Using Experimental Evolution to Study the Physiological Mechanisms of Desiccation Resistance in *Drosophila melanogaster*

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Accepted 12/21/2006; Electronically Published 5/8/2007

Online enhancement: appendix tables.

ABSTRACT

Data from populations undergoing experimental evolution can be used to make comparisons between physiologically differentiated populations and to determine evolutionary trajectories. Comparisons of long-established laboratory populations of *Drosophila melanogaster* that are strongly differentiated with respect to desiccation resistance are used to test alternative hypotheses concerning the mechanisms that fruit flies use to survive bouts of extreme desiccation. This comparative study supports the hypothesis that, in at least one case, *D. melanogaster* can evolve increased resistance to desiccation by decreasing water loss rates and by increasing bulk water content but not by increasing metabolic water content or dehydration tolerance. While glycogen was involved in water storage, its primary role was in water binding, not the production of metabolic water. Measurement of the trajectories of these component mechanisms during selection for desiccation resistance is used to demonstrate that water loss rate quickly plateaus in response to selection, while water content continues to improve. This disparity reveals the value of studying evolutionary trajectories and the need for longer-term selection studies in evolutionary physiology.

Introduction

The ability to withstand dry environments is one of the most important limiting factors for small insects. The relatively high ratio of surface area to body mass in small insects increases the amount of water lost by evaporation relative to the amount of water they can store, making them susceptible to dehydration. There are three physiological mechanisms by which an insect can overcome this problem: (1) increase the amount of water stored as both bulk water and metabolic water, (2) decrease the rate at which water is lost, or (3) increase the ability to tolerate water loss. By means of comparative studies, a variety of physiological traits that enhance the ability to resist desiccation have been associated with species from dry environments (reviewed by Hadley 1994). For example, Parsons (1970) showed that populations collected in dry environments have higher wet and dry weights than populations collected in more humid environments. In addition, lower water loss rates have been associated with *Drosophila* species found in dry environments (Eckstrand and Richardson 1980, 1981). Specifically, cuticular permeabilities have been shown to be lower in species located in dry environments than in those from more humid environments (Hadley 1994).

Results from selection studies investigating the mechanisms of desiccation resistance in *Drosophila* species are conflicting. In general, it is well established that desiccation resistance responds to selection in *Drosophila* species (Hoffman and Parsons 1989*a*, 1993; Rose et al. 1990, 1992). Even so, the extent to which particular physiological mechanisms contribute to desiccation resistance in *Drosophila* species is in dispute. Although most selection studies find that water loss rates are reduced in *Drosophila* populations selected for increased desiccation resistance and that dehydration tolerance does not contribute to desiccation resistance in these populations, there is some question as to whether increased water storage is involved in desiccation resistance (Hoffman and Parsons 1989*a*, 1993; Graves et al. 1992; Blows and Hoffman 1993; Gibbs et al. 1997; Gibbs 2002). There are two ways to store water: bulk water storage (water molecules that are obtainable from sources other than catabolism itself) and metabolic water storage (water molecules obtainable directly from catabolism). Hoffman and Parsons (1989*a*, 1993) and Blows and Hoffman (1993) found no differences in bulk water content storage, wet weight, or dry weight between selected and control lines for *Drosophila melanogaster* and *Drosophila simulans*. By contrast, Gibbs et al. (1997) found that populations of *D. melanogaster* selected for desiccation resistance had 30% more bulk water than their control populations. In addition, increased glycogen content, a possible source of metabolic water, has been associated with desiccation resistance (Graves et al. 1992; Gibbs et al. 1997), although

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Physiological and Biochemical Zoology 80(4):386–398. 2007. 1522-2152/2007/8004-6115$15.00

Hoffman and Parsons (1993) and Blows and Hoffman (1993) did not find this association. Extractable lipid, another possible source of metabolic water, has not been associated with desiccation resistance in *Drosophila* (Hoffman and Parsons 1989*b*; Graves et al. 1992; Gibbs et al. 1997). In this study, we determine the extent to which these possible physiological mechanisms contribute to desiccation resistance in *D. melanogaster* using both comparative data from the end-products of an artificial radiation produced by experimental evolution and data obtained using selection trajectories.

Material and Methods

Populations

In 1980, five O populations were derived from a large outbred laboratory population of *Drosophila melanogaster* originally collected in South Amherst, Massachusetts, by P. T. Ives, the base population being designated IV. These O populations are selected for late-life fertility (Rose 1984). In 1988, five D and five C populations were derived from each O population (Rose et al. 1990, 1992). The D populations are selected for desiccation resistance, and the C populations are their controls. In 1989, the SO populations were derived from each O population and selected for starvation resistance (Rose et al. 1992). Briefly, each of these involves a selection regime in which individuals had to survive a specific test, such as survival to late ages (O selection), survival under desiccating conditions (D selection), or survival under conditions of complete starvation but without desiccation (SO and C selection). However, fecundity and mating success were not controlled, making these procedures different from traditional artificial selection (Rose et al. 1990). See Rose et al. (2004) for detailed descriptions of these populations and their selection regimes.

In 1995, five NDO (new desiccation-resistant populations derived from the O population) and five NDCO (new desiccation-resistant control populations derived from the O population) populations were derived from each O population. The NDO and NDCO populations have the same selection regimes as the D and C populations, respectively. There are two reasons why the NDO and NDCO populations were created: (1) these populations were used to trace the initial evolutionary trajectory of each trait assayed and (2) the NDO populations were also used as populations for the comparative study.

Assay Procedures

Rearing of Experimental Flies for Each Assay. Before each assay was conducted, the experimental flies were taken off selection for two generations in order to establish a common rearing environment and to remove any parental effects. Within each assay generation, the flies were reared in 8-dram vials at ca. 60 eggs per vial for 14 d, standardized densities being achieved by monitoring of egg laying and manual transfers of eggs. The flies were then placed in cages and given ample food for no more than 2 wk. Twenty vials were used per population, so the population size was maintained at ca. 1,200 flies per generation. After two generations, the flies used for the assay were collected at exactly 60 eggs per vial and reared in 8-dram vials at 25°C.

Handling procedures for the experimental flies after eclosion varied depending on the assay. The experimental flies used in the blowing-air desiccation assay, the water-content-stored assay, and the water-loss-rate assay were transferred into vials with fresh banana-molasses medium within 24 h after eclosion and then transferred again after 2 d into new vials with fresh medium. This ensured that all of the experimental flies were alive and 4–5 d old when these assays were conducted. The experimental flies used in the stored-glycogen-content and stored-lipid-content assays were also transferred into fresh medium at eclosion and again after 2 d. When the flies were 4–5 d old, they were sorted by sex on CO_2 plates, placed in 1.5-mL centrifuge tubes, and then frozen at −70°C until the glycogen and lipid assays were conducted. Thus, these flies were killed before they were assayed for their gross biochemistry. The desiccation assay with desiccant did not follow the transfer procedure described above, as the experimental flies used in that assay were ca. 3–5 d old when they were assayed

Desiccation Resistance Using Desiccant. This assay measured the length of time in which a fly is able to live under dry conditions in an 8-dram glass vial containing desiccant. This procedure is described in more detail in Service and Rose (1985). A total of 40 males and 40 females were assayed from each population.

Desiccation Resistance Using Blowing Air. This assay measured the length of time in which a fly is able to live under dry conditions in a 1-mL plastic syringe tube with constant air flowing over the fly. An air compressor was used to pump air through these tubes and was connected to a Sable Systems (Henderson, NV) multiplexor used to divide the airflow into eight chambers. Five of these chambers were used, one for each replicate. One string of 300 1-mL tubes linked together by plastic tubing was connected to each chamber used. The first 120 tubes were not used and the next 180 tubes contained one fly per tube. Initially, 30 flies for each population were measured. On average the flow rate through the tubes was 18.34 mL/min and ranged from 10.8 mL/min to 25.9 mL/min between replicates.

The flies were checked every 1–2 h. The time of death was determined as the midpoint between each check. After time of death was determined, the fly was removed from the 1-mL plastic tube and placed in a 1.5-mL centrifuge tube. It was then weighed within 0.75–2.25 h of the time of death and then frozen at −70°C. Sample size varied from 26–30 flies for each population. All flies were 4–5 d old.

This assay was used for the comparative study, instead of the desiccation resistance assay that used desiccant, so that

water loss rates and desiccation resistance could be measured under similar conditions of moving dry air.

Water Content before the Desiccation Stress (Stored Water Content). This procedure is a modified version of the Gibbs et al. (1997) method. Briefly, 10 adult flies were frozen on dry ice. After 90 s, the 10 flies were weighed on a Cahn microbalance. These flies were then dried at 55°C. After ca. 24 h, the 10 flies were reweighed. Water content was calculated as the difference between wet and dry weight. Five sets of 10 females were used for each population. All flies were 4–5 d old.

Bulk Water Content after the Desiccation Stress (Dehydration Tolerance). This procedure is similar to that for water content before the desiccation stress except that the experimental flies measured were taken at death from the desiccation resistance assay (using blowing air). After the death of the flies was determined, the flies were weighed individually on a Cahn microbalance and then frozen in a −70°C freezer. The amount of time between death and time of weighing ranged from ca. 45 min to 2 h, for an average of ca. 90 min. The frozen flies were then dried at 60°C for 45 min and reweighed. Water content was calculated as the difference between wet and dry weight. Sample size varied from 26–30 flies for each population.

This assay measured the total amount of bulk water each population used while desiccating. This value was calculated by taking the difference between mean water content storage and mean dehydration tolerance for each population.

Water Loss Rates. This procedure was similar to that of Gibbs et al. (1997). Briefly, this procedure measured water loss rates using a Sable Systems flow-through respirometry system. Dry air was passed through 5-mL glass and aluminum chambers that contained 20 female flies/chamber. Dry air was passed through the empty chambers for 1 h, 6 h before recording water loss rates of the experimental flies in these same chambers, and each chamber had a corresponding control chamber. This system was contained in a temperature-controlled room at 25°C. For each population, 3–4 chambers of 20 female flies were assayed. Flies were placed in the chambers 2–3 h before measurements began. The flow rate through all chambers, control or experimental, was 25 mL/min. Each chamber was continually flushed, thus preventing the accumulation of water vapor. Only data from the last 30 min of a 1-h run were used in the analysis.

Glycogen Content before the Desiccation Stress (Glycogen Content Stored). The procedure employed was similar to the modified version of the carbohydrate extraction method of Van Handel (1965) described in Djawdan et al. (1997). Three standardized glycogen samples and two duplicate standard curves were prepared for each assay. Although these standard curves did not differ significantly, the curve that best predicted the known amount of glycogen was used to calculate the carbohydrate content of the flies. The absorbencies were read on a Bausch and Lomb Spectronic 20 spectrophotometer. Five sets of five female flies were assayed per population. All populations within each replicate were assayed together. All flies were 4–5-d-old adults.

Glycogen Content after the Desiccation Stress (Glycogen Content at Death). This assay is identical to the above assay except that the flies were taken at death from the desiccation assay (using blowing air). At death, these flies were frozen in a −70°C freezer until the assay was conducted. In addition, five sets of three female flies were used per population. Each replicate was assayed in parallel with the corresponding replicate of flies assayed for glycogen content stored.

Glycogen Content Used during the Desiccation Stress (Glycogen Content Used). This assay measured the total amount of glycogen each population used while desiccating. This value was calculated by taking the difference between mean glycogen content stored and mean glycogen content at death for each population.

Lipid Content before the Desiccation Stress (Lipid Content Stored). The procedure employed was identical to that of Djawdan et al. (1997), except that five sets of five females were assayed per population. The difference between initial dry mass and dry mass after the petroleum ether extraction of lipid was used as the total lipid content of the flies. Again, each replicate was assayed at the same time. All flies were 4–5 d old.

Lipid Content after the Desiccation Stress (Lipid Content at Death). This assay was identical to the above assay except that the flies were taken at death from the desiccation assay. At death, these flies were frozen in a −70°C freezer until the assay was conducted. Three sets of five female flies were used per population. Each replicate was assayed in parallel with the corresponding replicate of flies assayed for lipid content stored.

Lipid Content Used during the Desiccation Stress (Lipid Content Used). This assay measured the total amount of lipid used while desiccating. This value was calculated by taking the difference between mean lipid content stored and mean lipid content at death for each population.

Statistical Analyses

Analysis for the Comparative Study. Although the populations within each lineage are closely related (e.g., O_1 is more closely related to D_1 than to O_2), preliminary results show that the mean phenotypic values for desiccation resistance (assay using desiccant) are significantly different among selection regimes, except that the difference in means for the SO and C popu-

Table 1: Replicate means, treatment means, and standard errors (SEs) of desiccation resistance (h) assayed using desiccant for the D, NDO, SO, C, NDCO, and O populations

Replicate	D	NDO	SO	C	NDCO	O
1	58.40	45.58	26.28	20.33	15.20	14.23
2	77.48	50.30	23.81	15.25	13.86	11.50
3	109.15	51.10	20.15	18.03	14.25	12.68
4	87.72	54.68	18.00	20.78	14.83	12.50
5	92.65	35.73	19.95	16.70	13.37	12.28
Mean	85.08	47.48	21.64	18.22	14.30	12.64
SE	8.41	3.28	1.49	1.05	.33	.45

Note. Generation 184 of the D and C populations, generation 37 of the NDO and NDCO populations, generation 116 of the SO populations, and generation 105 of the O populations were used.

lations is marginally significant (Table 1, one-way ANOVA comparing treatment differences gave the following results: $P_{D vs NDO} = 0.003$; $P_{NDO vs SO} < 0.001$; $P_{SO vs C} = 0.098$; $P_{C vs NDCO} = 0.007$; $P_{NDCO vs O} = 0.017$). Furthermore, the populations that are not closely related but were subjected to the same selection regime (e.g., O_1, O_2, O_3, O_4, and O_5) do not differ substantially with respect to the amount of time able to survive desiccation stress relative to populations subjected to other selection regimes (Table 1). Because of this distribution, we conclude that our data group has five statistically independent but parallel evolutionary radiations.

We include the SO and NDO populations so that the distribution of dependent values has intermediate values. This prevents the regression analysis from being driven by the extreme values (Garland and Adolph 1994); desiccation resistance using blowing air (the dependent variable) was individually regressed on water content used, glycogen content used, lipid content used, water loss rates, and dehydration tolerance (the independent variables) in order to reveal the individual relationship between each of these traits and desiccation resistance. A plot of the studentized residuals versus the predicted values revealed that the variance of the dependent variable was not the same for all values of each independent variable. Log transformation of desiccation resistance was used to correct for this heterogeneity of variance.

Multiple regression analysis was used to examine the extent to which each trait contributes to the explanation of the variance observed in desiccation resistance (using blowing air) when all other traits are held constant. We used a forward selection method to choose predictor variables (Sokal and Rohlf 1995). Briefly, the forward selection method first chooses the independent variable that has the lowest P value for the t statistic testing the null hypothesis that the partial regression coefficient is 0. Next, for each variable not in the equation, the partial regression coefficients are calculated as if that variable were added to the model next. The variable with the lowest P value is then added to the model. For this analysis, the P value at which variables are no longer added is 0.05. The Pearson correlation coefficients are reported here to show the correlative relationship between all variables. These analyses used SPSS (ver. 6.1).

A priori Models. We developed an a priori model to predict the amount of time a fly can survive desiccation, using basic physiological assumptions. This model is different from the multiple regression analysis in that the model directly describes the relationship between all variables. The equation for this model is

$$\frac{(a + b + c)}{d} = e,$$

where a = water content used during the desiccation stress, b = all metabolic water possibly obtained from glycogen used during the desiccation stress (0.56 g water/1 g glycogen metabolized), c = all metabolic water possibly obtained from lipid used during the desiccation stress (1.07 g water/1 g of lipid metabolized), d = water loss rates, and e = number of hours predicted for resistance to the desiccation stress. This model is referred to as model 1. This model is then compared with a model not including variable c (model 2: $e = [a + b]/d$) and also to a model not including variables b and c (model 3: $e = a/d$).

The P value for the ANOVA, the adjusted R^2 values, the slopes, and the PRESS statistics were compared to determine which model best fits the data. The PRESS statistic is the sum of the squared differences between the predicted and observed value for each observation with that observation deleted from the computation of the regression equation (Allen 1971). In addition, Cook's distance was used to examine the data for points that may influence the results of the regression model. This statistic is a measure of how much the residuals of all cases would change if the current case were omitted from the calculations. If the Cook's distance is 4 or more, the data point is taken into consideration as a highly influential point. This analysis used Sigma Plot Regression Wizard (ver. 4.01).

In order to test if a point substantially influences a model, we first calculated the squared difference between the predicted and observed value of the influential point. The predicted value is calculated using the regression model that does not include this point ($[y$ (predicted from the regression model without influential point included) $- y$ (observed)$]^2$). Then we compared this squared deviation with the PRESS statistic calculated for the regression model that does include the influential point. If all points are equally influential, then this squared deviation is expected to be about $1/n$ of the PRESS statistic calculated for the model that includes the influential point. Here we call this test the "influential point test."

Analysis for the Responses to Selection. One-tailed paired *t*-tests are used to compare NDO and NDCO means for desiccation resistance in vials, water content stored, water loss rates, and glycogen content stored at each generation assayed. A paired *t*-test was used instead of a one-way ANOVA because the five pairs of NDO and NDCO populations were each recently derived from a different O population. A one-tailed test was used because there is an a priori reason to expect the selected populations will increase in mean value for each trait, whereas the controls will not.

Linear regression analyses were used for each trait to determine if the slope of the line describing the response to selection was significantly different from 0. The treatment means and standard errors for the treatment means were based on the five population means within each treatment ($N = 5$). These analyses used SPSS (ver. 6.1), with generation number as the independent variable.

A one-way ANOVA analysis was used to determine if the NDO populations were significantly different from the D populations for each trait assayed. The treatment means and standard errors for the treatment means were based on the five population means within each treatment ($N = 5$). These analyses used SPSS (ver. 6.1).

Results

Multiple Regression Analysis

Table 2 shows the replicate means, treatment means, and standard errors for the treatment means of desiccation resistance using blowing air, water content used, water loss rates, glycogen content used, lipid content used, and dehydration tolerance, the last quantified by total water content at death. In this table, the treatments are in order from those in which flies are least able to resist dessication stress to those in which flies are the most resistant. Figure 1 shows the hours of resistance to the desiccation stress using blowing air (log transformed) regressed on each additional trait measured: (A) water content used, (B) water loss rates, (C) glycogen content used, (D) lipid content used, and (E) dehydration tolerance. Table 3 shows the equation for each regression line, the R^2 value, and the F statistic and P value for the ANOVA. The R^2 value for the regression model including the variable for water content used was the highest ($R^2 = 0.608$) but was only slightly higher than the R^2 value for the regression model including the variable for glycogen content used ($R^2 = 0.568$). In addition, the regression model including the variable for lipid content used had the lowest R^2 value ($R^2 = 0.063$).

The Pearson correlation coefficients between all variables are

Table 2: Treatment means (Avg) and standard errors (SE) of treatment means for desiccation resistance using blowing air, water content used, water loss rate, glycogen content used, lipid content used, and dehydration tolerance

Type	Desiccation Resistance (h)	Water Content Used (mg/fly)	Water Loss Rate (μL/h/fly)	Glycogen Content Used (mg/fly)	Lipid Content Used (mg/fly)	Dehydration Tolerance (mg/fly)
C:						
Avg	9.919	.520	.034	.053	.058	.418
SE	.797	.028	.002	.004	.021	.018
NDCO:						
Avg	9.980	.450	.034	.049	.048	.378
SE	.791	.018	.002	.003	.009	.016
O:						
Avg	10.290	.503	.036	.049	.015	.319
SE	.875	.033	.002	.004	.010	.020
SO:						
Avg	14.590	.581	.032	.165	−.004	.447
SE	1.653	.043	.002	.020	.016	.025
NDO:						
Avg	19.881	.583	.022	.135	−.009	.426
SE	1.461	.010	.001	.011	.014	.008
D:						
Avg	31.302	.931	.025	.190	.030	.451
SE	1.649	.094	.002	.032	.015	.028

Note. Refer to Table A1 in the online edition of *Physiological and Biochemical Zoology* for replicate means.

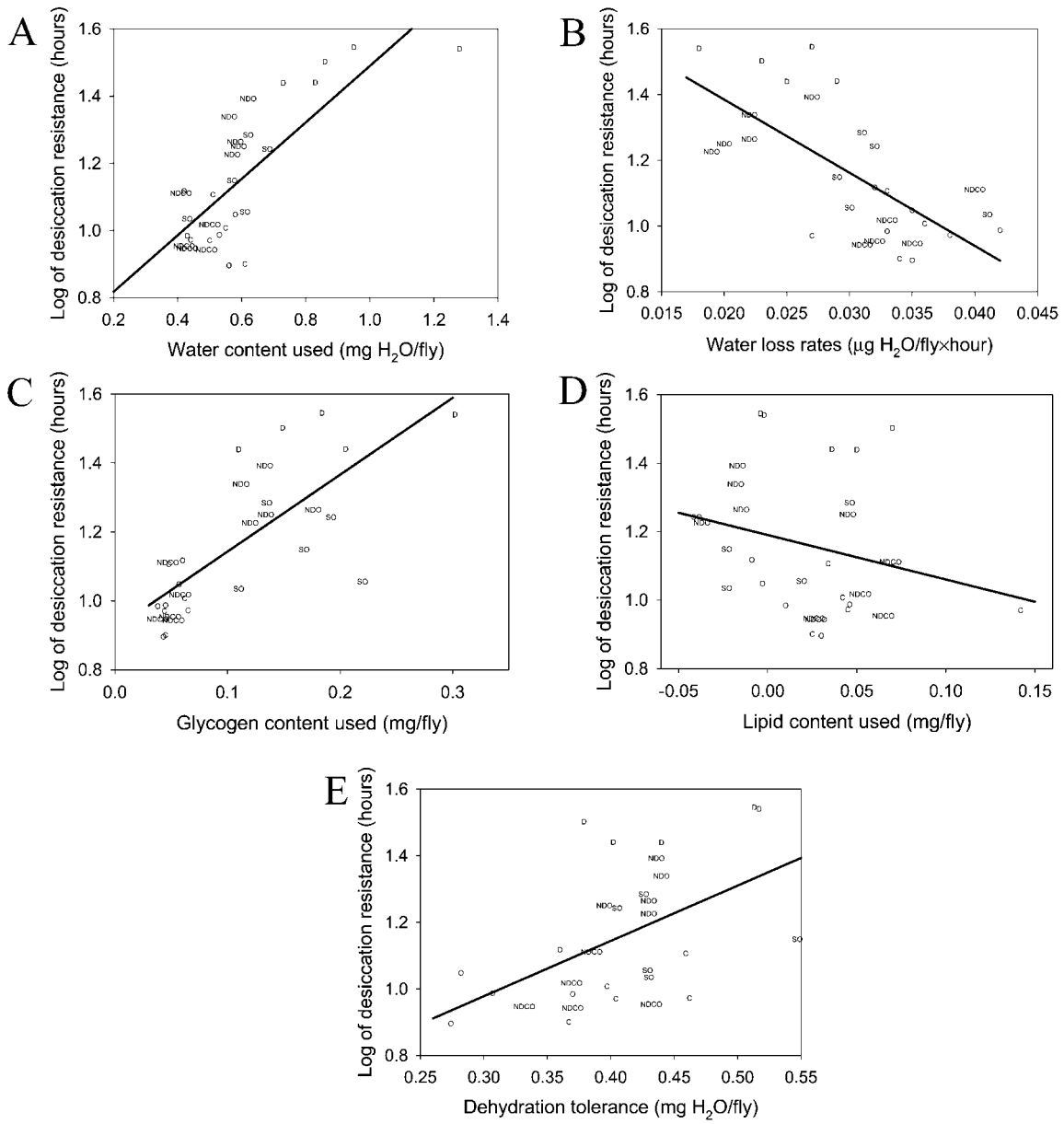

Figure 1. Linear regressions of the log of desiccation resistance against the indicated subsidiary physiological characters. Individual replicate populations are indicated by letter. *A*, Regression on water content used: regression formula $y = 0.65 + 0.84x$, $R^2 = 0.608$, $P < 0.001$. *B*, Regression on water loss rate: regression formula $y = 1.83 - 22.27x$, $R^2 = 0.472$, $P < 0.001$. *C*, Regression on glycogen content used: regression formula $y = 0.92 + 2.23x$, $R^2 = 0.568$, $P < 0.0001$. *D*, Regression on lipid content used: regression formula $y = 1.19 - 1.30x$, $R^2 = 0.063$, $P = 0.18$. *E*, Regression on dehydration tolerance: regression formula $y = 0.48 + 1.66x$, $R^2 = 0.262$, $P < 0.004$.

shown in Table 4. All variables are correlated with each other, except that the variable for lipid content used is not correlated with any other variables. When comparing only independent variables, the highest correlation coefficient is between glycogen content used and water content used ($r = 0.79$).

In order to determine which variables best predict desiccation resistance, a multiple regression analysis was used. The dependent variable was desiccation resistance (log transformed), and the independent variables were water content used, water loss rates, glycogen content used, lipid content used, and dehydration tolerance. The step-by-step results of this analysis are shown in Table A2 in the online edition of *Physiological and Biochemical Zoology*. Water content used was the first variable used in the regression model because this was the variable that resulted in the largest change in the R^2 value compared with the model that contains only the constant term. The partial

regression coefficients were calculated for each variable not in the equation as if this variable were the next variable to enter the regression model. Water loss rate had the lowest P value ($P = 0.012$) and was therefore added to the model. After this variable was entered into the model, the remaining variables (glycogen content used, lipid content used, and dehydration tolerance) had partial regression coefficients that were insignificant. Therefore, water content used and water loss rate were the variables that jointly best predicted desiccation resistance ($R^2 = 0.69$).

Predictive Model

The three a priori models described in "Material and Methods" were used to determine which variables best predict observed desiccation resistance. In Table 3, observed values are regressed on the predicted values from each model in order to determine which model best predicts observed desiccation resistance. The R^2 values for each model were very similar; therefore, model 1 and model 2 did not explain any more of the variation observed in desiccation resistance than model 3 (Table 3). Since model 3 includes fewer variables than model 1 and model 2, this model predicts the observed desiccation resistance most efficiently (Fig. 2*A*).

The D_3 population was an outlier in each of the regression models, as shown by the Cook's distances. When the D_3 population was removed from each regression model, the R^2 values increased for each model, but the relative R^2 values remained similar (Table 3). In addition, the PRESS statistic for each regression model decreased substantially.

The "influential point test shows that the D_3 population accounts for 43%, 49%, and 51% of the PRESS statistics for models 1, 2 and 3, respectively. This is much higher than the expected 3.3% (1/30) of the PRESS statistic. Although excluding the D_3 population from the analysis increases the predictability of model 3 (Fig. 2*B*), there is no biological justification for excluding this datum.

Even though model 3 best predicts the observed desiccation resistance in these populations, the slope of this model is 0.58. If this model accurately predicted the amount of time a fly can resist desiccation, then the slope should be close to 1. This may be due to the variation generated by using the population means instead of the treatment means. If treatment means are used, the R^2 value and the slope show a substantial increase (Fig. 3, $R^2 = 0.99$, slope = 0.814), but the 95% confidence intervals [CIs] do not contain the value of 1 (lower 95% CI = 0.70; upper 95% CI = 0.93).

Evolutionary Trajectories for the NDO Populations

The NDO and NDCO populations were assayed at generations 0, 5, 8, 11, 17, 31, and 37 for desiccation resistance using desiccant, water content stored, and water loss rates. Glycogen content stored was assayed at generations 0, 5, 8, 17, and 39.

Desiccation Resistance Using Desiccant. Significant differentiation in mean desiccation resistance between the NDO and NDCO populations occurs at generations 5, 8, 11, 17, 31, and 37 (Table A3 in the online edition of *Physiological and Biochemical Zoology*). The increase in the mean difference in desiccation resistance between the NDO and NDCO populations as selection proceeds is also supported by using a linear regression analysis (Fig. 4*A*; $y = -0.884 + 0.794x$, $R^2 = 0.924$, $P < 0.001$). Comparing mean desiccation resistance between the longer-selected D populations at generation 184 and the NDO populations at generation 37 shows that on average the D populations resist desiccation twice as long as the NDO populations (Table 5; D = 85.08 h and NDO = 47.48 h).

Water Content Stored. Significant differentiation between the NDO and NDCO populations in mean water content stored occurs at generations 8, 11, 17, 31, and 37 (Table A4 in the online edition of *Physiological and Biochemical Zoology*). The linear regression analysis shows that the NDO populations con-

Table 3: Linear regression results of observed desiccation resistance against predicted desiccation resistance based on the model indicated

Model	Equation of Line	R^2	F	P	PRESS	D_3 Cook's Distance
All 30 populations:[a]						
1. $(a + b + c)/d$	$y = 3.63 + .51x$	.669	56.56	<.0001	1,147.84	6.54
2. $(a + b)/d$	$y = 4.03 + .51x$	.68	59.61	<.0001	1,187.4	7.83
3. a/d	$y = 3.60 + .58x$	.682	60.11	<.0001	1,201.23	8.17
Eliminating D_3:[b]						
1. $(a + b + c)/d$	$y = -1.40 + .74x$	.718	68.69	<.0001	535.68	
2. $(a + b)/d$	$y = -1.13 + .76x$	.747	79.54	<.0001	486.68	
3. a/d	$y = -1.90 + .88x$	.753	60.11	<.0001	473.53	

Note. See "Material and Methods" for definitions of each variable included in these models.

[a] Analysis is based on all 30 population means.

[b] Analysis based on all population means except the D_3 population mean.

Table 4: Pearson correlation coefficients between all independent variables and desiccation resistance

	Water Content Used	Water Loss Rates	Lipid Content Used	Dehydration Tolerance	Glycogen Content Used	Desiccation Resistance
Water content used	1.00					
Water loss rates	−.575, $P <$ .001	1.00				
Lipid content used	−.145, P = .444	.183, P = .333	1.00			
Dehydration tolerance	.390, P = .017	−.441, P = .007	−.237, P = .104	1.00		
Glycogen content used	**.790**, $P <$ .001	−.610, $P <$ .001	−.404, P = .027	.560, $P <$ .001	1.00	
Desiccation resistance	.778, $P <$ .001	−.685, $P <$ .001	−.251, P = .091	.515, P = .002	.754, $P <$ .001	1.00

Note. The bold coefficient indicates the highest value.

tinue increasing in water content stored compared with their controls as selection continues (Fig. 4*B*; $y = -0.01 + 0.0047x$, $P < 0.001$, $R^2 = 0.945$). Comparing mean water content between the D populations at generation 184 and NDO populations at generation 37 shows that the D populations have 37% more water stored than the NDO populations (Table 5).

Water Loss Rates. Significant differentiation in mean water loss rates between the NDO and NDCO populations occurs at generations 8, 11, 17, 31, and 37 (Table A5 in the online edition of *Physiological and Biochemical Zoology*). The linear regression analysis indicates that the NDO populations, compared with their controls, continue to decrease in water loss rates as selection proceeds (Fig. 4*C*; $y = -0.0024–0.0004x$, $P = 0.0436$, $R^2 = 0.59$). Although this is the case, the relatively low R^2 value for this regression suggests that this decrease ends. In fact, there is no significant mean difference in water loss between generations 17, 31, and 37 ($P = 0.480$). In addition, the curve that best fits this data is a quadratic regression (Fig. 4*C*; $y = 0.0049 - 0.00176x + 0.0000355x^2$, $R^2 = 0.908$). Moreover, the water loss rates of the D populations are not statistically different from the water loss rates of the NDO populations (Table 5; $P = 0.31$).

Glycogen Content Stored. Significant differentiation in mean glycogen content stored between the NDO and NDCO populations occurs at generations 5, 8, 17, and 39 (Table A6 in the online edition of *Physiological and Biochemical Zoology*). The difference in glycogen content stored between the NDO and NDCO populations increases as selection proceeds (Fig. 4*D*; $y = -0.002 + 0.0031x$, $P < 0.001$, $R^2 = 0.988$). Although this increase in glycogen content appears to continue in the NDO population, the NDO populations at generation 39 and the D populations at generation 184 do not statistically differ in mean glycogen content stored (Table 5; $P = 0.360$).

Discussion

The Extent to Which Water Content Storage and Usage, Water Loss Rates, and Dehydration Tolerance Contribute to Desiccation Resistance

Based on multiple regression analysis, water used during the desiccation stress and water loss rates were the variables that best predicted the survival of a fly under desiccating conditions. Even though glycogen content used during the desiccation stress and dehydration tolerance were related to desiccation resistance, these variables did not explain significantly more of the variation observed in desiccation resistance. In addition, lipid content used during the desiccation stress was not related to desiccation resistance and thus did not explain any more of this variation.

Water Content Storage and Water Content Used. Our multiple regression analysis suggests that differences in the use of water are critical for determining survival under desiccating conditions. In addition, we found that water content stored is 47% higher in the D populations than their controls and 21% higher in the NDO populations than their controls. Although these findings are consistent with those of Gibbs et al. (1997), they are not consistent with the conclusions of Hoffman and Parsons (1989*a*, 1993) or Blows and Hoffman (1993). Gibbs et al. (1997) measured water content stored and found that the D lines have 30% more water than their controls, while Folk et al. (2001) estimated the increase to be about 34%. Although these percentages are lower than the 47% estimated here, these results are qualitatively similar in that desiccated-selected populations store much more water than their control populations. Folk et al. (2001) demonstrated that about 68% of this additional water is located in the hemolymph of the D flies. Blows and Hoffman (1993) found no difference in water content storage between desiccated-selected and control lines in populations of *Drosophila serrata*. Hoffman and Parsons (1989*a*) found no difference in water content used during a desiccation stress or in water content storage between selected and control lines in

Drosophila melanogaster. In addition, Hoffman and Parsons (1993) also found this result in populations of *D. melanogaster* and *Drosophila simulans*.

The differences between our findings and those from other laboratories could be due to differences in selection intensities combined with the number of generations of selection. In our study, selection on desiccation in each generation was stopped at 80% die off, and differentiation between selected and control lines for increased water storage occurred by generation 8. In the Blows and Hoffman (1993) study, for example, selection was stopped at 50% dying, and water-content differentiation between selected and control lines was not evident at generation 14. Because their selection intensity was less, after only 14 generations of selection, Blows and Hoffman may have not produced enough selection response to detect differentiation for water content storage between their control and selected lines. In most cases, our experiments use larger populations, greater selection intensities, and more generations of selection, making it easier for us to detect the underlying physiological mechanisms responsible for the response to selection. It is noteworthy that increased stored water content has not been found among desert populations of *Drosophila* (Gibbs and Matzkin 2001; Gibbs 2002). It may be that under some conditions in nature, trade-offs arise between water content and other functional characters, such as flight performance, that may limit the response to selection.

Water Loss Rates. We found that a low rate of water loss is another physiological mechanism that significantly accounts for the observed variation in desiccation resistance. Similar results have been shown in comparative studies using natural populations of *Drosophila* species (e.g., Eckstrand and Richardson 1980). In addition, this result is prevalent in other selection studies (Hoffman and Parsons 1989*a*, 1993; Graves et al. 1992; Gibbs et al. 1997).

Glycogen Content. Our study found that glycogen content on its own did not significantly explain variation in desiccation resistance even though this trait has a strong linear relationship with desiccation resistance. This is because glycogen content used is highly correlated with water content used. Therefore, this trait does not add any extra explanatory value to a model that already contains water content used.

Gibbs et al. (1997) suggest that stored glycogen and stored water are highly associated because glycogen acts as a "sponge" for water. Glycogen binds 3–5 times its weight in water (Schmidt-Nielsen 1990). In the D populations at generation 184, the average glycogen content stored was 0.19 mg/fly, and the average water content stored was 0.93 mg/fly. Therefore, glycogen may be responsible for holding 61%–100% of the bulk water. Thus, when water is needed, glycogen is catabolized in order to release this water. The amount of water released from the catabolism of each milligram of glycogen is 0.56 mg, so the amount of water contributed by the catabolism of glycogen is 0.11 mg of water. Because lipid is not found to be associated with desiccation resistance, catabolism of glycogen contributes only 11% of the total possible water used to resist desiccation (total water = 0.11 mg + 0.93 mg = 1.04 mg; [0.11 mg/1.04 mg] × 100 = 11%). Therefore, we concur with Gibbs (2002) that the "sponge" association between bulk water and glycogen is more important than the actual use of metabolized glycogen as a source of water.

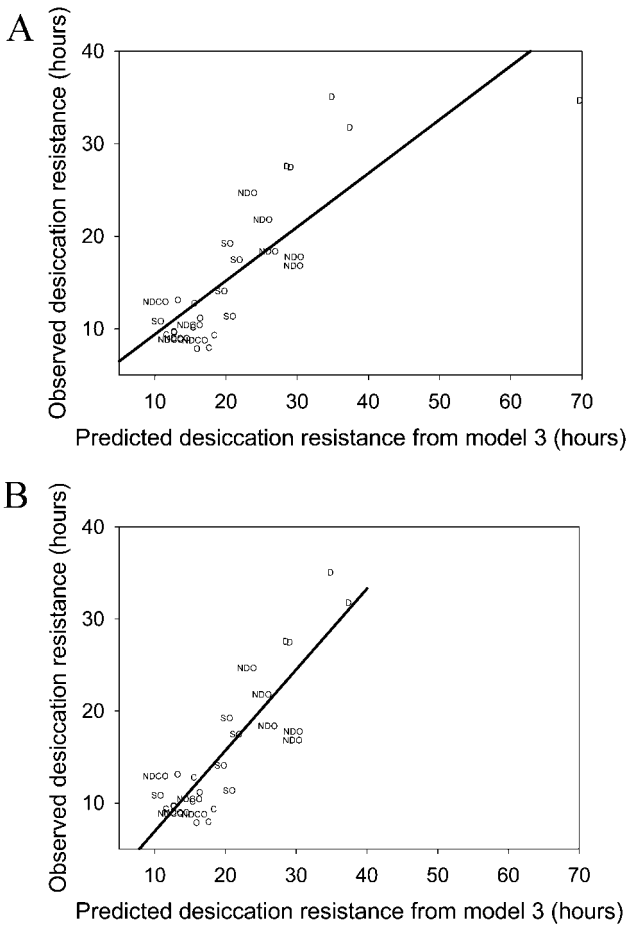

Figure 2. Linear regression of observed desiccation resistance against predicted desiccation resistance using model 3, based only on water content used and water loss rate. *A*, Using all 30 replicates: regression formula $y = 3.60 + 0.58x$, $R^2 = 0.682$, $P < 0.001$. *B*, Using 29 replicates, excluding replicate D_3: regression formula $y = -1.90 + 0.88x$, $R^2 = 0.753$, $P < 0.001$.

Lipid Content Used. On average, all populations used an insignificant amount of lipid, if any, during the desiccation stress. These results confirm other *Drosophila* studies in which extractable lipid is not found to be associated with desiccation resistance (Hoffman and Parsons 1989*b*; Blows and Hoffman 1993; Gibbs et al. 1997).

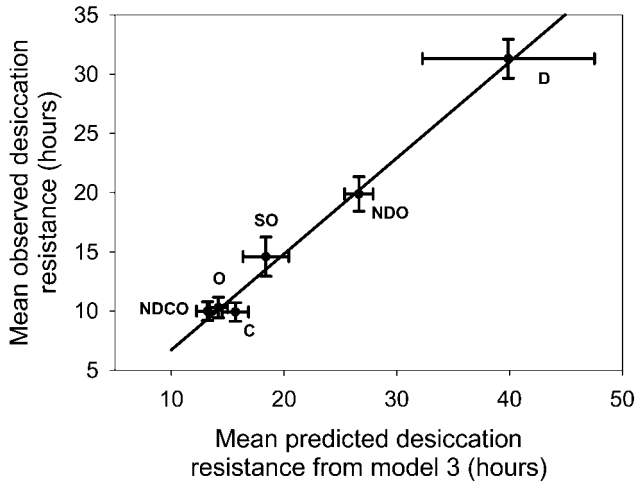

Figure 3. Linear regression of observed desiccation resistance against predicted desiccation resistance using model 3, based only on water content used and water loss rate. Regression uses treatment means with standard errors for the two observed characters indicated around the treatment mean values. Regression formula $y = -1.38 + 0.81x$, $R^2 = 0.99$, PRESS = 6.66.

Dehydration Tolerance. Although dehydration tolerance was not found to be associated with desiccation resistance using multiple regression analysis, there was a significant linear relationship found between these two traits. This result appears to contradict that of Gibbs et al. (1997), in which no differences in dehydration tolerance were found between the D and C populations at generation 120. But comparing only the D and C populations at generation 184 in our data reveals the same result as that of Gibbs et al. for the same D and C populations (one-way ANOVA, $P = 0.40$, analysis not shown). But the linear relationship found between these two traits in our study among all populations, not just the D and C populations, has a low R^2 value ($R^2 = 0.262$), which suggests that this relationship is not very strong in any case.

Three a priori models were compared to determine which variables were needed to predict desiccation resistance in laboratory-selected populations of *D. melanogaster*. Briefly, model 1 uses the variables for water content used, water loss rates, glycogen content used, and lipid content used to predict desiccation resistance. Model 2 and model 3 are similar, except in model 2, the variable for glycogen content used is excluded, and in model 3, the variables for glycogen content used and lipid content used are excluded. Model 1 and model 2 do not predict the observed desiccation resistance any better than model 3. Therefore, it is a reasonable inference from this analysis to conclude that the more water a fly has and the more it can reduce its rate of water loss, the longer the fly can survive desiccation stress. This result is consistent with the multiple regression analysis discussed above.

Although model 3 is the best predictor of desiccation resistance, the fitted slope of the model to all replicate populations is only 0.58. If this model were a good predictor of desiccation resistance, then the slope is expected to be ca. 1. The lower slope value could be due to the variation produced by using the replicate population means. When only the treatment means are used, not only does the R^2 value increase dramatically but the slope increases as well ($R^2 = 0.99$; slope = 0.81). This suggests that our model is indeed a good model for the evolutionary response to selection on desiccation, even if it does not perfectly predict all variation between replicate populations, variation that could be due to genetic drift or handling effects during assays.

Relative Rates of Evolution for the Characters Associated with Desiccation Resistance

The extent to which a response to selection occurs can help resolve the relationship between the two mechanisms that best predict desiccation resistance in our *D. melanogaster* populations: bulk water content and water loss rates. The response to selection for water loss rates in the NDO populations stops at generation 17, whereas the response to selection for water content stored shows no signs of stopping over the course of selection. This result is also evident in that mean water loss rates are not different between NDO and D populations, but these two sets of populations are quite different in both desiccation resistance and mean water content stored. This suggests that the physiological machinery that controls water loss rate is more constrained than the machinery that controls water storage. Among the limits affecting water loss rates might be constraints on the opening and closing of spiracles (Hoffman and Parsons 1989*a*, 1989*b*; see also Clark and Doanne 1983 or Williams et al. 1998) or on cuticle impermeability (Gibbs et al. 1997). These material limits may prevent further decreases in water loss rate by selection, while stored water may not face such limitations.

In addition to water content stored, we traced the evolutionary trajectory of glycogen content stored in the NDO populations in order to compare this trajectory with the trajectory of water content stored. Although the response to selection for water content stored is apparently still continuing, the response to selection for glycogen content has stopped. Therefore, even though these flies have accumulated most of the glycogen they apparently can handle, they still have the capacity to add water to their bodies. Considering that D populations resist desiccation for twice the amount of time as the NDO populations, it is apparent that increased water storage is the most important physiological mechanism involved in the progression to high levels of desiccation resistance in *D. melanogaster*.

Conclusion and Future Prospects

This study showed that in laboratory-selected populations of *D. melanogaster*, increased bulk water content and lower water

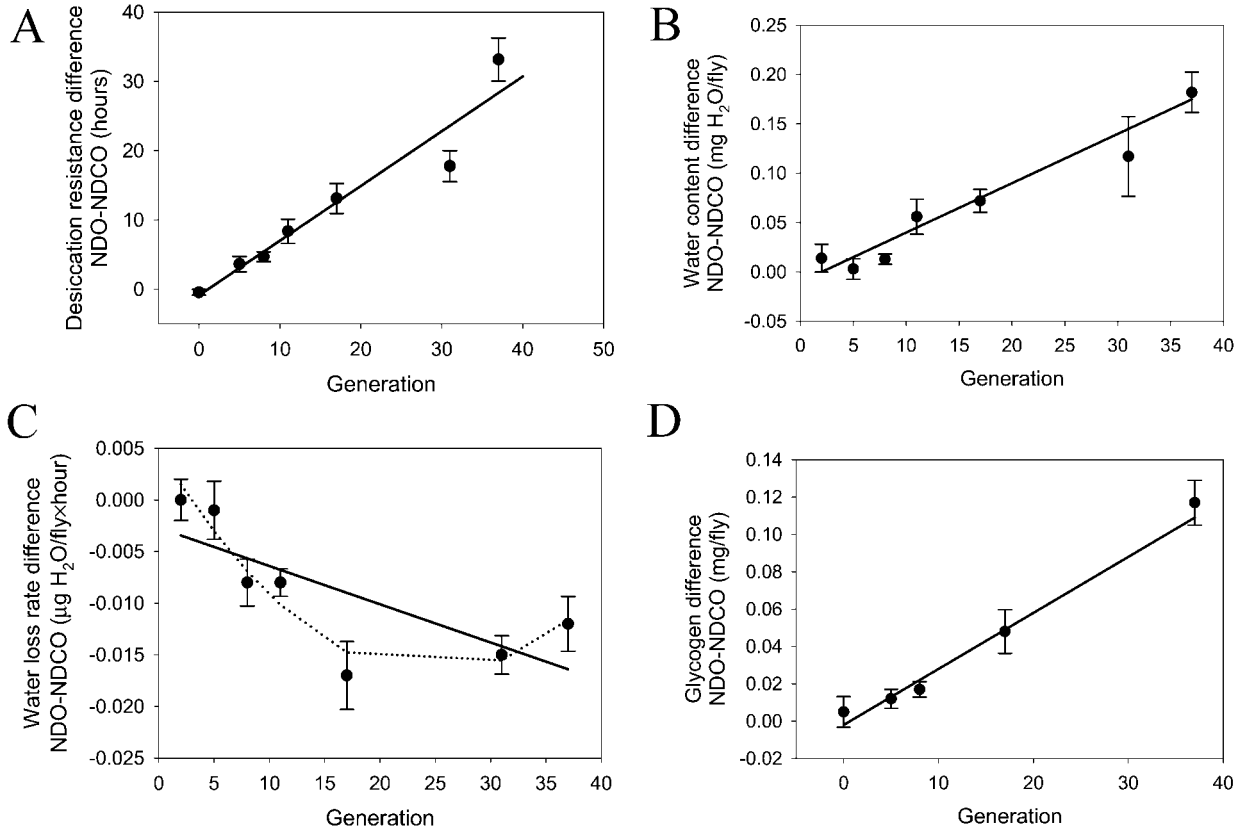

Figure 4. Linear regression of mean physiological differences between NDO and NDCO treatment means over the indicated generations. *A*, Desiccation resistance: regression formula $y = -0.88 + 0.79x$, $R^2 = 0.923$, $P < 0.001$. *B*, Water content storage: regression formula $y = -0.01 + 0.005x$, $R^2 = 0.923$, $P < 0.001$. *C*, Water loss rate: Linear regression formula $y = -0.0027 + 0.00037x$, $R^2 = 0.59$, $P = 0.044$; quadratic regression formula $y = 0.0049 - 0.00176x + 0.0000355x^2$, $R^2 = 0.908$, $P = 0.009$. *D*, Glycogen content storage: regression formula $y = -0.002 + 0.003x$, $R^2 = 0.988$, $P < 0.001$.

loss rates rather than increased dehydration tolerances or metabolic water content are the most important physiological mechanisms determining resistance to desiccation. This study also used experimental evolution to test a quantitative model for the physiology of desiccation resistance. This methodology, we suggest, has more analytical power than the use of comparative data collected from different species.

In this kind of study, the different "physiological types" of populations are replicated and readily regenerated, as our new desiccation-selected lines were. As we show by example, the combination of multiple kinds of replicated selection lines and assays of multiple physiological characters allows evolutionary physiologists to differentiate between physiological mechanisms according to their importance for the evolution of functional characters. Our analysis further suggests that some subsidiary physiological mechanisms may respond to selection initially but then plateau during further selection, while other physiological mechanisms continue to respond to selection. This suggests that short-term selection or genetic studies may not reveal the long-term course of evolution for the physiological underpinnings of particular functional characters, a finding that may prove to be a general rule for functional evolution (cf. Archer et al. 2003; Phelan et al. 2003; Rose et al. 2005). This suggests a need for selection studies of hundreds of generations in research on evolutionary physiology.

The approach we have exemplified here does, however, have clear limitations. Most importantly, its experimental power comes at the expense of not necessarily studying the actual processes of selection or the physiological response(s) to selection that occur in nature, outside of our laboratory. Thus, we cannot claim that any particular *Drosophila* population in nature evolves in response to desiccation selection in precisely the manner that we analyze in this study. Undoubtedly, any wild population will be subjected to a much more complex and less temporally stable pattern of natural selection than the selection that we have imposed on our laboratory populations. Furthermore, the physiological response(s) of such a wild population to such selection will probably be subject to different constraints than those facing our laboratory populations. This

Table 5: Treatment means and standard errors (SE) of desiccation resistance assayed using desiccant, water content stored, water loss rates, and glycogen content stored in the NDO and D populations at generations 37 and 184, respectively

	Desiccation Resistance ($P = .0030$)		Water Content Stored ($P = .0120$)		Water Loss Rates ($P = .3100$)		Glycogen Content Stored ($P = .3593$)	
	NDO	D	NDO	D	NDO	D	NDO	D
Mean	47.476	85.080	1.009	1.382	.022	.025	.174	.216
SE	3.277	8.406	.012	.115	.001	.002	.014	.041

Note. The *P* values are based on one-way ANOVA analyses. Refer to Table A7 in the online edition of *Physiological and Biochemical Zoology* for replicate means.

is a general problem impinging on all studies of experimental evolution conducted in laboratories.

Acknowledgments

This research was supported in part by grants from the National Institutes of Health and National Science Foundation to M.R.R. The authors are grateful to the many undergraduate research students who contributed to this research, particularly Ali Jamehdor and Brian Wakabioshi.

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