# Note

# Antioxidant Gene Expression in Active and Sedentary House Mice (*Mus domesticus*) Selected for High Voluntary Wheel-Running Behavior

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## ABSTRACT

We present liver mRNA levels of the two antioxidant enzymes catalase (CAT) and Mn-superoxide dismutase (SOD2) in four treatment groups of house mice assayed by RNase protection at 20 months of age. These groups were mice from four replicate selection and four replicate control lines from the sixteenth generation of selective breeding for high voluntary wheel running, housed with or without running wheels from age 3 weeks through 20 months. Exercising control females had induced CAT expression; SOD2 exhibited a similar pattern in females from two of the four control lines. Exercising male mice had induced CAT expression, but not SOD2 expression, irrespective of genetic background. We discuss these results with respect to both evolutionary (genetic) and training (exercise-induced) adaptations and explore predictions of these results in relation to the oxidative-damage theory of senescence.
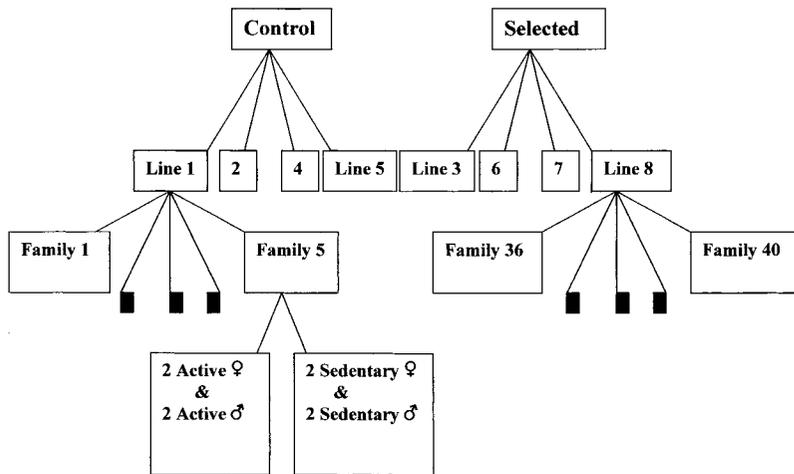
EVOLUTIONARY senescence theory argues that organisms senesce because the power of natural selection decreases with age (reviewed in ROSE 1991). Thus, selection is less effective against deleterious mutations expressed after reproductive peaks and, indeed, is virtually powerless against mutations expressed at postreproductive ages. This age-specific selection differential may result in the fixation of deleterious late-acting mutations ("mutation accumulation"; MEDAWAR 1952), particularly if the mutations are beneficial early in life ("antagonistic pleiotropy"; WILLIAMS 1957; reviewed in CHARLESWORTH 1994; MARTIN *et al.* 1996). Laboratory studies, primarily on Drosophila spp., support this life history theory tenet (*e.g.*, PLETCHER *et al.* 1998, 1999) and furthermore point to the prevalence of pleiotropic genes involved in trade-offs between survival and reproduction (ROSE 1984, 1990; SGRO and PARTRIDGE 1999; STEARNS *et al.* 2000) and survival and stress resistance (*e.g.*, ARKING *et al.* 1991; TATAR *et al.* 1997).

The oxidative stress hypothesis of aging states that the senescent phenotype, the intrinsic physiological and biochemical decline with age resulting in decreasing survival, results from the accumulation of oxidative damage to cellular components (*e.g.*, SOHAL *et al.* 1990; BARJA *et al.* 1994; reviewed in FINKEL and HOLBROOK 2000). Such damage is caused by an imperfect balance between the production of highly reactive oxygen species (ROS; *e.g.*, in the mitochondrial electron transport pathway) and their breakdown by enzymatic and nonenzymatic antioxidants (SOHAL and WEINDRUCH 1996). Specifically, the antioxidant enzymes in this study are involved in the removal of two ROS: Mn-superoxide dismutase (SOD2), present within mitochondria, converts superoxide anions into $H_2O_2$, and catalase (CAT) converts $H_2O_2$ into water and oxygen in the cytosol. Genetic mutations or environmental interventions that increase an organism's ability to either break down ROS or decrease their production can have the consequence of prolonging both average and maximum life span (*e.g.*, WEINDRUCH and WALFORD 1988; SOHAL *et al.* 1995; reviewed in BECKMAN and AMES 2000).

One such environmental intervention that remains equivocal with respect to its effects on ROS production, aging, and life span is exercise. Moderate exercise unequivocally improves overall health (*e.g.*, increased cardiovascular performance, BLAIR *et al.* 1995; decreased collagen degradation, THOMAS *et al.* 1992; balanced neuroendocrine function, TUMER *et al.* 1997), offsets the risk of age-related disease (ASTRAND 1992), and increases median life span (HOLLOSZY 1988, 1993; reviewed in MCCARTER 2000). But through increased oxygen con-

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FIGURE 1.—The nested, hierarchical colony design. Activity group and genetic background are crossed, line is nested with genetic background (four lines per background), and family is nested within line (5 families per line). Eight full sibs (four males and four females) from each of 40 families were used to construct the colony. Parental mice were from the fifteenth generation of a selective-breeding experiment for increased voluntary wheel-running exercise (SWALLOW *et al.* 1998). Pups were weaned at 21 days of age and placed in treatment groups at $28 \pm 3$ days of age in the mouse facility at Washington State University. Four males and four females from each of the 5 families in each line were used, with one-half placed in the active group and one-half placed in the sedentary group. Each activity group thus contained two females and two males from each of the 5 families within each of the eight lines, for a total of 160 individuals per activity group and 320 individuals in the new study colony. Mice in the active group were placed individually in cages with a 10-cm-radius running wheel and electronic wheel-revolution counter built into the cage top. Mice in the sedentary group were placed individually in standard rodent cages. Extra sibs were placed in similar housing and were used as sentinel mice to monitor the colony for the presence of specific-pathogen exposure; monthly panels were all negative for exposure during this study. Mice were checked daily; food and water were available ad libitum. Cage bottoms were cleaned once every 2 weeks, wheels were cleaned once every 4 weeks, and clean wheels were randomly assigned to active mice. At ~20 months of age, one active male, one sedentary male, one active female, and one sedentary female from each of the 40 families were decapitated, exsanguinated, and dissected. Tissues and organs were placed in a $-80°$ freezer.

sumption and metabolism, exercise can increase the production of highly damaging ROS (*e.g.*, JI *et al.* 1998; BEJMA and JI 1999) and is associated with increased antioxidant enzyme activity and upregulation of these enzymes in skeletal muscle, heart, and liver (HOLLANDER *et al.* 1999; BEJMA *et al.* 2000; JI 2000). However, because the balance between ROS production and cellular antioxidant defenses is imperfect, increased ROS production may result in increased cellular oxidative damage and thus limit maximum life span (SOHAL and WEINDRUCH 1996).

To test proximate and evolutionary mechanisms of aging related to exercise and oxidative damage, we measured antioxidant gene expression in active and sedentary middle-aged mice from the sixteenth generation of a replicated artificial selection experiment for high voluntary wheel running behavior (Figure 1; details of the selection experiment in SWALLOW *et al.* 1998). By generation 15, selection lines were running on average 150% more than control lines (11.3 km/day *vs.* 4.5 km/day; BRONIKOWSKI *et al.* 2001), primarily by increasing running speed (rpm; GARLAND 2002). We hypothesize that selectively bred mice will have levels of antioxidant gene expression lower than those of control mice, but that within each selection group, active mice will have levels of antioxidant gene expression higher than those of sedentary mice. We discuss these results in the context of evolutionary theories of senescence, oxidative stress mechanisms of aging, and an ongoing aging experiment involving full siblings of the individuals used herein.

**Quantitative assays for mRNA expression:** Gene expression levels (CAT, SOD2, β-actin), body mass, liver

mass, age, and wheel-running revolutions are in Table 1. Mice from selection lines with access to running wheels averaged higher daily revolutions over the 2 weeks prior to measuring antioxidant expression than did active mice from control lines (Table 2). Daily revolutions translate to selection and control mice running, on average, 4.0 and 2.7 km per day, respectively, for females, and 3.5 and 2.5 km per day, respectively, for males in the 2 weeks prior to sacrifice. These values compare to 7.7 and 4.5 km per day (selection and control, respectively) for the same individual females at 2 months of age and 6.2 and 4.8 per day for these same individual males at age 2 months. Thus, both females and males of selection lines continued running more than those of control lines through middle age, but the differences and the magnitudes were considerably smaller.

Although our selection regime did not influence CAT expression in sedentary animals, running did induce enhanced transcription in control, but not in selected, female mice and in both control and selected male mice (Table 2). Posthoc analysis of least-squares means from the full-model ANCOVA indicated that active control females had CAT expression significantly higher than that of the other female groups (Figure 2a). The magnitude of the effect of running in males was similar in both selection and control genetic backgrounds (Figure 2b).

In contrast to CAT, neither selection regime nor activity group affected SOD2 expression in either males or females (Table 2). For females, running was positively associated with SOD2 expression in two control lines (Table 2 and Figure 3a). For males, SOD2 expression

## TABLE 1

### Descriptive statistics of traits for females ($N = 68$) and males ($N = 71$)

| | Mean ± SD | |
|---|---|---|
| | Selection ($n = 36$) | Control ($n = 32$) |
| **Females** | | |
| Body mass (g) | 32.3 ± 3.7 | 35.4 ± 4.7 |
| Age (days) | 596 ± 1.8 | 596 ± 2.0 |
| Daily revolutions[a] | 6455 ± 3274 | 4144 ± 2493 |
| | ($n = 19$) | ($n = 15$) |
| Liver mass (mg) | 158 ± 31 | 167 ± 32 |
| CAT expression[b] | 1.30 ± 0.51 | 1.52 ± 0.66 |
| SOD2 expression | 1.25 ± 0.56 | 1.42 ± 0.61 |
| β-ACT expression | 1.03 ± 0.40 | 1.32 ± 0.52 |
| | Selection ($n = 34$) | Control ($n = 37$) |
| **Males** | | |
| Body mass (g) | 37.9 ± 4.5 | 41.0 ± 5.1 |
| Age (days) | 596 ± 1.4 | 596 ± 2.3 |
| Daily revolutions | 5941 ± 2251 | 3588 ± 1432 |
| | ($n = 17$) | ($n = 19$) |
| Liver mass (mg) | 209 ± 57.6 | 206 ± 46.5 |
| CAT expression | 1.32 ± 0.48 | 1.47 ± 0.65 |
| SOD2 expression | 1.13 ± 0.38 | 1.18 ± 0.40 |
| β-ACT expression | 1.13 ± 0.36 | 1.14 ± 0.39 |

[a] Daily revolutions are the mean daily wheel revolutions averaged over the 2 weeks prior to sacrifice.

[b] Mouse-specific antisense probes were made by first making template cDNA with a T7 RNA promoter site attached to the antisense cDNA strand and, second, using this cDNA as template in an *in vitro* transcription reaction. CAT and SOD2 genes were reverse transcribed (Advantage kit; Clontech, Palo Alto, CA) from total RNA extracted from livers (Trizol reagent; GIBCO BRL, Grand Island, NY). The resultant cDNA was amplified with PCR using the following primers: SOD2 sense 5′-TTCAATGGTGGGG GACATAT-3′, antisense with appended T7 promoter site 5′-TAA TACGACTCACTATAGGGGAGACTGTAAGCGACCTTGC TCCT; CAT sense 5′-GCAGATACCTGTGAACTGTC, antisense with appended T7 promoter site 5′-TAATACGACTCACTAT AGGGAGAGTAGAATGTCCGCACCTGAG (CAT sense and antisense primers from EL MOUATASSIM *et al.* 1999). $^{32}$P-labeled CTP RNA probes for SOD2 and CAT were made with *in vitro* transcription (Maxi-Script; Ambion, Austin, TX), using these amplified template T7 cDNAs. Radioactive probe for β-actin was made with cDNA supplied by the manufacturer. The final probe sizes were SOD2, 219 nucleotides (nt); CAT, 235 nt; β-actin, 304 nt. RNase protection assay reagents were used per manufacturer instructions (HybSpeed RPA kit; Ambion) to hybridize to SOD2, CAT, and β-actin in 20 μg of total RNA per individual mouse liver, 20 mice per assay group. In each assay group, we probed three replicate samples of 20 μg of a pooled liver total RNA (pooled from $n = 25$ individuals) to allow comparison of results among assays (*i.e.*, gel-to-gel comparison). To quantify mRNA levels for the three transcripts in each liver sample, radioactivity scores (counts per minute) from each of CAT, SOD2, and β-actin were divided by the average counts per minute of CAT, SOD2, and β-actin, respectively, from the three replicate lanes of pooled liver sample included in each assay and therefore as the proportion of these averaged gel RNA standards. We assayed a sample of 32 individuals twice to check for repeatability and loading consistency. On the basis of this paired correlation test, loading technique was determined to be repeatable; samples were consistent in their relative ordering in the two assays (Pearson $r = 0.65$, $n = 32$, $P < 0.0001$). Expression values are relative to a 20-μg RNA standard run on every gel.

varied among lines within both the selection and control groups (Table 2 and Figure 3b). Finally, β-actin was affected neither by genetic background nor by activity group (Table 2), but the *a priori* contrast between active selection and control females indicated higher β-actin expression in active control females.

**Selection for increased voluntary wheel running and long-term exercise training and antioxidant expression:** An understanding of the relationship among selective breeding for exercise, exercise level, and antioxidant expression is fundamental for revealing pleiotropic effects of genes involved in running behavior on oxidative damage and life span and hence may shed light on both proximal and evolutionary mechanisms of senescence (MARTIN *et al.* 1996). In females the selection group by activity group interaction on CAT expression was significant and was driven by control females with wheel access having CAT expression higher than that of selected females with wheel access, despite significantly higher levels of exercise in females from selected lines. In males, CAT expression was higher in active mice irrespective of selection group, despite significantly higher levels of running in selected males with wheel access. Finally, no significant effects of selection group or activity group were identified for SOD2 expression.

The results of this study bear on several questions relating exercise to aging. First, wheel running was significantly higher in selection mice than in control mice, both at the normal testing age (2 months) and at sacrifice (20 months, Table 1), although the amount of running declined significantly between ages 2 and 20 months. This result from analyses of "landmark age" data is not surprising in light of identified differences in both the position and shape of the entire wheel-running ontogeny between these same selected and control individuals; selected mice run more throughout ontogeny but also have a greater rate of decline in running as they age (T. J. MORGAN and P. A. CARTER, unpublished data). Recent comparative reviews of diverse taxa (insects, rodents, and primates) suggest that age-related decline in physical activity (including rodent wheel revolutions; *e.g.*, HOLLOSZY 1993) is widespread in various animal taxa and has a definite physiological basis (SALLIS 2000). Additionally, INGRAM (2000) summarized diverse evidence that changes in the dopaminergic system correlated with decreased activity with age. Thus we hypothesize that early-age selection on voluntary exercise and persistence of this differential through (at least) midlife reflect a persistent underlying difference in the dopamine/motivation system between genetic backgrounds (see RHODES *et al.* 2001).

Second, young selected mice from generation 14 had liver SOD2 enzyme activity lower than that of control mice (THOMSON *et al.* 2002); herein, no significant effects of selection were measured for SOD2 gene expression. The difference between the results of these two studies may be caused by post-translational modification

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TABLE 2

**Probability values from mixed-model, hierarchical ANCOVA *F*-tests**

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| Source of variation | CAT expression[a] Females | Males | SOD2 expression Females | Males | β-Actin expression Females | Males | Daily revolutions (averaged over 2 wk prior to sacrifice) Females | Males |
|---|---|---|---|---|---|---|---|---|
| Activity | 0.22 | **0.03** | 0.26 | 0.82 | 0.08 | 0.14 | NA | NA |
| Line type | 0.58 | 0.79 | 0.88 | 0.88 | 0.24 | 0.73 | **0.05** | **0.02** |
| Line (Line type) | 0.50 | 0.13 | **0.03** | **0.02** | **0.02** | 0.93 | 0.13 | 0.70 |
| Activity × line type | **0.04** | 0.61 | 0.41 | 0.22 | 0.32 | 0.18 | NA | NA |
| Activity × line (line type) | **0.01** | 0.52 | **0.02** | 0.62 | 0.82 | 0.48 | NA | NA |
| Body mass | 0.10 | 0.11 | 0.11 | 0.60 | 0.29 | 0.07 | 0.63 | 0.19 |
| Age | 0.06 | 0.42 | **0.01** | 0.44 | 0.98 | **0.002** | 0.90 | 0.92 |
| Time | 0.83 | 0.14 | 0.59 | 0.29 | **0.02** | 0.08 | 0.28 | 0.85 |
| N | 69 | 71 | 69 | 71 | 69 | 71 | 34 | 36 |
| Sedentary: selected *vs.* control[b] | 0.55 | 0.94 | 0.70 | 0.52 | 0.38 | 0.29 | NA | NA |
| Active: selected *vs.* control | **0.03** | 0.55 | 0.44 | 0.32 | **0.02** | 0.47 | NA | NA |

NA, not applicable.

[a] Expression variables were all normally distributed about their respective means and therefore were not transformed. Gene expression levels were analyzed separately for males and females (because females ran significantly more than males even before selection was applied) in mixed-model, hierarchical two-way nested analysis of covariance with Proc GLM in Program SAS (v6.12; SAS Institute, Cary, NC). Activity group (either active or sedentary) and genetic background (either selection or control) were the two main effects. Nested replicate line within genetic background and nested replicate family within line were random effects. Because family was never significant (all $P > 0.05$), we removed nested family from the model, thereby increasing the sample size by allowing inclusion of unbalanced families. Initial covariates included in the model were body mass, time of death, squared *z*-transformed time of death (to allow for a curvilinear relationship with time of day), age at death (days), and squared *z*-transformed age at death. Because squared *z*-transformed time and age were never significant, they were removed from the model. Also included in the statistical models were higher-order interactions (activity group by genetic background and activity group by nested line). Tests of main effects and interactions involving main effects were conducted with synthesized error terms as is appropriate for mixed models (implemented with the "random effects/test" option in SAS). Significant effects ($P < 0.05$) appear in boldface type.

[b] *A priori* contrasts.

in SOD2 enzymes or other molecular events that are not reflected in SOD2 expression (*e.g.*, mRNA stability). Alternatively, SOD2 expression and/or activity may change with age, duration of exercise, and selection generation. The Thomson *et al.* (2002) mice from selection generation 14 were housed with running wheels for 8 weeks beginning at 3 weeks of age and thus were assayed at 11 weeks of age. The mice herein were from generation 16, were housed with running wheels for almost 20 months beginning at 3 weeks of age, and were assayed at 20 months of age. Further investigation into the age specificity and effect of exercise duration on antioxidant expression and activity is warranted to address these hypotheses.

Third, active males had higher CAT expression, regardless of selection group. The effects of long-term exercise on the steady-state dynamics of the enzymatic antioxidant defense system are not clear. For example, Ji (1993) found significant increases in antioxidant enzyme activity in heart, liver, and skeletal muscle after a single (acute) exhaustive exercise bout, but not after 12 weeks of training. On the other hand, in a separate study employing 10 weeks of endurance training Hollander *et al.* (1999) found increased SOD2 and CAT expression in skeletal muscle. In addition, Holloszy and

colleagues (*e.g.*, Holloszy *et al.* 1985; Holloszy and Schechtman 1991; Holloszy 1993) documented an extension of average life span, but not maximum life span, in exercising rodents (see also Mlekusch *et al.* 1996). These latter studies suggest that the upregulation of antioxidant defenses in response to increased ROS production is not costly or at least is less costly than the assorted beneficial effects of exercise on overall health. Thus we predict that within a given selection group, active mice may have longer average life spans.

Finally, active-selected females had CAT expression lower than that of active-control females, which is similar to the SOD2 enzyme activity patterns in young mice reported in Thomson *et al.* (2002). In general, lower antioxidant expression rates are thought to correlate with lower ROS production (reviewed in Finkel and Holbrook 2000). Some studies have shown an increase in ROS production with exercise (*e.g.*, Jackson *et al.* 1985; Alessio 1993; Bejma and Ji 1999), and many others have shown that short-term exercise training increases antioxidant enzyme activity and/or expression in skeletal muscle and other organs in rats and mice (*e.g.*, Leeuwenburgh *et al.* 1994; Nakao *et al.* 2000). Indeed, in studies that have measured all components of the oxidative system (oxidants, antioxidants, and actual
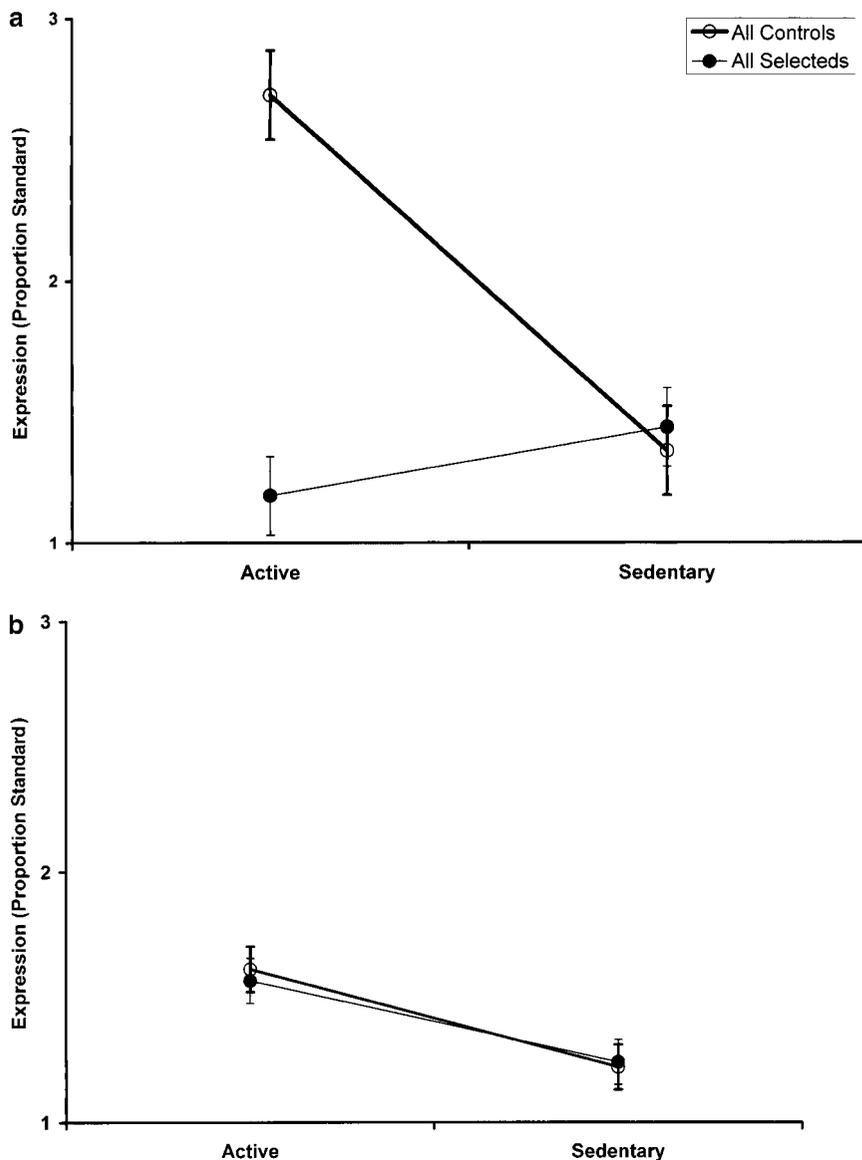
a



b



FIGURE 2.—Least-squares mean expression levels ($\pm$SE) of CAT by activity group and genetic background from the two-way nested ANCOVA for females (a) and males (b).

damage to cellular components), increased levels of oxidants tend to predict levels of induction of the antioxidant defense system (*e.g.*, AGARWAL and SOHAL 1993; SOHAL *et al.* 1994); thus lower CAT expression in exercising selection females may reflect lower ROS production and hence may reflect a beneficial pleiotropic effect of selection on voluntary exercise. However, an imperfect match between ROS production and antioxidant induction can result in increased oxidative damage; thus if lower CAT expression does not reflect lower ROS production, then active selected females should be experiencing more oxidative damage. This suggests a costly pleiotropic effect of early-age exercise selection on antioxidant defenses, which is consistent with the antagonistic pleiotropy theory of aging: genes with beneficial early effects having detrimental effects on life span through the physiological trade-off between exercise and oxidative damage. The ongoing study of whether early-age selection for voluntary exercise has positive or negative

genetic correlations with average and maximal life span will aid in distinguishing between these two predictions.

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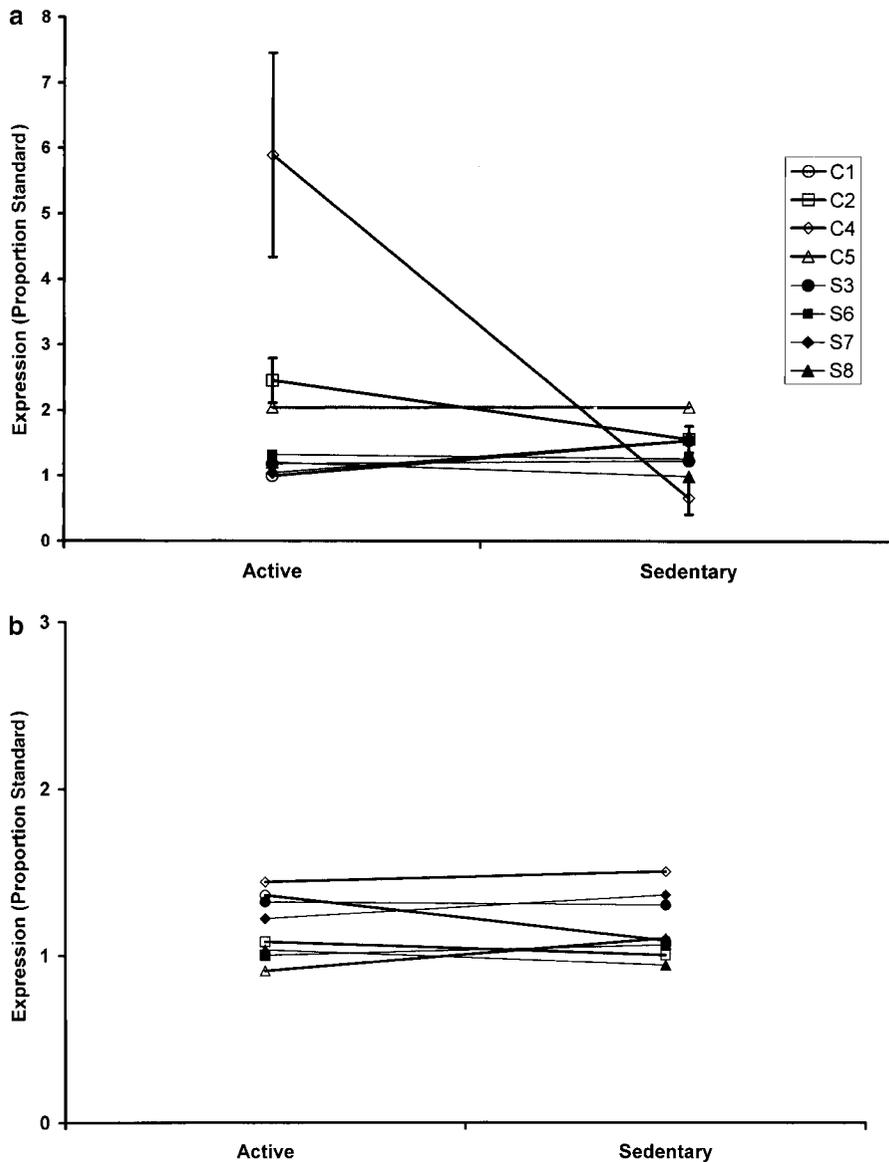
FIGURE 3.—Least-squares mean expression levels ($\pm$SE) of manganese-superoxide-dismutase (SOD2) by activity group and genetic background from the two-way nested ANCOVA for (a) females, where active control females had higher SOD2 in lines two and four, and for (b) males.

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