*), we wanted to investigate whether other species of *Drosophila* would also respond to larval crowding by evolving essentially the same set of traits. If the genetic architecture of traits relevant to fitness under larval

crowding is reasonably conserved across congeners, then we should see a similar pattern of correlated responses to selection for adaptation to larval crowding in other *Drosophila* species. We report results from two selection experiments, on *D. ananassae* and *D. nasuta nasuta*, involving selection for adaptation to larval crowding. *D. ananassae* is ecologically and phylogenetically closer to *D. melanogaster* (*Sophophora* Subgenus, Melanogaster Group, Melanogaster Subgroup), being a cosmopolitan human commensal and belonging to the Melanogaster Group, Ananassae Subgroup and Ananassae Species Complex, whereas *D. n. nasuta* belongs to the *Drosophila* Subgenus, Immigrans Group, Nasuta Subgroup and Frontal Sheen Complex, and is found primarily in orchards and open land. Our results showed that selected populations of both *D. ananassae* and *D. n. nasuta* exhibited similar patterns of correlated responses to selection for adaptation to larval crowding, but that these populations evolved greater competitive ability through traits very different from those seen earlier in *D. melanogaster* populations subjected to similar selection.

MATERIALS & METHODS

Experimental populations

This study used eight laboratory populations each of *D. ananassae* and *D. n. nasuta*. Four control *D. ananassae* populations (AB_{1-4}: **A**nanassae **B**aseline) were derived from a single population, initiated in May-June 2001 with about 300 wild-caught females from Bangalore, India, and maintained as a single population on a 21-day discrete generation cycle for 34 generations (Sharmila Bharathi *et al*. 2003). The four AB populations were maintained on a 21-day discrete generation cycle at 25º ± 1 C, ~90% relative humidity,

131 constant light and on cornmeal medium. Larval density was regulated at 60-80 larvae per
132 vial (9 cm × 2.4 cm) with 6 mL food. Forty vials were set up per replicate population.
133 Twelve days after egg-collection, eclosed adults (1500-1800) from all 40 vials were
134 collected into Plexiglas cages ($25 \times 20 \times 15$ cm^3) containing a petridish of food that was
135 changed every alternate day, and a moistened ball of cotton. On day 18 after egg-
136 collection, the flies were given food along with a generous smear of live yeast-acetic acid
137 paste. On day 21 from the previous egg-collection, fresh food plates were put into the
138 cages and eggs collected from them after 18 h to initiate the next generation.

139 From the AB populations, four populations (ACU_{1-4}: **A**nanassae **C**rowded as
140 larvae and **U**ncrowded as adults) were derived, one each from each of AB_{1-4}, two
141 generations after the AB populations were established. The ACU populations were
142 selected for adaptation to larval crowding by subjecting them to a density of 550-600 eggs
143 per vial with 1.5 mL of food. In initial generations the density was lower; the final
144 densities were attained by generation 15 of ACU selection. The ACU populations were
145 otherwise maintained the same as the AB controls, except that only 20 vials of eggs were
146 collected each generation (to keep the number of breeding adults similar to controls: 1500-
147 1800), and the collection of eclosed adults into the cages continued until day 18 from
148 previous egg-collection.

149 Four control *D. n. nasuta* populations (NB_{1-4}: **N**asuta **B**aseline) were derived, after
150 24 generations as a single population on a 21 day discrete generation cycle, from a
151 laboratory population established using about 70 females collected from orchards and
152 domestic garbage dumps in different parts of Bangalore, India, during October-November
153 2001 (Sharmila Bharathi *et al*. 2003). The four NB populations were maintained on a 21-

154 day discrete generation cycle at 25º ± 1 C, ~90% relative humidity, constant light and on
155 cornmeal medium. The larval density was regulated at 60-80 larvae per vial (9 cm × 2.4
156 cm) with 6 mL food. Fifty two vials were set up per replicate population to keep the
157 number of breeding adults at about 1500-1800. Twelve days after egg-collection, eclosed
158 adults from all 40 vials per replicate population were collected into Plexiglas cages (25 ×
159 20 × 15 cm^3) containing a petridish of food that was changed every alternate day, and a
160 moistened ball of cotton. On day 18 after egg-collection, the flies were given food along
161 with a generous smear of live yeast-acetic acid paste. On day 21 from the previous egg-
162 collection, fresh food plates were put into the cages and eggs collected from them after 18
163 h to initiate the next generation.

164 From the NB populations, four populations (NCU_{1-4}: **N**asuta **C**rowded as larvae
165 and **U**ncrowded as adults) were derived, one each from each of NB_{1-4}, two generations
166 after the NB populations were established. The NCU populations were selected for
167 adaptation to larval crowding by subjecting them to a density of 350-400 eggs per vial
168 with 2 mL of food. In initial generations the density was lower; the final densities were
169 attained by generation 15 of NCU selection. This difference from ACU in larval density is
170 to compensate for the larger size at each stage of the *D. nasuta* larvae. The NCU
171 populations were otherwise maintained in the same way as the NB controls, with similar
172 adult population size, except that the collection of eclosed adults into the cages continued
173 until day 18 from previous egg-collection, and only 40 vials with eggs were set up.

174 Since each ACU or NCU population was derived from one control population,
175 selected and control populations bearing identical numerical subscripts are more related to
176 each other than to other populations with which they share the selection regime.

177 Therefore, control and selected populations with identical subscripts were treated as
178 blocks, representing ancestry, in the statistical analyses.

179 **Collection of flies for assays**

180 All control and selected populations were maintained under common (control-type)
181 rearing conditions for one complete generation prior to assays, to eliminate non-genetic
182 parental effects. The progeny of these flies, hereafter 'standardized flies', were then used
183 for the various assays. To obtain progeny for assays, standardized flies in cages were
184 provided yeast-acetic acid paste on food for three days before egg collection. A fresh
185 petridish with food was then placed in the cages and the flies were allowed to lay eggs for
186 ~14 h, after which eggs were removed from the food with a moistened paintbrush and
187 placed into vials for setting up the various assays. All assays were conducted at 25 ± 1^{o}C,
188 under constant light.

189 **Pre-adult survivorship**

190 After 42 generations of ACU selection, eggs laid by standardized flies were placed into
191 vials at a density of either 70 or 600 per vial containing 1.5 mL of food. Eight such vials
192 were set up for each replicate AB and ACU population at each density in single-species
193 culture. Eight such vials at each density were also set up in two-species cultures, in
194 competition with a common competitor, a white eyed mutant population of *D.*
195 *melanogaster*, maintained for about 90 generations in the laboratory on a three week
196 discrete generation cycle. The white eyed population was derived from spontaneously
197 occurring mutant individuals in the JB populations (Sheeba et al. 1998) in our laboratory.
198 For the two-species cultures, eggs laid by standardized ACU, AB or white eyed flies were

collected and placed into vials at a density of either 70 (35 ACU or AB eggs and 35 eggs from the white eyed mutant population) or 600 (300 ACU or AB eggs and 300 eggs from the white eyed mutant population) per vial containing 1.5 mL of food. Eight such vials were set up for each replicate AB and ACU population at each density. The number of flies eclosing in each vial was recorded and used to calculate pre-adult survivorship.

At generation 76 of NCU selection, an assay similar to that described above was set up using the NB and NCU populations and the white eyed *D. melanogaster*. The only difference from the *D. ananassae* assay was that the low and high density treatments comprised of 70 or 350 eggs in vials with 2 mL of food.

Duration of pre-adult life-stages and pre-adult development time

After 53 generations of ACU selection, pre-adult development time was assayed at a high density of 600 eggs per vial with 1.5 mL of food. Eggs from standardized AB and ACU 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 every 6 h and the number of eclosing flies recorded.

After 71 generations of ACU selection, pre-adult development time was assayed at a low density of 30 eggs per vial with 6 mL of food (An earlier study at generation 41 of ACU selection had shown that pre-adult development time did not differ significantly between low densities of 30 or 70 eggs per vial with 6 mL of food: data not shown). Eggs from standardized AB and ACU flies were dispensed into each vial using a moistened paintbrush. Ten vials were set up per replicate population. After the pupae darkened, the vials were monitored for eclosion at 2 h intervals, and the number of eclosing flies

recorded. As part of the same assay, egg hatching time and the duration of each larval instar and the pupal stage were also determined. For assaying egg hatching time, 30 eggs from the standardized flies were arranged on a small agar cube in a food vial in six rows of five eggs each. Ten such vials were set up per population. Fifteen hours after egg laying, the vials were checked for any hatched eggs once every hour, till no eggs hatched for three consecutive hours. For assaying instar and pupal duration, eggs of approximately identical age were harvested over a three hour period from the standardized flies and dispensed into vials with 6 mL of food at a density of 30 eggs per vial. Sixty such vials were set up per population. Forty three hours after the midpoint of the three hour egg laying period, four vials per population were removed from the incubators and immersed in hot water. The dead larvae were removed and kept in 70% ethanol for subsequent examination. Every two hours, this process was repeated. The larval instars were differentiated based on the number of 'teeth' in the larval mouth hooks. From these data, the number of larvae of each instar present in each two-hourly sample was determined, and the median time of each molt was obtained by interpolation. For pupal duration and pre-adult development time, ten vials per population were set up with 30 eggs in 6 mL of food. After the first pupa was seen, the vials were screened every two hours and any new pupae that had formed were marked on the vial with different coloured marker pens. Thereafter, the vials were monitored for eclosion and the number of eclosing males and females in each vial was determined every two hours. These observations yielded data on egg to pupa and egg to adult development time, from which the pupal duration could be calculated.

After 84 generations of NCU selection, pre-adult development time was assayed at a high density of 350 eggs per vial with 2 mL of food. Eggs from standardized NB and NCU flies were dispensed into each vial using a moistened paintbrush. Eight such vials were set up per population. After the pupae darkened, the vials were checked every 6 h and the number of eclosing flies recorded.

After 62 generations of NCU selection, egg hatching time, larval instar duration, pupal duration and pre-adult development time of the NB and NCU populations were assayed exactly as described above for the ACU and AB populations.

Larval feeding rate

After 71 generations of ACU selection, the feeding rates of AB and ACU larvae were measured at physiologically equalized ages, based on the difference in AB and ACU development time. This was done by collecting eggs from the standardized ACU flies 5 h later than the AB flies. Thus, at the time of assay, ACU larvae were 58 h old, whereas AB larvae were 63 h old and thus approximately in the same relative stage of their larval development. Following Joshi and Mueller (1996), about a hundred eggs laid over a four hour period were collected from standardized flies and placed into two petridishes with non-nutritive agar each for AB and ACU populations. Twenty-four hours later, twenty-five newly hatched larvae were transferred from these agar petridishes to a petridish containing a thin layer of non-nutritive agar overlaid with 1.5 mL of 37.5% yeast suspension. Four such petridishes were set up per population. The larvae were then allowed to feed for 58 (ACU) or 63 (AB) h, by which time they were in the early third instar. At this point, 20 larvae from each population were assayed for feeding rate,

following the procedure of Joshi and Mueller (1996), by placing them individually in a small petridish (5 cm diameter) containing a thin layer of agar overlaid with a thin layer of 10% yeast suspension. After allowing for a 15 sec acclimation period, feeding rate was measured under a stereozoom 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. The same procedure was followed for assaying larval feeding rate of 20 larvae from each NCU and NB population, after 77 generations of NCU selection.

Larval foraging path length

Twenty early third instar larvae for each population were assayed for larval foraging path length. The collection of larvae for the assay was exactly as described above for larval feeding rate assays. For assaying foraging path length, individual larvae were placed in a small petridish (5 cm diameter) containing a thin layer of agar overlaid with a thin layer of 10% yeast suspension. After allowing for a 15 sec acclimation period, the larvae were allowed to move around on the petridish for 1 min. The path traversed by the larvae on the yeast surface was traced onto a transparency sheet and later measured with a thread and ruler. Selected and control populations, matched by subscripted indices, were assayed together at generations 52 and 81 of ACU and NCU selection, respectively.

Pupation height

Eggs from standardized flies were collected and placed into vials with 6 mL of food at a density of 50 (ACU/AB) or 70 (NCU/NB) eggs per vial. Ten and eight such vials were set

287 up per population for *D. ananassae* and *D. n. nasuta*, respectively. Once all pupae had
288 formed, pupation height of each pupa was measured, following Joshi and Mueller (1993),
289 as the distance between the surface of the food medium to the point between the anterior
290 spiracles of the pupa. Any pupa on or touching the surface of the food was given a
291 pupation height of zero. Assays were conducted at generations 39 and 34 of ACU and
292 NCU selection, respectively.

293 **Larval urea and ammonia tolerance**

294 After 49 generations of ACU selection, eggs laid over a four hour period by standardized
295 flies were collected and exactly 30 eggs per 6 mL food were placed into vials at three
296 concentrations each of urea (0, 14 and 18 g/L) or ammonia (0, 15 and 30 g/L NH_4Cl) in
297 the food. These are values that allowed the detection of differences in urea/ammonia
298 tolerance between selected and control populations in previous studies on *D. melanogaster*
299 (Shiotsugu et al. 1997; Borash et al. 1998). Ten vials were set up per population at each
300 concentration of either urea or ammonia, and the number of eclosing flies in each vial was
301 recorded to calculate pre-adult survivorship. Both urea and ammonia were used as there is
302 some confusion about which compound actually increases in concentration in crowded
303 larval cultures, although it is likely that it is ammonia in the case of *D. melanogaster*
304 (Botella et al. 1985; Borash et al. 1998). The NCU and NB populations were assayed in an
305 identical manner after 76 generations of NCU selection, except that the egg-laying period
306 was 12 hours, and the concentrations used were 0, 9 and 11 g/L for urea and 0, 15 and 20
307 g/L for ammonia, as earlier studies had shown that *D. n. nasuta* larvae were more sensitive
308 to urea and ammonia than their *D. ananassae* counterparts, and *D. n. nasuta* females are
309 not as fecund as *D. ananassae* females (data not shown).

Minimum feeding time and dry weight after minimum feeding

These assays were conducted only on the ACU and AB populations. After 45 generations of ACU selection, critical minimum feeding time for pupation was assayed by setting up freshly hatched larvae, from eggs laid by standardized flies, onto petridishes with agar overlaid with 1.5 mL of 37.5% yeast suspension, as described above for the feeding rate and foraging path length assays. Twenty five larvae were placed into each petridish, and sixty such petridishes were set up per population. At 46, 49, 52 and 55 h after egg hatch, a total of 150 larvae per population per time point were removed from the yeast, gently washed in water to remove any yeast sticking to their bodies, and placed in ten vials containing 5 mL of non-nutritive agar, at a density of 15 larvae per vial. These vials were subsequently monitored for pupation and eclosion, and the pre-adult survivorship after feeding for different periods of time noted. Using the information from this assay, a second assay was carried out after 68 generations of ACU selection to measure the dry weights at eclosion of ACU and AB flies that had fed as larvae for different durations of time, roughly corresponding to pre-adult survivorship of 13, 25, 50 and 60%, respectively (feeding for 54, 56, 59 and 62 h for AB, and 50, 55, 58 and 60 h for ACU populations, respectively). Freshly hatched larvae were collected, shifted to yeast and then removed and placed into agar vials after feeding for different durations corresponding to pre-adult survivorship of 13, 25, 50 and 60% for ACU and AB populations, exactly as described above. Once eclosions began in the agar vials, flies were collected every 4 h and frozen for subsequent weighing. Frozen flies were sorted into batches of five males or five females each and dried at 70^{o}C for 36 h before weighing in batches.

Statistical analyses

Since assays on ACU/AB and NCU/NB populations were conducted at different times, data from the two species were analyzed separately. All traits were subjected to completely randomized block analyses of variance (ANOVA). All ANOVAs treated replicate population 1..4 (representing ancestry) as random blocks crossed with the fixed factor selection regime. Additional fixed factors, crossed with both selection regime and block, were included when relevant. These factors were larval density and type of culture (single- or two-species) for pre-adult survivorship, pre-adult life-stage for life-stage 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 of dry weight at eclosion after feeding for different time durations corresponding to four different pre-adult survivorship levels. Development time data from low and high density were analyzed separately, rather than incorporating larval density as a factor, because the low and high density assays were conducted at different times for each species. Data on pre-adult survivorship were arcsine-squareroot transformed before ANOVA. For all traits, ANOVAs were done on replicate population mean values and, therefore, only fixed factor effects and interactions could be tested for significance. All analyses were implemented using Statistica for Windows rel.5.0 B, (Stat Soft 1995). Multiple comparisons were done using Tukey's HSD test.

RESULTS

Pre-adult survivorship

Pre-adult survivorship at high larval density is the primary trait expected to be under direct selection in populations exposed to high larval crowding each generation. With regard to

355 survivorship at low versus high larval density, the broad pattern of results was very similar
356 in the two species (Figure 1). In both species, pre-adult survivorship was, on an average,
357 significantly higher at low rather than high larval density, and in selected compared to
358 control populations (Figure 1, Table 1). The trend was for survivorship to be higher, on
359 average, in single-species cultures than in two-species cultures, but the difference was
360 significant only in the case of *D. ananassae* (Table 1). Both species showed a significant
361 selection regime × larval density interaction (Table 1), with selected and control
362 populations' survivorship not differing significantly at low density, and with selected
363 populations showing significantly higher survivorship than controls at high density
364 (Figure 1). Both species also showed a significant culture type × larval density interaction
365 (Table 1). In *D. ananassae*, averaged over selection regimes, survivorship at low density
366 in both types of cultures was similar whereas survivorship at high density was
367 significantly greater in single-species than two-species cultures (Figure 1). In *D. n. nasuta*,
368 a somewhat opposite pattern was seen. Averaged over selection regime, survivorship at
369 low density in single-species cultures was higher than in two-species cultures (Figure 1),
370 but not significantly so. However, at high density, survivorship in two-species cultures
371 was higher than in single-species cultures but, again, not significantly so. In fact, the only
372 clear significant difference in the multiple comparisons was that between single-species
373 cultures at low and high densities (Figure 1.). In the case of *D. ananassae*, there was also a
374 significant culture type × selection regime interaction (Table 1), but the only clearly
375 significant differences in multiple comparisons were between survivorship in two-species
376 cultures at high density on the one hand, and in the other three culture type × selection
377 regime combinations on the other (Figure 1).

Pre-adult development time

In general, pre-adult development time in *D. n. nasuta* was higher than that in *D. ananassae*, as also noted earlier (Sharmila Bharathi et al. 2004). Pre-adult development time was, in general, greater in males than in females, and greater at high rather than low larval density, as is usually the case in *Drosophila* (Figures 2,3, Table 2).

Interestingly, in both species at both larval densities, there was a significant main effect of selection regime, with selected populations consistently showing substantially lower development time than control populations at both densities (Figures 2,3, Table 2). On an average, AB development time was higher than that of ACU populations by about 14 and 29 h at low and high larval density, respectively (Figures 2A, 3A), whereas that of NB populations was greater than that of NCU populations by about 17 and 29 h at low and high larval density, respectively (Figures 2B,3B). In three of the four assays, there was no significant selection regime × sex interaction (Table 2). In *D. n. nasuta* at high larval density, control males and females had similar development times whereas NCU males had significantly higher development time than NCU females, albeit by just about 1 h (Figure 3B).

Pre-adult life-stage duration

The overall pattern of pre-adult life-stage durations in both species was similar to what is seen in *Drosophila* spp. in general. Consistent with the pre-adult development time results, both species showed a main effect of selection regime (Table 3), with life-stage durations being greater in control rather than selected populations, on an average (Figure 4). In *D. ananassae*, a 20 h egg stage was followed by two larval instars of about 24 h each, a third

larval instar of about 46 h and a pupal stage lasting about 87 h (Figure 4A); there was no significant selection regime × life-stage duration interaction. In *D. n. nasuta*, all pre-adult life-stages differed significantly from one another in duration, with a 23 h egg stage, followed by two larval instars of about 27 and 29 h, respectively, a longer third larval instar of about 63 h and a pupal stage lasting about 87 h (Figure 4B). The longer larval instar durations in *D. n. nasuta* are consistent with their larger adult size compared to *D. ananassae* (Sharmila Bharathi et al. 2004). *D. n. nasuta* also showed a significant selection regime × life-stage duration interaction (Table 3), with a large (~12 h) reduction in third instar duration in the NCU populations (Figure 4B). We note that, given the time interval between subsequent observations (1 h for egg-hatch; 2 h for larval instars), our screening might be too coarse to pick up small but consistent differences on the order of 30 min – 1 h. However, the differences between selected and control populations in the durations of different pre-adult life-stages, though often not significant, did add up roughly to the overall difference seen in pre-adult development time between selected and control populations in both species.

Larval traits

Surprisingly, larval feeding rate did not differ significantly between selected and control populations in either species (Table 4). Mean (± s.e.) larval feeding rates in sclerite retractions per min were, in fact, extremely similar between selected and control populations of both species (AB: 128.33 ± 3.13; ACU: 131.55 ± 3.05; NB: 112.76 ± 5.29; NCU: 113.03 ± 4.34). In both species, selected populations showed significantly greater mean (± s.e.) pupation height in cm than control populations (AB: 1.02 ± 0.28; ACU: 1.60

± 0.20; NB: 0.44 ± 0.12; NCU: 1.54 ± 0.11) (Table 4). In case of larval foraging path length, the results varied between species, with *D. ananassae* showing a significant main effect of selection regime while *D. n. nasuta* did not (Table 4). AB populations had mean (± s.e.) foraging path length in cm of 5.52 ± 0.61, compared to 7.38 ± 0.60 in the ACU populations. NB and NCU populations had mean (± s.e.) foraging path length in cm of 4.24 ± 0.53 and 4.08 ± 0.43, respectively.

Larval urea and ammonia tolerance

There was no clear pattern to the results of assays on pre-adult survivorship in the presence of metabolic wastes like urea (Figure 5) and ammonia (Figure 6) in the food medium, except for strong evidence for the toxic effects of these compounds, reflected in significant main effects of concentration in all four ANOVAs (Table 5).

ACU populations had lower survivorship than AB controls across all levels of urea, including 0 g/L, and thus showed no evidence of greater or lesser urea tolerance than the AB controls (Figure 5A), with a significant main effect of selection regime but no significant selection regime × concentration interaction (Table 5). There was suggestive evidence for greater ammonia tolerance in the ACU compared to the AB populations (Figure 6A). At 0 g/L of ammonia in the food, ACU populations had significantly lower survivorship than AB controls, whereas at 30 g/L, the ACU survivorship was higher, though not significantly so, than the AB survivorship (Figure 6A). The selection regime × concentration interaction was also significant for this assay (Table 5).

In the case of *D. n. nasuta*, there was no significant effect of selection regime or of the selection regime × concentration interaction in the urea tolerance assay (Table 5).

However, there was a non-significant trend of NCU survivorship being lower than NB at 0 g/L and higher than NB at 11 g/L (Figure 5B). In case of ammonia, NCU populations had consistently lower survivorship than NB controls at all concentrations (Figure 6B) and there was no significant effect of selection regime or of the selection regime × concentration interaction, yielding no suggestion of increased ammonia tolerance in the NCU populations.

Minimum critical feeding time

In the minimum critical feeding time assay, only significant main effects of selection regime and feeding duration were seen (Table 6), with pre-adult survivorship increasing from feeding durations from 46-55 h, and with ACU survivorship being higher than that of the AB populations at every feeding duration (Figure 7A). At 55 h of feeding as larvae, ACU survivorship was over 50% while that of the AB populations was less than 30% (Figure 7A). Overall, ACU populations seemed to attain similar levels of survivorship approximately 6 h before the AB controls (Figure 7A), which is commensurate with the 5.48 h difference between their first and second instar durations combined. These results suggest that larvae in the ACU populations attain their minimum critical size for pupation approximately 5-6 h before their AB controls.

In the ANOVA on data from the assay of dry weight at eclosion of individuals that had fed for different durations of time as larvae, corresponding to four different levels of pre-adult survivorship, only the main effects of sex and survivorship were significant (Table 6). On an average, females were significantly heavier than males, and dry weight at eclosion tended to increase with survivorship (Figure 7B). At no survivorship level was

there a significant difference between the dry weights of AB and ACU flies (Figure 7B), suggesting that the minimum critical size for pupation in the selected and control populations is probably not different, even though the ACU populations attain it faster than the AB controls.

DISCUSSION

It is clear that both the ACU and NCU populations did evolve adaptations to larval crowding over the course of selection: in both species, pre-adult survivorship at high density and competitive ability was greater in selected than in control populations (Figure 1, Table 1). The overall pattern of correlated responses to selection in the two species was also similar, and, more importantly, different from that reported earlier in case of *D. melanogaster* in that it did not involve the evolution of higher feeding rates (Table 4).

In clear contrast to what was seen earlier in *D. melanoagster*, both ACU and NCU populations evolved faster pre-adult development time than their controls (Table 2), with the difference being apparent at both low (Figure 2) and high (Figure 3) assay density. The pre-adult development time difference between selected and control populations is entirely due to reduction in the duration of larval instars (Figure 4). In absolute terms, the reductions in pre-adult development time in the selected populations (~14 h in ACU, ~17 h in NCU) are quite large. For comparison, populations of *D. melanogaster* subjected to strong directional selection for reduced pre-adult development time showed a reduction of ~16 h in the larval duration, and ~10 h in pupal duration, after 50 generations of selection (Prasad et al. 2001). In the earlier studies on *D. melanogaster*, CU populations showed no difference from controls in development time at low density, whereas they were faster

488 developing than controls when assayed at high density (Borash and Ho 2001). While the
489 *K*-populations did show faster development than *r*-populations when assayed at low
490 density (Bierbaum et al. 1989), this cannot be unequivocally ascribed to density-dependent
491 selection. The *K*-populations were on an overlapping generation maintenance regime and,
492 relative to a discrete generation regime like that of the *r*-populations, this itself would
493 impose direct selection for faster development. If we put the results on life-stage duration
494 (figure 4) and development time (Figures 2,3) together with those from the minimum
495 feeding time assay (Figure 7), the ACU and NCU populations have evolved faster
496 development, especially in the first two larval instars, enabling them to reach the critical
497 minimum size for pupation about 6 h earlier than controls. This is particularly impressive
498 considering that *D. melanogaster* populations selected directly for rapid development
499 reached the critical size only about 2 h before controls after 50 generations of selection
500 (Prasad et al. 2001). At the same time, the weights of ACU and NCU flies are not different
501 from controls after feeding for different durations close to the minimum feeding time
502 (Figure 7, Table 6). Thus, in terms of time, ACU and NCU larvae are clearly more
503 efficient at converting food to biomass than controls during the first two (and an early part
504 of the third) larval instars, becoming equally heavy adults as controls after feeding for
505 about 6 h less. Of course, whether they are more efficient also in terms of food consumed
506 cannot be determined from these experiments. However, these results do suggest that
507 unlike the *K*- and CU populations, (Mueller 1990; Joshi and Mueller 1996), the ACU and
508 NCU populations have evolved to become more rather than less efficient than controls.
509 The faster development of the ACU and NCU populations, relative to controls, is also
510 interesting in the context of observations that faster development correlates with greater

competitive ability across species of *Drosophila* (Krijger et al. 2001), even though direct selection for rapid development leads to the evolution of reduced competitive ability (Shakarad et al. 2005) through reduced larval feeding rate, foraging path length and pupation height (Prasad et al. 2001) as well as reduced larval urea tolerance (Joshi et al. 2001).

Unlike in *D. melanogaster*, larval feeding rates did not evolve to become greater in the ACU and NCU populations (Table 4), although these populations clearly evolved greater competitive ability than controls (Figure 1). In fact, we measured feeding rates at various points during ACU and NCU selection, and also at various larval stages, and there was never any difference between selected and control populations in either species (data not shown). Interestingly, larval foraging path length was greater in ACU populations than in AB populations, whereas NCU and NB populations had very similar foraging path lengths (Table 4). Thus, at least in *D. ananassae*, the consistent positive relationship between larval feeding rate and foraging path length seen in *D. melanogaster* (Joshi and Mueller 1988, 1996; Sokolowski et al. 1997; Borash et al. 2000; Prasad et al. 2001; Mueller et al. 2005) seems to be uncoupled. Pupation height evolved to become greater in both ACU and NCU populations, relative to controls, mirroring results from the *K*-populations (Mueller and Sweet 1986), but not the CU populations (Joshi and Mueller 1996; but see also Mueller et al. 1993 and Joshi et al. 2003). One possible reason for the evolution of increased pupation height in the *K*-populations but not the CU populations has been speculated to be the greater hardness and dryness of the cornmeal based food (*r*- and *K*-populations) compared to banana based food (UU and CU populations). It was suggested that banana food being much softer and more fluid compared to cornmeal food,

534 perhaps the UU populations were also under selection for increased pupation height due to
535 greater risk of pupal drowning on the food surface even at their relatively low rearing
536 density (Joshi et al. 2003). In this context, we note that our ACU/AB and NCU/NB
537 populations are maintained on a relatively hard and dry cornmeal food and this might be
538 the reason for the evolution of greater pupation height in selected populations, relative to
539 controls.

540 Compared to other traits, the results on urea and ammonia tolerance in the selected
541 and control populations were not very clear. While pre-adult survivorship clearly
542 decreased with increasing amounts of urea or ammonia in the food (Figures 5,6, Table 5),
543 there was no clear evidence for greater urea or ammonia tolerance in the selected
544 populations, except for greater ammonia tolerance in the ACU populations, relative to the
545 AB controls (Figure 6A). There was a slight suggestion of a trend towards increased urea
546 tolerance in the NCU populations, relative to NB controls in that NCU survivorship at 0
547 g/L was below that of NB populations and became slightly higher than NB populations at
548 11 g/L (Figure 5B). However, there was no significant selection regime × urea
549 concentration interaction (Table 5), indicating that this is at best a suggestive result. It
550 might be that crowded *D. ananassae* and *D. n. nasuta* cultures have experienced
551 differential levels of urea versus ammonia build-up to which they have adapted.
552 Moreover, we have seen, in other studies with *D. melanogaster*, that the detection of
553 between-population differences in urea/ammonia tolerance is often affected by an
554 interaction between the specific concentrations used and the larval density (Avani Mital,
555 Gitanjali P. Vaidya and Amitabh Joshi, *unpubl. data*). It is, therefore, possible that we
556 were unable to detect significant differences between selected and control populations due

557 to the specific concentrations and larval densities used in our assays of urea and ammonia
558 tolerance. It is also known that larval exposure to urea markedly reduces subsequent
559 fecundity in *D. melanogaster*, and that populations selected for increased larval urea
560 tolerance undergo smaller fecundity declines than controls when reared as larvae on food
561 with urea (Shiotsugu et al. 1997). Thus, it is also possible that the ACU and NCU
562 populations might have evolved greater tolerance to the detrimental effects of urea on
563 fecundity; our experiments did not explore this possibility.

564 Overall, the crowded *D. ananassae* and *D. n. nasuta* populations seem to evolve
565 greater competitive ability and pre-adult survivorship at high density primarily through a
566 combination of reduced duration of the larval stage, faster attainment of minimum critical
567 size for pupation, greater efficiency of food conversion to biomass, increased pupation
568 height and, perhaps, greater urea/ammonia tolerance. This is in contrast to *D.*
569 *melanogaster*, in which crowding adapted populations evolve greater competitive ability
570 and pre-adult survivorship at high density primarily through a combination of increased
571 larval feeding rate and foraging path length, at the cost of reduced efficiency of food
572 conversion to biomass, and greater urea/ammonia tolerance (Mueller 1997; Prasad and
573 Joshi 2003; Mueller 2009). Thus, the ACU and NCU populations appear to have
574 responded to crowding in a manner closer to the canonical notion of *K*-selection, in
575 contrast to the primarily α-selection responses shown by the *K*- and CU populations of *D.*
576 *melanogaster* (see also Dey et al. 2012). Thus, the results underscore the fact that there
577 are, in principle, multiple routes to the evolution of greater competitive ability (Joshi et al.
578 2001; Dey et al. 2012), something that has also been experimentally demonstrated in the
579 context of inter-specific competition in *Drosophila* (Joshi and Thompson 1995).

580 At this point, we can only speculate about the reason(s) for why the evolution of
581 increased competitive ability in the ACU and NCU populations occurred in a manner so
582 different from that seen earlier in *D. melanogaster*. We believe there are three possible
583 reasons for the observed discrepancy in correlated responses to selection for adaptation to
584 larval crowding across the three species, and the three proposed explanations are not
585 mutually exclusive. First, it is possible that the pattern of genetic variances and
586 covariances among traits relevant to survival in a crowded culture is different from that in
587 *D. melanogaster* in the two species studied here (*D. ananassae* and *D. n. nasuta*). For
588 example, maybe the AB and NB populations do not harbor additive genetic variance for
589 larval feeding rate. Such differences of genetic architecture could simply reflect between-
590 species differences, being the result of historical selection pressures and contingencies.
591 However, if that were the case, we would have expected the results from *D. ananassae* to
592 be closer to *D. melanogaster*, as compared to *D. n. nasuta*, given the phylogenetic and
593 ecological relationships of these species (see Introduction, last paragraph). A second, more
594 likely, possibility is that these differences of genetic architecture between *D. ananassae*
595 and *D. n. nasuta* on the one hand, and *D. melanogaster* on the other, are because the ACU
596 and NCU populations represent selection on relatively recently wild-caught populations
597 (see Materials and methods) whereas the *K*- and CU populations involved selection on
598 populations that had already been in the laboratory for a large number of generations
599 (Mueller and Ayala 1981; Joshi and Mueller 1996). There might be significant differences
600 in the genetic architecture of fitness-related traits between wild and laboratory
601 populations. Finally, it is possible that the discrepancies between the results of this study
602 and those from earlier work on *D. melanogaster* are due to differences in the specific

603 details of the respective maintenance regimes of selected populations in the various
604 studies. Such small differences in selection regimes have been implicated in the different
605 trajectories of pupation height in the *K*- versus the CU populations (Joshi et al. 2003). The
606 *K*-populations were maintained on a serial transfer system in half-pint milk bottles with
607 about 350 mL food per bottle (Mueller and Ayala 1981), while the CU populations were
608 maintained in 6 dram vials (2.2 cm dia) at a density of about 1000-1500 eggs per vial in
609 about 5 mL of food. Unlike in our ACU and NCU populations, neither food amount nor
610 number of eggs per vial were exactly controlled in CU maintenance. In the CU
611 populations, eggs were laid by adult females onto thin films of food on four watch glasses
612 per cage. These films of food with eggs on them were then sliced up and divided roughly
613 equally among 40 vials that each contained about 3 mL of food (A. Joshi, *pers. obs*.).
614 Thus, our ACU and NCU populations had a smaller absolute amount of food per rearing
615 container (bottle/vial) than the *K*- and CU populations, and also differed from the latter in
616 specific densities of eggs per mL of food. It is, therefore, possible that the time course of
617 food depletion and nitrogenous waste build-up in the ACU and NCU cultures is somewhat
618 different from that in the *K*- and CU populations. It has been shown theoretically that
619 optimal feeding rates are likely to decline as the concentration of nitrogenous waste in the
620 food increases (Mueller et al. 1995). Thus, at least in principle, it is possible that the
621 optimal feeding rates in the ACU and NCU populations are actually less than they were
622 for the *K*- and CU populations, and that is why increased feeding rates did not evolve in
623 our experiments. Subsequent studies will attempt to discriminate between these various
624 explanations. However, for the time being, we believe that these results underscore the
625 need for a more nuanced understanding of adaptations to larval crowding in *Drosophila*,

626 with a greater appreciation for the fact that increased competitive ability can be attained
627 through the evolution of fairly different suites of traits. We also need to be cognizant of
628 the fact that seemingly small differences of maintenance in otherwise similar selection
629 regimes might mediate the evolution of very different trajectories in phenotypic space.

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FIGURES

Figure 1. Mean pre-adult survivorship at low and high larval density in the selected and control populations of the two species, when cultured alone or in the presence of equal numbers of eggs of white-eyed *D. melanogaster* competitors. Error bars are standard errors around the mean of the four replicate population mean values. Significant differences between pairs of means are indicated by labeling them with different letters. For both species, as the three-way interaction between type of culture, selection regime and larval density was not significant, only the difference between the mean pre-adult survivorships of selected and control populations at high and low larval densities, averaged over type of culture (single- or two-species), is highlighted.

Figure 2. Mean male and female pre-adult development time at low larval density in the selected and control populations of the two species. Error bars are standard errors around the mean of the four replicate population mean values. For both species, significant differences between pairs of means are indicated by labeling them with different letters. For *D. ananassae*, as the interaction between sex and selection regime was not significant, only the difference between the mean pre-adult development time of males and females, averaged over selection regimes is highlighted.

Figure 3. Mean male and female pre-adult development time at high larval density in the selected and control populations of the two species. Error bars are standard errors around the mean of the four replicate population mean values. Significant differences between

pairs of means are indicated by labeling them with different letters. For both species, as the interaction between sex and selection regime was not significant, only the difference between the mean pre-adult development time of males and females, averaged over selection regimes is highlighted.

Figure 4. Mean duration in hours of different pre-adult life-stages at low larval density in the selected and control populations of the two species. Error bars are standard errors around the mean of the four replicate population mean values. For each species, significant differences between pairs of means are indicated by labeling them with different letters. For *D. ananassae*, the interaction between selection regime and life-stage was not significant. Consequently, only the differences in mean duration between life-stages, averaged over block and selection regime, have been highlighted.

Figure 5. Mean pre-adult survivorship at low density with three different levels of urea in the food medium in the selected and control populations of the two species. Error bars are standard errors around the mean of the four replicate population mean values. For each species, significant differences between pairs of means are indicated by labeling them with different letters. For both species, the interaction between selection regime and urea level was not significant. Consequently, only the differences between mean survivorship at different urea levels, averaged over block and selection regime, have been highlighted.

Figure 6. Mean pre-adult survivorship at low density with three different levels of ammonia in the food medium in the selected and control populations of the two species. Error bars are standard errors around the mean of the four replicate population mean values. For each species, significant differences between pairs of means are indicated by labeling them with different letters. For *D. n. nasuta* the interaction between selection regime and ammonia level was not significant. Consequently, only the differences between mean survivorship at different ammonia levels, averaged over block and selection regime, have been highlighted.

Figure 7. (A) Mean pre-adult survivorship after larval feeding for different amounts of time, and (B) mean dry weight (in 10^{-3} g) at eclosion after larval feeding for different amounts of time corresponding to four different mean pre-adult survivorship levels, in the *D. ananassae* selected and control populations. Error bars are standard errors around the mean of the four replicate population mean values. For both traits, significant differences between pairs of means are indicated by labeling them with different letters. For both traits, the interaction between selection regime and feeding duration/survivorship was not significant. Consequently, only the differences in mean survivorship between the different levels of feeding duration, and differences in mean dry weight at eclosion at different levels of survivorship, averaged over block, selection regime and sex, have been highlighted.

Table 1. Summary of results of four-way ANOVA on mean arcsin squareroot transformed pre-adult survivorship at low and high density in the two sets of selected populations and controls, in single-species cultures and in two-species competitive cultures with equal numbers of white-eye *D. melanogaster* eggs. Since the analysis was done on population means, block and interactions involving block were not tested for significance.

	ACU/AB		NCU/NB	
Effect	$F_{1,3}$	P	$F_{1,3}$	P
culture type	17.44	0.03	1.54	0.30
selection	14.11	0.033	43.67	<0.01
density	1621.07	<0.01	51.54	<0.01
culture type × selection	11.81	0.04	5.50	0.10
culture type × density	10.03	0.05	17.46	0.03
selection × density	525.03	<0.01	60.88	<0.01
culture type × selection × density	2.94	0.19	2.12	0.24

Table 2. Summary of results of two-way ANOVA on mean pre-adult development time, assayed at low and high larval density at different generations in the two sets of selected populations and controls. Since the analysis was done on population means, block and interactions involving block were not tested for significance.

	ACU/AB		NCU/NB	
Low density				
Effect	$F_{1,3}$	P	$F_{1,3}$	P
selection	49.23	<0.01	115.50	<0.01
sex	82.32	<0.01	3.75	0.15
selection × sex	1.93	0.26	18.61	0.02
High density				
Effect	$F_{1,3}$	P	$F_{1,3}$	P
selection	60.37	<0.01	364.65	<0.01
sex	41.18	<0.01	13.09	0.04
selection × sex	8.58	0.06	5.49	0.10

Table 3. Summary of results of three way ANOVA on mean pre-adult lifestage (egg, L1, L2, L3, pupa) duration in the two sets of selected populations and controls. Since the analysis was done on population means, block and interactions involving block were not tested for significance.

	ACU/AB		NCU/NB	
Effect	$F_{1,3 \text{ sel}/ 4,12 \text{ else}}$	P	$F_{1,3 \text{ sel}/ 4,12 \text{ else}}$	P
selection	168.38	<0.01	115.50	<0.01
life-stage	1778.55	<0.01	9485.70	<0.01
selection × life-stage	1.46	0.28	37.05	<0.01

Table 4. Summary of results of two way ANOVA on mean larval feeding rate, foraging path length and pupation height in the two sets of selected populations and controls. Since the analysis was done on population means, block and interactions involving block were not tested for significance; only the *F* and *P* values for the main effect of selection regime are shown.

	ACU/AB		NCU/NB	
Trait	$F_{1,3}$	*P*	$F_{1,3}$	*P*
feeding rate	0.54	0.52	0.06	0.82
foraging path length	94.51	<0.01	0.48	0.54
pupation height	18.12	0.02	51.62	<0.01

Table 5. Summary of results of three way ANOVA on mean arcsin squareroot transformed pre-adult survivorship at three different concentrations of urea or ammonia in the food medium in the two sets of selected populations and controls. Since the analysis was done on population means, block and interactions involving block were not tested for significance.

	ACU/AB		NCU/NB	
Urea tolerance				
Effect	$F_{1,3 \text{ sel}/ 2,6 \text{ else}}$	P	$F_{1,3 \text{ sel}/ 2,6 \text{ else}}$	P
selection	20.39	0.02	0.14	0.73
concentration	54.77	<0.01	24.88	<0.01
selection × concentration	0.51	0.62	0.32	0.74
Ammonia tolerance				
Effect	$F_{1,3 \text{ sel}/ 2,6 \text{ else}}$	P	$F_{1,3 \text{ sel}/ 2,6 \text{ else}}$	P
selection	0.14	0.73	45.29	0.73
concentration	199.34	<0.01	417.76	<0.01
selection × concentration	14.97	0.02	0.88	0.46

Table 6. Summary of results of three way ANOVA on mean arcisn squareroot transformed pre-adult survivorship (factors: block, selection regime and larval feeding duration) and mean dry weight (factors: block, selection regime, sex and pre-adult survivorship) of eclosing flies after larvae were allowed to feed for different time periods in the AB and ACU populations. Since the analysis was done on population means, block and interactions involving block were not tested for significance.

	Survivorship		Dry weight	
Effect	$F_{1,3 \text{ sel}/ 3,9 \text{ else}}$	P	$F_{1,3 \text{ sel}/ 3,9 \text{ else}}$	P
Selection	174.79	<0.01	0.01	0.94
feeding duration (for survivorship)	37.07	<0.01		
Survivorship (for dry weight)			16.39	<0.01
Sex (for dry weight)			223.27	<0.01
selection × feeding duration (for survivorship)	0.35	0.78		
selection × survivorship (for dry weight)			2.76	0.10
selection × sex (for dry weight)			3.25	0.17
survivorship × sex (for dry weight)			1.40	0.30
selection × survivorship × sex (for dry weight)			0.48	0.71

Figure 1.

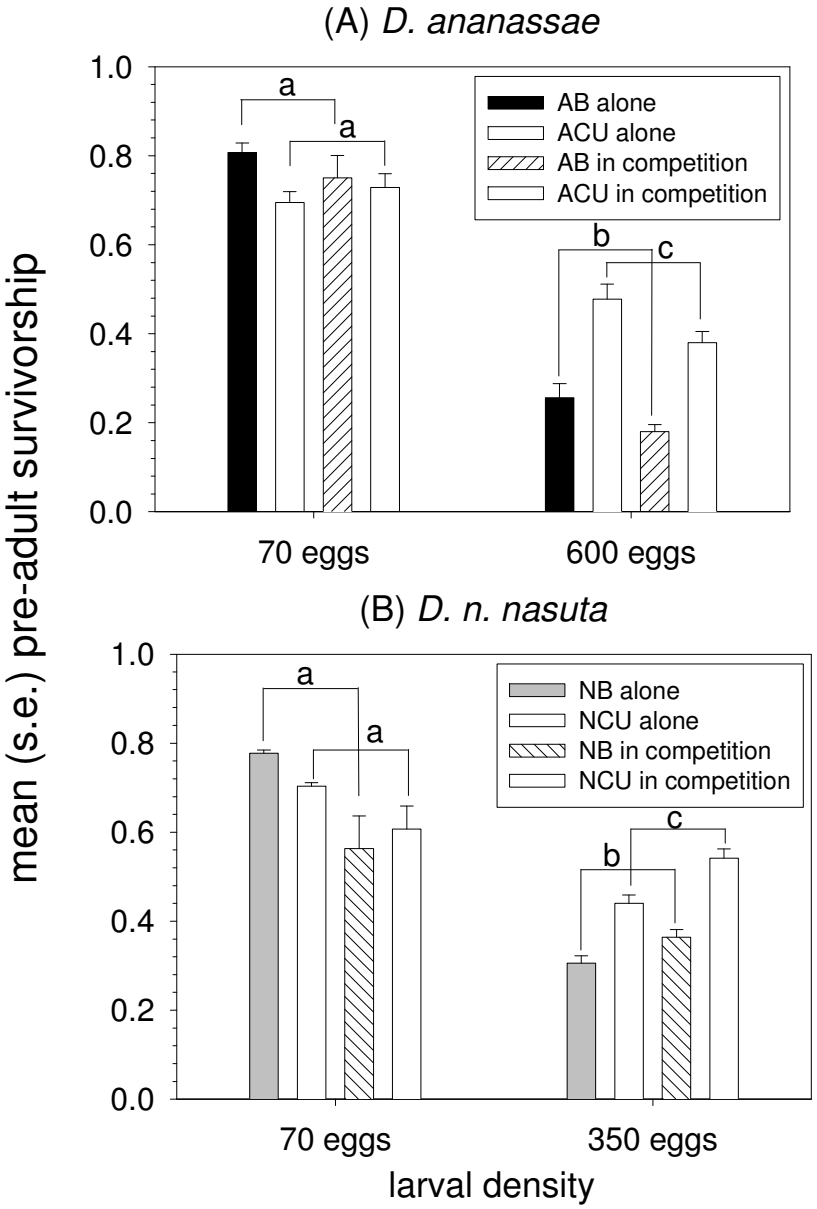
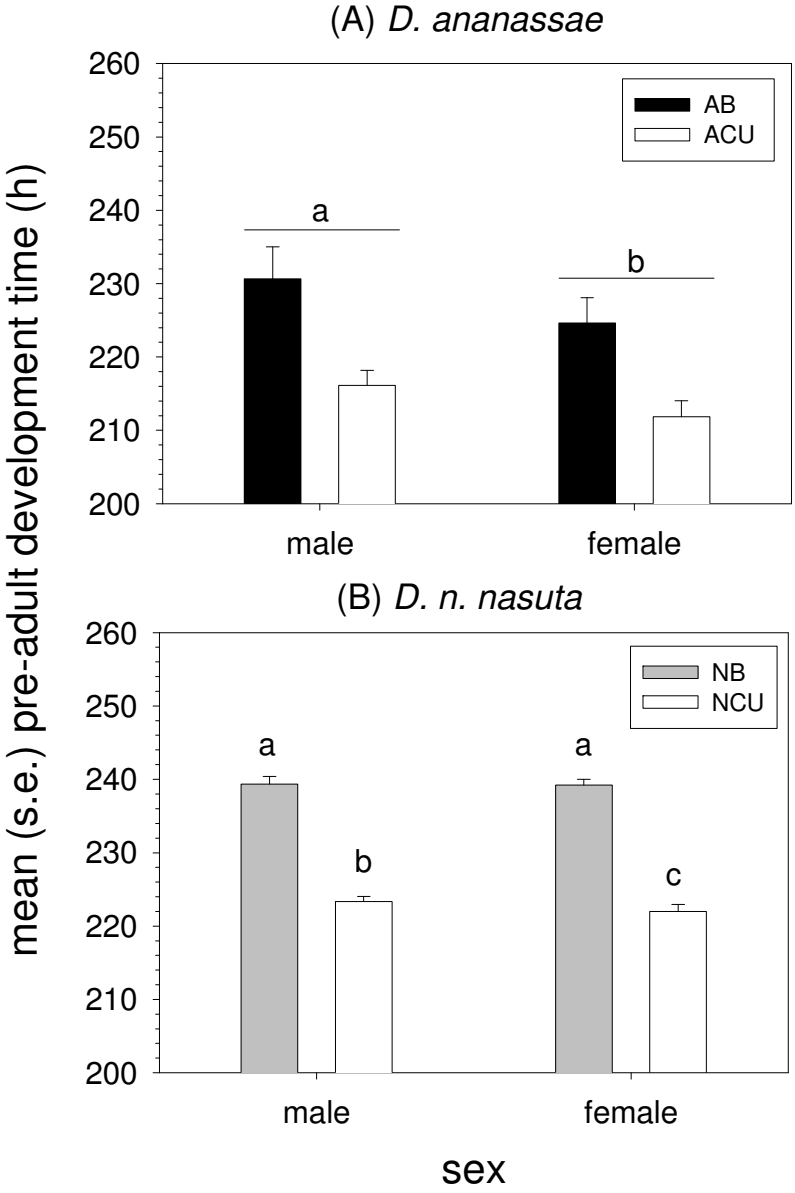


Figure 2.



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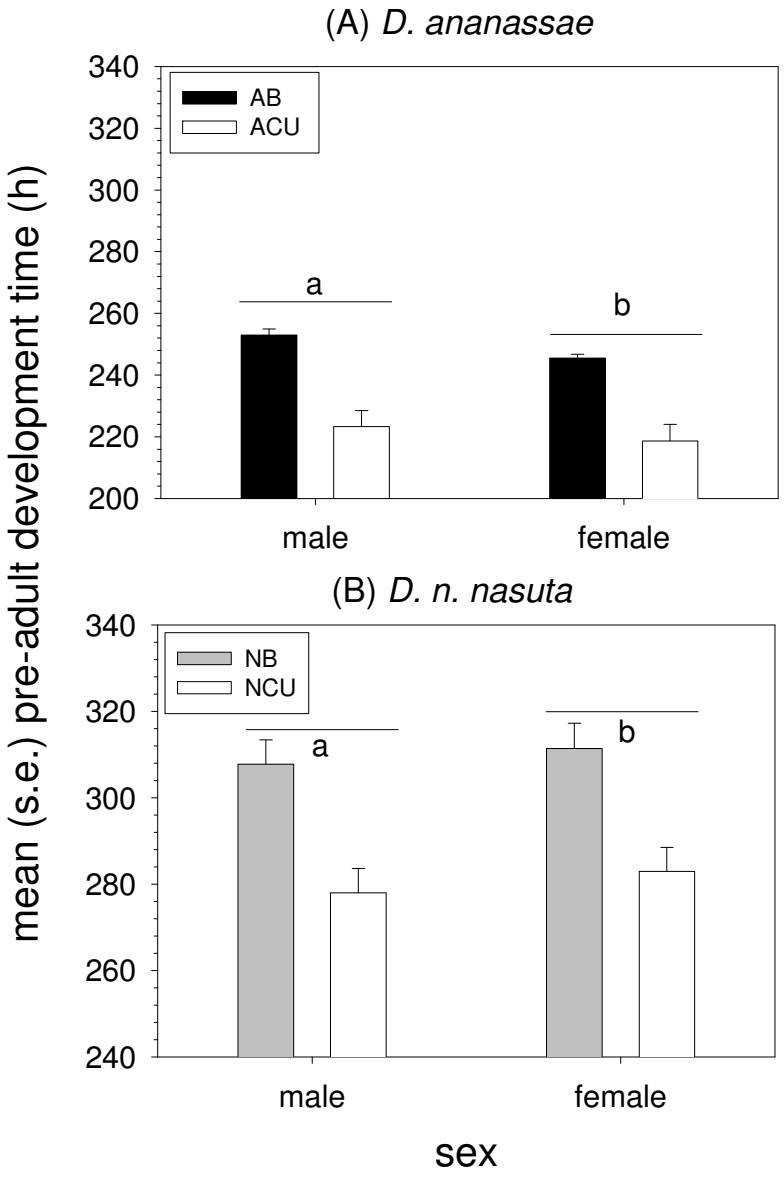

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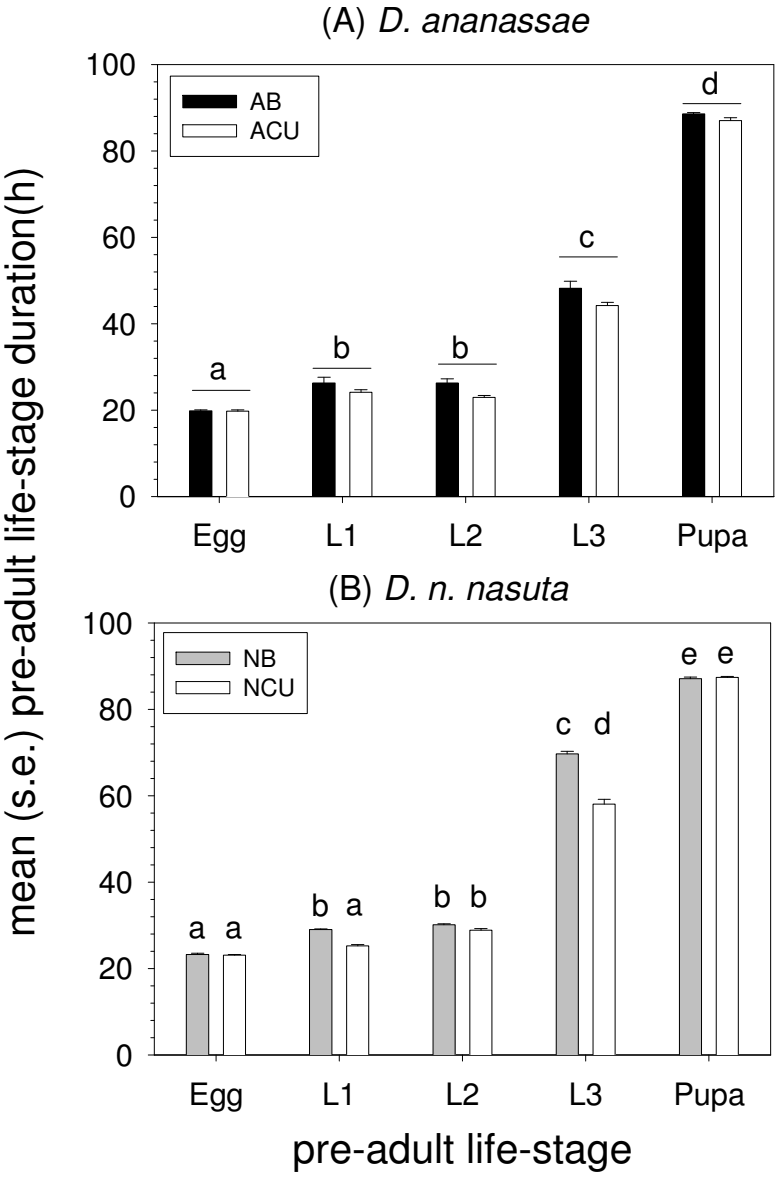

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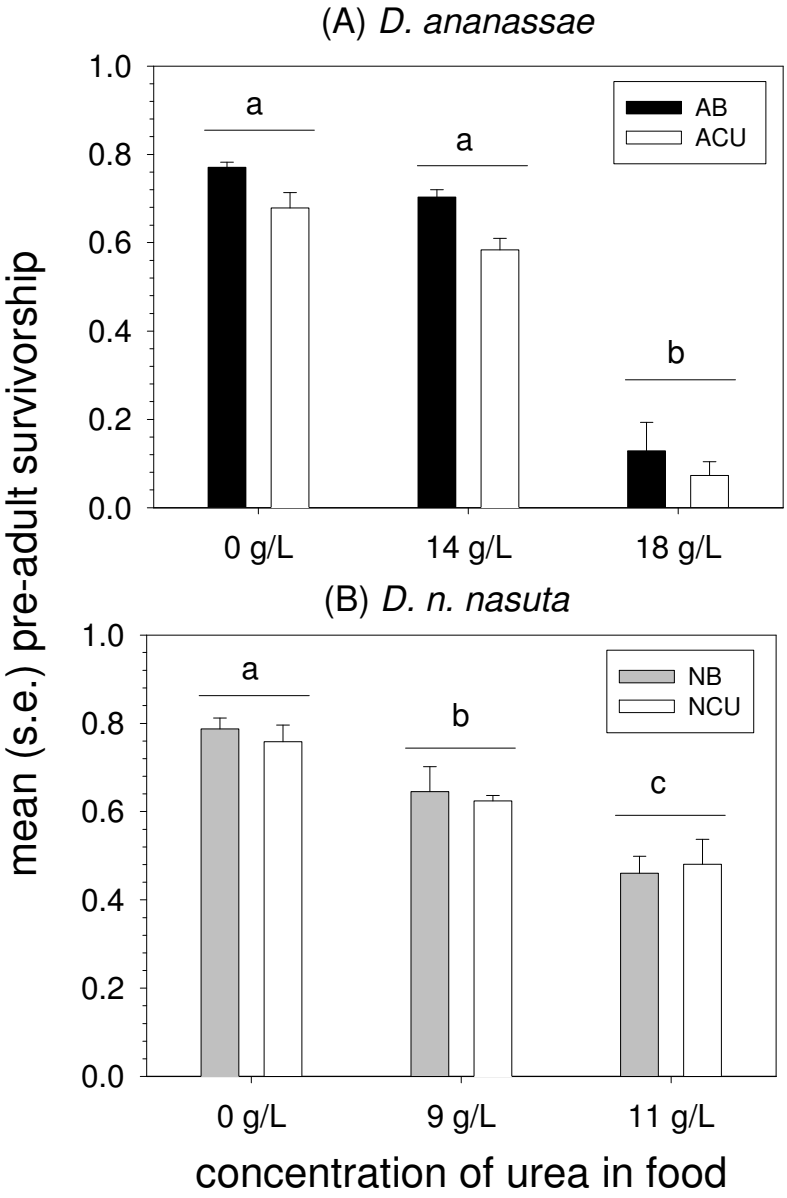

Figure 6.

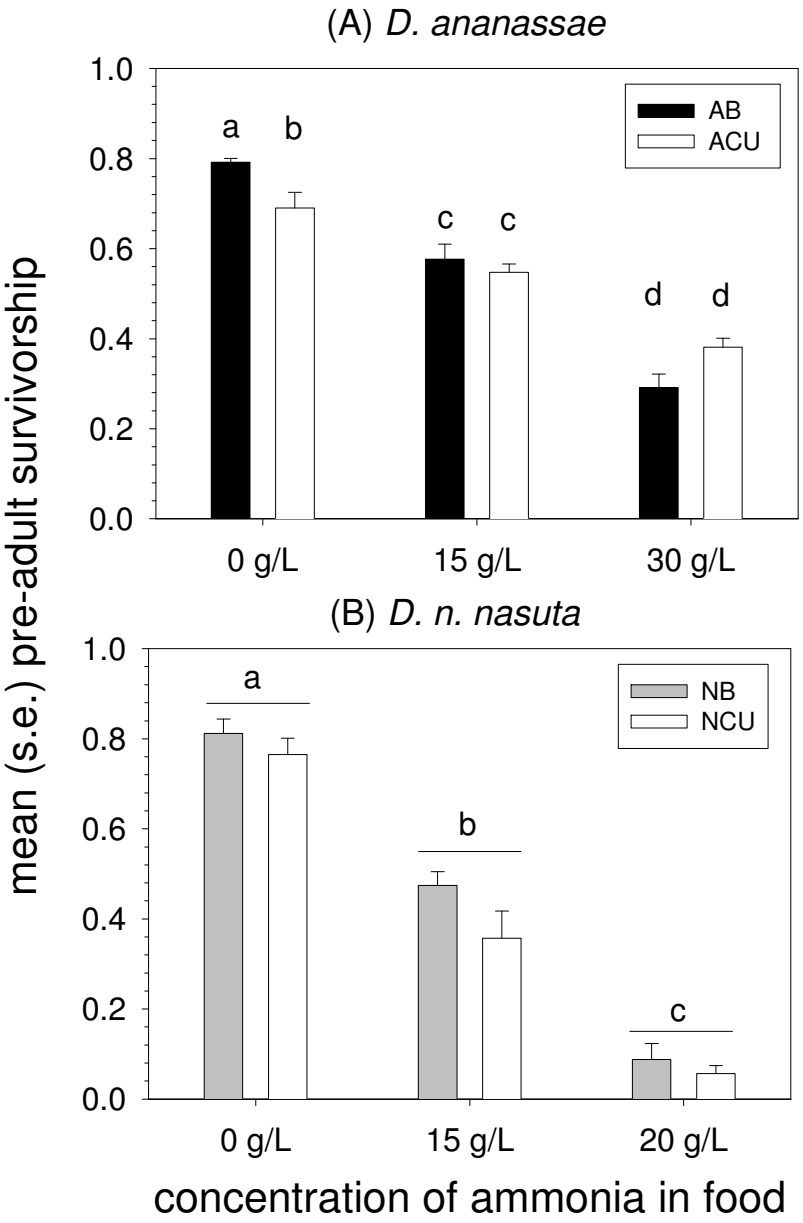

Figure 7.