Selection for desiccation resistance in adult *Drosophila melanogaster* affects larval development and metabolite accumulation

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Summary

We studied larval development and acquisition of metabolic resources in *Drosophila melanogaster* selected for adult desiccation resistance. Desiccation-selected (D) flies had longer developmental times in comparison with control populations, resulting in significantly higher body mass. No differences were found in larval growth rates, suggesting that increased body mass results from the extended larval feeding period. Larvae from both D and control lines molted from second to third instar 81–84 h after egg laying, whereas D larvae pupated 5–6 h later than controls. This indicates that selecting adults for desiccation resistance results in longer third larval instar. Newly eclosed D flies had higher carbohydrate and water contents in comparison with control populations. No differences were found in body mass, water or metabolite contents of newly eclosed selected and control flies after larvae were prevented from further feeding from 96 h after egg laying onwards. This shows that differences in accumulated carbohydrate and water stores are a result of evolved differences in the duration of third larval instar. The contribution of third instar feeding to desiccation resistance of adult selected flies is higher than that of controls. Thus, selection for adult stress resistance has resulted in correlated changes in larval physiology.

Key words: *Drosophila melanogaster*, desiccation resistance, developmental time, critical weight, life history, water, carbohydrates.

Introduction

Insects face a major challenge to conserve body water as a result of their high surface area to body mass ratio. Together with behavioral avoidance strategies (e.g. burrowing and nocturnal activity), desiccation resistance can be enhanced by one or more of the following physiological adaptations: increased water stores, lowered water loss rate and/or enhanced dehydration tolerance. Similarly, starvation resistance can be achieved by increased energy stores and/or decreased metabolic rates.

Drosophilidae have been employed in stress resistance studies of both natural populations and laboratory selected lines (Gibbs et al., 1997; Gibbs et al., 2003; Chippindale et al., 1998; Hoffmann and Harshman, 1999; Gibbs, 2002; Marron et al., 2003). These have shown that evolved differences in management of body water and energy stores are correlated with stress resistance.

Stress (desiccation and starvation)-selected *Drosophila melanogaster* are heavier than unselected controls immediately after eclosion, and maintain this advantage during early adulthood (Chippindale et al., 1998). Soon after eclosion, desiccation-selected flies (D) have greater carbohydrate content in comparison with starved controls, whereas the latter have relatively high lipid content. It has been suggested that the increased accumulation of glycogen in larvae of D flies is advantageous in contributing to increased body water content (Gibbs et al., 1997; Chippindale et al., 1998) (but see Folk et al., 2001), as glycogen binds three to five times its weight in bulk water (Schmidt-Nielsen, 1997). Carbohydrates also serve as the primary source of energy in adult flies during desiccation stress (Marron et al., 2003). Therefore, increased accumulation of carbohydrates during larval feeding could result in enhanced desiccation resistance of the newly eclosed fly. However, Chippindale et al. (Chippindale et al., 1998) collected adult flies at 0–6 h following eclosion, and therefore their reported body metabolite contents may reflect both accumulated larval stores and early adult feeding. These authors also reported significantly longer egg to adult developmental times for desiccation-selected *D. melanogaster* in comparison with control populations. Although selected for stress resistance as adults, differences between newly eclosed selected and control flies may indicate that selection affects their developmental physiology. However, no significant relationship was found between selection for desiccation resistance in adults and larval developmental time in other reports (Hoffmann and Parsons, 1993; Bubliy and Loeschcke, 2005).

Adult insects do not grow, and their size is determined by the size at which the last instar larva begins metamorphosis

(Nijhout, 2003). The timing of metamorphosis is determined by reaching a critical developmental stage, characterized by a small pulse of ecdysteroid secretion (Nijhout, 1981), after which completion of metamorphosis no longer depends on resource availability (Bakker, 1959). The development of *D. melanogaster* is divided into a flexible stage until larvae commit to pupation early in the third instar, followed by a fixed growth period until pupariation (Bakker, 1959). Reaching the critical developmental stage of committing to pupation has been associated with the attainment of a critical body mass, or 'critical weight' (de Moed et al., 1999).

In this study we examined the growth and developmental pattern of desiccation-selected *D. melanogaster* and compared it with those of starved and fed control populations. Previous studies varied in methodology, most notably in different time intervals used for determination of developmental time (counting eclosing adults every 6–24 h). We report more precise developmental time values under our experimental conditions by collecting emerging adults at hourly intervals. The main objectives of this study were to: (i) determine whether selected and control lines differ in larval developmental time; (ii) if these differences occur, determine whether they are also reflected in a delayed commitment to pupation in larvae of desiccation selected flies; (iii) establish whether a possible delay in the commitment of D larvae to pupation reflects lower growth rate earlier in development, or a shift in the critical mass associated with initiation of metamorphosis; and (iv) assess the contribution of longer larval development to the overall higher desiccation resistance of adult D flies.

Materials and methods
Fly selection and maintenance

All populations of *Drosophila melanogaster* Meigen were maintained at 24.5°C under constant light. We used a fly population founded from ~400 females collected in New Jersey, USA in 1999. Flies were maintained as a large outbred population in the laboratory until selection began. To minimize the possibility of artifacts due to adaptation to a new environment, the populations were maintained on a standard 3-week stock cycle for 12 generations before selection was started (Chippindale, 2006). Pre-adult stages were reared at densities of ~60 larvae in vials containing 10 ml of corn meal–sucrose–yeast medium. After 2 weeks, adult flies (approximately 4 days post-eclosion) were transferred to 5.5-l Plexiglas population cages containing two Petri dishes of food. A cloth sleeve covered one end and allowed access to the cage. The medium was changed every 2 days. After 4 days, yeast paste was added to stimulate egg production. Approximately 1200 eggs were collected after 7 days to found the next generation.

Selection for desiccation resistance was performed by removing food from the cages 1–4 h after transferring the flies. A cheesecloth-covered dish containing ~200 g of silica gel desiccant was placed inside, and the open end of the cage was loosely covered with plastic wrap to allow gas exchange while reducing influx of water vapor from the surroundings. Initially, the cages contained ~7500 flies. They were checked hourly until 80–85% of the flies had died. The desiccant was then removed and fresh food was provided for the survivors. The flies were given several days to recover before egg collection for the next generation.

Because desiccation selection required the removal of both food and water, each selection line (D) was matched to a starved control line (S), whose cage received two agar plates instead of desiccant. At each generation, each of these stocks was starved for the same length of time. To control for the effects of starvation stress, each pair of stressed populations had a matched, unstressed, fed control population (F). Population sizes in all treatments were maintained to provide an estimated 1000–1500 adults after selection. Flies were selected as described above for the first 30 generations following the initiation of selection. This was followed by 25–30 generations of less severe selection before the experiments were carried out. During this time, desiccation resistance was maintained by subjecting flies to desiccation for 24 h after transferring them to the cages, a treatment that kills nearly all control flies (A.G.G., personal observation). It has been shown previously that desiccation resistance of similar populations was not compromised even after 35 generations of relaxed selection (Passananti et al., 2004). Three replicate populations (D_A–D_C, S_A–S_C, F_A–F_C), sharing a common ancestry, were maintained from each of the selected and control populations.

Larval growth and development

Egg collection for each assay was carried out using selected and control flies of the same age (usually 4–14 days), and of the same selection generation. Adults from each of the nine fly populations were transferred to empty 175-ml bottles for a 1 h. The bottles were covered with a 35×10 mm Petri dish containing grape agar as a substrate for egg laying. Sets of 80 eggs were then collected and placed in food vials containing approximately 10 ml of cornmeal food. The vials were incubated in a controlled temperature chamber at 24.5°C and constant light, and larvae were collected at 6-h intervals, between 72 and 108 h after egg laying (AEL). Thirty larvae were transferred to 1% agar vials for determination of commitment to pupation (see below). Ten sets of three larvae were rinsed in water to remove food particles, and immediately frozen at –20°C for future measurements of body mass and water contents (N=30 for each of the nine populations, for each time point). After thawing, the larvae were blotted dry on an absorbent tissue and weighed to the nearest 0.001 mg (C30 Cahn microbalance, Cerritos, CA, USA). The larvae were then dried at 60°C overnight for dry body mass and water content determination. Wet and dry body mass of individual wandering larvae was measured after male and female larvae from each of the experimental populations (N=8 for each sex) were collected from the inner surface of the food vials. Vials were checked every 1.5 h for emergence of wandering larvae from

the food. Gender identification of wandering larvae was based on gonad morphology (Folk et al., 2001).

Larvae from additional vials (N=60 for each population) were frozen for determination of developmental stage as a function of larval age. Larval instar was determined based on the morphology of the mouthparts and anterior spiracles (Ashburner, 1989). We staged larvae at 3-h intervals from 72 and 84 h AEL, after preliminary experiments indicated that all larvae were second instars at 72 h AEL, whereas practically all larvae had molted to third instar by 84 h AEL.

Rates of commitment to pupation during development were measured by transferring 30 larvae from each population to vials containing non-nutritious agar at the same 6-h intervals (72–108 h AEL). The agar vials were then placed back in the incubator, and the eventual pupation rates reflected the rates of commitment to pupation at time of transfer. Additional sets of food vials with 80 larvae each (two vials for each of the nine populations) were used to determine pupation rates at 6-h intervals, ranging from 118 to 160 h AEL.

Larval feeding regimes

In order to quantify the contribution of third instar feeding to evolved desiccation resistance, newly eclosed flies from the nine experimental populations (three replicates each of D, S and F) were collected following one of two larval feeding regimes. In one treatment, larvae were transferred from food to agar vials at 96 h AEL after preliminary data (see Results) had shown that larvae from all nine populations commit to pupation prior to this time point. In the control treatment, larvae were allowed to feed *ad libitum* throughout larval development.

Developmental time analysis

Egg collection and incubation was carried out as described above. Newly eclosed flies were collected at hourly intervals, and egg to adult developmental time was calculated.

Desiccation resistance analysis

Following eclosion, the flies were transferred to empty vials individually, and restricted to the lower half of the vials by a foam stopper. Silica gel was then added above the stopper to maintain low humidity, and the vial was sealed with Parafilm. The vials were placed back in the incubator and mortality was recorded at hourly intervals, after which the dead flies were sexed.

Metabolic fuel assays

Newly eclosed flies, from both feeding experimental groups, were collected as described above and immediately frozen at –20°C. After thawing, the flies were sexed, and individual flies were weighed (±0.001 mg) and then homogenized in microfuge tubes containing 200 µl 0.05% Tween 20 (in water) using a hand-held grinder. The tubes were then incubated at 70°C for 5 min to prevent lipase activity. The samples were then centrifuged for 1 min at 16 000 g, and the supernatant was removed to new tubes which were frozen until measurements.

Carbohydrates

Triplicates (10 µl) from each sample were loaded on 96-well microplates, and 10 µl of *Rhizopus* amyloglucosidase (0.8 mg ml^{-1}; A-7255, Sigma-Aldrich Co., St Louis, MO, USA) were added to each to catalyze the conversion of glycogen and trehalose into glucose (Parrou and Francois, 1997). The plates were then left overnight at room temperature. The following day, 90 µl of liquid glucose reagent (Pointe Scientific Inc., Canton, MI, USA) were added to each sample, and absorbance at 340 nm was measured using a SpectraMax Plus^{384} microplate reader (Molecular Devices, Sunnyvale, CA, USA). Carbohydrate concentrations were determined using standards of known glycogen concentration.

Triglycerides

Triglyceride content was measured using Serum Triglyceride Determination Kits (TR 0100, Sigma-Aldrich Co.). Triplicate samples (30 µl each) were placed in microplates, and 100 µl free glycerol reagent was added, before absorbance was read at 540 nm. Then 25 µl of triglyceride reagent was added, and the plates were allowed to sit in room temperature for 15 min, before absorbance was read again at 540 nm. The amount of triglycerides was calculated as the difference between free glycerol levels before and after the use of the triglyceride reagent, using standards of known glycerol concentration.

Protein

Supernatants were diluted with water at 1:1 and 1:2 ratios for the food deprivation and *ad libitum* feeding treatments, respectively. To measure protein content, 8 µl triplicates from each sample were loaded on a microplate, and 200 µl of protein assay reagent (50 parts bicinchoninic acid solution to 1 part 4% CuSO$_4$) were added. The plates were then incubated overnight at room temperature, and absorbance at 562 nm was measured the following day. Protein concentrations were determined using standards of bovine serum albumin (82516, Sigma-Aldrich Co.).

Water content

Newly eclosed flies were collected as described above following the two larval feeding regimes, and frozen at –20°C for future measurements. The flies were then thawed, and their wet mass was measured (±0.001 mg). The flies were then dried at 60°C overnight before dry mass was determined. Water content was calculated as the difference between the two measurements.

Statistics

Unless stated otherwise, data were analyzed as follows. Larval wet and dry mass were determined without sexing the larvae first, and therefore analyzed by two-way ANOVA, with replicate populations treated as a random effect and selection treatments as a fixed effect. Wandering larvae were sexed before mass determination and therefore three-way ANOVAs were used with sex and selection treatment as fixed effects and

replicate populations as a random effect. Developmental and desiccation times, body mass and water, and metabolic fuel contents of newly eclosed flies were analyzed similarly. Protein content was used as a covariate for comparisons of carbohydrate and triglyceride contents. Dry body mass was used as a covariate for comparisons of body water contents. Tukey's HSD tests were used for *post-hoc* comparisons of means following ANOVA/ANCOVAs. In some of the assays (as stated in the Results section), when high variability between replicate populations prevented drawing clearer conclusions, data were pooled before further between-treatments analyses.

Frequencies of second molt, commitment to pupation and pupation rates at different time points during development were analyzed using a χ^2 test, with selection treatment and replicate population as main effects. Statistical analysis was carried out using STATISTICA for Windows software (version 7.1).

Results
Larval growth and development

No significant differences ($P>0.05$) were found in either wet or dry body mass (Figs 1, 2) of desiccation selected (D), starved (S) and fed (F) control larvae between 72–108 h AEL. One exception was a lower wet body mass of S larvae in comparison with that of F larvae at 96 h AEL. Wandering D larvae tended to have higher wet and dry masses (1.983±0.043 g and 0.529±0.011 g, respectively; mean ± s.e.m.; N=48) than S (1.850±0.037 g; 0.501±0.010 g) and F (1.773±0.037 g and 0.478±0.010 g) larvae, although these differences were not statistically significant (F=5.7 and 5.8 respectively; P=0.07). As a result of their longer developmental time (see below), D larvae emerged from the food later than larvae of both control populations.

Second molt frequencies at different time points, presented in Fig. 3, show that larvae from all three selection treatments reach 50% molt rate between 81 and 84 h AEL. No significant
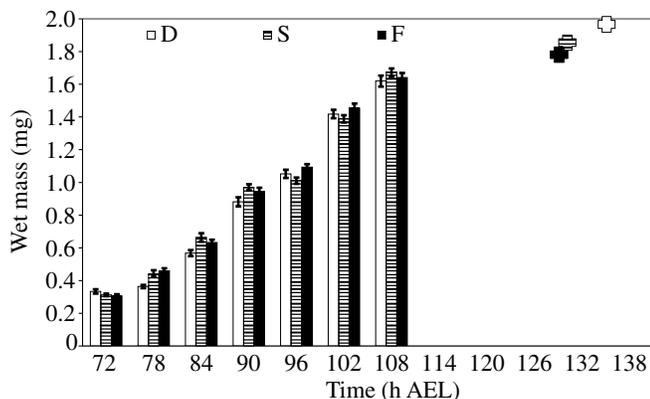
differences in frequencies of second molt were found among the three populations at either 81 or 84 h AEL ($P>0.05$).

Timing of commitment to pupation was expressed by the pupation rates of larvae transferred from food to agar vials at 6-h intervals throughout larval development. Pupation rates were relatively low (<10%), and similar among the three fly lines, when larvae were transferred to agar up to 78 h AEL (Fig. 4). Rates of commitment to pupation increased rapidly afterwards, and when transferred to agar 96 h AEL pupation rates exceeded 95% in larvae of all three experimental lines. Interestingly, pupation rates of D larvae were significantly lower than those of S and F larvae when transferred to agar 90 h AEL (P=0.02; Fig. 4).

D larvae had significantly longer egg to pupae developmental time, with significantly lower pupation frequencies at 130 and 136 h AEL (Fig. 5; $P<0.001$). The mean (± s.e.m.) egg to pupa developmental time, calculated by a logistic regression of pupation rates at the different time points,

Fig. 2. Larval dry mass (mean ± s.e.m.; mg) of desiccation selected (D), and starved (S) and fed (F) controls as a function of developmental time. Crosses indicate mean dry masses of wandering larvae at the estimated time of 50% pupation.
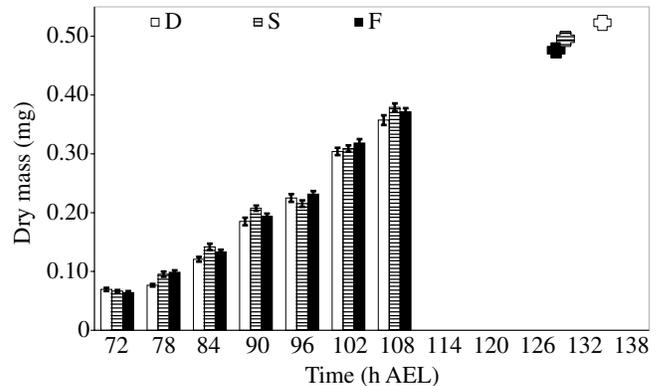
Fig. 1. Larval wet mass (mean ± s.e.m.; mg) of desiccation selected (D), and starved (S) and fed (F) controls as a function of developmental time. Crosses indicate mean wet masses of wandering larvae at the estimated time of 50% pupation.
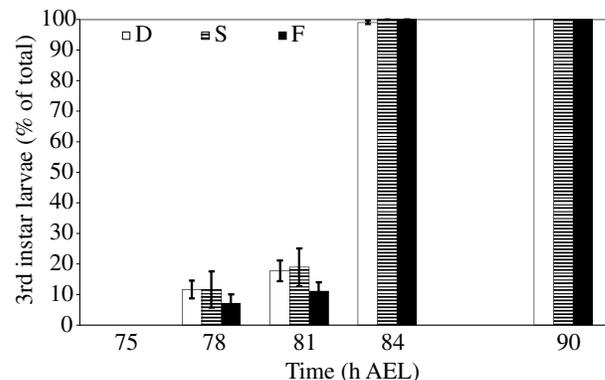
Fig. 3. Second molt rates, expressed as the percentage (± s.e.m.) of third instar larvae from the sampled population, as a function of developmental time. D, desiccation selected; S, starved and F, fed controls.
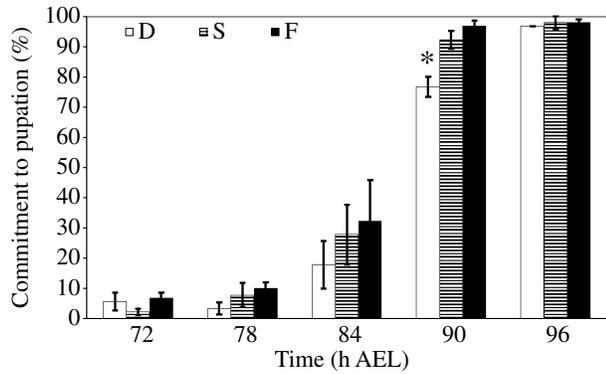
Fig. 4. Mean rates of larvae commitment to pupation, of desiccation selected (D), and starved (S) and fed (F) controls expressed as percentages (± s.e.m.), as a function of developmental time. *$P<0.05$.
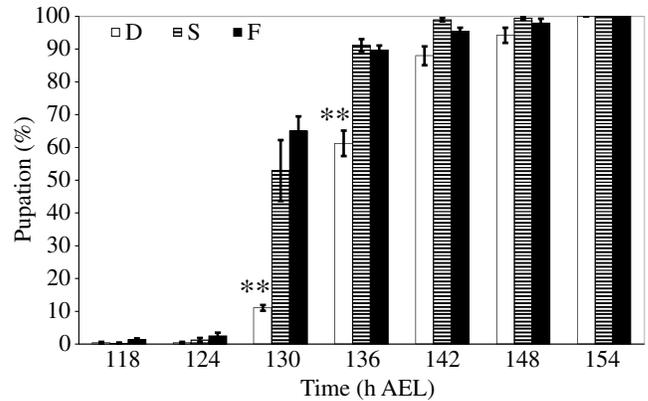

Fig. 5. Pupation rates (mean ± s.e.m.) of desiccation selected (D), and starved (S) and fed (F) controls as a function of developmental time. **$P<0.001$.

was 134.9±0.2 h for D larvae, compared with 129.8±0.3 h and 128.9±0.2 h for S and F larvae, respectively.

Larval feeding regimes

Developmental time

Egg to adult developmental time of both males (241.3±1.1 h, $N=35$) and females (235.6±0.9 h, $N=52$) was significantly longer in D larvae compared with S (236.5±0.8 h, $N=58$; 231.9±0.8 h, $N=66$) and F (235.2±0.8 h, $N=69$; 231.6±0.8 h, $N=69$) control groups following *ad libitum* larval feeding ($F=17.6$; $P<0.001$). No significant differences were found between replicate populations of each of the selection treatments ($F=2.0$; $P>0.05$). Developmental time of male flies was significantly longer than that of females ($F=40.7$; $P<0.001$).

Desiccation resistance

Significant differences in desiccation resistance of newly eclosed flies were detected under both larval feeding regimes in the order D>S>F (Table 1) ($F=31.8$; $P<0.001$). As in developmental time measurements, no differences were found between replicate populations of each of the selection treatments ($F=0.1$ and 0.2 for the two feeding regimes; $P>0.05$). Transfer of D larvae to agar vials 96 h AEL resulted in a decrease of 13–14 h in desiccation resistance time compared with 7.5–9.3 h in S and F flies.

Body mass and metabolite content

Table 2 summarizes wet mass and metabolic fuel contents of newly eclosed flies. When allowed to feed throughout larval development, the newly eclosed D flies had significantly higher wet mass in comparison with starved and fed controls ($F=108$; $P<0.001$). Note that a similar, albeit statistically insignificant pattern was seen in wandering larvae (see above). Female flies were significantly larger than males ($F=811$; $P<0.001$). No significant differences were found between selection treatments in carbohydrate contents when using replicate populations as a random effect ($F=2.1$; $P=0.23$). However, D flies consistently contained more carbohydrates than their replicate controls. The mean (± s.e.m.) values of pooled samples were 35.6±1.2 μg for D (35.7±2.5, 37.9±1.7 and 33.2±1.7, for D_A-D_C, respectively), 29.3±1.3 μg for S (27.2±2.3, 34.9±2.1, 25.8±1.4) and 30.4±1.1 μg for F (24.3±1.3, 33.3±1.6, 33.6±1.7). Pooling replicate populations' data resulted in significantly higher carbohydrate levels for newly eclosed D flies in comparison with S and F ($F=7.9$; $P<0.001$). No differences were found in triglyceride contents of selected and control flies ($F=1.4$; $P>0.05$). Males and females did not differ significantly in either triglyceride or carbohydrate contents ($F=3.3$ and 0.2 respectively; $P>0.05$).

Mean body mass of newly eclosed female flies was significantly higher than that of males even when larvae were

Table 1. *Desiccation resistance of desiccation selected, starved and fed controls following the two experimental larval feeding treatments*

| | Desiccation resistance (h) | | | | | |
| | *Ad libitum* | | | 96 h AEL | | |
Sex	D	S	F	D	S	F
Male	34.5±0.8 (35)	26.3±0.7 (58)	24.3±0.7 (69)	21.5±0.7 (46)	18.8±0.7 (47)	16.1±0.7 (54)
Female	35.8±0.7 (52)	27.9±0.7 (66)	26.0±0.6 (69)	22.1±0.6 (62)	18.6±0.7 (45)	17.1±0.7 (42)

Ad libitum, larvae left to feed until pupation; 96 h AEL, larvae removed from food at 96 h AEL; D, desiccation selected; S, starved; F, fed. Values are means ± s.e.m. (*N*).

Table 2. *Wet body mass, protein, carbohydrate and triglyceride contents of newly eclosed male and female flies following the two larval feeding regimes*

	Ad libitum			96 h AEL		
	D	S	F	D	S	F
Males						
Wet body mass (mg)	0.964±0.014	0.917±0.017	0.881±0.017	0.476±0.019	0.498±0.018	0.481±0.011
Protein (µg)	36.4±2.0	34.0±1.5	32.3±1.8	20.6±0.8	24.5±0.8	24.6±1.2
Carbohydrates (µg)	34.0±1.8	29.0±2.0	27.6±1.5	21.3±2.0	18.1±2.3	19.5±1.8
Triglycerides (µg)	18.7±0.9	15.6±0.7	14.8±1.0	1.8±0.3	3.3±0.7	3.9±0.7
Females						
Wet body mass (mg)	1.245±0.032	1.169±0.022	1.113±0.014	0.598±0.034	0.560±0.023	0.588±0.011
Protein (µg)	45.8±1.9	44.1±2.0	41.1±1.6	24.0±1.4	25.6±0.9	23.3±0.6
Carbohydrates (µg)	37.2±1.4	29.6±1.6	33.2±1.3	21.0±2.3	19.8±1.8	25.9±2.7
Triglycerides (µg)	20.5±0.9	20.7±1.4	17.8±1.0	2.5±0.6	3.2±0.5	2.7±0.3

Ad libitum, larvae left to feed until pupation; 96 h AEL, larvae removed from food at 96 h AEL; D, desiccation selected; S, starved; F, fed. Values are means ± s.e.m.; N=24 for all groups.

deprived of further feeding at 96 h AEL (F=29.9; P<0.05), but the effect of sex on triglyceride and carbohydrate contents was not significant (F=1.5; P>0.05). This larval feeding regime resulted in similar mean body mass values (F=0.01; P=0.99), as well as similar triglyceride and carbohydrate contents (F=1.1 and 1.5 respectively; P>0.05) for newly eclosed selected and control flies (Table 2).

Water content

Mean wet and dry body mass and water contents are summarized in Table 3. Significant differences in wet body mass of newly eclosed flies were found in the order D>S>F (F=12.4; P<0.05). Selection treatment also had a significant effect on dry body mass (F=17.0; P<0.05). However, no significant difference in dry body mass was found between newly eclosed D and S flies, whereas both were significantly larger than F (α=0.05). As in wet body mass data, differences in water content were in the order D>S>F (F=7.2; P<0.05).

No differences were found between selection treatments in wet body mass (F=0.5; P=0.6), dry body mass (F=0.8; P=0.5) or water content (F=0.1; P=0.9) of newly eclosed flies when larvae were transferred to non-nutritious agar vials at 96 h AEL.

Discussion

Mechanisms of desiccation resistance in *Drosophila*, in both wild and laboratory selected populations, have been extensively studied [for a review of studies, see Gibbs (Gibbs, 2002)]. Selection for desiccation resistance in laboratory-reared populations has been performed on adult flies, and previous studies have largely focused on the evolved responses of adult flies to stressful environment. Nevertheless, there is some evidence that selection for desiccation resistance of adult flies results in altered larval developmental physiology (Chippindale et al., 1998).

Our results show longer developmental time for D larvae in comparison with that of their controls. This is in agreement with the findings of Chippindale et al. (Chippindale et al., 1998), but not with other studies (Hoffmann and Parsons, 1993; Bubliy and Loeschcke, 2005). However, previous studies differed in methodology, including nutrition, temperature, selection regime (duration of exposure to stress, number of generations) and larval rearing density. Differences in these environmental factors result in varying patterns of responses to selection in life history traits (Prasad and Joshi, 2003), and could be responsible for the apparent inconsistent responses of larval developmental time to desiccation selection. Moreover, we assayed eclosion at hourly intervals and were able to detect a ~5–6 h difference in developmental time between selected and control flies. These differences could have been masked in previous studies where developmental time was determined by collecting eclosing adults every 6 h (Bubliy and Loeschcke, 2005) or 24 h (Hoffmann and Parsons, 1993).

The size of adult insects is determined by larval developmental time and growth rate. Prolonged development results in increased food consumption (assuming unchanged growth rate), thus increasing resources for the adult insect. Wet and dry mass measurements at 6-h intervals throughout larval development (Figs 1, 2) indicate that the higher body mass of newly eclosed D flies results from their extended larval developmental time, which is not coupled with increased growth rate. It is important to note that despite their shorter development times, body mass of newly eclosed females is significantly higher than that of males (Tables 2, 3). This could be explained by a higher growth rate of females during late developmental stages (Partridge et al., 1994).

Despite their longer egg to adult developmental time, D larvae did not have a delayed second molt (Fig. 3). Our data show that larvae of both desiccation selected and control lines molt from second to third instar 81–84 h AEL on average. Egg to pupa (Fig. 5) and egg to adult developmental times show similar differences between selected and control lines, suggesting a similar length of pupal stage for all three lines.

Thus, the observed differences in total developmental time between selected and control populations result from an extended third larval instar stage.

Larvae of *D. melanogaster* reach a 'critical stage' early in the third instar, after which they will pupate and then eclose even if prevented from further feeding (Bakker, 1959). It was therefore interesting to determine whether the longer developmental time of D larvae coincides with a delay in reaching the irreversible developmental stage of commitment to pupation. Our results show (Fig. 4) that D larvae have significantly lower rates of commitment to pupation 90 h AEL in comparison with control populations. The critical stage for pupation is associated with attaining a relatively constant 'critical weight' for pupation (Robertson, 1963). Interestingly, no significant difference was found in either wet or dry mass of D, S and F larvae 90 h AEL (*P*=0.32 and 0.33, respectively). Therefore, it appears that selection for desiccation resistance in adult *D. melanogaster* has resulted in a shift in the 'critical weight' associated with the commitment of the larvae to complete development and pupate. Observed differences in total developmental times, together with the fact that the growth period following commitment to pupation is fixed in *D. melanogaster* (Santos et al., 1997), support the notion of a shift in the critical stage as a result of selection for desiccation resistance. It is interesting to note that differences in the 'critical weight' have been shown to occur between natural populations of *D. melanogaster* from different geographical regions, highlighting the existence of genetic variation in this developmental trait (de Moed et al., 1999). An increase in body size threshold and an extended last larval instar have also been shown to occur following the introduction of the dung beetle *Onthophagus taurus* to a new habitat, and were shown to result from an evolutionary modification of sensitivity to juvenile hormone (Moczek and Nijhout, 2002).

In this study, wet body mass of newly eclosed D flies was significantly higher than that of control populations. However, no significant difference was found between the dry mass of D and S flies. This is in agreement with a previous report in which wet body mass of newly eclosed D flies was higher than that of starved controls, but no differences were found in dry mass (Chippindale et al., 1998). Therefore, it appears that selecting adult flies for desiccation resistance affects water management during earlier developmental stages, resulting in higher body water content of newly eclosed flies (Table 3).

Although larval development of *D. melanogaster* was clearly affected by adult selection, it was still unclear whether the observed developmental changes contributed to the increased fitness of the selected flies under desiccation conditions. Results from the desiccation assay emphasize the contribution of the third instar larval feeding to stress resistance performance of adult flies. As was expected for all three experimental fly lines, our results indicate that newly eclosed flies are considerably more desiccation resistant when fed *ad libitum* during larval stages in comparison with newly eclosed flies from the food deprivation treatment. However, newly eclosed D flies are significantly more resistant to desiccation than their controls, even when prevented from completing the full course of normal larval feeding (Table 1, 96 h AEL). This highlights a component of stress resistance that is independent of metabolite acquisition during larval development, as no differences in either triglyceride or carbohydrate contents were found among flies from selected and control lines. This component of evolved resistance to desiccation in D flies is probably a result of relatively low rates of water loss of desiccation-selected flies (Gibbs et al., 1997). Still, our results show that the extended third instar larval development of D larvae contributes even further to the overall higher desiccation resistance in comparison with control groups. The contribution of late third instar larval feeding to desiccation resistance is 50% higher in D flies compared to their controls (13.0–13.7 h and 7.5–9.3 h, respectively). Overall, the feeding period extending from 96 h AEL through to the wandering stage results in a 60% increase in desiccation resistance of newly eclosed D flies and 40–50% in newly eclosed control flies, as reflected by comparing *ad libitum* larval feeding with the starvation treatment.

Increased desiccation resistance in selected *D. melanogaster* has been associated with higher carbohydrate contents

Table 3. *Wet and dry body mass and water contents of newly eclosed flies*

	Ad libitum			96 h AEL		
	D	S	F	D	S	F
Males						
Wet mass (mg)	1.020±0.014	0.976±0.018	0.916±0.015	0.493±0.024	0.459±0.019	0.478±0.020
Dry mass (mg)	0.287±0.005	0.279±0.005	0.259±0.005	0.118±0.006	0.115±0.006	0.120±0.006
Water content (mg)	0.733±0.012	0.697±0.013	0.657±0.011	0.375±0.018	0.344±0.013	0.358±0.015
Females						
Wet mass (mg)	1.347±0.025	1.265±0.019	1.204±0.017	0.488±0.012	0.553±0.025	0.567±0.025
Dry mass (mg)	0.373±0.007	0.362±0.005	0.339±0.005	0.113±0.004	0.133±0.009	0.136±0.007
Water content (mg)	0.974±0.019	0.903±0.015	0.865±0.012	0.375±0.010	0.420±0.017	0.431±0.018

Ad libitum, larvae left to feed until pupation; 96 h AEL, larvae removed from food at 96 h AEL; D, desiccation selected; S, starved; F, fed.
Values are means ± s.e.m.; *N*=24 for all groups.

(Djawdan et al., 1998; Chippindale et al., 1998; Folk et al., 2001) (but see Hoffmann and Harshman, 1999). Glycogen storage has been suggested as a mechanism of increasing intracellular water contents (Gibbs et al., 1997), but other evidence supports carbohydrate storage in the haemolymph, possibly in the form of trehalose (Folk et al., 2001). Results in this study show a trend for higher carbohydrate accumulation during pre-adult development of D flies in comparison with their controls (Table 2). Despite the variability among replicate populations, the trend shown is in agreement with previously reported carbohydrate contents of newly eclosed flies (3 h post eclosion) (Chippindale et al., 1998). This correlates with their longer developmental time and enhanced resistance of adults to desiccation stress (Table 1). The relatively high variability associated with the carbohydrate assay could explain the lack of statistical difference when replicate populations were treated as a random effect, but pooling selection treatment data resulted in significantly higher carbohydrate contents in newly eclosed desiccation selected flies.

Interestingly, newly eclosed flies from all three experimental lines had similar carbohydrate contents when resource accumulation was prevented from 96 h AEL onwards (Table 2). This indicates that the higher carbohydrate content of newly eclosed D flies results from increased carbohydrate accumulation in the late third instar larval stage (assuming catabolism of comparable ratios of metabolic fuels during the pupal stage).

The lack of statistical difference between starved (S) and fed (F) control flies in rates of commitment to pupation (Fig. 4) suggests that desiccation stress rather than food deprivation is the selective force responsible for the shift in larval 'critical weight' of the D flies. This developmental change results not only in increased body size, but also in higher carbohydrate (Table 2) and water contents (Table 3). Hence, increased carbohydrate accumulation as a result of an extended third instar stage may provide the necessary mechanism for increasing larval water storage, thus increasing the fitness of newly eclosed D flies under desiccating conditions. The carbohydrate content of newly eclosed D flies was ~6 µg higher than that of their controls (Table 2). Glycogen can only bind three to five times its own mass water, and therefore water bound to accumulated intracellular glycogen cannot fully account for the higher water content of newly eclosed D flies (Table 3). This may suggest that the extra water accumulated during larval development of D flies is stored both as intracellular glycogen-bound water and in the haemolymph (Folk et al., 2001).

In conclusion, this study shows that selecting adult flies for desiccation resistance results in major developmental changes. A shift in the 'critical weight' of the larvae contributes to an extended third larval instar, which in turn provides the newly eclosed adult fly with increased body mass and carbohydrate and water contents. These developmental changes significantly affect adult fitness under stressful conditions.

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