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

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Abstract

The standard view of adaptation to larval crowding in fruitflies, built on results from 25 years of multiple 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. These results, moreover, were at odds from the predictions of classical *K*-selection theory, notably the expectation that selection at high density should result in the increase of efficiency of conversion of food to biomass, and were better interpreted through the lens of α-selection. We show here 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 time efficiency of food conversion to biomass, increased pupation height with a relatively small role of increased urea/ammonia tolerance, if at all. 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.

57 **Introduction**

58 Adaptation to crowding leading to greater competitive ability, first conceptualized as *r*-
59 and *K*-selection theory (MacArthur and Wilson 1967), is an important phenomenon in
60 ecology and evolution that was extensively studied in *Drosophila melanogaster* over the
61 past three decades, using experimental evolution approaches (reviewed in Joshi 1997;
62 Mueller 1997; Prasad and Joshi 2003). With lifespan, fecundity, starvation resistance and
63 dessication resistance, some of the other traits extensively examined via experimental
64 evolution in *D. melanogaster*, there were conflicting reports of differing patterns of
65 correlated responses to selection between laboratories (Ackermann et al. 2001; Prasad and
66 Joshi 2003; Rose et al. 1996), that led, in part, to a growing appreciation that evolution is
67 often "local" (Rose et al. 2005). In the case of adaptation to crowding, however, the
68 pattern of correlated responses to selection was largely consistent over two separate
69 selection experiments, involving *D. melanogaster* populations originating from different
70 geographical sources (*r*- and *K*-populations: Mueller and Ayala 1981; UU and CU
71 populations: Joshi and Mueller 1996), and the suite of traits through which selected
72 populations evolved greater competitive ability was very different from the canonical
73 version of *r*- and *K*-selection theory that emphasized the centrality of increased food to
74 biomass conversion efficiency as an adaptation to chronic crowding (Mueller 2009).

75 Results from the earlier selection studies on adaptation to crowding in *D.*
76 *melanogaster* have been reviewed extensively (Joshi 1997; Mueller 1997; Joshi et al.
77 2001; Prasad and Joshi 2003; Dey et al. 2012) and, consequently, we will just summarize
78 the major observed correlated responses of pre-adult traits to selection. Relative to the low
79 density *r*-populations, the crowding adapted *K*-populations exhibited greater (i) pre-adult

80 survivorship, when assayed at high larval density (Bierbaum et al. 1989), (ii) pre-adult
81 competitive ability (Mueller 1988), (iii) larval feeding rate (Joshi and Mueller 1988), (iv)
82 larval foraging path length (Sokolowski et al. 1997), (v) pupation height (Mueller and
83 Sweet 1986; Joshi and Mueller 1993), and (vi) minimum food requirement for pupation
84 (Mueller 1990). Pre-adult survivorship of the *r*- and *K*-populations did not differ
85 significantly when assayed at low larval density (Bierbaum et al. 1989). Pre-adult
86 development time in the *K*-populations was lower than that of the *r*-populations at low (30
87 larvae per vial) and moderately high (160 larvae per vial) density, but greater at very high
88 (320 larvae per vial) density (Bierbaum et al. 1989). The evolution of higher larval feeding
89 rate and pupation height in crowding adapted populations was subsequently verified with
90 a separate set of populations (*rK* and *r*×*rK* populations), by Guo et al (1991).

91 Relative to the control UU populations, the larval crowding adapted CU
92 populations exhibited greater (i) pre-adult survivorship, when assayed at high larval
93 density (Mueller et al. 1993; Shiotsugu et al. 1997), (ii) larval feeding rate (Joshi and
94 Mueller 1996), (iii) larval foraging path length (Sokolowski et al. 1997), (iv) minimum
95 food requirement for pupation (Joshi and Mueller 1996), and (v) tolerance to nitrogenous
96 wastes like urea (Shiotsugu et al. 1997; Borash et al. 1998) and ammonia (Borash et al.
97 1998). Pre-adult survivorship of the CU and UU populations did not differ significantly
98 when assayed at low larval density (Mueller et al. 1993; Shiotsugu et al. 1997). CU pre-
99 adult development time was similar to the UU populations when assayed at low larval
100 density, but lower than the UU populations when assayed at high larval density (Borash
101 and Ho 2001). Pupation height of the CU populations was greater than the UU controls
102 during early generations of CU selection (Mueller et al. 1993), but did not differ

103 significantly from the controls after about 60 generations of selection (Joshi and Mueller
104 1996); possible reasons for this are extensively discussed by Joshi et al. (2003). Urea and
105 ammonia tolerance were not assayed on the *r*- and *K*-populations, whereas competitive
106 ability was not assayed on the CU and UU populations.

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

123 The notion that faster feeding is a strong correlate of pre-adult competitive ability
124 in *Drosophila* has a lot of support. Populations selected for faster feeding rate were also
125 found to be better competitors (Burnet et al. 1977)), and populations selected for rapid

126 pre-adult development evolved both reduced feeding rate and reduced competitive ability
127 (Prasad et al. 2001; Shakarad et al. 2005; Rajamani et al. 2006). Similarly, populations
128 selected for increased parasitoid resistance evolved both reduced feeding rate and reduced
129 competitive ability (Fellowes et al. 1998, 1999). The notion that there is a cost to faster
130 feeding (Joshi and Mueller 1996) was supported by the rapid return of feeding rates to
131 control levels when CU populations were maintained at moderate densities (Joshi et al.
132 2003). There is also evidence for a trade-off between urea/ammonia tolerance and larval
133 feeding rate. Populations selected for greater urea and ammonia tolerance, respectively,
134 also showed reduced larval feeding rate (Borash et al. 2000) and larval foraging path
135 length (Mueller et al. 2005), and populations selected for greater urea tolerance did not
136 show higher survivorship than controls at high larval density (Shiotsugu et al. 1997). In
137 general, larval feeding rate and forgaing path length appear to be positively correlated
138 (Joshi and Mueller 1988, 1996; Sokolowski et al. 1997; Borash et al. 2000; Prasad et al.
139 2001; Mueller et al. 2005). Thus, the evolution of competitive ability in *Drosophila* seems
140 to be the outcome of a balance between mutually antagonistic traits like increased larval
141 feeding and forgaing behaviour (and perhaps pupation height), greater tolerance to
142 nitrogenous wastes, and a reduced efficiency of conversion of food to biomass. Indeed, the
143 CU populations exhibited a temporal polymorphism for two of these traits: offspring of
144 early eclosing flies in a crowded culture showed higher feeding rates, whereas offspring of
145 late eclosing flies showed greater urea/ammonia tolerance than controls (Borash et al.
146 1998).

147 Given that the above view of adaptation to larval crowding in *Drosophila* was built
148 around studies on a single species (*D. melanogaster*), we wanted to investigate whether

149 other species of *Drosophila* would also respond to larval crowding by evolving essentially
150 the same set of traits. If the genetic architecture of traits relevant to fitness under larval
151 crowding is reasonably conserved across congeners, then we should see a similar pattern
152 of correlated responses to selection for adaptation to larval crowding in other *Drosophila*
153 species. We report results from two selection experiments, on *D. ananassae* and *D. nasuta*
154 *nasuta*, involving selection for adaptation to larval crowding. *D. ananassae* is ecologically
155 and phylogenetically closer to *D. melanogaster* (*Sophophora* Subgenus, Melanogaster
156 Group, Melanogaster Subgroup), being a cosmopolitan human commensal and belonging
157 to the Melanogaster Group, Ananassae Subgroup and Ananassae Species Complex,
158 whereas *D. n. nasuta* belongs to the *Drosophila* Subgenus, Immigrans Group, Nasuta
159 Subgroup and Frontal Sheen Complex, and is found primarily in orchards and open land.
160 Our results showed that selected populations of both *D. ananassae* and *D. n. nasuta*
161 exhibited similar patterns of correlated responses to selection for adaptation to larval
162 crowding, but that these populations evolved greater competitive ability through traits
163 very different from those seen earlier in *D. melanogaster* populations subjected to similar
164 selection.

165 Materials and methods

166 *Experimental populations*

167 This study used eight laboratory populations each of *D. ananassae* and *D. n. nasuta*. Four
168 control *D. ananassae* populations (AB_{1-4}: **A**nanassae **B**aseline) were derived from a single
169 population, initiated in May-June 2001 with about 300 wild-caught females from
170 Bangalore, India, and maintained as a single population on a 21-day discrete generation

171 cycle for 34 generations (Sharmila Bharathi *et al*. 2003). The four AB populations were
172 maintained on a 21-day discrete generation cycle at 25º ± 1 C, ~90% relative humidity,
173 constant light and on cornmeal medium. Larval density was regulated at 60-80 larvae per
174 vial (9 cm × 2.4 cm) with 6 mL food. Forty vials were set up per replicate population.
175 Twelve days after egg-collection, eclosed adults (1500-1800) from all 40 vials were
176 collected into Plexiglas cages (25 × 20 × 15 cm^3) containing a Petridish (9 cm diameter) of
177 food that was changed every alternate day, and a moistened ball of cotton. On day 18 after
178 egg-collection, the flies were given food along with a generous smear of live yeast-acetic
179 acid paste. On day 21 from the previous egg-collection, fresh food plates were put into the
180 cages and eggs collected from them after 18 h to initiate the next generation.

181 From the AB populations, four populations (ACU_{1-4}: **A**nanassae **C**rowded as
182 larvae and **U**ncrowded as adults) were derived, one each from each of AB_{1-4}, two
183 generations after the AB populations were established. The ACU populations were
184 selected for adaptation to larval crowding by subjecting them to a density of 550-600 eggs
185 per vial with 1.5 mL of food. In initial generations the density was lower; the final
186 densities were attained by generation 15 of ACU selection. The ACU populations were
187 otherwise maintained the same as the AB controls, except that only 20 vials of eggs were
188 collected each generation (to keep the number of breeding adults similar to controls: 1500-
189 1800), and the collection of eclosed adults into the cages continued until day 18 from
190 previous egg-collection, as eclosion in a crowded culture is staggered over several days.

191 Four control *D. n. nasuta* populations (NB_{1-4}: **N**asuta **B**aseline) were derived, after
192 24 generations as a single population on a 21 day discrete generation cycle, from a
193 laboratory population established using about 70 females collected from orchards and

194 domestic garbage dumps in different parts of Bangalore, India, during October-November
195 2001 (Sharmila Bharathi *et al*. 2003). The four NB populations were maintained on a 21-
196 day discrete generation cycle at 25º ± 1 C, ~90% relative humidity, constant light and on
197 cornmeal medium. The larval density was regulated at 60-80 larvae per vial (9 cm × 2.4
198 cm) with 6 mL food. Fifty two vials were set up per replicate population to keep the
199 number of breeding adults at about 1500-1800. Twelve days after egg-collection, eclosed
200 adults from all 40 vials per replicate population were collected into Plexiglas cages (25 ×
201 20 × 15 cm^3) containing a Petridish of food that was changed every alternate day, and a
202 moistened ball of cotton. On day 18 after egg-collection, the flies were given food along
203 with a generous smear of live yeast-acetic acid paste. On day 21 from the previous egg-
204 collection, fresh food plates were put into the cages and eggs collected from them after 18
205 h to initiate the next generation.

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

216 Since each ACU or NCU population was derived from one control population,
217 selected and control populations of each species bearing identical numerical subscripts are
218 more related to each other than to other populations with which they share the selection
219 regime. Therefore, control and selected populations with identical subscripts were treated
220 as blocks, representing ancestry, in the statistical analyses.

221 ***Collection of flies for assays***

222 All control and selected populations were maintained under common (control-type)
223 rearing conditions for one complete generation prior to assays, to eliminate non-genetic
224 parental effects. The progeny of these flies, hereafter 'standardized flies', were then used
225 for the various assays. To obtain progeny for assays, standardized flies in cages were
226 provided yeast-acetic acid paste on food for three days before egg collection. A fresh
227 Petridish with food was then placed in the cages and the flies were allowed to lay eggs for
228 ~14 h, after which eggs were removed from the food with a moistened paintbrush and
229 placed into vials for setting up the various assays. All assays were conducted at 25 ± 1°C,
230 under constant light.

231 ***Pre-adult survivorship***

232 After 42 generations of ACU selection, eggs laid by standardized flies were placed into
233 vials at a density of either 70 or 600 per vial containing 1.5 mL of food. Eight such vials
234 were set up for each replicate AB and ACU population at each density in single-species
235 culture. Eight such vials at each density were also set up in two-species cultures, in
236 competition with a common competitor, a white eyed mutant population of *D.*
237 *melanogaster*, maintained for about 90 generations in the laboratory on a three week

238 discrete generation cycle. The white eyed population was derived from spontaneously
239 occurring mutant individuals in the JB populations (Sheeba et al. 1998) in our laboratory.
240 For the two-species cultures, eggs laid by standardized ACU, AB or white eyed flies were
241 collected and placed into vials at a density of either 70 (35 ACU or AB eggs and 35 eggs
242 from the white eyed mutant population) or 600 (300 ACU or AB eggs and 300 eggs from
243 the white eyed mutant population) per vial containing 1.5 mL of food. Eight such vials
244 were set up for each replicate AB and ACU population at each density. The number of
245 flies eclosing in each vial was recorded and used to calculate pre-adult survivorship.

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

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

251 After 53 generations of ACU selection, pre-adult development time was assayed at a high
252 density of 600 eggs per vial with 1.5 mL of food. Eggs from standardized AB and ACU
253 flies were dispensed into each vial using a moistened paintbrush. Eight such vials were set
254 up per replicate population. After the pupae darkened, vials were checked every 6 h and
255 the number of eclosing flies recorded.

256 After 71 generations of ACU selection, pre-adult development time was assayed at
257 a low density of 30 eggs per vial with 6 mL of food (An earlier study at generation 41 of
258 ACU selection had shown that pre-adult development time did not differ significantly
259 between low densities of 30 or 70 eggs per vial with 6 mL of food: data not shown). Eggs

260 from standardized AB and ACU flies were dispensed into each vial using a moistened
261 paintbrush. Ten vials were set up per replicate population. After the pupae darkened, the
262 vials were monitored for eclosion at 2 h intervals, and the number of eclosing flies
263 recorded. As part of the same assay, egg hatching time and the duration of each larval
264 instar and the pupal stage were also determined. For assaying egg hatching time, 30 eggs
265 from the standardized flies were arranged on a small agar cube in a food vial in six rows
266 of five eggs each. Ten such vials were set up per population. Fifteen hours after egg
267 laying, the vials were checked for any hatched eggs once every hour, till no eggs hatched
268 for three consecutive hours. For assaying instar and pupal duration, eggs of approximately
269 identical age were harvested over a three hour period from the standardized flies and
270 dispensed into vials with 6 mL of food at a density of 30 eggs per vial. Sixty such vials
271 were set up per population. Forty three hours after the midpoint of the three hour egg
272 laying period, four vials per population were removed from the incubators and immersed
273 in hot water. The dead larvae were removed and kept in 70% ethanol for subsequent
274 examination. Every two hours, this process was repeated. The larval instars were
275 differentiated based on the number of 'teeth' in the larval mouth hooks. From these data,
276 the number of larvae of each instar present in each two-hourly sample was determined,
277 and the median time of each molt was obtained by interpolation. For pupal duration and
278 pre-adult development time, ten vials per population were set up with 30 eggs in 6 mL of
279 food. After the first pupa was seen, the vials were screened every two hours and any new
280 pupae that had formed were marked on the vial with different coloured marker pens.
281 Thereafter, the vials were monitored for eclosion and the number of eclosing males and
282 females in each vial was determined every two hours. These observations yielded data on

283 egg to pupa and egg to adult development time, from which the pupal duration could be
284 calculated.

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

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

293 ***Larval feeding rate***

294 After 71 generations of ACU selection, the feeding rates of AB and ACU larvae were
295 measured at physiologically equalized ages, based on the difference in AB and ACU
296 development time. This was done by collecting eggs from the standardized ACU flies 5 h
297 later than the AB flies. Thus, at the time of assay, ACU larvae were 58 h old, whereas AB
298 larvae were 63 h old and, thus, approximately in the same relative stage of their larval
299 development. Following Joshi and Mueller (1996), about a hundred eggs laid over a four
300 hour period were collected from standardized flies and placed into two Petridishes with
301 non-nutritive agar each for AB and ACU populations. Twenty-four hours later, twenty-
302 five newly hatched larvae were transferred from these agar Petridishes to a Petridish
303 containing a thin layer of non-nutritive agar overlaid with 1.5 mL of 37.5% yeast
304 suspension. Four such Petridishes were set up per population. The larvae were then

305 allowed to feed for 58 (ACU) or 63 (AB) h, by which time they were in the early third
306 instar. At this point, 20 larvae from each population were assayed for feeding rate,
307 following the procedure of Joshi and Mueller (1996), by placing them individually in a
308 small Petridish (5 cm diameter) containing a thin layer of agar overlaid with a thin layer of
309 10% yeast suspension. After allowing for a 15 sec acclimation period, feeding rate was
310 measured under a stereozoom microscope as the number of cephalopharyngeal sclerite
311 retractions in a 1 min period. Selected and control populations, matched by the subscripted
312 indices, were assayed together, with one larva from the selected population and one from
313 the control population being assayed alternately. The same procedure was followed for
314 assaying larval feeding rate of 20 larvae from each NCU and NB population, after 77
315 generations of NCU selection.

316 ***Larval foraging path length***

317 Twenty early third instar larvae for each population were assayed for larval foraging path
318 length. The collection of larvae for the assay was exactly as described above for larval
319 feeding rate assays. For assaying foraging path length, individual larvae were placed in a
320 small Petridish (5 cm diameter) containing a thin layer of agar overlaid with a thin layer of
321 10% yeast suspension. After allowing for a 15 sec acclimation period, the larvae were
322 allowed to move around on the Petridish for 1 min. The path traversed by the larvae on the
323 yeast surface was traced onto a transparency sheet and later measured with a thread and
324 ruler. Selected and control populations, matched by subscripted indices, were assayed
325 together at generations 52 and 81 of ACU and NCU selection, respectively.

326 ***Pupation height***

327 Eggs from standardized flies were collected and placed into vials with 6 mL of food at a
328 density of 50 (ACU/AB) or 70 (NCU/NB) eggs per vial. Ten and eight such vials were set
329 up per population for *D. ananassae* and *D. n. nasuta*, respectively. Once all pupae had
330 formed, pupation height of each pupa was measured, following Joshi and Mueller (1993),
331 as the distance between the surface of the food medium to the point between the anterior
332 spiracles of the pupa. Any pupa on or touching the surface of the food was given a
333 pupation height of zero. Assays were conducted at generations 39 and 34 of ACU and
334 NCU selection, respectively.

335 ***Larval urea and ammonia tolerance***

336 After 49 generations of ACU selection, eggs laid over a four hour period by standardized
337 flies were collected and exactly 30 eggs per 6 mL food were placed into vials at three
338 concentrations each of urea (0, 14 and 18 g/L) or ammonia (0, 15 and 30 g/L NH_4Cl) in
339 the food. These are values that allowed the detection of differences in urea/ammonia
340 tolerance between selected and control populations in previous studies on *D. melanogaster*
341 (Shiotsugu et al. 1997; Borash et al. 1998). Ten vials were set up per population at each
342 concentration of either urea or ammonia, and the number of eclosing flies in each vial was
343 recorded to calculate pre-adult survivorship. Both urea and ammonia were used as there is
344 some confusion about which compound actually increases in concentration in crowded
345 larval cultures, although it is likely that it is ammonia in the case of *D. melanogaster*
346 (Botella et al. 1985; Borash et al. 1998). The NCU and NB populations were assayed in an
347 identical manner after 76 generations of NCU selection, except that the egg-laying period
348 was 12 hours, and the concentrations used were 0, 9 and 11 g/L for urea and 0, 15 and 20
349 g/L for ammonia, as earlier studies had shown that *D. n. nasuta* larvae were more sensitive

350 to urea and ammonia than their *D. ananassae* counterparts, and *D. n. nasuta* females are
351 not as fecund as *D. ananassae* females (data not shown).

352 ***Minimum feeding time and dry weight after minimum feeding***

353 These assays were conducted only on the ACU and AB populations. After 45 generations
354 of ACU selection, critical minimum feeding time for pupation was assayed by setting up
355 freshly hatched larvae, from eggs laid by standardized flies, onto Petridishes with agar
356 overlaid with 1.5 mL of 37.5% yeast suspension, as described above for the feeding rate
357 and foraging path length assays. Twenty five larvae were placed into each Petridish, and
358 sixty such Petridishes were set up per population. At 46, 49, 52 and 55 h after egg hatch, a
359 total of 150 larvae per population per time point were removed from the yeast, gently
360 washed in water to remove any yeast sticking to their bodies, and placed in ten vials
361 containing 5 mL of non-nutritive agar, at a density of 15 larvae per vial. These vials were
362 subsequently monitored for pupation and eclosion, and the pre-adult survivorship after
363 feeding for different periods of time noted. Using the information from this assay, a
364 second assay was carried out after 68 generations of ACU selection to measure the dry
365 weights at eclosion of ACU and AB flies that had fed as larvae for different durations of
366 time, roughly corresponding to pre-adult survivorship of 13, 25, 50 and 60%, respectively
367 (feeding for 54, 56, 59 and 62 h for AB, and 50, 55, 58 and 60 h for ACU populations,
368 respectively). Freshly hatched larvae were collected, shifted to yeast and then removed
369 and placed into agar vials after feeding for different durations corresponding to pre-adult
370 survivorship of 13, 25, 50 and 60% for ACU and AB populations, exactly as described
371 above. Once eclosions began in the agar vials, flies were collected every 4 h and frozen

372 for subsequent weighing. Frozen flies were sorted into batches of five males or five
373 females each and dried at 70°C for 36 h before weighing in batches.

374 ***Statistical analyses***

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

393 **Results**

394 ***Pre-adult survivorship***

395 Pre-adult survivorship at high larval density is the primary trait expected to be under direct
396 selection in populations exposed to high larval crowding each generation. With regard to
397 survivorship at low versus high larval density, the broad pattern of results was very similar
398 in the two species (Figure 1). In both species, pre-adult survivorship was, on an average,
399 significantly higher at low rather than high larval density, and in selected compared to
400 control populations (Figure 1, Table 1). The trend was for survivorship to be higher, on
401 average, in single-species cultures than in two-species cultures, but the difference was
402 significant only in the case of *D. ananassae* (Table 1). Both species showed a significant
403 selection regime × larval density interaction (Table 1), with the survivorship of selected
404 and control populations not differing significantly at low density, and with selected
405 populations showing significantly higher survivorship than controls at high density
406 (Figure 1). Both species also showed a significant culture type × larval density interaction
407 (Table 1). In *D. ananassae*, averaged over selection regimes, survivorship at low density
408 in both types of cultures (single- or two-species) was similar, whereas survivorship at high
409 density was significantly greater in single-species than two-species cultures (Figure 1). In
410 *D. n. nasuta*, the opposite pattern was seen. Averaged over selection regime, survivorship
411 at low density in single-species cultures was higher than in two-species cultures (Figure
412 1), but not significantly so. However, at high density, survivorship in two-species cultures
413 was higher than in single-species cultures but, again, not significantly so. In fact, the only
414 clear significant difference in the multiple comparisons was that between single-species
415 cultures at low and high densities (Figure 1.). In the case of *D. ananassae*, there was also a
416 significant culture type × selection regime interaction (Table 1), but the only clearly

417 significant differences in multiple comparisons were between survivorship in two-species
418 cultures at high density on the one hand, and in the other three culture type × selection
419 regime combinations on the other (Figure 1).

420 ***Pre-adult development time***

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

425 Interestingly, in both species at both larval densities, there was a significant main
426 effect of selection regime, with selected populations consistently showing substantially
427 lower development time than control populations at both densities (Figures 2,3, Table 2).
428 On an average, AB development time was higher than that of ACU populations by about
429 14 and 29 h at low and high larval density, respectively (Figures 2A, 3A), whereas that of
430 NB populations was greater than that of NCU populations by about 17 and 29 h at low and
431 high larval density, respectively (Figures 2B,3B). In three of the four assays, there was no
432 significant selection regime × sex interaction (Table 2). In *D. n. nasuta* at high larval
433 density, control males and females had similar development times whereas NCU males
434 had significantly higher development time than NCU females, albeit by just about 1 h
435 (Figure 3B).

436 ***Pre-adult life-stage duration***

437 The overall pattern of pre-adult life-stage durations in both species was similar to what is
438 seen in *Drosophila* spp. in general. Consistent with the pre-adult development time results,
439 both species showed a main effect of selection regime (Table 3), with life-stage durations
440 being greater in control rather than selected populations, on an average (Figure 4). In *D.*
441 *ananassae*, a 20 h egg stage was followed by two larval instars of about 24 h each, a third
442 larval instar of about 46 h and a pupal stage lasting about 87 h (Figure 4A); there was no
443 significant selection regime × life-stage duration interaction. In *D. n. nasuta*, all pre-adult
444 life-stages differed significantly from one another in duration, with a 23 h egg stage,
445 followed by two larval instars of about 27 and 29 h, respectively, a longer third larval
446 instar of about 63 h and a pupal stage lasting about 87 h (Figure 4B). The longer larval
447 instar durations in *D. n. nasuta* are consistent with their larger adult size compared to *D.*
448 *ananassae* (Sharmila Bharathi et al. 2004). *D. n. nasuta* also showed a significant
449 selection regime × life-stage duration interaction (Table 3), with a large (~12 h) reduction
450 in third instar duration in the NCU populations (Figure 4B). We note that, given the time
451 interval between subsequent observations (1 h for egg-hatch; 2 h for larval instars), our
452 screening might be too coarse to pick up small but consistent differences on the order of
453 30 min – 1 h. However, the differences between selected and control populations in the
454 durations of different pre-adult life-stages, though often not significant, did add up
455 roughly to the overall difference seen in pre-adult development time between selected and
456 control populations in both species.

457 ***Larval traits***

458 Surprisingly, larval feeding rate did not differ significantly between selected and control
459 populations in either species (Table 4). Mean (± s.e.) larval feeding rates in sclerite
460 retractions per min were, in fact, extremely similar between selected and control
461 populations of both species (AB: 128.33 ± 3.13; ACU: 131.55 ± 3.05; NB: 112.76 ± 5.29;
462 NCU: 113.03 ± 4.34). In both species, selected populations showed significantly greater
463 mean (± s.e.) pupation height in cm than control populations (AB: 1.02 ± 0.28; ACU: 1.60
464 ± 0.20; NB: 0.44 ± 0.12; NCU: 1.54 ± 0.11) (Table 4). In case of larval foraging path
465 length, the results varied between species, with *D. ananassae* showing a significant main
466 effect of selection regime while *D. n. nasuta* did not (Table 4). AB populations had mean
467 (± s.e.) foraging path length in cm of 5.52 ± 0.61, compared to 7.38 ± 0.60 in the ACU
468 populations. NB and NCU populations had mean (± s.e.) foraging path length in cm of
469 4.24 ± 0.53 and 4.08 ± 0.43, respectively.

470 ***Larval urea and ammonia tolerance***

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

475 ACU populations had lower survivorship than AB controls across all levels of
476 urea, including 0 g/L, and thus showed no evidence of greater or lesser urea tolerance than
477 the AB controls (Figure 5A), with a significant main effect of selection regime but no
478 significant selection regime × concentration interaction (Table 5). There was suggestive
479 evidence for greater ammonia tolerance in the ACU compared to the AB populations

480 (Figure 6A). At 0 g/L of ammonia in the food, ACU populations had significantly lower
481 survivorship than AB controls, whereas at 30 g/L, the ACU survivorship was higher,
482 though not significantly so, than the AB survivorship (Figure 6A). The selection regime ×
483 concentration interaction was also significant for this assay (Table 5).

484 In the case of *D. n. nasuta*, there was no significant effect of selection regime or of
485 the selection regime × concentration interaction in the urea tolerance assay (Table 5).
486 However, there was a non-significant trend of NCU survivorship being lower than NB at 0
487 g/L and higher than NB at 11 g/L (Figure 5B). In case of ammonia, NCU populations had
488 consistently lower survivorship than NB controls at all concentrations (Figure 6B) and
489 there was no significant effect of selection regime or of the selection regime ×
490 concentration interaction, yielding no suggestion of increased ammonia tolerance in the
491 NCU populations.

492 ***Minimum critical feeding time***

493 In the minimum critical feeding time assay, only significant main effects of selection
494 regime and feeding duration were seen (Table 6), with pre-adult survivorship increasing
495 from feeding durations from 46-55 h, and with ACU survivorship being higher than that of
496 the AB populations at every feeding duration (Figure 7A). At 55 h of feeding as larvae,
497 ACU survivorship was over 50% while that of the AB populations was less than 30%
498 (Figure 7A). Overall, ACU populations seemed to attain similar levels of survivorship
499 approximately 6 h before the AB controls (Figure 7A), which is commensurate with the
500 54.8 h difference between their first and second instar durations combined. These results

501 suggest that larvae in the ACU populations attain their minimum critical size for pupation
502 approximately 5-6 h before their AB controls.

503 In the ANOVA on data from the assay of dry weight at eclosion of individuals that
504 had fed for different durations of time as larvae, corresponding to four different levels of
505 pre-adult survivorship, only the main effects of sex and survivorship were significant
506 (Table 6). On an average, females were significantly heavier than males, and dry weight at
507 eclosion tended to increase with survivorship (Figure 7B). At no survivorship level was
508 there a significant difference between the dry weights of AB and ACU flies (Figure 7B),
509 suggesting that the minimum critical size for pupation in the selected and control
510 populations is probably not different, even though the ACU populations attain it faster
511 than the AB controls.

512 Discussion

513 It is clear that both the ACU and NCU populations did evolve adaptations to larval
514 crowding over the course of selection: in both species, pre-adult survivorship at high
515 density and competitive ability was greater in selected than in control populations (Figure
516 1, Table 1). The overall pattern of correlated responses to selection in the two species was
517 also similar, and, more importantly, different from that reported earlier in case of *D.*
518 *melanogaster* in that it did not involve the evolution of higher feeding rates (Table 4).

519 In clear contrast to what was seen earlier in *D. melanoagster*, both ACU and NCU
520 populations evolved faster pre-adult development time than their controls (Table 2), with
521 the difference being apparent at both low (Figure 2) and high (Figure 3) assay density. The
522 pre-adult development time difference between selected and control populations is entirely

523 due to reduction in the duration of larval instars (Figure 4). In absolute terms, the
524 reductions in pre-adult development time in the selected populations (~14 h in ACU, ~17
525 h in NCU) are quite large. For comparison, populations of *D. melanogaster* subjected to
526 strong directional selection for reduced pre-adult development time showed a reduction of
527 ~16 h in the larval duration, and ~10 h in pupal duration, after 50 generations of selection
528 (Prasad et al. 2001). In the earlier studies on *D. melanogaster*, CU populations showed no
529 difference from controls in development time at low density, whereas they were faster
530 developing than controls when assayed at high density (Borash and Ho 2001). While the
531 *K*-populations did show faster development than *r*-populations when assayed at low
532 density (Bierbaum et al. 1989), this cannot be unequivocally ascribed to density-dependent
533 selection. The *K*-populations were on an overlapping generation maintenance regime and,
534 relative to a discrete generation regime like that of the *r*-populations, this itself would
535 impose direct selection for faster development. If we put the results on life-stage duration
536 (Figure 4) and development time (Figures 2,3) together with those from the minimum
537 feeding time assay (Figure 7), the ACU and NCU populations have evolved faster
538 development, especially in the first two larval instars, enabling them to reach the critical
539 minimum size for pupation about 6 h earlier than controls. This is particularly impressive
540 considering that *D. melanogaster* populations selected directly for rapid development
541 reached the critical size only about 2 h before controls after 50 generations of selection
542 (Prasad et al. 2001). At the same time, the weights of ACU and NCU flies are not different
543 from controls after feeding for different durations close to the minimum feeding time
544 (Figure 7, Table 6). Thus, in terms of time, ACU and NCU larvae are clearly more
545 efficient at converting food to biomass than controls during the first two (and an early part

546 of the third) larval instars, becoming equally heavy adults as controls after feeding for
547 about 6 h less. Of course, whether they are more efficient also in terms of food consumed
548 cannot be determined from these experiments. However, these results do suggest that
549 unlike the *K*- and CU populations, (Mueller 1990; Joshi and Mueller 1996), the ACU and
550 NCU populations have evolved to become more rather than less efficient than controls.
551 The faster development of the ACU and NCU populations, relative to controls, is also
552 interesting in the context of observations that faster development correlates with greater
553 competitive ability across species of *Drosophila* (Krijger et al. 2001), even though direct
554 selection for rapid development leads to the evolution of reduced competitive ability
555 (Shakarad et al. 2005) through reduced larval feeding rate, foraging path length and
556 pupation height (Prasad et al. 2001) as well as reduced larval urea tolerance (Joshi et al.
557 2001).

558 Unlike in *D. melanogaster*, larval feeding rates did not evolve to become greater in
559 the ACU and NCU populations (Table 4), although these populations clearly evolved
560 greater competitive ability than controls (Figure 1). In fact, we measured feeding rates at
561 various points during ACU and NCU selection, and also at various larval stages, and there
562 was never any difference between selected and control populations in either species (data
563 not shown). Interestingly, larval foraging path length was greater in ACU populations than
564 in AB populations, whereas NCU and NB populations had very similar foraging path
565 lengths (Table 4). Thus, at least in *D. ananassae*, the consistent positive relationship
566 between larval feeding rate and foraging path length seen in *D. melanogaster* (Joshi and
567 Mueller 1988, 1996; Sokolowski et al. 1997; Borash et al. 2000; Prasad et al. 2001;
568 Mueller et al. 2005) seems to be uncoupled. Pupation height evolved to become greater in

569 both ACU and NCU populations, relative to controls, mirroring results from the *K*-
570 populations (Mueller and Sweet 1986), but not the CU populations (Joshi and Mueller
571 1996; but see also Mueller et al. 1993 and Joshi et al. 2003). One possible reason for the
572 evolution of increased pupation height in the *K*-populations but not the CU populations
573 has been speculated to be the greater hardness and dryness of the cornmeal based food (*r*-
574 and *K*-populations) compared to banana based food (UU and CU populations). It was
575 suggested that banana food being much softer and more fluid compared to cornmeal food,
576 perhaps the UU populations were also under selection for increased pupation height due to
577 greater risk of pupal drowning on the food surface even at their relatively low rearing
578 density (Joshi et al. 2003). In this context, we note that our ACU/AB and NCU/NB
579 populations are maintained on a relatively hard and dry cornmeal food and this might be
580 the reason for the evolution of greater pupation height in selected populations, relative to
581 controls.

582 Compared to other traits, the results on urea and ammonia tolerance in the selected
583 and control populations were not very clear. While pre-adult survivorship clearly
584 decreased with increasing amounts of urea or ammonia in the food (Figures 5,6, Table 5),
585 there was no clear evidence for greater urea or ammonia tolerance in the selected
586 populations, except for greater ammonia tolerance in the ACU populations, relative to the
587 AB controls (Figure 6A). There was a slight suggestion of a trend towards increased urea
588 tolerance in the NCU populations, relative to NB controls in that NCU survivorship at 0
589 g/L was below that of NB populations and became slightly higher than NB populations at
590 11 g/L (Figure 5B). However, there was no significant selection regime × urea
591 concentration interaction (Table 5), indicating that this is at best a suggestive result. It

592 might be that crowded *D. ananassae* and *D. n. nasuta* cultures have experienced
593 differential levels of urea versus ammonia build-up to which they have adapted.
594 Moreover, we have seen, in other studies with *D. melanogaster*, that the detection of
595 between-population differences in urea/ammonia tolerance is often affected by an
596 interaction between the specific concentrations used and the larval density (Avani Mital,
597 Gitanjali P. Vaidya and Amitabh Joshi, *unpubl. data*). It is, therefore, possible that we
598 were unable to detect significant differences between selected and control populations due
599 to the specific concentrations and larval densities used in our assays of urea and ammonia
600 tolerance. It is also known that larval exposure to urea markedly reduces subsequent
601 fecundity in *D. melanogaster*, and that populations selected for increased larval urea
602 tolerance undergo smaller fecundity declines than controls when reared as larvae on food
603 with urea (Shiotsugu et al. 1997). Thus, it is also possible that the ACU and NCU
604 populations might have evolved greater tolerance to the detrimental effects of urea on
605 fecundity; our experiments did not explore this possibility.

606 Overall, the crowded *D. ananassae* and *D. n. nasuta* populations seem to have
607 evolved greater competitive ability and pre-adult survivorship at high density primarily
608 through a combination of reduced duration of the larval stage, faster attainment of
609 minimum critical size for pupation, greater efficiency of food conversion to biomass,
610 increased pupation height and, perhaps, greater urea/ammonia tolerance. This is in
611 contrast to *D. melanogaster*, in which crowding adapted populations evolve greater
612 competitive ability and pre-adult survivorship at high density primarily through a
613 combination of increased larval feeding rate and foraging path length, at the cost of
614 reduced efficiency of food conversion to biomass, and greater urea/ammonia tolerance

615 (Mueller 1997; Prasad and Joshi 2003; Mueller 2009). Thus, the ACU and NCU
616 populations appear to have responded to crowding in a manner closer to the canonical
617 notion of *K*-selection, in contrast to the primarily α-selection responses shown by the *K*-
618 and CU populations of *D. melanogaster* (see also Dey et al. 2012). Thus, the results
619 underscore the fact that there are, in principle, multiple routes to the evolution of greater
620 competitive ability (Joshi et al. 2001; Dey et al. 2012), something that has also been
621 experimentally demonstrated in the context of inter-specific competition in *Drosophila*
622 (Joshi and Thompson 1995).

623 At this point, we can only speculate about the reason(s) for why the evolution of
624 increased competitive ability in the ACU and NCU populations occurred in a manner so
625 different from that seen earlier in *D. melanogaster*. We believe there are three possible
626 reasons for the observed discrepancy in correlated responses to selection for adaptation to
627 larval crowding across the three species, and the three proposed explanations are not
628 mutually exclusive. First, it is possible that the pattern of genetic variances and
629 covariances among traits relevant to survival in a crowded culture is different from that in
630 *D. melanogaster* in the two species studied here (*D. ananassae* and *D. n. nasuta*). For
631 example, maybe the AB and NB populations do not harbour additive genetic variance for
632 larval feeding rate. Such differences of genetic architecture could simply reflect between-
633 species differences, being the result of historical selection pressures and contingencies.
634 However, if that were the case, we would have expected the results from *D. ananassae* to
635 be closer to *D. melanogaster*, as compared to *D. n. nasuta*, given the phylogenetic and
636 ecological relationships of these species (see Introduction, last paragraph). A second, more
637 likely, possibility is that these differences of genetic architecture between *D. ananassae*

638 and *D. n. nasuta* on the one hand, and *D. melanogaster* on the other, are because the ACU
639 and NCU populations represent selection on relatively recently wild-caught populations
640 (see Materials and methods) whereas the *K*- and CU populations involved selection on
641 populations that had already been in the laboratory for a large number of generations
642 (Mueller and Ayala 1981; Joshi and Mueller 1996). There might be significant differences
643 in the genetic architecture of traits related to fitness under larval crowding between wild
644 and laboratory populations. Finally, it is possible that the discrepancies between the results
645 of this study and those from earlier work on *D. melanogaster* are due to differences in the
646 specific details of the respective maintenance regimes of selected populations in the
647 various studies. Such small differences in selection regimes have been implicated in the
648 different trajectories of pupation height in the *K*- versus the CU populations (Joshi et al.
649 2003). The *K*-populations were maintained on a serial transfer system in half-pint milk
650 bottles with about 350 mL food per bottle (Mueller and Ayala 1981), while the CU
651 populations were maintained in 6 dram vials (2.2 cm diameter) at a density of about 1000-
652 1500 eggs per vial in about 5 mL of food. Unlike in our ACU and NCU populations,
653 neither food amount nor number of eggs per vial were exactly controlled in CU
654 maintenance. In the CU populations, eggs were laid by adult females onto thin films of
655 food on four watch glasses per cage. These films of food with eggs on them were then
656 sliced up and divided roughly equally among 40 vials that each contained about 3 mL of
657 food (A. Joshi, *pers. obs*.). Thus, our ACU and NCU populations had a smaller absolute
658 amount of food per rearing container (bottle/vial) than the *K*- and CU populations, and
659 also differed from the latter in specific densities of eggs per mL of food. It is, therefore,
660 possible that the time course of food depletion and nitrogenous waste build-up in the ACU

661 and NCU cultures is somewhat different from that in the *K*- and CU populations. It has
662 been shown theoretically that optimal feeding rates are likely to decline as the
663 concentration of nitrogenous waste in the food increases (Mueller et al. 1995). Thus, at
664 least in principle, it is possible that the optimal feeding rates in the ACU and NCU
665 populations are actually less than they were for the *K*- and CU populations, and that is
666 why increased feeding rates did not evolve in our experiments. Subsequent studies will
667 attempt to discriminate between these various explanations. However, for the time being,
668 we believe that these results underscore the need for a more nuanced understanding of
669 adaptations to larval crowding in *Drosophila*, with a greater appreciation for the fact that
670 increased competitive ability can be attained through the evolution of fairly different
671 suites of traits. We also need to be cognizant of the fact that seemingly small differences
672 of maintenance in otherwise similar selection regimes might mediate the evolution of very
673 different trajectories in phenotypic space.

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791 **FIGURES**

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

801

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

809

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

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

817

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

825

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

833

834

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

843

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

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

860

	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

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

874

	ACU/AB		NCU/NB	
875				
876 Low density				
877 Effect	$F_{1,3}$	P	$F_{1,3}$	P
878 selection	49.23	<0.01	115.50	<0.01
879 sex	82.32	<0.01	3.75	0.15
880 selection × sex	1.93	0.26	18.61	0.02
881 High density				
882 Effect	$F_{1,3}$	P	$F_{1,3}$	P
883 selection	60.37	<0.01	364.65	<0.01
884 sex	41.18	<0.01	13.09	0.04
885 selection × sex	8.58	0.06	5.49	0.10

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

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

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

905

906

	ACU/AB		NCU/NB	
907 Trait	$F_{1,3}$	P	$F_{1,3}$	P
908 feeding rate	0.54	0.52	0.06	0.82
909 foraging path length	94.51	<0.01	0.48	0.54
910 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	$F_{1,3\ sel/\ 2,6\ else}$	P	$F_{1,3\ sel/\ 2,6\ else}$	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	$F_{1,3\ sel/\ 2,6\ else}$	P	$F_{1,3\ sel/\ 2,6\ else}$	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

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

934

	Survivorship		Dry weight	
Effect	$F_{1,3\ sel/\ 3,9\ else}$	P	$F_{1,3\ sel/\ 3,9\ else}$	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

948 Figure 1.

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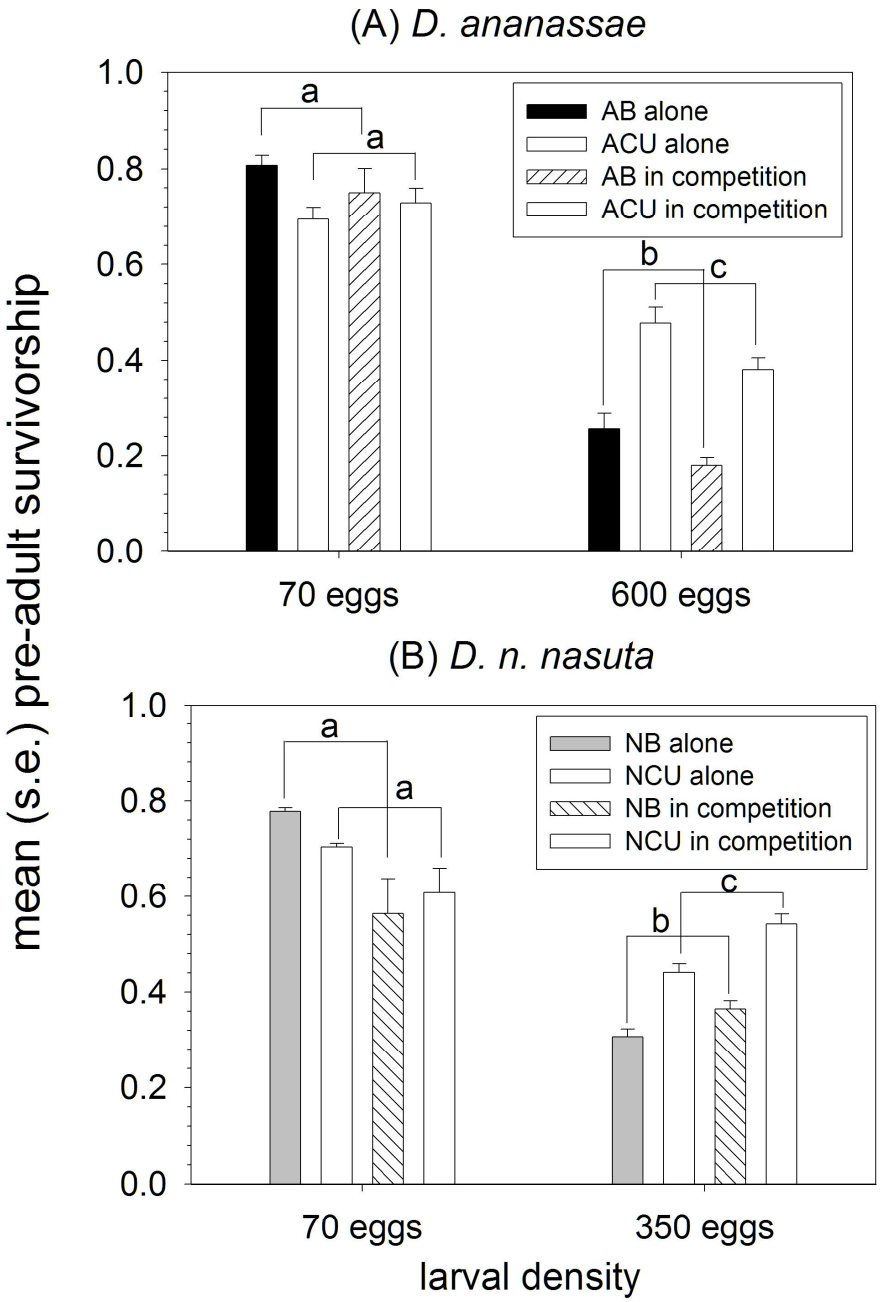

965 Figure 2.

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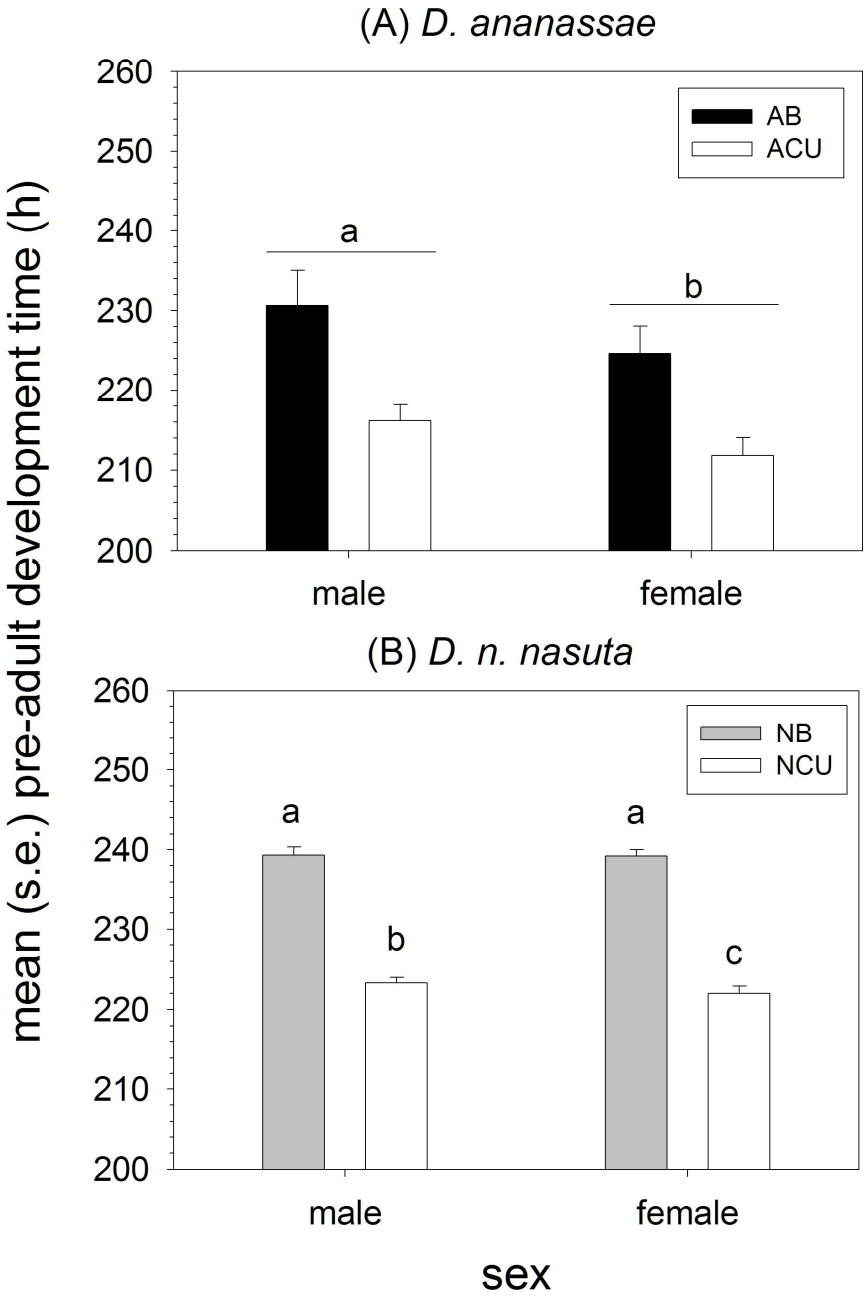

984 Figure 3.

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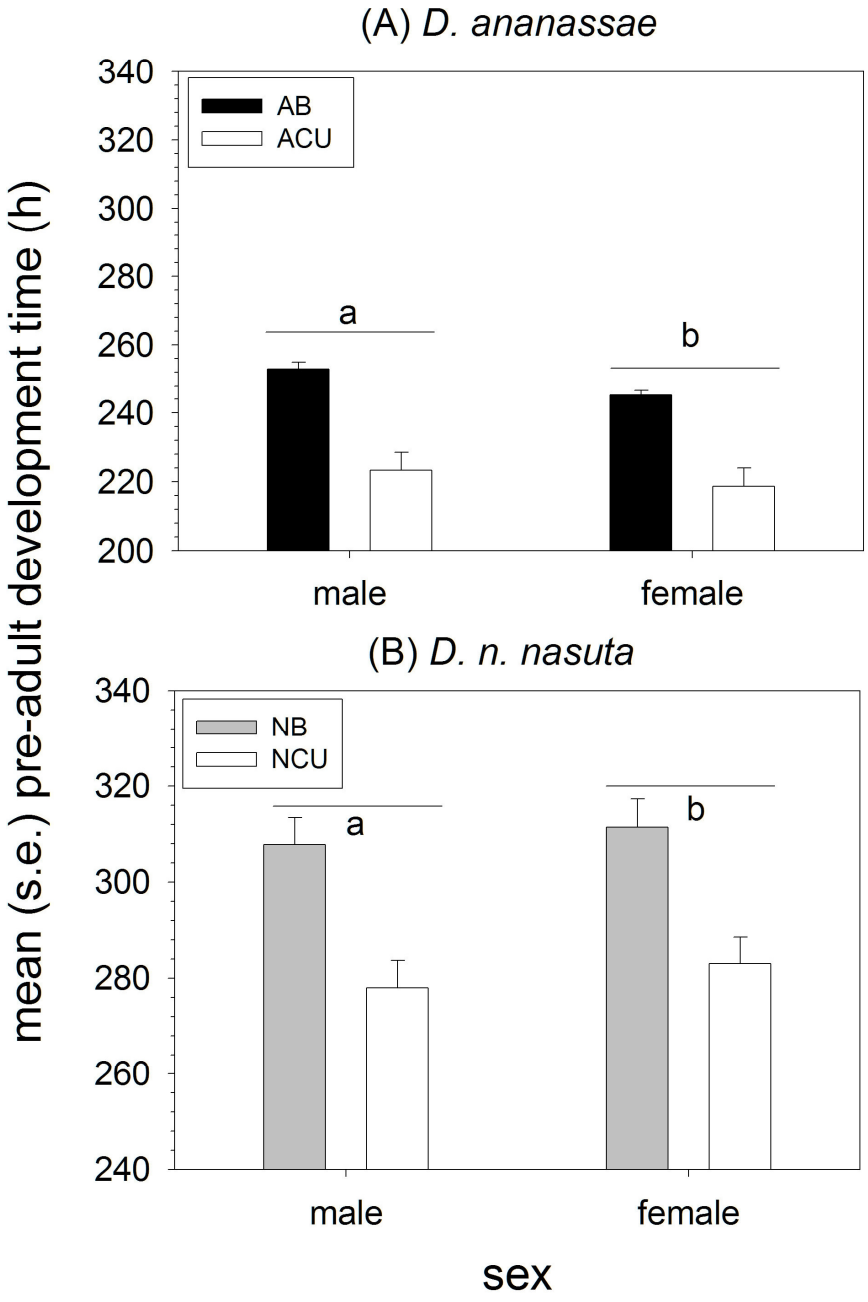

1004 Figure 4.

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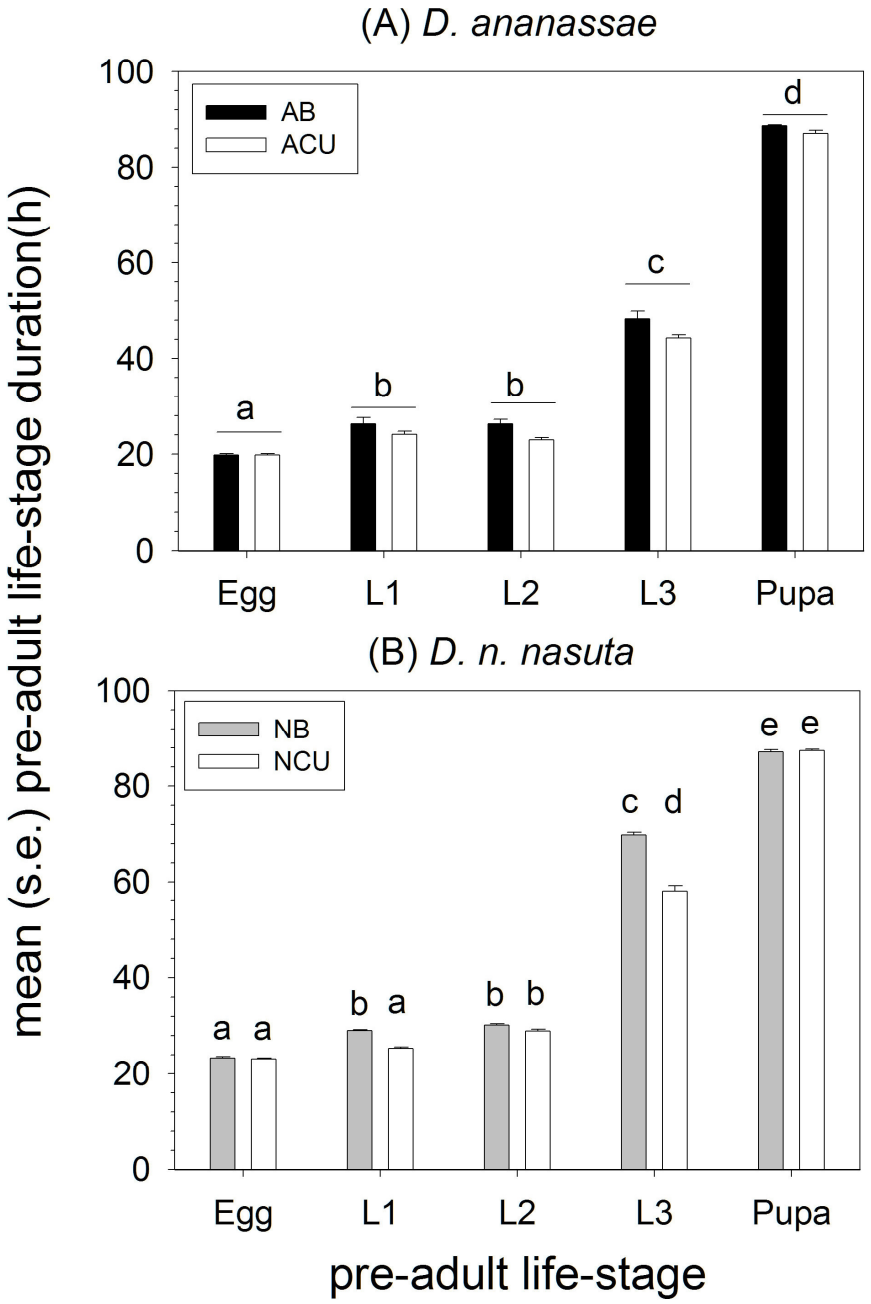

1021 Figure 5.

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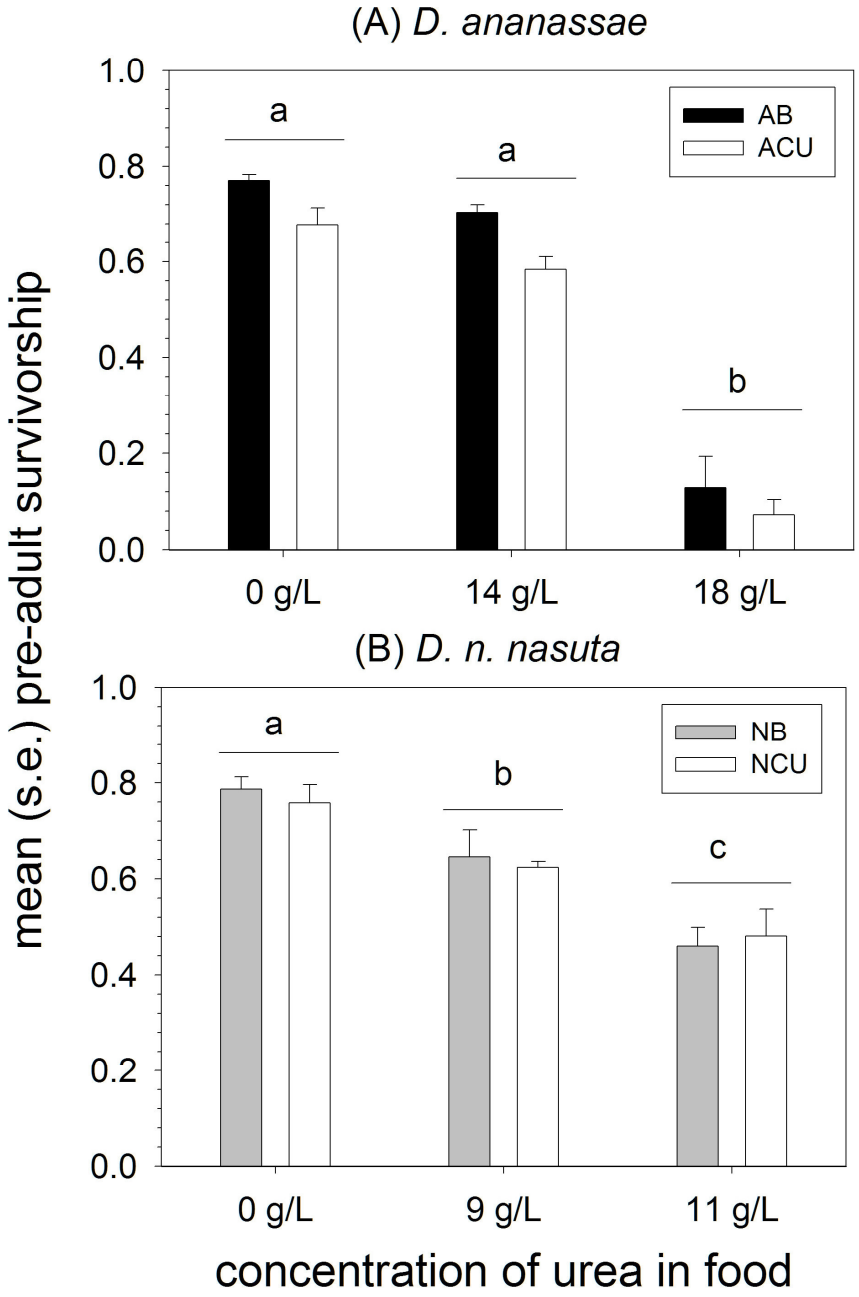

1040 Figure 6.

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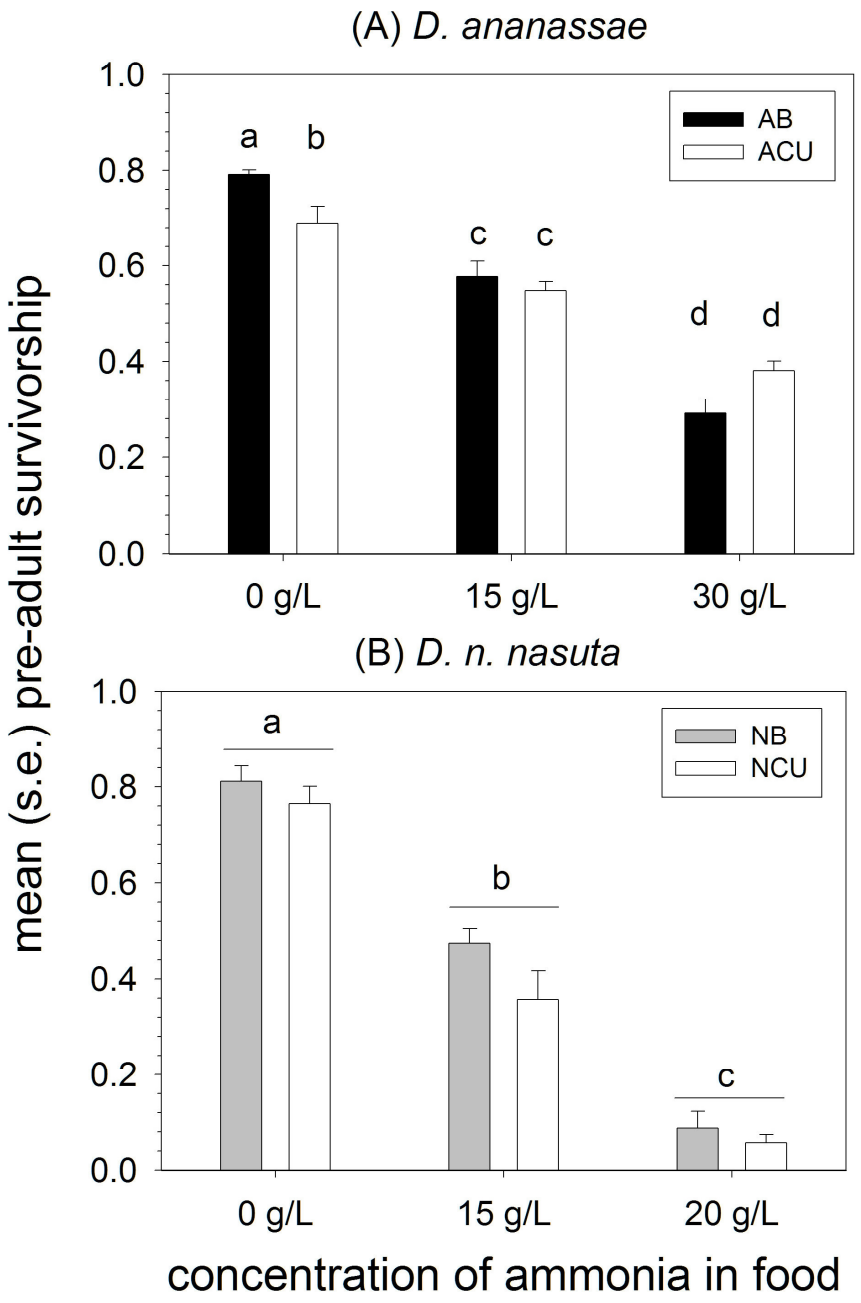

1059 Figure 7.

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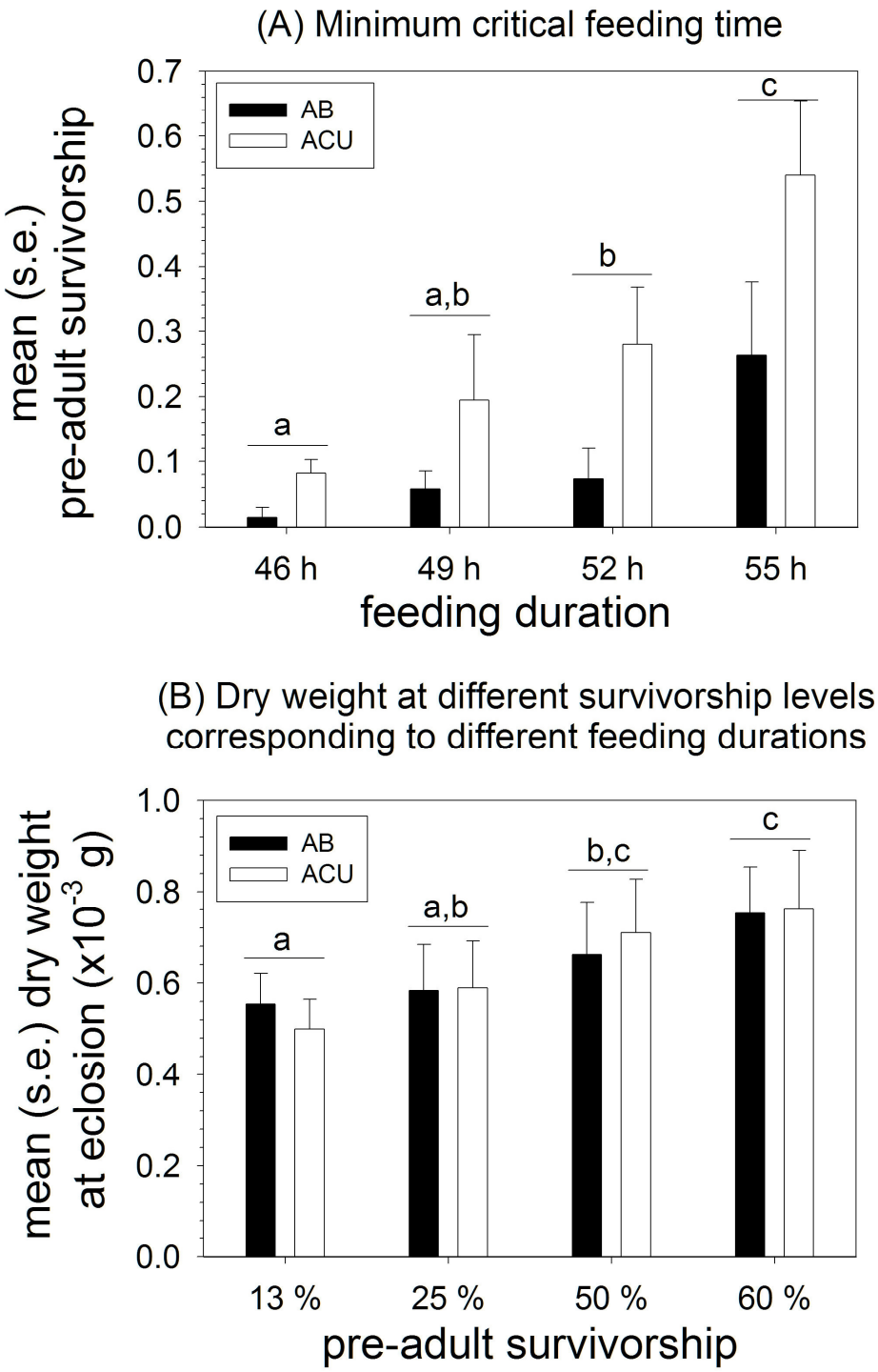
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