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A new set of laboratory-selected *Drosophila melanogaster* lines for the analysis of desiccation resistance: response to selection, physiology and correlated responses

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Summary

Artificial selection experiments provide insights into the evolutionary factors that can shape adaptive responses and have previously been utilized to examine the physiological adaptations that can improve survival to desiccation in *Drosophila melanogaster*. While such studies demonstrate that multiple resistance mechanisms may arise *via* different base populations and selection regimes, water retention emerges as a key mechanism for desiccation survival. Here, we present the physiological, correlated response and life history data for a new set of selection lines designed for the genetic dissection of desiccation resistance. After 26 generations of selection for desiccation resistance, female survival increased twofold. In contrast to previous studies, the altered resistance was associated primarily with enhanced dehydration tolerance and increased mass and less consistently with decreased rates of water loss. Life history tradeoffs and correlated selection responses were examined and overlap with previously published data. We crossed the resistant selected lines to desiccation-sensitive lines from the same control background to examine how each heterozygous resistant chromosome (excluding four) may improve desiccation resistance and observed that most of the resistance was due to genes on the third and first chromosomes, although interaction effects with the second chromosome were also detected. Results are compared to other selection responses and highlight the multiple evolutionary solutions that can arise when organisms are faced with a common selection pressure, although water loss rate remains a common mechanism in all studies.

Key words: *Drosophila melanogaster*, desiccation resistance, laboratory selection, physiology, dehydration tolerance.

Introduction

Insects in arid environments face the challenge of evaporative water loss owing to their small size and high surface area:volume ratio (Hadley, 1994; Schmidt-Neilson, 1990; Williams et al., 1998). This has led to a number of adaptations to minimize water loss, particularly in desert species (reviewed in Addo-Bediako et al., 2001; Gibbs, 2002; Hadley, 1994). The evolution of water balance in insects has been widely investigated using the insect model *Drosophila*, which is amendable to both natural and artificial selection for resistance to desiccation stress. For example, in natural populations of *D. melanogaster*, desiccation resistance often tends to be greater in populations from temperate zones compared with tropical zones (Hoffmann and Harshman, 1999), while in the laboratory, different *Drosophila* respond to selection for desiccation resistance (Hoffmann and Parsons, 1989a; Hoffmann and Parsons, 1993; Rose, 1996). By utilizing laboratory selection to investigate the life-history, physiological and genetic changes associated with selection responses, insights can be gained into the evolutionary factors that shape adaptive responses, as well as the types of traits that can increase desiccation resistance. These studies may also aid in elucidating the genetic basis of physiological changes over longer evolutionary timeframes, while comparative physiology between different selected populations may highlight common resistance mechanisms and trait interactions (Gibbs, 1999; Harshman and Hoffmann, 2000; Zera and Harshman, 2001).

In *D. melanogaster*, previous studies based on two different populations have examined the physiological adaptations, correlated responses and life-history tradeoffs associated with the desiccation selection response (Chippindale et al., 1998; Folk and Bradley, 2000; Folk et al., 2001; Gibbs et al., 1997; Hoffmann and Parsons, 1989a; Hoffmann and Parsons, 1989b; Rose et al., 1990; Rose et al., 1992; Williams et al., 1998). More recently, Bubliy and Loeschcke selected for desiccation resistance among other stress traits in order to examine

correlated responses with longevity (Bubliy and Loeschcke, 2005) but did not examine physiological responses to selection for desiccation. Direct comparison of the Hoffmann and Parsons (Hoffmann and Parsons, 1989a; Hoffmann and Parsons, 1989b) and Rose et al. (Rose et al., 1990; Rose et al., 1992) lines illustrates that enhanced desiccation resistance following selection can be associated with different evolutionary trajectories (reviewed in Hoffmann and Harshman, 1999). The three mechanisms by which insects can increase desiccation resistance include (1) reducing the rate of water lost, (2) increasing bulk water and (3) tolerating greater amounts of water loss (dehydration tolerance) (Gibbs et al., 1997; Gibbs and Matzkin, 2001; Hoffmann and Parsons, 1989a). While increased desiccation resistance in both sets of selected lines has consistently been associated with reduced rates of water loss, patterns of resource storage and partitioning as well as life history trait associations have varied markedly. For example, while the Rose et al. (Rose et al., 1990; Rose et al., 1992) lines (from now referred to as D and C lines) evolved increased wet weight (attributable to extra water and carbohydrate stores) and reduced rates of water loss, desiccation resistance in the Hoffmann and Parsons lines (Hoffmann and Parsons, 1989a; Hoffmann and Parsons, 1989b; Hoffmann and Parsons, 1993) was primarily associated with both reduced water loss and metabolic rates. Harshman and Hoffmann discussed several causes for these dissimilar responses, in particular the use of different base populations, selection regimes and degrees of adaptation to laboratory environments (Harshman and Hoffmann, 2000). For example, Hoffmann and Parsons derived their selected and control lines (Hoffmann and Parsons, 1989a) from a mass-bred population founded by 30 inseminated field females following three years of laboratory adaptation. By contrast, the D lines were derived from a mass-bred population pre-selected for postponed age of reproduction (O stocks) in 1980, five years following initial laboratory culture (Service et al., 1985). Despite inherent experimental differences, the studies have produced evidence of some robust traits, evolutionary constraints and potential for multiple evolutionary pathways available for desiccation resistance.

The primary aim of the present study was to establish a new set of desiccation-resistant selected lines of *D. melanogaster* for the analysis of the selection response in terms of physiology, correlated responses and life history traits, in addition to providing lines for understanding the genetic basis of the response to desiccation resistance selection, which remains poorly defined. Here, we present the physiological and correlated response data from the new lines selected for 26 generations. The traits assessed were primarily based on previous studies to form a comparative basis for understanding the mechanisms underlying artificially evolved resistance. We describe several components of water balance and partitioning, including measures of water loss rates, dehydration tolerance, bulk water levels, glycogen content and hemolymph volume. The selection response only partially overlapped with previous studies, including reduced water loss and increased wet mass; however, further dissection revealed that alternative mechanisms underlie the increased mass in these lines compared with the D lines. Here, increased desiccation resistance was primarily associated with an increase in dehydration tolerance, a physiological mechanism previously unobserved in selected studies, providing further evidence of the many ways *D. melanogaster* may evolve desiccation resistance, as well as an opportunity to further study a lesser understood adaptation to desiccation stress. We examined changes in development time and fecundity, as well as correlated responses with other climatic stresses; these traits overlapped with at least one previous study. The selection response was further characterized at the chromosome level by partitioning to each major chromosome the combined effects of dominant genes underlying desiccation resistance.

Materials and methods

Fly cultures

All rearing and assays were at 25°C, under constant light. Culture media comprised sucrose (5.4% w/w), dried yeast (3.6% w/w) and agar (1.8% w/w), with nipagin and propionic acid as preservatives.

Directional selection: desiccation resistance

The selected and control lines were founded from a mass-bred population comprising pooled isofemale lines collected from 10 locations along the Australian east coast in 2000. For the selection regime, 1000 non-virgin 3–6-day-old flies (mixed sexes) were randomly allocated into vials of 50 using CO_2 anesthesia and allowed 1 day for recovery prior to desiccation. Flies were desiccated in empty glass vials covered in gauze in a large glass chamber containing silica gel at a relative humidity (RH) of <10%, and the final 10% of surviving flies were used to found the following generation. The selected lines underwent this regime 26–29 times at the time of most assays, unless noted otherwise. The control lines were not subjected to any treatment and were maintained in comparable densities to the selected lines on media in discrete two-week cycles. In this selection regime, flies are starved as well as desiccated, although flies die from desiccation stress well before they die from starvation after several days. The two desiccation-resistant selected lines will be referred to as S1 and S2, and the control lines as C1 and C2.

Physiological assays

For the following assays, fourth generation progeny (F_4) from selection 26 were tested to counter any paternal and grandparental effects of desiccation. Batches of eggs were placed into vials containing 25 ml of fresh media, and emerging flies were collected within 24 h. The flies were aged for 3 days, separated by sex using CO_2 anesthesia, and held at a density of 10 or 20 flies per vial for another day. Weight loss in groups and individuals, mortality, water content and dehydration tolerance were conducted following Hoffmann and Parsons (Hoffmann and Parsons, 1989a) with minor modifications.

Detailed analyses of desiccation resistance

To assess desiccation resistance in the selected and control lines, a mortality curve was generated by desiccating five replicates of 10 females and 10 males (sexes tested separately). The flies were placed in empty vials sealed with gauze and were affixed with Parafilm® to another vial containing approximately 10 g of silica desiccant. Vials were scored at hourly intervals until all flies in a group had died. The time for half the flies to die (LT_{50}) was determined by linear interpolation.

Weight loss measured in groups

Using flow-through respirometry to detect rates of water loss in *D. melanogaster*, Gibbs et al. reported that water loss was high in the first two hours of desiccation before stabilizing for the next few hours (Gibbs et al., 1997). Consequently, in the present study, weight loss due to short-term desiccation was measured in groups of 20 females desiccated for 3 h (7–8 replicates). Groups were weighed on a Satorius microbalance to the nearest 0.1 mg before and after desiccation. Weight loss, expressed as the initial percentage of weight lost, was used as an estimate of water loss rates, although it may also reflect weight lost through metabolism and, to a much lesser extent, defecation (Gibbs, 2002).

Water content, weight loss and mortality of individual flies

To assay total water content and dehydration tolerance, individual C and S females were placed in vials as described above, and mortality was scored at hourly intervals. Females were weighed as described above immediately prior to desiccation and again after drying overnight at 60°C. Water content of the females was estimated from the difference between wet and dry weights. Dehydration tolerance was assessed as the percentage of total water lost at death (wet weight – weight at death)/(wet weight – dry weight).

Water partitioning, carbohydrate and lipid content

Altered patterns of resource storage, including water partitioning (Chippindale et al., 1998; Folk et al., 2001) and whole-body lipid content (Djawdan et al., 1998; Hoffmann and Parsons, 1989b), were examined in previous studies of desiccation-resistant selected lines. For example, Chippindale et al. observed that, relative to their controls, the D selected lines stored extra water and had high levels of the energy storage carbohydrate glycogen (Chippindale et al., 1998) and that the extra water was partitioned to the hemolymph (Folk et al., 2001). In the present study, we tested glycogen, hemolymph and gross lipid content in females. To measure hemolymph volume, blotting assays were conducted after Folk et al. (Folk et al., 2001) with slight modifications. Five replicate groups of 10 four-day-old females from each of the four populations were anaesthetized with CO_2 and weighed as a group. The abdomen of each was gently torn with surgical forceps, and hemolymph was blotted from the opening with a piece of Kimwipe® slightly moistened with isotonic saline. Within a maximum of 10 min, the 10 blotted flies were reweighed as a group and dried for 1 h at 60°C and weighed a third time. Hemolymph volume was estimated from the reduction in mass following blotting.

Glycogen levels were determined following the methods described by Clark and Keith (Clark and Keith, 1988) using the Sigma Glucose assay kit (product number GAGO-20; Castle Hill, NSW, Australia). Five replicates of 10 females per line were weighed to the nearest 0.1 mg and homogenised (on ice) in 250 μl of homogenisation buffer (0.01 mol l^{-1} KH_2PO_4, 1 mmol l^{-1} EDTA, pH 8). The homogenates were centrifuged at 17 949 ***g*** for 2 min at 4°C. The supernatant was then removed and placed in a microcentrifuge tube on ice. The pellet was resuspended in 100 μl of homogenisation buffer and recentrifuged, and the second supernatant was added to the first. The test reagent contained 0.1 U ml^{-1} amyloglucosidase, 5 U ml^{-1} glucose oxidase, 1 U ml^{-1} peroxidase and 0.04 mg ml^{-1} *o*-dianisidine dihydrochloride. The reagent is buffered by salts contained in the Sigma preparation of glucose oxidase and peroxidase (Clark and Keith, 1988). Aliquots of 10 μl of homogenate were added to 1.5 ml of test reagent and incubated at 37°C for 30 min. Optical density was read at 450 nm, and concentration of glycogen was estimated from a standard curve prepared using glucose standards.

Gross lipid content in females was measured following Hoffmann and Parsons (Hoffmann and Parsons, 1989b) with minor modifications. Briefly, females were dried at 60°C for 48 h in groups of 20 and weighed as described above. Lipid was extracted by placing whole flies in ether for 24 h. Flies were reweighed after drying again for 48 h. Five replicates of 20 flies from each line were extracted.

Correlated stress responses

To examine correlated abiotic stress responses associated with selection for desiccation resistance, we assayed the S and C lines for starvation resistance, cold and heat mortality. For starvation resistance, three replicates of 10 four-day-old females were tested using the two-vial method described for desiccation, substituting the desiccant with cotton wool soaked in 10 ml of water. Flies were scored at 8-h intervals until at least 50% in each vial were dead.

Cold mortality was examined by exposing S and C flies to subzero conditions until vials reached around 50% mortality. To determine this point, females were submerged in a –2°C cold bath filled with ethylene glycol for increments of 30 min ranging from 30 min to 2.5 h. Mortality was scored after 24 h. Initially three replicate vials of 10 flies were stressed at each time point, and the assay was repeated using 10 replicates of 10 females per line at 2.5 h. These assays were treated as separate blocks.

For heat stress, females were assessed for survival by subjecting three replicates of 10 females to a 39°C water bath for 30 min and scoring mortality after 24 h.

Correlated life history changes

The effect of selection for desiccation resistance on development time and early fecundity was assessed after 26

generations of selection. For development time, 20 replicates of 10 eggs were placed on 25 ml of fresh media. Egg to adult development was scored at 6-h intervals until all flies had eclosed; males and females were recorded separately to test for sex specific differences. Early fecundity was examined by placing pairs of virgin females and males (0–1 days old) into empty vials containing a small plastic spoon filled with treacle media coated with a 10% yeast suspension. Egg production within a 24-h period was recorded at the same time daily for 5 days, and early fecundity was calculated as the number of eggs produced, averaged over 15–18 replicates per line.

Chromosomal analysis

Desiccation resistance was mapped at the chromosome level in the resistant selected lines. The selected lines were each crossed to a line derived from the control lines (representing the same genetic background) that were briefly and weakly selected for six generations for desiccation susceptibility. We have also utilized this combination of lines to identify quantitative trait loci affecting survival to desiccation stress (K. M. Guthridge, M. Telonis-Scott, R. J. Hallas and A. A. Hoffmann, unpublished).

Indirect selection: desiccation susceptibility

Selection for desiccation susceptibility was carried out as described by Quintana and Prevosti (Quintana and Prevosti, 1990) with minor modifications. Thirty isofemale lines were established from the mass-bred population described above. From each isofemale line, 30 inseminated 4–7-day-old females were sorted and desiccated (as described above) until all lines reached 100% mortality. From the original 30 isofemale lines, the eight lines with the lowest desiccation resistance were chosen (for their siblings) to found the next generation; these lines all produced abundant offspring (minimizing the likelihood that they carried deleterious alleles with large effects). Untreated siblings were crossed (30 crosses between the eight isofemale lines) and offspring were reselected as described above. This fairly weak selection process was repeated for six generations and eventually resulted in eight isofemale lines with a lowered level of desiccation resistance. The two most desiccation-susceptible lines were used for the chromosome mapping crosses and were not included in the physiology assays.

To examine the effect of each resistant chromosome on resistance in heterozygous form (excluding chromosomes 4 and Y), crosses were set up between S1 and S2 and the two most susceptible of the indirectly selected lines (data not shown). Prior to crossing, heterozygosity in the selection/ susceptible lines was decreased by full sib mating for 16 generations (lines that maintained the resistant/susceptible desiccation phenotypes were maintained). The crosses were designed to isolate each chromosome in turn in heterozygous form in the desiccation-susceptible background. Four lines were used in the crosses, representing two replicates of each desiccation-resistant and -susceptible background. The three major chromosomes (X, 2 and 3) from the selection and susceptible lines were identified using three polymorphic microsatellite repeats that distinguished the inbred lines: for the X chromosome a repeat from the *period* locus was used (GenBank accession number AE003425), MS:AC004516 was used for chromosome 2 (AC004516) and MS:AC008193 for chromosome 3 (AC008193). For brevity, the lines established from the resistant selected lines with the high levels of resistance are referred to as H, and those lines from the susceptible selected lines with a low level of resistance are L. Resistant H males were mated to virgin L females and the reciprocal cross was also set up. The F_1 males from each cross were backcrossed to virgin H females, yielding eight classes of progeny (Fig. 4). F_2 females from each cross were phenotyped for desiccation resistance (as described for the selection regimes) and genotyped using a LiCor IR^2 DNA analyzer (Lincoln, NE, USA). Fecundity varied markedly between the crosses derived from the four inbred replicate selected lines, at least 15–20 F_2 females were assayed from each of the crosses derived from S1, and 48–50 females were assayed from each of the crosses derived from S2.

Analyses

Proportional data were arcsine transformed for normality whereas other data were analyzed untransformed, using ANOVAs, and differences between the lines were tested with planned contrasts, with three comparisons performed: (1) between the selected and control lines, (2) between controls and (3) between selected lines. Chromosomal effects on desiccation resistance and susceptibility were analyzed in a three-way ANOVA, with chromosomes X, 2 and 3 as fixed factors. Data from the two sets of crosses derived from S1 and S2 were pooled after preliminary analysis revealed similar chromosomal effects between the lines.

Results

Response to selection

Selection for desiccation resistance for 26 generations increased the LT_{100} on average by 1.9-fold in females and 1.7-fold in males (Fig. 1A,B). There was a highly significant line term in the LT_{50} ANOVA ($F_{3,32}$=83.690, P<0.001), as well as a significant line by sex interaction ($F_{3,32}$=14.458, P<0.001). Selected females reached LT_{50} on average 13.2 h later than their controls. While males also responded to selection, they were less resistant to desiccation than females, as the control males reached LT_{50} at around 8 h as opposed to an average of 13 h in the selected lines, representing an average increase in survival of 5 h.

Physiological assays

Weight loss after 3 h

Females were desiccated for three hours to test whether increased water conservation contributed to the selection response. Results and analyses for wet weight, dry weights and weight loss in groups of 20 after 3 h are given in Table 1. Females from the selected lines tended to lose less weight than control females, with a significant line term in the ANOVA

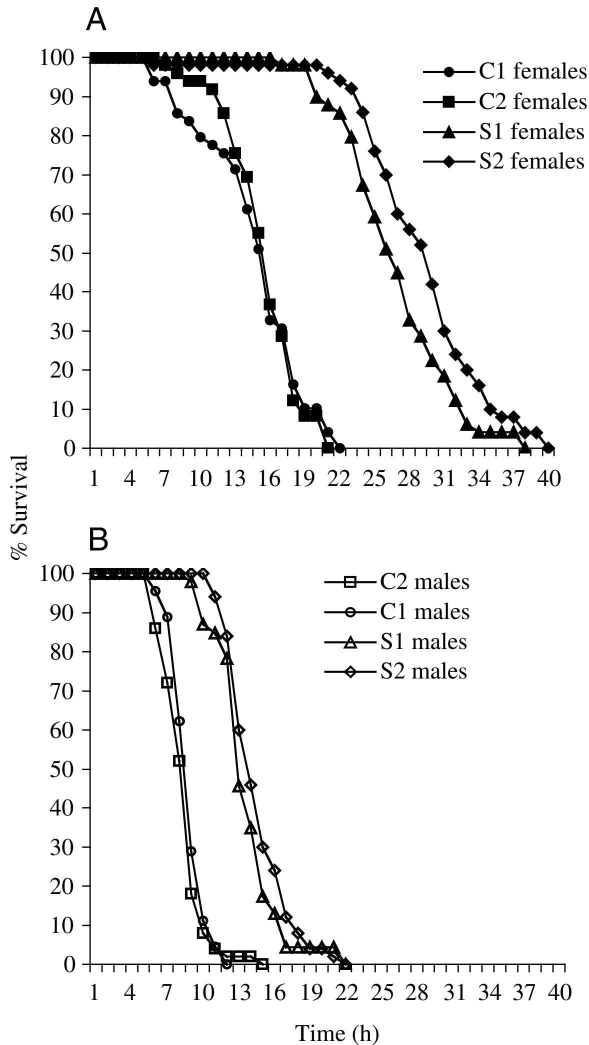

Fig. 1. Survival curve of the two selected and control lines (shown as average percentage survival per hour) when stressed, in groups of 10. (A) Females (filled markers); (B) males (open markers).

Table 1. *Wet weight, dry weight and percentage of weight lost by females after 3 h of desiccation*

		Wet weight ($g\times10^{-2}$)	Dry weight ($g\times10^{-3}$)	% Weight lost after 3 h
Control lines	1	2.88±0.09	7.06±0.33	11.18±0.99
	2	2.76±0.11	6.43±0.27	9.80±1.89
Selected lines	1	3.30±0.09	7.20±0.83	9.07±0.88
	2	3.08±0.08	7.25±0.45	6.37±1.57
Mean squares for ANOVAs[a] (d.f.)				
Line (3)		4.17×10^{-5}***	1.12×10^{-6}***	31.24***
Error (25)		1.03×10^{-6}	2.64×10^{-7}	1.88

[a]ANOVAs on weight loss were carried out on arcsin-transformed proportions (×100); ***P<0.001.

Values are means ± s.d. and are based on 7–8 replicates of 20 flies.

(P<0.001) and when tested with a planned contrast (P<0.001). In addition, the planned contrast between selected lines was significant, with S2 losing around 30% less weight after three hours than S1. For the wet weight of groups of 20 females, there was a highly significant line term in the ANOVA (P<0.001), and the larger selected females were significantly different from the controls when tested with a planned contrast (P<0.001). Moreover, there was significant within-line variation for group wet weight; planned contrasts were significant between the controls (P<0.05) and between the selected lines (P<0.001), with S1 weighing around 7% more than S2. There was a significant line term for dry weight (P<0.05), and the planned contrast between the control and selected lines was significant (P<0.05), as well as the contrast between the control lines (P<0.05).

Mortality and weight change of individual females

Data and analyses for mortality and weight changes of individual females are presented in Table 2. When flies were desiccated individually, there was a highly significant line term in the ANOVA (P<0.001), as individual S1 and S2 females survived twice as long as control females, and this was significant by a planned contrast (P<0.001). There was variation among the controls that was significant when tested with a planned contrast (P<0.05); C2 survived desiccation approximately 23% longer than C1 (an outcome not observed when groups of flies were tested). The data for body weight of individuals was consistent with the data collected in groups (Table 1), with a significant line term in the ANOVA (P<0.001), also evident when tested with a planned contrast (P<0.001). As in groups, S1 weighed around 8% more than S2.

We measured the percentage of total water lost at death to determine whether the selection response was associated with an increase in dehydration tolerance. There was a significant line term in the ANOVA (P<0.001), as the selected lines tolerated on average 10% more total water loss than the controls, and this was also significant when tested with a planned contrast (P<0.001).

To examine variation in water content, individuals were dried until they stopped losing weight. The line term was significant in the ANOVA (P<0.05), as well as a planned contrast (P<0.05). This variation may be attributable to differences between the control lines (planned contrast, P<0.05). There was a significant line effect for dry weight (P<0.0001), and the differences between the control and selected lines were significant when tested with a planned contrast (P<0.001), in addition to significant differences between the selected lines (P<0.05).

Water partitioning

Females were assayed for glycogen levels and weighed before and after hemolymph blotting to examine their potential for extracellular water storage. The proportional quantities of hemolymph volume, carbohydrate per fly and gross lipid content in groups of 20 flies are given in Table 3. The average hemolymph content per fly was 13%, while the average

Table 2. *Desiccation resistance (time to death), wet weight and weight loss of individual females*

		Time to death (h)	Wet weight ($g\times10^{-3}$)	% Weight loss: Total water loss at death	% Weight loss: After drying	Dry weight ($g\times10^{-4}$)
Control lines	1	10.60±2.28	1.40±0.13	47.36±5.71	74.60±2.63	3.57±0.57
	2	13.85±2.01	1.40±0.08	50.43±6.79	76.48±1.98	3.29±0.36
Selected lines	1	23.70±6.07	1.76±0.15	60.39±5.81	76.64±2.05	4.10±1.24
	2	26.25±4.73	1.61±0.11	59.22±5.67	76.52±2.27	3.79±0.43
Mean squares for ANOVAs[a] (d.f.)						
Line (3)		1140.63***	6.15×10^{-7}***	278.91***	8.35*	2.35×10^{-4}***
Error (76)		17.15	1.41×10^{-8}	12.18	2.28	2.15×10^{-5}

[a]ANOVAs on mass loss were carried out on arcsin-transformed proportions (×100); *P<0.05; ***P<0.001.
Values are means ± s.d. and are based on 20 replicates.

glycogen content per fly was 17%. There was no significant difference between the selected and control lines for hemolymph and glycogen content. Whole-body lipid content tended to be higher in the selected lines, and there was a significant line term in the ANOVA (P<0.001) as well as significant variation between the selected and control lines when tested with a planned contrast (P<0.001). Multiple comparisons also revealed intra-line variation for both the control comparisons (P<0.001) and selected line comparisons (P<0.001).

Life history traits

To determine the effect of selection for desiccation resistance on development time, egg to adult development was quantified for both males and females (Fig. 2A) and was found to increase in response to selection. Females eclosed earlier than males, and there was a significant line and sex term in the ANOVA ($F_{3,72}$=27.48, P<0.001 and $F_{1,72}$=12.80, P<0.001), respectively.

Early fecundity was examined by placing pairs of males and females in vials and scoring the total egg output over five days (Fig. 2B). There was a significant line term in the ANOVA ($F_{3,64}$=12.13, P<0.001), and all three planned comparisons

Table 3. *Hemolymph and glycogen content as a percent of wet weight per fly, and percent lipid content based on 20 flies*

		% Hemolymph	% Glycogen	% Lipid content
Control lines	1	13.62±3.52	14.42±4.38	14.04±1.08
	2	14.01±1.51	16.81±1.66	12.50±0.40
Selected lines	1	12.97±3.01	17.85±2.04	15.70±1.20
	2	12.60±1.02	19.74±4.39	18.70±0.70
Mean squares for ANOVAs[a] (d.f.)				
Line (3)		1.123	12.05	84.10***
Block (1)		15.18		
Error		3.249±12	6.834±12	8.45±15

[a]ANOVAs were carried out on arcsin-transformed proportions (×100); ***P<0.001.
Values are means ± s.d. and are based on 4–5 replicates of 10 flies.

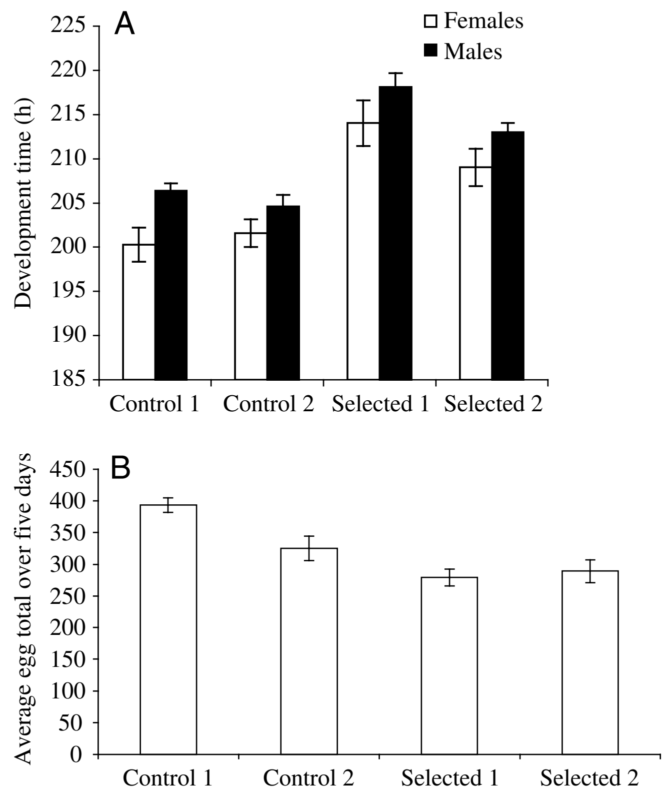

Fig. 2 (A) Egg to adult development time (25°C). Ten eggs were placed in vials and scored at 6-h intervals until all flies had eclosed. Open bars indicate females, while filled bars represent males. The error bars are standard errors around the mean of 20 replicates. (B) Early fecundity. Patterns of early fecundity were observed in pairs of 0–1-day-old flies, where egg production within a 24 h period was recorded at the same time daily for 5 days and was assessed as the mean number of eggs produced in total per line. The error bars are standard errors around the mean of 15–18 replicate pairs.

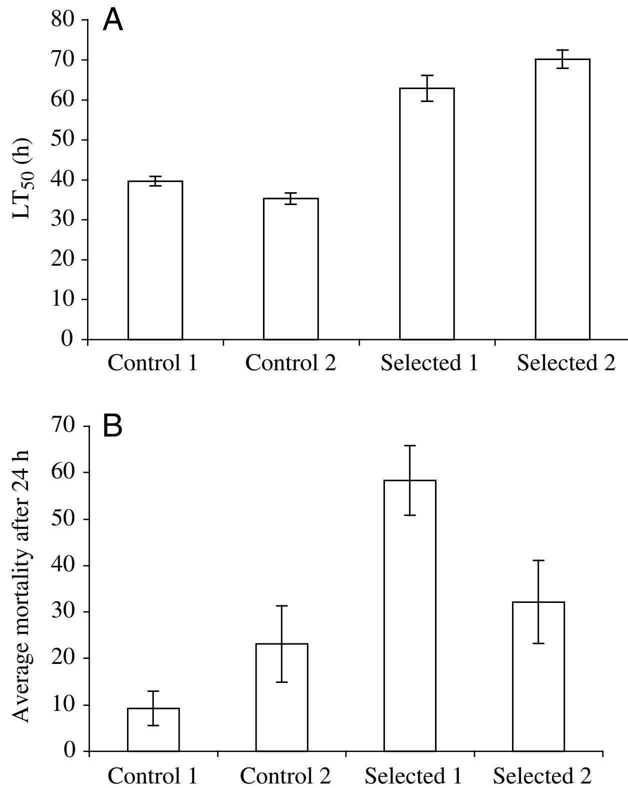

Fig. 3. Correlated stress responses. (A) Starvation resistance scored every 8 h until 50% mortality, with error bars as standard errors around the mean of three replicates of 10 females. (B) Cold mortality at –2°C for 2.5 h, average mortality after 24 h, with error bars as standard errors around the mean of 13 replicates of 10 females.

between lines were significant (P<0.001), while the overall trend was for a reduction in egg output in the selected lines.

Correlated stress trait responses

The increase in desiccation resistance was strongly correlated with increased resistance to starvation stress in the selected lines, which survived starvation twice as long as the controls (Fig. 3A). There was a significant line term in the ANOVA ($F_{3,8}$=881.59, P<0.001) as well as a highly significant planned comparison between the selected and control lines (P<0.001). Mortality 24 h after cold stress at –2°C for 2.5 h differed significantly between the lines ($F_{3,43}$=8.74, P<0.001), but this was due to increased mortality in only one of the selected lines compared with the other lines (Fig. 3B). Mortality after 24 h following heat stress at 39°C for 30 min was over 50% in all lines, and there was no difference for survival in the ANOVA ($F_{3,8}$=1.66, P>0.05).

Chromosome resistance

The ANOVA comparing LL (desiccation-susceptible) females with LH females (heterozygous for resistant selected line chromosomes) showed that when isolated in heterozygous form, chromosome 3 significantly contributed to the variation between the genotypes (P<0.001), with a significant interaction

Table 4. *ANOVA comparing LL homozygotes and HL heterozygotes for desiccation resistance*

Chromosome	d.f.	MS	F	P
1	1	63.70	3.605	0.059
2	1	15.38	0.877	0.350
3	1	265.60	15.150	<0.001
1×2	1	1.87	0.011	0.744
1×3	1	6.63	0.378	0.539
2×3	1	232.90	13.280	<0.001
1×2×3	1	28.79	1.642	0.202
Error	133	17.52		

effect between chromosomes 2 and 3 (P<0.001) (Table 4). Fig. 4 presents the average desiccation resistance of the eight genotypes generated by the crosses described in the Materials and methods. Desiccation resistance was highest when all chromosomes were heterozygous, while individuals heterozygous for chromosome 2 (SS RS SS) or both X and 2 (RS RS SS) showed no increase in resistance, with a very similar phenotype to the susceptible homozygotes (SS SS SS), although both chromosomes X and 2 increased resistance when in combination with chromosome 3. Both chromosome 3 (SS SS RS) and the X chromosome (RS SS SS) increased desiccation resistance in the susceptible background, although we did not detect significant effects for the X chromosome in the ANOVA (Table 4).

Discussion

Response to selection and physiology

We observed a substantial increase in both male and female survival to desiccation stress following selection for resistance, consistent with earlier studies of *D. melanogaster* (reviewed in Hoffmann and Harshman, 1999). There was sexual dimorphism for desiccation survival at the onset of our selection regime, and males were invariably culled before the

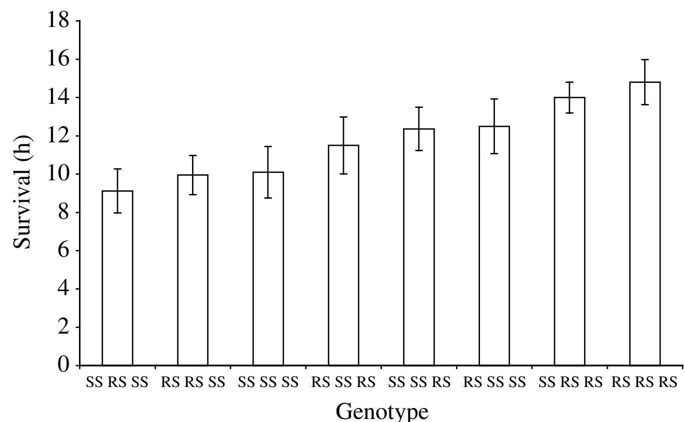

Fig. 4. Average desiccation resistance of the eight F_2 genotypes (females tested) generated from the crosses described in the Materials and methods.

Table 5. *Summary of genetic associations between traits involved in selection for desiccation resistance in D. melanogaster from three sets of lines of different founders*

Correlated trait	Hoffmann and Parsons	Rose and coworkers	Present study	References
Water loss rate	↓	↓	↓	1, 2
Metabolic rate	↓	↓	–	3, 4, 5*
Wet weight	NC	↑	↑	3, 2, 6
Dry weight	NC	↓	↑	3, 2
Glycogen/carbohydrate	–	↑	NC	5, 6
Hemolymph volume	–	↑	NC	7
Lipid level	NC	↓	↑	4, 5, 6
Water content	NC	↑	NC	1, 6, 7
Dehydration tolerance	NC	NC	↑	3, 2
Development time	NC	↑	↑	6, 1
Early fecundity	↓	NC	↓	3, 8
Starvation resistance	↑	↑	↑	3, 9

Reference abbreviations: 1, Hoffmann and Parsons (1993); 2, Gibbs et al. (1997); 3, Hoffmann and Parsons (1989a); 4, Hoffmann and Parsons (1989b); 5, Djawdan et al. (1998); 6, Chippindale et al. (1998); 7, Folk et al. (2001); 8, Chippindale et al. (1993); 9, Rose et al. (1992).

*The associations are for mass-specific metabolic rate. Djawdan et al. (1998) controlled for the amount of non-metabolizing mass (such as lipid and carbohydrate) and found that the negative association between metabolic rate and desiccation resistance disappeared.

↑, increase; ↓, decrease, NC, no change; –, not measured.

90% mortality mark, as also reported by Chippindale et al. (Chippindale et al., 1998), resulting in only inseminated females founding subsequent generations. Despite the different fitness requirements of the sexes, the presence of resistant males implies that there is a partially common genetic basis between the sexes underlying the selection response.

Given the fitness advantage of females over males for desiccation resistance, we were most interested in characterizing the physiological basis of the selection response in mature females. Table 5 presents a summary of genetic associations between traits involved in selection for desiccation resistance, both for the lines described in this study and those previously published. Here, selection was associated with an increase in wet weight. One obvious explanation for increased weight is that larger flies have a smaller surface area across which to lose water. This outcome is consistent with lines directly selected for desiccation resistance (Gibbs et al., 1997) and in lines selected indirectly in response to very mild desiccation stress (Kennington et al., 2003), but not in other direct-selection lines (Bubliy and Loeschcke, 2005; Hoffmann and Parsons, 1989b). Size and desiccation resistance covary in natural populations of *Drosophila*; van Herrewege and David observed that temperate species were on average heavier and survived desiccation longer than their tropical counterparts (van Herrewege and David, 1997). At first glance, the different sets of desiccation-selected lines have converged on similar size phenotypes, but further examination of the mechanisms underlying the selection responses reveals very different adaptations. For example, relative to their controls, the D lines are larger, due primarily to post-pupation increases in bulk water, shown to be partitioned to the hemolymph (extracellular accumulation) rather than associated with augmented glycogen levels (intracellular accumulation) (Chippindale et al., 1998; Folk et al., 2001). Conversely, we observed that resistant females do not sequester extra water as a resistance strategy; accumulated water could not account for the increase in wet mass, and consistent with this observation is the lack of association with hemolymph and glycogen content in resistant females compared with their controls.

The primary mechanism by which *Drosophila* survive desiccation is increased water retention, a response consistently demonstrated across multiple studies, including natural adaptation in desert species (Gibbs, 2002; Gibbs et al., 2003), artificial selection experiments (Hoffmann and Harshman, 1999) and a mutagenesis study (Telonis-Scott and Hoffmann, 2003). We also observed decreased water loss rates in the selected lines, although this was not consistent in comparisons between all control and selected lines, and the selected lines also varied (S2 lost approximately 30% less water than S1 in the same 3-h period). In contrast to previous selection studies (see Table 5), as well as data from xeric *Drosophila* (Gibbs and Matzkin, 2001), the selected populations also endured desiccation longer by increasing dehydration tolerance by around 10%, measured as total water loss prior to death. Some mechanisms that insects may employ to tolerate low water content include compartmentalizing water, regulating osmotic effects, rendering a higher proportion of water osmotically inactive or, in the severest form of dehydration, some insects undergo anhydrobiosis (Danks, 2000). These selected lines provide an opportunity to further explore the physiological basis of enhanced dehydration tolerance in *D. melanogaster* from a laboratory evolution perspective. In terms of the primary physiological adaptations insects may evolve in response to desiccation stress, the selected females do not accumulate extra water but show increased water conservation in addition to tolerating greater water loss during desiccation. Table 5 illustrates that these particular adaptive responses in concert are unique to this

set of lines, further emphasizing that the abundant variation for desiccation resistance in *D. melanogaster* may lead to many potential evolutionary pathways.

Evidence suggests that in *D. melanogaster*, body size, water content and carbohydrate content are genetically correlated (Clark and Doane, 1983; Clark et al., 1990; Folk et al., 2001). In the present study, we tested whole-body lipids and found an overall trend for increased lipid storage in the selected lines. Clark et al. observed that body size tended to decrease as lipid content increased in *D. melanogaster* artificially selected for lipid storage (Clark et al., 1990); interestingly, S2 stored more lipids and weighed significantly less than S1. Previous studies have demonstrated that water volume and lipid storage are inversely proportional (Clark and Doane, 1983; Clark et al., 1990; Folk et al., 2001); for example, Folk et al. proposed that, in response to selection, the D flies accumulated more water and therefore may be constrained to reduce lipid content (Folk et al., 2001). Differences in lipid levels between the two different sets of selected lines are not unexpected given the different control treatments employed, as the D control lines (C lines) accumulated more lipids, most likely owing to an evolutionary history of mild starvation stress (Chippindale et al., 1998). The selected lines developed here may be constrained to evolve increases in water volume due to increased lipid storage and/or correlations with other biochemical characters involved in dehydration tolerance.

Correlated stress responses

The association between starvation and desiccation resistance following artificial selection has been well documented in *D. melanogaster* (Hoffmann and Parsons, 1993; Harshman et al., 1999), as well as in natural populations of temperate *Drosophila* (van Herrewege and David, 1997). We also report a strong positive correlation between starvation and desiccation resistance following selection. Previous studies suggested that this association might be in part due to shared patterns of glycogen storage (Hoffmann and Harshman, 1999) although this seems an unlikely scenario here. In the present study, the positive correlation between starvation and desiccation might be associated with increased lipid reserves; starvation resistance in *D. melanogaster* is correlated with increased lipids, evident from data collected from adipose^{60} mutants (Clarke and Doane, 1983) as well as selected lines (Chippindale et al., 1996; Harshman et al., 1999). However, this hypothesis requires further rigorous physiological examination; the effect of mild starvation stress on the selected lines in contrast to the untreated controls needs to be considered, although Hoffmann and Parsons did not observe augmented lipids following selection *via* an identical selection regime (Hoffmann and Parsons, 1989b).

We did not observe a correlation with heat mortality and increased desiccation resistance, in contrast to Bubliy and Loeschcke, who suggest that heat shock proteins may underlie the positive correlation between the traits (Bubliy and Loeschcke, 2005). However, we did observe a negative correlation with cold mortality and desiccation resistance in S1. There is no physiological data to suggest a tradeoff for cold tolerance in desiccation-resistant females, although Hoffmann et al. reported the same tradeoff in females selected for starvation resistance and cold resistance, respectively, potentially related to lipid metabolism (Hoffmann et al., 2005). As the starvation lines from the latter study were derived from the controls here, there may be some common mechanism related to lipid metabolism underlying both starvation and desiccation resistance and contributing to the tradeoff with cold survival. However, the complex antagonistic pleiotropy observed here is difficult to interpret given the response in only one selected line. Clearly, the replicate lines have exhibited variation in responses, despite efforts to maintain identical treatments. This can occur when two replicates (present study) or five replicates (Bradley and Folk, 2004) are compared and may reflect levels of genetic variation in the base stocks expressed through replicate lines.

Life history tradeoffs

Evidence from populations of *D. melanogaster* selected for stress resistance suggests that energy allocation may influence tradeoffs between survival and reproduction (Borash and Ho, 2001). Studies have directly tested this hypothesis and shown that there is a reproductive cost to stress resistance for oxidative stress (Wang et al., 2001) and both oxidative and starvation stress (Salmon et al., 2001) as well as resistance to cold (Watson and Hoffmann, 1996). For desiccation resistance, tradeoffs between resistance and early fecundity were reported in one study (Hoffmann and Parsons, 1989a) but not in another (Chippindale et al., 1993). Zera and Zhao demonstrated that lipid accumulation (in the form of triglycerides) is a key component in the tradeoff between increased early reproduction and reduced flight capability in the short-winged morph of *Gryllus firmus* (Zera and Zhao, 2003). The negative association between lipid biosynthesis and early ovarian development may occur for starvation-selected lines as well. We observed significant variation between the control and selected lines for total egg number over five days, with egg number reduced in the selected lines, in addition to significant variation in intra-group comparisons. Hoffmann and Parsons suggested that the decline in early fecundity in their lines was related to reduced metabolic rates because they found no changes in lipid levels (Hoffmann and Parsons, 1989a). It is possible that the correlated fecundity response in the present study may be associated with lipid levels, potentially of the same nature as those utilized for starvation resistance, however more detailed physiological assays are required, and basal metabolic rate also needs to be considered. Selected flies of both sexes tended to take longer to develop; this result was reported by Chippindale et al. (Chippindale et al., 1998) but not by Hoffmann and Parsons (Hoffmann and Parsons, 1993) or Bubliy and Loeschcke (Bubliy and Loeschcke, 2005), although these lines were selected for a shorter duration and the expression of different patterns of resource acquisition may require longer selection.

Prolonged development time has also been documented in lines selected for starvation resistance in both long-term laboratory-adapted lines (Chippindale et al., 1996) and recently derived field lines (Harshman et al., 1999), suggesting a general association between starvation resistance, increased lipid and increased development time (Harshman et al., 1999). If increased lipids contribute to both the correlation between starvation resistance and other life history traits, it is possible that we may have selected for an overlapping pathway of general stress resistance in these desiccation-resistant lines.

Chromosome mapping

The outcome of the chromosome mapping crosses suggests that autosomal (chromosome 3) and (to a lesser extent) X-linked genes improve desiccation resistance in a desiccation-susceptible background derived from the same control population as the resistant-selected lines. A direct comparison of the selected and untreated control lines would provide information as to which chromosomes carry genes to improve desiccation resistance, however our comparison of both resistant and susceptible lines derived from the same background is likely to provide similar information. Deleterious alleles might contribute to decreased resistance in susceptible lines compared with the controls, but we only undertook weak selection for susceptibility for a short duration (six generations) among family groups that all produced offspring, making it less likely that deleterious genes contributed to the selection response. We also did not find any reduction in fitness of the susceptible lines that might have indicated the presence of such genes. In concurrence with our results, Hoffmann and Parsons found that the response to selection for desiccation resistance was not sex specific and reported that both autosomal and X-linked genes acted mostly additively (Hoffmann and Parsons, 1989b).

The interactions between chromosomes detected in the present study suggest some epistasis among genes related to stress resistance; widespread epistasis was also observed at the gene expression level during starvation stress (Harbison et al., 2005) and may be expected for complex traits such as stress resistance.

In summary, our results highlight the diversity of mechanisms that can underlie responses to selection for desiccation resistance. We have found a new mechanism (tolerance of degree of total water loss) not detected in previous studies and confirmed the central role of water conservation in selection responses for increased desiccation resistance. Correlated responses in life history traits show overlap with those detected in other studies. Altered desiccation resistance is associated with genes on the third chromosome but there are also interactions with genes on chromosome 2.

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