Heredity (2002) 88, 52–61

www.nature.com/hdy

Response of Sod-2 enzyme activity to selection for high voluntary wheel running

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The objective of this study was to examine the correlated response of anti-oxidant enzyme activity to selective breeding for increased voluntary wheel running in house mice. Activity of liver superoxide dismutase-2 (Sod-2), a free radical scavenger, was measured in four groups of mice. 'Active' individuals were housed in cages with attached wheels for 8 weeks beginning at weaning; 'sedentary' individuals were housed in cages with attached wheels that were prevented from rotating. Both of these treatments were applied to male and female mice from generation 14 of a replicated artificial selection experiment, which is composed of four lines selected for high wheel running and four randomly bred lines that serve as controls. In females, Sod-2 activity was significantly lower in selected *vs* control animals, regardless of presence/absence of a free-turning wheel. This difference suggests a trade-off between early-age voluntary wheel-running activity and Sod-2 activity. In males, Sod-2 activity was significantly affected by an interaction between selection group and activity group, with males from selected lines having lower Sod-2 activity relative to control males only in the sedentary treatment. These negative correlated responses of Sod-2 activity to selection on wheel running are discussed in the context of antagonistic pleiotropy models of aging and with respect to potential effects on lifespan.
Heredity (2002) **88,** 52–61. DOI: 10.1038/sj/hdy/6800008

Keywords: artificial selection; evolution; exercise; *Mus domesticus*; senescence; Sod

Introduction

Correlated response to selection is being increasingly examined as an important process by which traits evolve (eg, Lande, 1979; Lande and Arnold, 1983; Hayes and Garland, 1995; Carter *et al*, 2000). A correlated response to selection occurs when one trait responds to selection on a second trait, and is necessarily dependent on the existence of an additive genetic correlation between the two traits (Falconer and Mackay, 1996). Genetic correlations are most commonly caused by pleiotropy, although linkage disequilibrium can also play a role (Wright, 1968).

Genetic correlations and correlated responses to selection can be important in the occurrence of evolutionary constraints: if one trait has a negative genetic correlation with a second trait that is itself positively genetically correlated with fitness, then positive responses to selection by the first trait will be constrained (review in Schwenk, 1995). On the other hand, the speed of evolution can be enhanced by a positive genetic correlation between two traits, one of which is experiencing selection and the other of which is also positively correlated with fitness. In addition, the genetic and mechanistic architecture of complex traits can be revealed by correlated responses to selection. For example, a correlated response in a physiological trait to selection on a behavioral trait indicates that the two traits are genetically correlated and likely share some of the same genes, perhaps because the physiological trait at least partly causes the expression of the behavioral trait (Garland and Carter, 1994).

Several research programs have used replicated artificial selection experiments as a method to study correlated responses to selection (eg, Rose, 1984; Lynch, 1994; Zera *et al*, 1998; Carter *et al*, 2000). Replicated artificial selection is a powerful tool to study evolutionary processes and the genetic architecture and mechanistic underpinnings of complex traits. Any trait that responds in correlated fashion to the selection must be genetically correlated with the trait of selection (Lynch, 1994; Falconer and Mackay, 1996; Swallow *et al*, 1998a). In addition, because replicate selected and control lines are used, trait comparison can be made within any generation to test for correlated responses to selection (Lynch, 1994; Falconer and Mackay, 1996; Swallow *et al*, 1998a). Finally, this approach has the advantage of not needing to derive estimates of the genetic correlations, which in practice can be quite difficult because of large sampling variances (Falconer and Mackay, 1996); rather, the correlated response can be identified by comparing selected lines to control lines for the trait of interest (Lynch, 1994; Carter *et al*, 2000).

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Received 9 March 2001; accepted 6 August 2001

Locomotion underlies many different behaviors, is dependent on physiological and biochemical traits, is ecologically relevant, and therefore is ideal for studies of correlated evolution (eg, Garland and Carter, 1994; Boggs and Frappell, 2000; Miles *et al*, 2000; Kelt and Van Vuren, 2001; Kramer and McLaughlin, 2001). We have selected for increased voluntary wheel running in house mice to examine the correlated evolution of locomotor, behavioral, physiological, and biochemical traits. Four lines selected for high voluntary wheel-running activity and four randomly bred control lines were produced from a base population of outbred Hsd:ICR house mice (Swallow *et al*, 1998a). After 10 generations of selection, the high-selected lines ran on average 75% more total revolutions per day than did the control lines (Swallow *et al*, 1998a; Koteja *et al*, 1999), which exceeds the activity of wild house mice born and raised under the same conditions (Dohm *et al*, 1999). Body mass (Swallow *et al*, 1999) and food consumption at 76 days of age showed a correlated response to selection by generation 10, with mice from selected lines being smaller and eating more food (on a mass-adjusted basis) than those from control lines (Koteja *et al*, 1999). Mice from selected lines build smaller thermoregulatory nests (at generation 10, Carter *et al*, 2000), have higher night-time body temperatures when active on wheels (generation 17, Rhodes *et al*, 2000), and have more symmetrical hindlimb bone lengths (Garland *et al*, 2000). Mature males from the selected lines have a maximal aerobic capacity (oxygen consumption elicited during forced treadmill exercise) approximately 6% higher than in control lines under some housing conditions (Swallow *et al*, 1998b), and males from selected lines also exhibit elevated insulin-stimulated glucose uptake in some hindlimb muscles (Dumke *et al*, 2001). Pharmacological studies suggest differences in the dopaminergic neuromodulatory system between selected and control lines, which may influence the motivational basis of elevated wheel running in the selected lines (Rhodes *et al*, 2001). Finally, activities of several muscle enzymes showed genotype-by-environment interactions: selected mice had higher enzyme activity levels, but only when housed with access to a running wheel that was free to rotate, thus demonstrating that selection has not diminished the capability of muscle aerobic capacity to respond to training (Houle-Leroy *et al*, 2000).

Other biochemical traits that might evolve in concert with increased wheel-running activity include those involved in the production of free radicals as well as anti-oxidant enzymes. The free superoxide and hydroxyl radicals produced during aerobic metabolism react to create a number of toxic reactive oxygen metabolites (ROMs; Sohal and Weindruch, 1996), which can damage essential components of cells, including proteins, membranes, and DNA (Weindruch *et al*, 1993). The hypothesis that generation of free radicals is elevated during strenuous exercise is supported by numerous studies (eg, Davies *et al*, 1982; Jackson *et al*, 1985; Alessio, 1993). However, the destructiveness of ROMs can be countered through the action of anti-oxidant enzymes. For example, superoxide dismutase (Sod) and catalase are enzymes which eliminate O_2^- and H_2O_2 respectively, and consequently reduce the amount of oxidative damage that occurs (Weindruch *et al*, 1993). Some evidence supports the idea that long-term exercise training may also increase the level of anti-oxidant enzyme activity, as well as generally improving the health of an individual while aging (Jenkins *et al*, 1984; Ji *et al*, 1986, 1990, 1991). Indeed, much evidence now supports oxidative damage as a probable proximate mechanism of senescence in mammals and insects (Sohal and Orr, 1992; Agarwal and Sohal, 1993; Ku and Sohal, 1993; Ku *et al*, 1993; Barja *et al*, 1994; Orr and Sohal, 1994; Sohal *et al*, 1995; Sohal and Weindruch, 1996; Beckman and Ames, 1998).

We hypothesize that traits related to oxidative damage have responded in correlated fashion to selection on voluntary wheel-running activity. As a first test of this hypothesis, we measured activity of the anti-oxidant enzyme Sod-2 in 150 mice from the four selected lines and the four control lines of generation 14 from the selection experiment described above. We focused on the activity of Sod-2 in liver because Sod-2 can be easily measured from large numbers of individuals and because livers are large enough to provide the quantity of tissue needed. In addition, because exercise itself may have an effect on Sod-2 activity, mice from all eight lines were individually housed for 8 weeks either in cages with functional running wheels attached ('active' group) or in cages with attached wheels that were prevented from rotating ('sedentary' group) (Swallow *et al*, 1999; Houle-Leroy *et al*, 2000). This two-way experimental design allowed us to test for potential genotype by environment interactions.

Materials and methods

Animals and design

A full description of the selection experiment that produced the mice can be found in Swallow *et al* (1998a). Briefly, 112 male and 112 female outbred Hsd:ICR mice were purchased from Harlan Sprague Dawley and paired randomly. From these litters, one male and one female were randomly paired (with the stipulation of no sib matings), and each pair was randomly assigned to one of eight closed lines; the offspring of these pairings were designated generation 0. At generation 0, each of the eight lines were randomly assigned to either the control or selection treatment group. Beginning at generation 0, all lines were maintained by establishing 13 pairs within each line each generation; within each line, the first 10 litters weaned with at least two pups of each sex were used to maintain that line. Also beginning at generation 0, all mice each generation spent 6 days in cages attached to running wheels, so that mice would have to voluntarily leave the housing cage and enter the wheel to run. Beginning at generation 1, breeders within each of the four selection lines were chosen based on total wheel running on days 5 plus 6. Within family selection was used, to reduce inbreeding, so the highest running male and highest running female from each family were chosen as breeders, and pairings made randomly, with the stipulation of no sib matings. In control lines, one male and one female were chosen randomly, and pairings made randomly, with the stipulation of no sib matings. By generation 10, females from selected lines were running 73% more than females from control lines, and for males the difference was 76%.

A full description of the mice used herein can be found in Swallow *et al* (1999), who report data on body mass for the same individuals, and in Houle-Leroy *et al* (2000),

who report data on mixed hindlimb skeletal muscle enzyme activities of these same individuals. Briefly, from second litters of generation 14 of the selection experiment, 40 selected mice and 40 control mice were housed from ages 3 to 11 weeks with access to running wheels that were free to rotate, while at the same time 40 selected mice and 40 control mice were housed in cages with wheels that were locked to prevent rotation. In the group with wheel access, mice from selected lines were more active than controls during these 8 weeks; during week 8, selected females ran 2.5-times as far as control females, and selected males ran 2.1-times as far as control males (Swallow *et al*, 1999). At the end of the 8 weeks mice were sacrificed, livers collected and weighed to the nearest 0.1 mg, and frozen at –80°C. Liver tissue was chosen to assay Sod-2 because of its high mitochondria content and therefore elevated cell respiration and potential free-radical production and Sod-2 enzyme activity.

Tissue preparation

Tissues were prepared following the methods of Paoletti and Macali (1990) and Paoletti *et al* (1986). Liver samples were homogenized in glass-on-glass in 5 volumes of ice cold 0.25 mM triethanolamine-diethanolamine (tea-dea) buffer. The homogenate was spun in a Beckman GS-15R centrifuge at 20 000 *g* at 4°C for 30 min to remove cellular debris, and the supernatant saved. To remove hemoglobin, which interferes with Sod-2 activity, 0.4 ml ethanol-chloroform (2:1 v:v) per ml of homogenate and 0.57 ml double distilled water per ml of homogenate was added. The homogenate was vortexed and incubated in a water bath at 37°C for 15 min with three additional vortexes. The homogenate was then centrifuged at 20 000 *g* at 4°C for 30 min, and the supernatant drawn off and dialyzed for 2 h in cold 0.25 mM tea-dea buffer using a Genotech Tube-O-Dialyzer (MWCO 50 000). The final dilution used in the Sod assay was 0.004 mg liver tissue to 4000 μl buffer. The homogenate was stored overnight at –80°C, and Sod-2 assays performed the following day.

Assay methods

Samples were assayed by measuring inhibition of NADH oxidation by mercaptoethanol in the presence of EDTA and Mn, following the methods of Paoletti and Macali (1990). NADH solution was made fresh daily, using 10 mg NADH dissolved in 2 ml double demineralized (dd) water. Mercaptoethanol solution was made fresh daily in a glass beaker, with 25 μl mercaptoethanol dissolved in 35.5 ml dd water. The EDTA/$MnCl_2$ solution can be stored at RT for up to 2 weeks, and was made by mixing 5.58 g EDTA to a final volume of 100 ml with dd H_2O, adjusting the pH to 7.0, then adding an equal volume of 1.62 g Mn(4 H_2O) dissolved in 100 ml dd water. The assays were run by adding sequentially to the cuvette: 0.70 ml of 0.1 mM tea-dea buffer, 25 μl EDTA-Mn solution, 40 μl NADH solution, and 100 μl Sod. The reaction was then initiated by adding 200 μl mercaptoethanol solution. Changes in absorbance were measured at 340 nm in a Shimadzu UV-120–02 spectrophotometer for 16 min; we chose activity at 8 min for analysis, following Paoletti and Macali (1990) and Paoletti *et al* (1986), because at this time Sod-2 had not yet saturated the mercaptoethanol. A control was run with each set of three duplicate samples; percent inhibition was calculated as (sample rate)/(control rate) × 100; 1 U of Sod-2 activity was defined as half-maximal inhibition (Paoletti and Macali, 1990).

Total protein content of each homogenate was determined using a Pierce BCA protein assay reagent kit. Protein content was measured on a Molecular Devices Thermomax microplate reader at 562 nm.

Statistics

The general linear models (GLM) procedure in SAS was used to estimate a split-plot nested ANCOVA model to test effects of Linetype (selected *vs* control) and Activity group (sedentary *vs* free wheel-access) on Sod-2 activity and liver mass. The two main grouping factors, Linetype and Activity, were considered fixed effects. Families were nested within lines, and were a random effect, and replicate Line ($n = 8$ total) nested within Linetype was also a random effect. In the foregoing mixed models (ie, with both random and fixed effects), we tested the effects over appropriate error terms as follows: in the two-way ANCOVA models, effects of Linetype were tested over the mean squares of Line, and effects of Line were tested over the Family mean squares. Effects of Activity and the Linetype*Activity interaction were tested over the mean squares of the Activity*Line interaction. The Linetype*Activity term tests for genotype-by-environment interactions (ie, differential effects of selection between the two environments). Sex was not modeled because females run significantly more on wheels than do males, and have done so since generation 0, ie, before selection was first applied (Swallow *et al*, 1998a); hence all of the analyses were performed separately for each sex, as has been done for all other analyses of mice from this project (eg, Swallow *et al*, 1998a, 1999; Carter *et al*, 2000; Houle-Leroy *et al*, 2000).

Additionally, a more powerful test for response to selection in one environment but not the other is *a priori* contrast testing, which was done between selected and control mice in the sedentary environment only, and between selected and control mice in the active environment only. These two tests are orthogonal, meet the criteria for *a priori* tests of specific groups within main effects (Sokal and Rohlf, 1981), and are obtained using the contrast command within SAS Proc GLM.

Age at sacrifice and time of day of sacrifice were used as covariates in the ANCOVA model because both of these variables can significantly affect physiological and biochemical traits in these mice (eg, Carter *et al*, 1999; Houle-Leroy *et al*, 2000). In addition, because both these variables can have non-linear effects on traits, the square of the z-transformed age and the square of the z-transformed time of day were also used as covariates; variables were z-transformed before squaring to eliminate the high correlation between a variable and its square (Swallow *et al*, 1999; Carter *et al*, 2000; Houle-Leroy *et al*, 2000). In the course of model derivation, plots of raw data and of residuals were inspected and statistical outliers (defined as individuals with standardized residuals greater than 2.5 or less than –2.5; Houle-Leroy *et al*, 2000) were dropped to ensure that assumptions of the model were fulfilled. Adjusted means and standard errors were calculated using the LSMEANS command in the SAS GLM procedure; all covariates in the model, regardless of statistical significance, were used to calculate adjusted means.

Because body mass was allowed to respond in corre-

Table 1 Descriptive statistics of traits for both females ($n = 76$) and males ($n = 74$)

	Mean	*Standard deviation*	*Range*
Females			
Body mass (g)	26.51	2.918	20.11–33.92
Age (days)	78.94	1.399	75–81
Sod activity (U/mg protein)	62.53	26.02	17.05–132.7
Liver protein (mg/mg liver)	0.320	0.0661	0.188–0.495
Liver mass (mg)	1,387	193.5	1,025–1,869
Males			
Body mass (g)	33.09	3.683	24.42–42.57
Age (days)	78.89	1.504	75–82
Sod activity (U/mg protein)	71.73	34.44	14.96–169.2
Liver protein (mg/mg liver)	0.323	0.0823	0.160–0.616
Liver mass (mg)	1,810	245.8	1,113–2,610

lated fashion to selection on wheel running (Swallow *et al*, 1998a, 1999), all three traits measured herein are analyzed twice: once using statistical models with body mass as a covariate, and once using statistical models which do not use body mass as a covariate (see also Carter *et al*, 2000). Together these two analyses demonstrate how a trait responds in correlated fashion to direct selection on voluntary wheel-running (body mass included as a covariate in the statistical model) and how a trait responds in correlated fashion to both the selection on voluntary wheel-running and the correlated evolution of body mass (body mass not included as a covariate in the statistical model). Only linear effects of body mass were tested because of the linear effect of body mass on wheel running (Swallow *et al*, 1998a, 1999; Carter *et al*, 2000; Houle-Leroy *et al*, 2000).

Results

Descriptive statistics for Sod-2 activity, liver mass, liver protein concentration, and relevant covariates are presented separately for males and females in Table 1. Split-plot ANCOVA of Sod-2 activity (Units/mg protein) in females, with body mass included as a covariate in the statistical model, revealed no significant effect of Activity ($P = 0.8559$; Table 2); however, Linetype had a marginally non-significant effect ($P = 0.0551$; Table 2). The *a priori* contrasts revealed significant differences between selected and control females in both the sedentary and active environments ($P = 0.0140$ and $P = 0.0262$, respectively), with selected females having significantly lower Sod-2 activity than control females in both environments (Figure 1). Body mass did not have a significant effect ($P = 0.7843$; Table 2), but age was significant ($P = 0.0035$; Table 2), with older mice tending to have higher levels of Sod-2, even though the range of age at sacrifice spanned only 6 days (Table 1). No other covariates were statistically significant. When Sod-2 activity in females was analyzed without body mass included as a covariate in the statistical model, Linetype was significant ($P = 0.0134$; Table 3; Figure 1), with selected females having lower Sod-2 activity than control females. Age was the only significant covariate in this model, with Sod-2 activity increasing with age ($P = 0.0016$; Table 3).

Split-plot ANCOVA of Sod-2 in males with body mass included in the model as a statistical covariate revealed no significant effects of Activity or Linetype, but the Linetype*Activity interaction (Figure 1) was marginally non-significant ($P = 0.0636$; Table 2). *A priori* contrasts revealed significantly lower Sod-2 activity in selected *vs* control males in the sedentary environment only ($P = 0.0149$; Figure 1). Both body mass ($P = 0.0345$; Table 2) and age ($P = 0.0473$; Table 2) were significant covariates, with Sod-2 activity decreasing significantly with body mass and increasing significantly with age. When Sod-2 activity in males was analyzed without body mass as a covariate in the statistical model, no significant effects of Linetype or Activity were measured, but a mar-

Table 2 *P* values from split-plot ANCOVA with body mass included as a covariate in the statistical model

	Sod activity (U/mg protein)		*Liver mass (mg)*		*Protein concentration (mg protein/mg liver)*	
	Females	*Males*	*Females*	*Males*	*Females*	*Males*
Activity	0.8559	0.2372	0.3730	0.9830	0.0870	0.2616
Linetype	0.0551	0.1841	0.8805	0.9029	0.4362	0.3541
Line (Linetype)	0.6197	0.1234	0.1359	0.4325	0.0820	0.2173
Family (Line(Linetype))	0.4834	0.3599	0.4540	0.3570	0.2513	0.5663
Linetype*Activity	0.9937	0.0636	**0.0438**	0.8206	0.2108	0.7741
Activity*Line(Linetype)	0.8936	0.6484	0.5460	0.7225	0.4706	0.8381
Body mass	0.7843	**0.0345**	**0.0001**	**0.0006**	0.6542	0.3985
Age	**0.0035**	**0.0473**	0.1517	0.6593	0.5996	0.2457
Age^2	0.9150	0.5764	0.4595	0.2984	0.3320	0.1338
Time	0.2346	0.5524	0.5621	**0.0364**	0.7879	0.2422
Time^2	0.2646	0.5568	0.3238	0.5142	0.5498	0.8031
n	76	74	74	78	76	78
Sedentary: selected *vs* control	**0.0140**	**0.0149**	0.2380	0.8126	0.0902	0.1164
Active: selected *vs* control	**0.0262**	0.1789	0.4943	0.9563	0.5073	0.1896

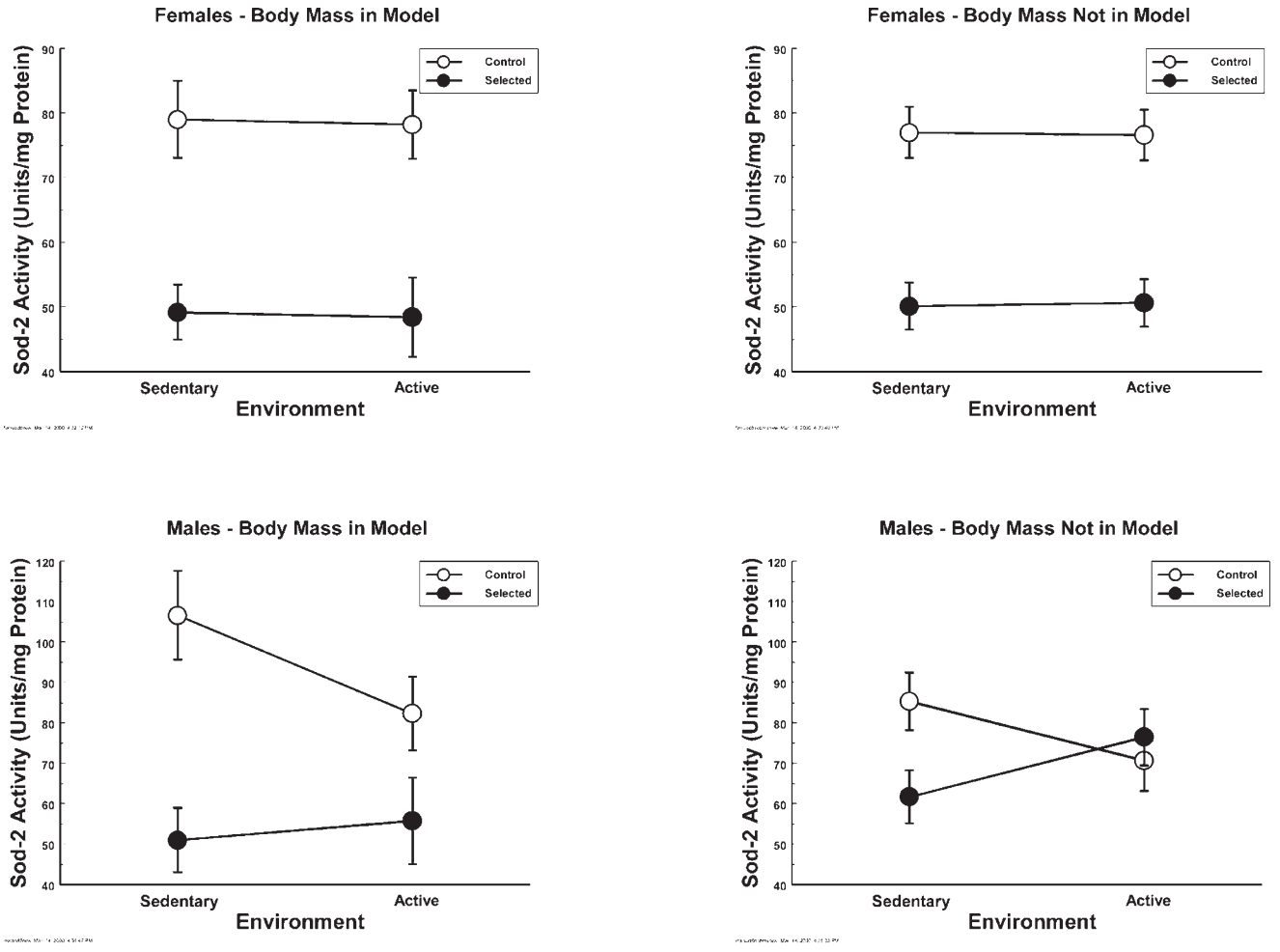

Figure 1 Least square means and standard errors from both ANCOVA models of Sod-2 activity (U/mg protein) for females and males respectively. See Tables 2 and 3 for covariates used in each model.

Table 3 P values from split-plot ANCOVA without body mass included as a covariate in the statistical model

	Sod activity (U/mg protein)		*Liver mass (mg)*		*Protein concentration (mg protein/mg liver)*	
	Females	*Males*	*Females*	*Males*	*Females*	*Males*
Activity	0.9813	0.9927	0.2429	0.0938	0.0857	0.4550
Linetype	**0.0134**	0.6551	**0.0367**	**0.0334**	0.3080	0.5809
Line (Linetype)	0.4459	0.0753	**0.0022**	0.4585	0.0689	0.0739
Family (Line(Linetype))	0.4430	0.5180	0.6762	**0.0196**	0.2186	0.5764
Linetype*Activity	0.9000	**0.0499**	**0.0128**	0.4643	0.1689	0.6925
Activity*Line(Linetype)	0.8921	0.7754	0.8746	0.1517	0.4459	0.8873
Age	**0.0016**	0.0915	**0.0159**	0.5854	0.7115	0.2801
Age^2	0.9500	0.5789	0.8027	0.5259	0.2823	0.1316
Time	0.2297	0.3083	0.8246	**0.0125**	0.7983	0.1530
$Time^2$	0.2167	0.4634	0.7638	0.4088	0.4614	0.7306
n	76	74	76	76	76	78
Sedentary: selected *vs* control	**0.0034**	0.0679	**0.0049**	0.0635	**0.0473**	0.1986
Active: selected *vs* control	**0.0038**	0.6405	**0.0001**	**0.0268**	0.5902	0.4088

ginally significant Linetype*Activity effect was identified ($P = 0.0499$; Table 3; Figure 1): in the sedentary environment, selected males had lower Sod-2 activity than control males, but in the active environment selected males had higher Sod-2 activity than control males. No covariates were statistically significant (Table 3).

Analysis of female liver mass (Figure 2) using split-plot ANCOVA with body mass as a covariate in the statistical model showed a significant Linetype*Activity interaction ($P = 0.0438$, Table 2), with selected females having larger livers in the sedentary environment, but smaller livers in the active environment, relative to control females. Body

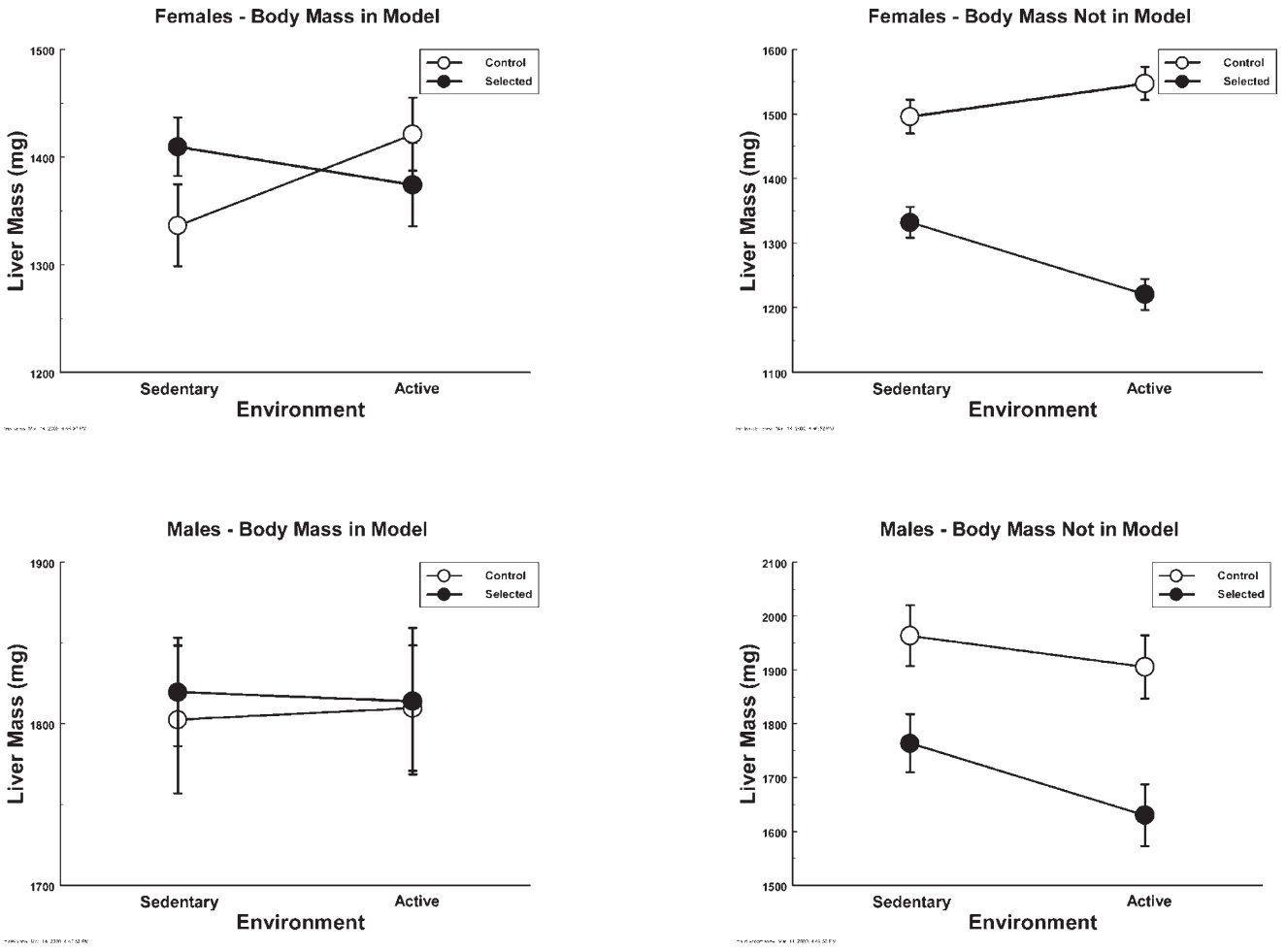

Figure 2 Least square means and standard errors from both ANCOVA models of liver mass (mg) for females and males respectively. See Tables 2 and 3 for covariates used in each model.

mass was a highly significant covariate, with liver mass increasing with body mass. When female liver mass was analyzed without body mass included as a covariate in the statistical model, Linetype was statistically significant ($P = 0.0367$; Table 3), with selected females having significantly smaller livers than control females. This latter effect was undoubtedly caused by the overall lower body mass of selected females relative to control females (Swallow *et al*, 1999). In addition, a significant interaction was measured between Linetype and Activity ($P = 0.0128$; Table 3): in selected females, sedentary individuals had a higher liver mass, whereas in control females, active individuals had a higher liver mass. Swallow *et al* (1999) did not identify a significant Linetype by Activity interaction on body mass, so the interaction measured herein can not be caused by body mass differences. Age was the only covariate that had a significant effect ($P = 0.0159$; Table 3).

Analysis of male liver mass (Figure 2) using split-plot ANCOVA with body mass as a covariate showed no significant differences in liver mass between selection or activity groups, nor any genotype-by-environment interactions (Figure 2; Table 2); as in females, body mass was a significant predictor of liver mass ($P = 0.0006$; Table 2). Liver mass in males analyzed without body mass as a covariate in the statistical model showed a significant effect of Linetype ($P = 0.0334$; Table 3); selected males had lower liver mass than control males (Figure 2), which, as in females, was almost certainly caused by the negative correlated response of body mass to selection on wheel running (Swallow *et al*, 1999).

Liver protein concentration was analyzed for females and males using statistical models that included and excluded body mass as a covariate (Figure 3). No main effects or covariates were significant in any analysis (Tables 2 and 3); however, in females analyzed without body mass as a covariate in the statistical model, *a priori* tests of differences between selection groups within each environment revealed significantly higher liver protein concentration in selected females relative to control females in the sedentary environment only ($P = 0.0473$; Table 3).

Discussion

We studied the effects of 14 generations of selection for increased voluntary wheel running and the effects of access to running wheels on liver Sod-2 enzyme activity and liver mass. Each trait was analyzed with and without body mass as a covariate in the statistical model, because by generation 14 body mass had responded in negative correlated fashion to selection on wheel running

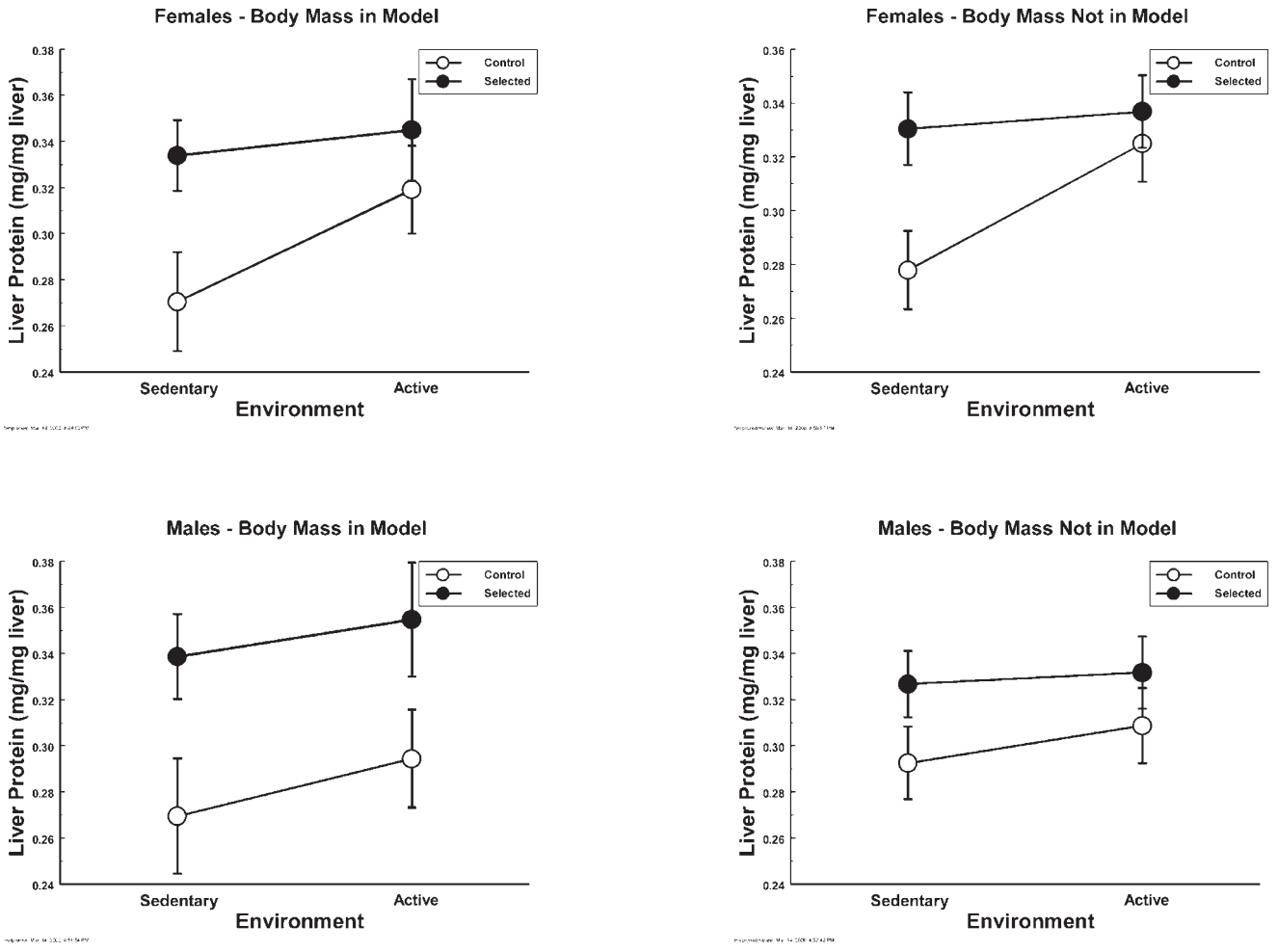

Figure 3 Least square means and standard errors from both ANCOVA models of protein concentration (mg protein/mg liver) for females and males respectively. See Tables 2 and 3 for covariates used in each model.

(Swallow *et al*, 1999). The analyses that included body mass as a covariate statistically removed the effect of body mass' response to selection and so estimated the correlated responses of each trait only to evolutionary changes in wheel running, whereas the analyses that did not include body mass as a covariate estimated the correlated response of each trait to selection on wheel running itself and the negative response of body mass to selection on wheel running.

In males, the response of Sod-2 activity to selection on wheel running was complex. Body mass was a significant covariate when included in the model ($P = 0.0345$; Table 2), with Sod-2 activity decreasing with increasing body mass; Linetype was not significant ($P = 0.1841$), and the Linetype*Activity interaction was marginally non-significant ($P = 0.0636$). However, the *a priori* contrast revealed a significant difference between selected and control males in the sedentary environment. When body mass was excluded from the model, a marginally significant interaction between Linetype and Activity was identified ($P = 0.0499$), with control males having higher Sod-2 activity in the sedentary group but having lower Sod-2 activity in the active group relative to selected males. Thus, males demonstrate a negative correlated response of Sod-2 activity to selection on wheel running that is dependent on the environment, regardless of the inclusion of body mass as a statistical covariate; hence, the response is caused by the direct selection on wheel running, and not by the negative correlated response in body mass. Analysis of liver mass revealed no additional information on this response of Sod-2 activity in males to selection on wheel running.

In females, Sod-2 activity showed a consistent negative correlated response to selection on wheel running. When body mass was included as a covariate in the statistical model, Linetype was marginally non-significant, but both *a priori* contrasts revealed significant negative effects of selection in both environments. Excluding body mass as a covariate in the statistical model caused the *a priori* contrasts to be slightly more significant, and caused Linetype to become significant from marginally non-significant. These differences indicate that Sod-2 activity in females demonstrated a negative response to selection on wheel running, with little effect of the negative correlated response in body mass.

This negative response of Sod-2 activity to selection on wheel running could have important ramifications for rates of senescence in mice from the selected lines relative to the unselected control lines. If both groups have equal ROM production, then selected mice will likely suffer higher levels of oxidative damage because of reduced Sod-2 activity, and hence may have shortened lifespans

(Beckman and Ames, 1998). This would suggest that antagonistic pleiotropy may be occurring between wheel running and Sod-2 activity: increasing wheel running results in lower Sod-2 activity, which may result in a shortened lifespan. Antagonistic pleiotropy is one genetic mechanism proposed to underlie the evolution of senescence (eg, Rose, 1991; Martin *et al*, 1996) which has strong support from numerous studies (Wattiaux, 1968a,b; Luckinbill *et al*, 1984; Rose, 1984; Service *et al*, 1985; Service, 1987; Partridge and Fowler, 1992; Arking *et al*, 1991). On the other hand, some evidence suggests that Sod activity may decline with decreases in ROM production (Finkel and Holbrook, 2000). If this is the case, then selected mice may be experiencing less ROM production and lower levels of oxidative damage, which may result in an increase in life span. Clearly, our results warrant further investigation of oxidative damage, antioxidant enzyme activity, and lifespan in this model system of mice.

As expected, body mass was a significant predictor of liver mass in females and males, with larger mice having larger livers. For females, models including and excluding body mass as a covariate demonstrated significant interactions between Linetype and Activity, with selected mice having larger livers in the sedentary environment, but control mice having larger livers in the active environment. In addition, when body mass was not included as a covariate, control mice had significantly higher liver mass than did selected mice in both males and females; such a result is hardly surprising, given the differences in body mass between selected and control mice (Swallow *et al*, 1999), and given the very strong effect of body mass on liver mass.

An increase in liver mass may lead to increased basal metabolic rate (BMR: Konarzewski and Diamond, 1995 on laboratory mice; Daan *et al*, 1990 on birds; see also Garland and Else, 1987; Garland, 1984 on lizards). Differences in BMR may have implications for oxidative damage and rates of senescence. Interspecific comparisons found that elevated levels of ROMs correlate positively with high BMR and negatively with maximum lifespan potential (MLSP), suggesting that animals with elevated BMR in general produce larger amounts of free radicals and live shorter lives (Sohal *et al*, 1989). Correspondingly, increased Sod activity was observed in the liver, heart, and brain of species that produce relatively higher amounts of free radicals (Sohal *et al*, 1989, 1990). When Sod activity is compared to MLSP, no relationship is found; however, the ratio of Sod activity to BMR is positively correlated with MLSP. In our mice, considering the total response to selection (wheel running and body mass) in females, selected mice had both lower Sod-2 activity and smaller livers, so the ratio of Sod-2 activity to BMR may not have changed. If this is the case, then the apparent antagonistic pleiotropic effect of increased wheel running (and also decreased body mass) on Sod-2 activity may be offset by the negative effect on BMR of smaller liver mass, so that MLSP is not influenced. On the other hand, selected mice have less total Sod-2 activity because activity per mg protein is lower and because the liver is smaller, so that if ROM production is not significantly reduced in selected mice, they will experience higher rates of oxidative damage, which might negatively influence MLSP.

The results presented herein clearly demonstrate a negative correlated response of Sod-2 enzyme activity to selection on wheel-running behavior in females with and without wheel access, and in males without wheel access. Such genetic change in activity of an enzyme in response to selection on a behavior is remarkable, and could be caused either by allelic substitutions at the Sod-2 locus or by allelic changes in loci that control expression of the Sod-2 gene. These results warrant additional investigations into the genetic mechanism underlying changes in Sod-2 activity, as well as the relationships between liver mass and BMR in these mice (see also Swallow *et al*, 1998b), the effect of selection on the activity of the antioxidant enzyme catalase, and the effect of selection for voluntary wheel running on longevity itself.

Acknowledgements

We thank Steve Austad and Gretchen Hoffmann for technical advice. This research was supported in part by a Washington State University Honors MiniGrant to SLT and by NSF grant IBN-9728434 to TG Research presented here was described in Animal Research Protocol No. A-48–9700-L00101–4-04–96, approved on 15 May 1996 by the Institutional Animal Care and Use Committee of the College of Letters and Science, University of Wisconsin, Madison.

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