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Water acquisition and partitioning in *Drosophila melanogaster*: effects of selection for desiccation-resistance

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Summary

We examined physiological features related to water balance in five replicate populations of *Drosophila melanogaster* that have undergone selection for enhanced resistance to desiccation (D populations) and in five replicate control (C) populations. Adult D flies contain 34 % more water than the control flies. We examined two hypotheses for increased water acquisition in the D flies: (i) that they accumulate more water early in development and (ii) that they have a reduced post-eclosion diuretic water loss. We found no evidence of differential water or dry mass acquisition between the C and D populations prior to adulthood. We also found no evidence of differential post-eclosion diuresis, i.e. both C and D groups showed insignificant changes in water volume in the 4 h period immediately after eclosion. In addition, we quantified water content in the intra- and extracellular compartments of the C and D populations and were able to identify the hemolymph as the primary storage site of the 'extra' water carried by the desiccation-resistant flies. We estimated that 68 % of the increased water volume observed in the D flies was contained in the hemolymph. Desiccation-resistance was strongly correlated with hemolymph volume and only weakly with intracellular water volume. Survival during desiccation was also strongly related to the carbohydrate content of the D flies. It has been presumed that the D flies accumulate carbohydrate primarily as intracellular glycogen, which would result in a significant increase in intracellular water volume. We found that carbohydrate content was weakly correlated with intracellular water volume and more strongly with hemolymph volume. The carbohydrate pool in the D flies may, therefore, be contained in the extracellular compartment as well as in cells. These results are suggestive of the importance of modifications in hemolymph volume and hemolymph solute concentrations in the evolution of enhanced desiccation-tolerance in populations of *Drosophila melanogaster*.

Key words: *Drosophila melanogaster*, water acquisition, water partitioning, desiccation-resistance, carbohydrate, hemolymph, laboratory selection, water balance.

Introduction

Regulation of water content is essential for all organisms. In insects, water can be gained through consumption, absorption of atmospheric water vapor and production of water during metabolism (Hadley, 1994). Simultaneously, water is lost through the processes of excretion, cuticular and respiratory transpiration and in bodily secretions. When living in desiccating environments, insects must be able to maintain water balance by restricting water loss (Edney, 1977). Water conservation is especially challenging for *Drosophila melanogaster*, in which there is no absorption of water from the atmosphere and net water loss occurs even in atmospheres exhibiting greater than 90 % relative humidity (Arlian and Eckstrand, 1975).

Investigators studying physiological responses in populations of *Drosophila melanogaster* that have undergone laboratory selection for enhanced desiccation-tolerance have proposed that resistance to dehydration is increased in adults primarily by a reduction in the rate of cuticular water loss and by an increase in body water content (Hoffman and Parsons, 1993; Gibbs et al., 1997; Djawdan et al., 1998). Some additional features, whose functional significances are currently unclear, appear to play a role in the evolution of desiccation-resistance in *Drosophila melanogaster*. These include changes in the respiratory pattern and behavior during dehydration as well as modifications of the rate of development and of the carbohydrate-to-lipid ratio of storage products (Williams et al., 1998; Williams and Bradley, 1998; Chippindale et al., 1998; Djawdan et al., 1998).

On the basis of the above studies, a model has been proposed describing the processes by which *Drosophila melanogaster* responds to desiccation (Gibbs et al., 1997; Chippindale et al., 1998; Bradley et al., 1999). At 4 days post-eclosion, desiccation-resistant adults are larger than control adults because of an increased water content. This increase in stored

water, coupled with a reduction in the rate of water loss, is thought to be responsible for all of the increased desiccation-resistance observed in desiccation-selected populations relative to their control populations. There is no difference in water content at death between the selected and control populations (Gibbs et al., 1997).

We have examined these same desiccation-selected populations and their controls in further detail with regard to four physiological characteristics: water acquisition during development, the role of post-eclosion diuresis in water balance, the anatomical site of water storage and the role of carbohydrate storage in water partitioning. The first of these involves the timing of water accumulation during development. It has been posited that the most of the 'extra' water in desiccation-resistant adults is accumulated during larval development and that the cost of increased resource acquisition leads to higher juvenile mortality (Chippindale et al., 1998; Gibbs, 1999). Because laboratory selection is imposed only on 4-day-old adults, differentiation in water content between the desiccation-tolerant and the control larvae, pupae or immature adults would provide evidence that water balance in juveniles and immature adults is affected by desiccation-selection, on the older adults. We wished to determine more precisely the stage at which the flies show differentiation in water content by examining third-instar larvae, pupae, recently eclosed adults and mature adult flies (at 4 days post-eclosion).

Second, it has been shown that many holometabolous insects release substantial quantities of water following eclosion and prior to adult flight and, as a consequence, blood volumes may be reduced by up to 66–75 % during the hours following the emergence of the imago (Lee, 1961; Cottrell, 1962; Mills and Whitehead, 1970; Nicolson, 1976; Strathie and Nicolson, 1993; Chapman, 1998). We hypothesized that, if a similar post-eclosion diuresis were to have occurred in *Drosophila melanogaster*, the desiccation-resistant populations may have blunted this diuresis as a means of conserving water for desiccation-resistance.

Third, we wished to determine the location of the 'extra' water stored in the desiccation-selected populations but absent from the control populations. In insects, pools of extracellular water are stored at various sites, including the gut, salivary glands, rectum and hemolymph, and are critical to maintenance of homeostasis during bouts of water stress (for a review, see Hadley, 1994). Generally, water is moved from the reservoir sites to dehydration-sensitive tissues to protect cells from irreversible damage during desiccation. Hemolymph, second only to intracellular water in volume, is the largest extracellular pool of water in insects. We hypothesized that hemolymph might be the primary site of water storage in desiccation-resistant flies.

Finally, a significant increase in carbohydrate stores has been observed in the desiccation-selected *Drosophila melanogaster* populations (Graves et al., 1992; Chippindale et al., 1998; Djawdan et al., 1998). It has been proposed that this carbohydrate exists principally in the form of glycogen and that storage of energy in this form allows the concurrent storage of water of hydration associated with glycogen. During periods of dehydration, water of hydration might be released as glycogen is metabolized, thus contributing to the maintenance of water balance. In this study, we estimated the carbohydrate and intracellular water content of the desiccation-resistant and control flies. We hypothesized that, if glycogen serves as a significant form of water storage in *Drosophila melanogaster*, we should observe a correlation between the glycogen content and intracellular water content of the flies.

Materials and methods

Fly populations

Five large, outbred populations of *Drosophila melanogaster*, designated D_1–D_5, have undergone laboratory selection for enhanced desiccation-resistance every generation since 1988 (Rose et al., 1990; Rose et al., 1992). Control populations (C_1–C_5), paired with the D populations by subscript number, have been concurrently maintained. At the time of this experiment, all populations had gone through more than 200 generations. Each of the five C_n/D_n pairs derived from one of five ancestral populations (O_1–O_5) that had undergone selection for postponed senescence since 1980 (Rose, 1984). For example, the O_1 population is the direct, common ancestor of both C_1 and D_1, while O_2 is the common ancestor of C_2 and D_2, etc.

Batches of eggs (60–80) were collected from each fly population and placed into each of 30–50 30 g food vials containing approximately 5 ml of banana-molasses food. The flies were allowed to develop at 25 °C and under conditions of 24 h light. Fourteen days following egg collection, all C and D flies were transferred from food vials into designated population cages made of Plexiglas. (Each cage has a single open end.) At this time, all D populations were placed under desiccation-selection. To lower relative humidity, a desiccant (Drierite) was placed into each cage housing the D flies, and the open end of the cage was covered with plastic wrap. Neither food nor water was supplied. Each D population was thus maintained until 80 % mortality had been reached, at which time the extant flies were provided with food, which was a source of both nutrients and water.

Prior to the selection treatment, the control populations had been maintained under identical conditions to the D populations. At the time that the D populations were placed under desiccation-selection, all C populations were placed under a control selection regime. Differences between the C and D selection treatments were as follows: (i) C flies had access to drinking water in the form of non-nutritional agar; (ii) desiccant was not placed in cages housing the C flies and (iii) the open end of the C population cage remained covered only with cloth, which allows cage humidity to equilibrate with ambient humidity. Each C population was released from the C selection treatment (i.e. food was supplied) at the same time that its paired D population was released from the D selection treatment. Following the period of selection and subsequent replenishment, eggs were collected from all C and D

populations for rearing of, and laboratory selection on, the next generation.

Prior to all experiments, C and D populations were removed from the laboratory selection regimes for two generations. Eggs were collected from females within the second generation, and the offspring were reared and used in the experiments. By releasing the flies from the influence of strong selection for two generations, we were able to conclude that the observed differences between the C and D populations were attributable to genetic differences and not to grandparental or parental effects (resulting from stress of selection) on offspring.

Rearing of flies for experiments began with the collection of batches of eggs (60–80) from each population; the eggs from each batch (10 per population) were placed in 30 g food vials containing 5 ml of food. Fourteen days after egg collection, adults were transferred to Plexiglas cages; 3–4 days later, eggs were collected from these adults for rearing of the next generation. Two days prior to egg collection, flies were given a boost in nutrition by the addition of a yeast paste to their regular food. Throughout development, flies were fed *ad libitum* and maintained at 25 °C and under conditions of 24 h light. Only females were used in all assays to eliminate differences due to gender. Unless noted otherwise, experiments on adults were performed on females that had been permitted to develop for 14 days following the egg stage, at which time the average female is a 4-day-old, mature adult.

Gravimetric method of estimating body water

Third-instar larvae and wanderers

Male larvae have relatively large, ovoid gonads that are visible through the integument and can be used for identification and culling of this gender. (Prior to our experiments, we ran trials in which we culled alleged male larvae, allowed the remaining larvae to develop and then determined the gender of the adults. We had 100 % accuracy in culling out males.) On approximately day 4 after the egg stage, third-instar female larvae were collected; female wanderers (i.e. late third-instar larvae that 'wander' up the inner surface of food vials prior to pupation) were collected 1 day later. To remove adhering particles of food, each larva was briefly rinsed in isotonic saline (350 mosmol l^{-1}) and then blotted on an absorbent tissue for approximately 3–5 s (Rizki, 1978). Each was then weighed on a Cahn 29 automatic electrobalance (Cerritos, CA, USA), dried at 60 °C for 1 h, and reweighed. Wet body mass and dry body mass were measured in 10 third-instar larvae and in 10 wanderers from each of the five C and five D populations. The mass of total body water was calculated by subtracting dry mass from wet mass. Body water was also expressed as a percentage of wet mass.

Pupae

Five days following egg collection, third-instar female larvae were identified, placed into fresh food vials and allowed to continue development for a further 3 days. At that time, the pupae were carefully scraped from the inner vial surface using a stainless-steel surgical scalpel to ensure that the puparium was removed intact. Each pupa was then weighed, dried at 60 °C for 24 h and weighed again. To ensure complete desiccation of pupae, the pupal cases were cracked prior to placing them in the drying oven. Wet mass and dry mass were measured for 10 pupae from each of the five C and five D populations. Water content and percentage body content were calculated as described above.

Adults

Immediately following eclosion, females were transferred to fresh food vials and allowed to continue maturing for 10 min, 1 h, 2 h or 4 h. After the prescribed time, each adult was anesthetized with CO_2, decapitated and immediately weighed. (Flies were decapitated to eliminate escape following recovery from anesthesia.) The flies were dried at 60 °C for 1 h and weighed again. Females used in the experiments involving 4-day-old, mature adults were maintained in food vials until 14 days following the egg stage, at which time each was anesthetized with CO_2 and treated according to the protocol used for the younger flies. At each prescribed time point, the wet body mass and dry body mass of 10 adults from each population were measured. Water content and percentage body water were calculated as described above.

Blotting technique to measure hemolymph volume

Ten 4-day-old, mature adult females from each of the five C and five D populations were anesthetized with CO_2 and weighed. The abdomen of each was gently torn with surgical forceps. Hemolymph was blotted from the abdominal opening with a Kimwipe that had been slightly moistened with isotonic saline (250 mosmol l^{-1}) (Singleton and Woodruff, 1994). Each fly was then reweighed, dried for 1 h at 60 °C and weighed a third time. Hemolymph volume was estimated by determining the reduction in mass following hemolymph blotting (Richardson et al., 1931; Wall, 1970; Nicolson et al., 1974; Cohen et al., 1986). Percentage total body water attributable to hemolymph volume was also estimated.

Desiccation performance assay

Four 4-day-old, mature adult females were briefly anesthetized with CO_2 and transferred to an empty 30 g vial. A foam stopper was placed approximately 3 cm down into the vial, and approximately 4.5 g of Drierite was placed on top of the stopper. The open end of the vial was then sealed with Parafilm. C flies were checked hourly for mortality, while D flies were checked every 4 h. Mortality was determined by the inability of the flies to resume an upright position after the vial had been shaken. Desiccation-resistance was expressed as survival time (h) during dehydration and was estimated for 40 flies (10 vials, each containing four flies) from each C and D population.

Carbohydrate assay

A modification of the colorimetric method of carbohydrate extraction (Djawdan et al., 1998) was used to measure the

carbohydrate content of five fly samples from each of the 10 populations (C_1–C_5 and D_1–D_5). Prior to sample preparation, 4-day-old adult females were anesthetized with CO_2, dried for 45 min at 60 °C and then weighed to obtain dry mass. Each sample was prepared by grinding five flies in 0.5 ml of distilled water for 1.5 min using a battery-operated, handheld Kontes grinder. The homogenized samples, contained in 1.5 ml microcentrifuge tubes, were placed in a boiling water bath for 20 min. Part of the sample (0.1 ml) was then added to 3 ml of anthrone reagent (150 mg of anthrone per 100 ml of 72 % sulfuric acid). This solution was incubated in a water bath at 90 °C for 20 min, and absorbance was measured at 620 nm. Carbohydrate concentration was estimated from a standard curve prepared using known concentrations of glycogen.

Statistical analyses

Differentiation between the C and D fly groups in wet mass, dry mass, absolute and percentage body water content, absolute hemolymph volume, post-eclosion water volume and carbohydrate content was analyzed using analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons of means (SYSTAT Software). Because each C_n–D_n population pair shared ancestry (and populations were maintained as pairs throughout selection), common ancestry was treated as a block in the ANOVAs. Blocks were treated as random effects, while selection treatment and developmental phase were treated as fixed effects. Percentages were transformed to arcsine values prior to inclusion in ANOVAs. To meet the assumptions of normality and homoscedasticity, hemolymph volume data were log-transformed.

Change in water content of the C and D fly groups during the 4 h post-eclosion period was analyzed using linear regression. Each point in the regression represented the mean from either all five C or all five D populations (i.e. five populations per selection treatment). Correlations between survival time during desiccation and hemolymph volume, intracellular water volume and carbohydrate content, as well as correlations between carbohydrate content and hemolymph and intracellular water volumes, were calculated using linear regression. Each point in the regressions represented the mean estimated from 10 individuals from each of the five C and five D populations. A two-tailed, paired Student's *t*-test was used to examine carbohydrate content, normalized by total dry mass, between C and D adult flies. The significance level for analyses was 0.05.

Results

To eliminate potential parental and grandparental effects, all flies used in experiments were released from laboratory selection and reared under identical conditions for two successive generations. Offspring from the second-generation parents were used in all experiments.

Dry and wet masses

Dry mass did not differ significantly between the C and D flies within the four developmental phases examined: wanderers, pupae, newly eclosed adults and mature 4-day-old adults (P=0.35, Fig. 1). Both C and D fly groups showed significant gains in dry mass (P<0.001, both groups) as wanderers metamorphosed into pupae and showed significant losses of dry mass (P<0.001, both groups) as young adults emerged from their puparia. Dry mass in all populations increased significantly (P<0.001, both groups) as growth continued over the 4 days following eclosion.

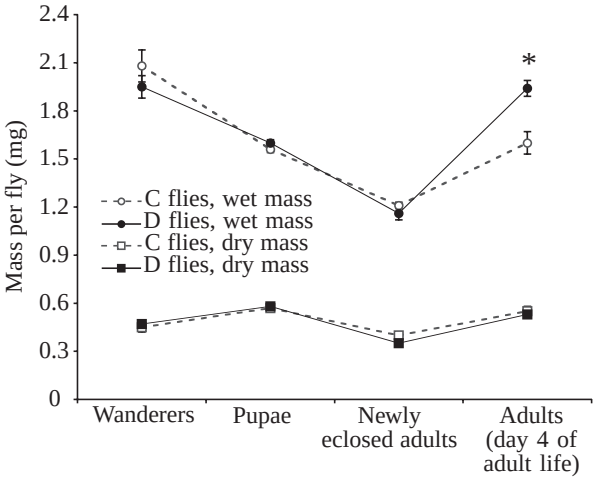

Fig. 1. Comparison of wet and dry masses of desiccation-selected flies (D flies) and their controls (C flies) at four phases of development. Values are means of five populations from either the C or D selection treatments ± S.E.M. The asterisk denotes a significant difference between the C and D flies, which was observed only in wet mass at 4 days post-eclosion (P<0.001).

Within the wanderer, pupal and immature adult phases, C and D flies did not differ in wet mass (P>0.08, Fig. 1). As development proceeded within the C and D fly groups from wanderer to pupa and then to newly eclosed adult, wet mass in C and D flies declined steeply (by approximately 40 % for all flies), primarily as a result of net water loss. Both groups had significant gains (P<0.001) in wet mass between eclosion and 4 days post-eclosion as a result of both an increase in dry mass and water accumulation. The wet mass of mature 4-day-old D flies was significantly greater (P<0.001) than that of C flies. On average, mature D-selected flies had an approximately 21 % greater wet mass than the non-selected flies.

Total body water volume during development

There was no difference between the C and D flies in total body water content at the wanderer, pupal and newly eclosed adult stages (P=1.0). A significant increase in total body water in the D flies, relative to the C flies, was detected only in 4-day-old adults (P=0.001).

Significant changes in total body water content were observed between successive phases of development within the C and D populations. Total body water declined significantly (P<0.001, both groups) between the wanderer and pupal stage. Only in the D flies was a significant increase (P<0.001)

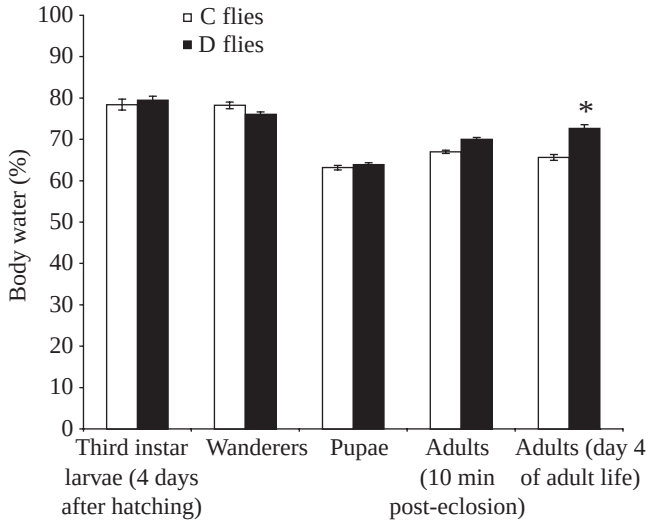

Fig. 2. Comparison of percentage body water content of desiccation-selected flies (D flies) and their controls (C flies) at five phases of development. Values are means of five populations from either the C or D selection treatments ± S.E.M. The asterisk denotes a significant difference between the C and D flies in percentage body water, which was observed only at 4 days post-eclosion (P<0.001).

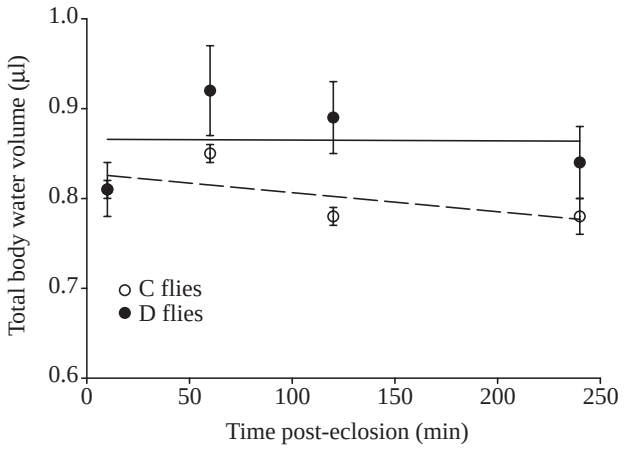

Fig. 3. Total body water volume measured at 10 min, 1 h, 2 h and 4 h following eclosion. The slopes of the regressions were not significantly different from zero for either the C flies (P=0.36) or the D flies (P=0.98). Values are means ± S.E.M. of five populations from either the C or D selection treatments.

observed as newly eclosed, immature flies matured into 4-day-old adults.

Percentage body water during development

The percentage of total wet mass attributable to water, expressed as percentage body water, was compared between the C and D flies during the four phases of development mentioned above and in non-wandering third-instar larvae. Measurements of non-wandering third-instar larvae were made 4 days after egg hatching. We could not be certain that the C and D larvae developed at the same rate and were, therefore, at exactly the same developmental phase. We have therefore decided to include the non-wandering third-instar larvae only in the estimation of percentage body water. Percentage body water was not significantly different (P>0.70) between the C and D flies during all stages prior to mature adults (Fig. 2). Differentiation in percentage body water was observed only in mature, 4-day-old adults (P<0.001), at which time percentage body water content was approximately 66 % in the C flies and 73 % in the D flies. Differentiation in percentage body water between mature C and D flies was primarily a result of a significant increase in total body water volume in the D flies and was not due to differences in dry mass between the two fly groups.

A significant change in percentage body water between successive phases of development within the C and D fly groups was observed only at one stage within the C group: percentage body water decreased between the wanderer and pupal stages (P<0.001). A significant decrease (P<0.001) in percentage body water was also observed in the D flies during this same period. This decline in both fly groups resulted from a decrease in absolute water volume and a concurrent increase in dry mass in both groups as larvae metamorphosed into pupae (Fig. 1). Unique to the D flies was a significant increase (P<0.001) in percentage body water during the period between the pupal and the newly eclosed adult stages. While both body water volume and dry mass were lost between the two stages, the proportion of dry mass (0.40) lost was almost twice that of water (0.21), resulting in an overall increase in percentage body water in the D flies.

Total body water post-eclosion

The data did not support the hypothesis that a reduction in urinary water loss at eclosion is a mechanism by which the desiccation-resistant populations differentially retain more body water. The rate of change in body water volume during the period between 10 min and 4 h post-eclosion was not significantly different from zero for either the C and or the D group (P=0.36 and P=0.98, for C and D flies, respectively, Fig. 3). The C flies showed an average net loss of 0.03 µl of water, while the D flies showed an average net gain of 0.03 µl, which represents a change of only 3.7 % in total body water volume in both fly groups over the entire 4 h period. In addition, the C and D populations did not differ significantly in body water volume at 10 min, 1 h, 2 h or 4 h following eclosion (P=0.28).

Hemolymph volume in C and D flies

The hemolymph volume in desiccation-resistant flies (0.323±0.022 µl) was significantly greater than that of control flies (0.078±0.009 µl, means ± S.E.M., N=10 flies per population, P<0.001). On average, D flies had 3.37±0.135 times (mean ± S.D.) more hemolymph than did C flies. The mean percentage of total body water contained within the hemolymph pool ranged from 6 to 10 % in the C population and from 18 to 27 % in the D population. Calculated as $[(D_{hv}-C_{hv})/(D_{total\,bw}-C_{total\,bw})100]$, we estimated that

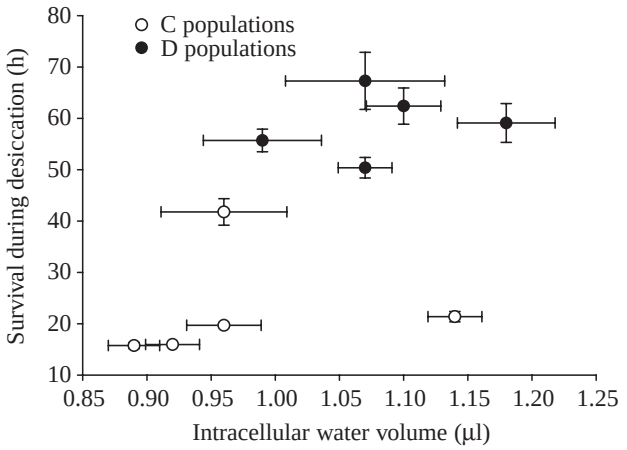

Fig. 4. Only a weak relationship was observed between survival during desiccation and intracellular water volume (r^2=0.34, P=0.054). Each filled circle represents the mean (± S.E.M.) from one of the five D populations, while each open circle represents the mean (± S.E.M.) from one of the five C populations (N=10 flies per population).

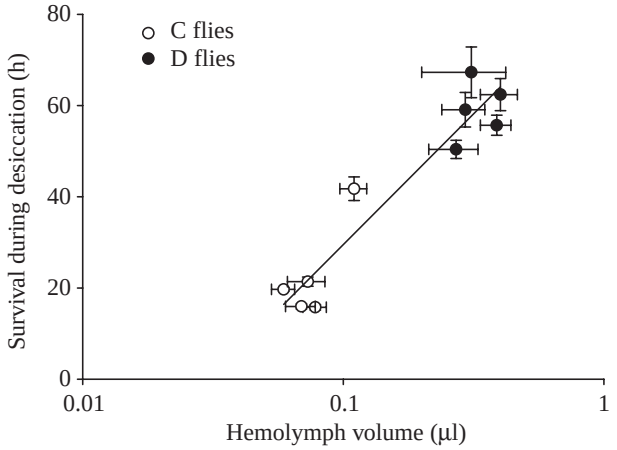

Fig. 5. A strong relationship was observed between survival during desiccation and hemolymph volume (r^2=0.85, P=0.0002). Each filled circle represents the mean value (± S.E.M.) from one of the five D populations, while each open circle represents the mean (± S.E.M.) value from one of the five C populations (N=10 flies per population). Hemolymph data were log-transformed prior to statistical analyses.

approximately 68 % of the 'extra' body water (bw) volume in the D flies could be accounted for by the increase in hemolymph volume (hv).

Water partitioning and resistance to desiccation

There was a significant relationship between total body water volume and tolerance to desiccation (P=0.0004, r^2=0.78, y=90.74x–70.85). We found a weak relationship between intracellular water content (calculated as total body water minus hemolymph volume) and desiccation-resistance (r^2=0.34, P=0.054, Fig. 4). While intracellular water was only mildly related to desiccation-tolerance, the relationship between extracellular water content, namely hemolymph volume, and desiccation-resistance was highly significant (P=0.0002, r^2=0.85, y=59.7x –146.3, Fig. 5).

Carbohydrate content

When compared with control populations, the desiccation-resistant populations had more absolute carbohydrate per fly (P<0.001) and more carbohydrate adjusted for total dry mass (P=0.02, Fig. 6). The C and D populations had an average of 94 µg and 168 µg carbohydrate per fly, respectively. Among the five D-selected populations, the relationship between desiccation-resistance and proportional carbohydrate content (calculated as carbohydrate content/dry mass) was significant (P=0.032, r^2=0.77, y=64.73x+41.39, Fig. 7). Our data suggest that, in the desiccation-tolerant flies, the ability to endure bouts of dehydration was correlated with carbohydrate content.

While the correlation between carbohydrate stores (adjusted for dry mass) and total body water volume among the C and D populations was significant (P=0.004, r^2=0.67, y= 1.89x+0.83), a significant relationship was not found between the intracellular water volume and carbohydrate content

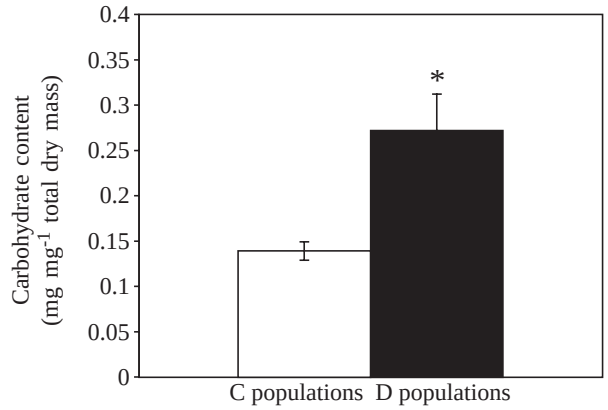

Fig. 6. Comparison of carbohydrate level adjusted for total dry mass between C and D populations at 4 days post-eclosion. Values are means ± S.E.M. of five populations from either the C or D selection treatments. The asterisk denotes a significant difference between the C and D flies in carbohydrate content (P=0.02).

(r^2=0.35, P=0.074). In contrast, there was a significant correlation between hemolymph volume and carbohydrate stores (P=0.03, r^2=0.48, y=3.71 x+2.63).

Discussion

Body water volume and wet mass

It has been hypothesized that, relative to the control flies, desiccation-selected flies have a slightly slower growth rate and a longer egg-to-adult developmental time, which purportedly lead to greater accumulation of resources (including water) during larval development and result in heavier adults (Chippindale et al., 1998). It has been further proposed that females, in particular, respond to selection for

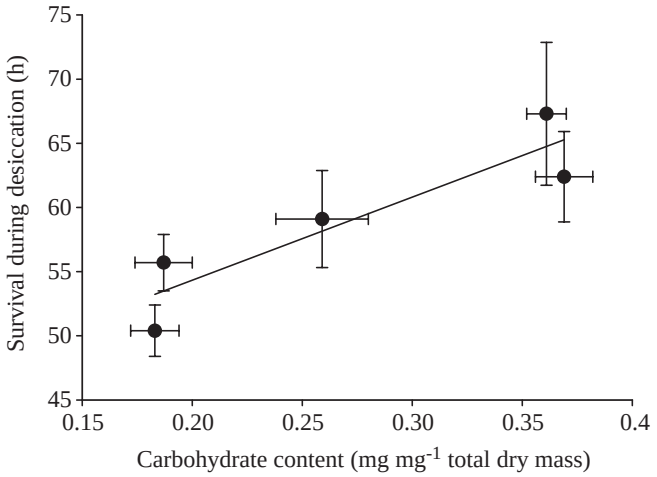

Fig. 7. The relationship between mean survival during desiccation and mean carbohydrate content adjusted for total dry mass (r^2=0.77, P=0.032). Each point is the mean value (± S.E.M.) from one of the five desiccation-selected populations (N=10).

enhanced desiccation-resistance by increasing pre-adult growth and resource acquisition. In the present study, we examined differentiation in body water volume and total wet mass (a measure of body size) between C and D females during development.

In the late larval phase, the total body water content and wet mass of the C and D wanderers did not differ. We conclude that the D larvae do not acquire 'extra' water that could then be carried into adulthood. Percentage body water (76–79 %) was also undifferentiated between the C and D larvae, suggesting that proportionality of water content and dry mass does not differ between the two fly groups during larval development. We examined body water in pupae, in immature adults at various times following eclosion (see below) and in mature adults at 4 days (96 h) post-eclosion. Differentiation in body water between the C and D flies occurred only in the mature adults. We established *a posteriori* that the period between 4 and 96 h following adult ecdysis was the critical period during which body water content diverged between the C and D adults.

Body water volume following eclosion

In some holometabolous insects, large volumes of urine are eliminated during the hours following eclosion, resulting in a significant reduction in hemolymph volume prior to first flight. Isolated Malpighian tubules of *Drosophila melanogaster* adult females are capable of high rates of fluid secretion (Dow et al., 1994), and we anticipated that the C and D populations would void significant amounts of urine following eclosion. We hypothesized that a dampening of post-eclosion diuresis might be a mechanism for the differential water retention observed in the desiccation-resistant populations compared with the control populations. Such a diminution in water loss might provide the D flies with a greater blood volume and an enhanced ability to resist stress of desiccation early in adulthood. We noted that flies from our laboratory-selected populations were capable of flight within 2 h following adult eclosion; hence, we predicted that diuresis would not continue past 4 h post-eclosion.

Flies from the C populations experienced a slight loss of water only between 1 and 2 h after eclosion. The gut contents (i.e. meconium) were generally voided within 2 h following adult emergence from the puparium. Urine mixed with meconium may result in a slight loss of water when the meconium is voided (Nicolson, 1976). The D flies had a very slight loss of water during between 1 and 4 h post-eclosion. Despite the slight decline in body water volume, neither group of flies had a statistically significant net change in body water content over the entire 4 h period (Fig. 3). Hence, we found no evidence of significant post-eclosion diuresis in the C or D populations of *Drosophila melanogaster*. Diuresis following adult ecdysis in drosophilids has not been carefully examined, and it may not occur in this group. We suggest that the water loss for subsequent flight may occur during the larval–pupal transition.

Dry mass, lipid and carbohydrate

The chief metabolic storage product in the C flies is lipid, whereas the D flies store metabolic reserves primarily as carbohydrate (Djawdan et al., 1998). In 1988, $C_{1–5}$ and $D_{1–5}$ populations were derived from five populations (i.e. $O_{1–5}$) of *Drosophila melanogaster* that had undergone selection for postponed senescence since 1980 (Rose et al., 1990; Rose et al., 1992). The O populations have responded to selection for delayed aging by increasing both lipid stores and glycogen content. In addition, the C populations have endured mild starvation-selection (during the treatment regime, C flies are provided with water but not food), which also results in an increase in lipid storage in *Drosophila melanogaster* (Service, 1987). The lipid-dense phenotype in the C flies represents both the ancestral response to selection for postponed aging and a response to mild starvation-selection.

In contrast, laboratory selection for enhanced desiccation-resistance has resulted in a variant phenotype in which carbohydrate stores are greatly increased. Our results support the hypothesis that the accumulation of carbohydrate is an evolutionary response to desiccation-selection of our five desiccation-tolerant populations because resistance to dehydration was significantly correlated with carbohydrate content (Fig. 7). The consistency of the response within all the D populations suggests that preferential storage of carbohydrates as metabolic fuel plays a role in desiccation-resistance. The mechanistic link between carbohydrate storage and tolerance to desiccation remains unclear.

Carbohydrate and water content in desiccation-resistant Drosophila melanogaster

Individual D flies have, on average, approximately 80 % more carbohydrate than control flies. Various studies have suggested that the primary carbohydrate involved is glycogen (Graves et al., 1992; Gibbs et al., 1997; Chippindale et al.,

1998; Djawdan et al., 1998). Glycogen can bind 3–5 times its weight in water (Schmidt-Nielsen, 1997). Although the exact water-to-glycogen ratio in our populations has not been established, it has been proposed that water of hydration, released during glycogenolysis, may help to maintain water balance during desiccation. In the present study, we examine this hypothesis further.

Djawdan et al. (Djawdan et al., 1998) examined the evolution of lipid and carbohydrate storage in the C and D flies and found that all five D populations traveled along the same evolutionary pathway, that of accumulating carbohydrates while reducing lipid content. It has been proposed that, by increasing glycogen stores, the desiccation-resistant flies augment intracellular water volume. We observed that the desiccation-resistant flies had a significantly greater intracellular water volume than the C flies (data not shown, P=0.03). However, we did not find a strong relationship between intracellular water volume and carbohydrate content among the C and D populations. Furthermore, when we regressed resistance to desiccation against water volume within the cells, we found only a weak relationship (Fig. 4). The absence of a strong correlation between carbohydrate level (as well as desiccation-resistance) and intracellular water volume led us to re-evaluate the assumption that glycogen is the primary carbohydrate responsible for enhanced desiccation-resistance.

Although carbohydrate content and intracellular water volume were not significantly correlated, we found that carbohydrate content was strongly correlated with extracellular water, namely hemolymph volume. These results suggest that the desiccation-resistant flies are accumulating (in addition to glycogen) additional carbohydrate, which may be in the hemolymph. Trehalose is the most common sugar in insect blood (Chapman, 1998). It is also an important osmolyte and carbohydrate reserve. We posit that a portion of the large carbohydrate stores found in the desiccation-resistant flies may be trehalose. The desiccation-resistant flies have a hemolymph volume that is, on average, more than 300 % greater than that of the C flies, so the increase in carbohydrate content in the D flies may reflect, in part, an increase in blood trehalose content in these flies. Precise measurement of trehalose and glycogen levels in flies from both treatments will require the development of enzymatic procedures for analyzing intra- and extracellular compartments. We are currently developing such techniques for our future studies.

High blood volume: implications for osmoregulation in desiccation-tolerant Drosophila melanogaster

The mature D-selected adults had 34 % more water volume than the C-selected flies. Approximately two-thirds of this ‘extra’ water volume in the D flies was located in the hemolymph. During periods of dehydration, intracellular water volume must be maintained. Excessive cellular water loss can cause irreparable damage to and precipitation of macromolecules, resulting in cell death (Hadley, 1994). The hemolymph presumably acts as a reservoir from which water can be removed and redistributed to cells. Yet removal of only water from the hemolymph would increase the osmotic pressure as solutes became more concentrated. Hence, osmotically active solutes must be removed from the blood to maintain a constant osmotic pressure if the volume is reduced.

Three general strategies are employed by insects during bouts of desiccation: (i) toleration of the fluctuations in blood osmotic pressure (simply ‘wait-it-out’), (ii) excretory regulation of blood osmotic pressure with the excretion of solutes as hemolymph volume is reduced and (iii) internal regulation of blood osmotic pressure during which solutes are sequestered within the insect as blood volume declines (Wall, 1970; Arlian, 1979; Nicolson, 1980; Cohen et al., 1986; Naidu, 1998). Of the two osmoregulatory strategies, the former is more disadvantageous because solute removal is irreversible. The animal must feed to replace excreted solutes. Osmoregulation of hemolymph coupled with internal storage of osmotically active solutes allows blood osmotic pressure to remain relatively constant despite significant reductions in volume, and then, upon rehydration, solutes can be transported from the storage sites and back into the blood. The insect is thus freed from the necessity of finding a solute-laden resource.

Body size, water content and storage of metabolic fuel in Drosophila melanogaster

In response to selection for enhanced desiccation-resistance, all five D populations have evolved a larger body size, increased whole-body water content and elevated carbohydrate content. There is evidence from other studies on *Drosophila* that these features are genetically correlated (Clark and Doane, 1983; Clark et al., 1990) and may, therefore, be constrained to covary to some degree in response to selection.

Clark et al. (Clark et al., 1990) subjected a large outbred population of *Drosophila melanogaster* to artificial selection on lipid storage. The response to selection on lipid stores was confounded by an unexpected allometric relationship between lipid content and body mass, i.e. body size decreased when the percentage of body mass composed of lipid increased. A regression of lipid content on body mass resulted in a slope of less than 1, indicating that, as body mass increased, the proportion that was lipid decreased. A possible explanation for covariance of lipid level and body size is that larger flies generally carry more water, and it has been shown that water volume is inversely proportional to lipid content in *Drosophila* (Clark and Doane, 1983; Clark et al., 1990). An inverse relationship between body water volume (high) and lipid content (low) was found in our desiccation-resistant flies. If the D flies respond to desiccation by increasing their water content, are they constrained to reduce lipid content? Reductions in the storage of calories as lipid may, by necessity, lead to a significant increase in the storage of calories as carbohydrate.

It is interesting to note that the characters that have evolved under laboratory selection for enhanced desiccation-selection are not always identical to those that have differentiated in wild populations from habitats varying in temperature and humidity (Gibbs, 1999; Harshman and Hoffman, 2000). However, a

positive correlation between body size and desiccation-tolerance has been found in wild populations of various drosophilid species, including *Drosophila melanogaster* (Karan et al., 1998; Parkash and Munal, 1999).

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References

Arlian, L. G. (1979). Significance of passive sorption of atmospheric water vapor and feeding in water balance of the rice weevil, *Sitophilus oryzae*. *Comp. Biochem. Physiol.* **62A**, 725–733.

Arlian, L. G. and Eckstrand, I. A. (1975). Water balance in *Drosophila pseudoobscura* and its ecological implications. *Ann. Ent. Soc. Am.* **68**, 827–832.

Bradley, T. J., Williams, A. E. and Rose, M. R. (1999). Physiological responses to selection for desiccation resistance in *Drosophila melanogaster*. *Am. Zool.* **39**, 337–345.

Chapman, R. F. (1998). *The Insects: Structure and Function*. Fourth edition. Cambridge: Cambridge University Press.

Chippindale, A. K., Gibbs, A. G., Sheik, M., Yee, K. J., Djawdan, M., Bradley, T. J. and Rose, M. R. (1998). Resource acquisition and the evolution of stress resistance in *Drosophila melanogaster*. *Evolution* **52**, 1342–1352.

Clark, A. G. and Doane, W. W. (1983). Desiccation tolerance of the *adipose*^{60} mutant of *Drosophila melanogaster*. *Hereditas* **99**, 165–175.

Clark, A. G., Szumski, F. M., Bell, K. A., Keith, L. E., Houtz, S. and Merriwether, D. A. (1990). Direct and correlated responses to artificial selection on lipid and glycogen contents in *Drosophila melanogaster*. *Genet. Res.* **56**, 49–56.

Cohen, A. C., March, R. B. and Pinto, J. D. (1986). Effects of water stress and rehydration in hemolymph volume and amino acid content in the blister beetle *Cysteodemus armatus*. *Comp. Biochem. Physiol.* **85A**, 743–746.

Cottrell, C. B. (1962). The imaginal ecdysis of blowflies. III. Observations on the hydrostatic mechanisms involved in digging and expansion. *J. Exp. Biol.* **39**, 431–448.

Djawdan, M., Chippindale, A. K., Rose, M. R. and Bradley, T. J. (1998). Metabolic reserves and evolved stress resistance in *Drosophila melanogaster*. *Physiol. Zool.* **71**, 584–594.

Dow, J. A. T., Maddrell, S. H. P., Görtz, A., Skaer, N. J., Brogan, S. and Kaiser, K. (1994). The Malpighian tubules of *Drosophila melanogaster*: fluid secretion and its control. *J. Exp. Biol.* **197**, 421–428.

Edney, E. B. (1977). *Water Balance in Land Arthropods*. New York: Springer-Verlag.

Gibbs, A. G. (1999). Laboratory selection for the comparative physiologist. *J. Exp. Biol.* **202**, 2709–2718.

Gibbs, A. G., Chippindale, A. K. and Rose, M. R. (1997). Physiological mechanisms of evolved desiccation resistance in *Drosophila melanogaster*. *J. Exp. Biol.* **200**, 1821–1832.

Graves, J. L., Toolson, E. C., Jeong, C., Vu, L. N. and Rose, M. R. (1992). Desiccation, flight, glycogen and postponed senescence in *Drosophila melanogaster*. *Physiol. Zool.* **65**, 268–286.

Hadley, N. F. (1994). *Water Relations of Terrestrial Arthropods*. San Diego, CA: Academic Press.

Harshman L. G. and Hoffman, A. A. (2000). Laboratory selection experiments using *Drosophila*: what do they really tell us? *Trends Ecol. Evol.* **15**, 32–36.

Hoffman, A. A. and Parsons, P. A. (1993). Direct and correlated responses to selection for desiccation resistance: a comparison of *Drosophila melanogaster* and *D. simulans*. *J. Evol. Biol.* **6**, 643–657.

Karan, D., Dahiya, N., Munjal, A. K., Gibert, P., Moreteau, B., Parkash, R. and David, J. R. (1998). Desiccation and starvation tolerance of adult *Drosophila*: opposite latitudinal clines in natural populations of three different species. *Evolution* **52**, 825–831.

Lee, R. M. (1961). The variation of blood volume with age in the desert locust (*Schistocerca gregaria* Forsk.). *J. Insect Physiol.* **6**, 36–51.

Mills, R. R. and Whitehead, D. L. (1970). Hormonal control of tanning in the American cockroach: changes in blood cell permeability during ecdysis. *J. Insect Physiol.* **16**, 331–340.

Naidu, S. G. (1998). Haemolymph amino acid, sugar and glycerol levels in the Namib Desert tenebriod *Stips stali* during dehydration and rehydration. *Comp. Biochem. Physiol.* **119**, 477–484.

Nicolson, S. W. (1976). Diuresis in the cabbage white butterfly, *Pieris brassicae*: fluid secretion by the Malpighian tubules. *J. Insect Physiol.* **22**, 1347–1356.

Nicolson, S. W. (1980). Water balance and osmoregulation in *Onymacris plana*, a tenebriod beetle from the Namib Desert. *J. Insect Physiol.* **26**, 315–320.

Nicolson, S. W., Horsfield, P. M., Gardiner, B. O. C. and Maddrell, S. H. P. (1974). Effects of starvation and dehydration on osmotic and ionic balance in *Carausius morosus*. *J. Insect Physiol.* **20**, 2061–2069.

Parkash, R. and Munjal, A. K. (1999). Climatic selection of starvation and desiccation resistance in populations in some tropical drosophilids. *J. Zool. Syst. Evol. Res.* **37**, 195–202.

Richardson, C. H., Burdette, R. C. and Eagleson, C. W. (1931). The determination of the blood volume of insect larvae. *Ann. Ent. Soc. Am.* **24**, 503–507.

Rizki, T. M. (1978). The circulatory system and associated cells and tissues. In *The Genetics and Biology of* Drosophila, vol. 2b (ed. M. Ashburner and T. R. F. Wright), pp. 397–452. New York, London: Academic Press.

Rose, M. R. (1984). Laboratory evolution of postponed senescence in *Drosophila melanogaster*. *Evolution* **38**, 1004–1010.

Rose, M. R., Graves, J. L. and Hutchison, E. W. (1990). The use of selection to probe patterns of peliotrophy in fitness characters. In *Insect Life Cycles* (ed. F. Gilbert), pp. 29–42. New York: Springer-Verlag.

Rose, M. R., Vu, L., Park, S. U. and Graves, J. L. (1992). Selection on stress resistance increases longevity in *Drosophila melanogaster*. *Exp. Gerontol.* **27**, 241–250.

Schmidt-Nielsen, K. (1997). *Animal Physiology: Adaptation and Environment*. Fifth edition. Cambridge: Cambridge University Press.

Service, P. M. (1987). Physiological mechanisms of increased stress resistance in *Drosophila melanogaster* selected for postponed senescence. *Physiol Zool.* **60**, 321–326.

Singleton, K. and Woodruff, R. I. (1994). The osmolarity of adult *Drosophila* hemolymph and its effect on oocyte–nurse cell electrical polarity. *Dev. Biol.* **161**, 154–167.

Strathie, L. W. and Nicolson, S. W. (1993). Post-eclosion diuresis in a flightless insect, the silkmoth *Bombyx mori*. *Physiol. Ent.* **18**, 435–439.

Wall, B. J. (1970). Effects of dehydration and rehydration on *Periplaneta americana*. *J. Insect Physiol.* **16**, 1027–1042.

Williams, A. E. and Bradley, T. J. (1998). The effect of respiratory pattern on water loss in desiccation-resistant *Drosophila*. *J. Exp. Biol.* **201**, 2953–2959.

Williams, A. E., Rose, M. R. and Bradley, T. J. (1998). Using laboratory selection for desiccation resistance to examine the relationship between respiratory pattern and water loss in insects. *J. Exp. Biol.* **201**, 2945–2952.