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Superoxide dismutase deficiency impairs olfactory sexual signaling and alters bioenergetic function in mice

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Oxidative stress (an overproduction of reactive oxygen species in relation to defense mechanisms) may restrict investment in life history traits, such as growth, reproduction, lifespan, and the production of sexual signals to attract mates. The constraint on sexual signaling by oxidative stress is of particular interest because it has been proposed as a mechanism ensuring that only good-quality males produce the most attractive sexual signals. Despite these predictions, evidence supporting this theory is, at best, equivocal. We used a superoxide dismutase knockout mouse to demonstrate that oxidative stress directly impairs investment in morphological (preputial glands) and molecular (major urinary proteins) components of olfactory signaling essential for mate attraction. By maintaining males in a much more competitive environment than usual for mouse laboratory experiments, we also revealed a range of phenotypes of superoxide dismutase deficiency not observed in previous studies of this mouse model. This range included impaired bioenergetic function, which was undetectable in the control environment of this study. We urge further examination of model organisms in seminatural conditions and more competitive laboratory environments, as important phenotypes can be exposed under these more demanding conditions.

sexual selection | condition dependence | antioxidant | male–male competition | Sod1

Life history theory is based on the premise that physiological constraints limit investment in life history components. These constraints are expected to generate the negative correlations commonly observed between these traits on an interspecies level (1). Within species, individuals also differ in their capacity to invest in different traits (2), although the physiological causes of this variation remain largely elusive.

Oxidative stress occurs as a consequence of an overproduction of reactive oxygen species (ROS) in relation to defense mechanisms (3). Because ROS are produced as a by-product of oxidative phosphorylation, investment in energetically demanding life history traits has been predicted to increase oxidative stress, which may then constrain investment in other areas (4, 5). Ecological research in wild animals has provided correlative evidence for a link between oxidative stress and investment in traits, such as growth (6), reproduction (7, 8), and lifespan (7). Early evidence linking oxidative stress to variation in lifespan within (9) and between species (10) led to the development of genetically modified antioxidant enzyme knockout animals, allowing precise tests of the effects of these pathways on lifespan under controlled laboratory conditions (11). These tests concluded that oxidative stress does not play a major role in governing the lifespan of laboratory animals (11).

As in the case of lifespan, sexual signals show considerable among-individual variation and can correlate with oxidative stress. Sexual signals are often condition-dependent in expression, such that only good-quality signalers express the largest or most elaborate sexual signals (12). These signaling systems only evolve and remain stable if poor quality signalers cannot invest as much in signaling as higher quality individuals (13). It has been suggested that oxidative stress might limit investment in sexual signaling, maintaining signal honesty. Oxidative stress is intimately linked with health (3) and immunocompetence (14). A signal that reveals a lack of oxidative stress may thus indicate a healthy mate of good genetic and phenotypic quality (14, 15).

We used a molecular-genetic model, developed to understand the role of oxidative stress in aging, to test whether this physiology constrains investment in sexual signaling. We used a genetically modified mouse that does not express copper-zinc superoxide dismutase (SOD1), an important antioxidant that has only one known biological function: protection against oxidative stress. Mice completely deficient in SOD1 show an elevation of oxidative damage in various tissues (16, 17) but show relatively few pathophysiological differences at younger ages (3–6 mo; as used in this study) (16–18) despite this increased oxidative stress.

In mice, the olfactory signaling system is particularly important for attraction of mates, with males scent-marking their territories extensively with urine to attract mates (19). Reductions in various aspects of olfactory signaling occur with senescence, and are correlated with reduced attractiveness of old male scent. It has been tentatively suggested that oxidative stress could be linked to this reduced sexual signaling with age (20). We started by examining the ability of homozygous, fully deficient *Sod1* mice ($Sod1^{-/-}$ mice) to scent-mark a territory area with urine and invest in various molecular and morphological scent signaling components with established roles in female attraction. Investment was compared with both wild-type males ($Sod1^{+/+}$) and heterozygous males ($Sod1^{+/-}$) in two different environments. In the first environment, males were isolated, corresponding to standard laboratory conditions for this strain. In the second environment, males were required to greatly increase their investment

Significance

Oxidative stress is expected to restrict investment in life history traits, including sexual signals used to attract mates. However, evidence supporting this prediction remains equivocal. We utilized superoxide dismutase knockout mice to provide, to our knowledge, the first direct evidence that oxidative stress impairs investment in important sexual signals, in this case major urinary protein pheromones with a well-established role in mate attraction. When males were housed in a competitive social environment, further morphological impairments in sexual signaling were revealed (through smaller preputial glands). Impairments in bioenergetic function, not previously known in this mouse model, also became apparent, highlighting how changes in the housing environment of model organisms can reveal previously undiscovered traits of interest.

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in scent-marking in response to regular exposure to intruders and potential mates over a 3-wk period (termed "territorial" environment). This process allowed us to assess whether effects of oxidative stress are revealed under more demanding conditions, which are probably closer to natural conditions in the wild.

With males housed in these two different environments, we took the opportunity to examine some of the oxidative costs and metabolic adaptations that occur with investment in territory defense, and the potential effects of SOD1 deficiency on these responses. This process also allowed us to address two hypotheses proposed to explain why males with high oxidative stress may reduce investment in sexual signaling. The first hypothesis suggests that metabolically costly sexual signals may reveal oxidative stress because individuals with poor antioxidant defense (associated with oxidative stress) are less able to protect against the additional ROS-induced damage generated by signal expression (5). This increased oxidative damage may produce costs to sexual signaling that outweigh the fitness benefits expected in return, making investment in these traits unprofitable for individuals with high oxidative stress (15). The second hypothesis suggests that oxidative stress may impair bioenergetic function, reducing the ability of animals to produce energy efficiently. This would be expected to limit the amount of energy available to allocate to metabolically costly sexual signals (15). By examining changes in markers of oxidative stress, whole-body metabolism, and bioenergetic function in both standard and territorial environments, we were able to explore the validity of these hypotheses in relation to mammalian scent communication.

Results

Olfactory Sexual Signaling. First, we examined the ability of *Sod1*$^{-/-}$ mice to scent-mark a territory area with urine. *Sod1*$^{-/-}$ males show a similar ability to scent-mark their territories compared with *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males in both unstimulated standard laboratory conditions and in the territorial environment, where males were required to greatly increase their investment in scent marking in response to conspecific exposure (Fig. 1*A*).

Although the ability to deposit scent-marks is important when patrolling and reapplying scent-marks regularly, the molecular components involved in conveying information are also particularly important for attraction of mates and deterring male intruders (19). Males excrete in their urine extremely large concentrations of major urinary proteins (MUPs). These MUPs have a number of roles in sexual signaling, including binding and releasing volatile odorants slowly over time (21) and inducing female attraction and memory of scent (22). Experimentally increasing the concentration of these proteins in urine increases the attractiveness of male scent to females (23), indicating that any changes in MUP concentration with oxidative stress are likely to influence male attractiveness. When examining urinary protein concentration, of which over 99% is MUP (24), *Sod1*$^{-/-}$ males showed lower urinary protein levels (Fig. 1*B*) ($F_{2,\ 40}$ = 9.35, $P < 0.0001$). This aspect of sexual signaling is therefore directly impacted by oxidative stress. MUP concentration also increased in response to territorial stimulation in all genotypes, as previously reported to occur in wild-derived mice (25), and the difference between the genotypes was reduced by the end of the experiment after 3 wk of the environmental manipulations.

At the end of the experiment we also examined the size of each male's preputial glands, which produce several important volatile signals also involved in mate attraction (19). *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males in the territorial environment showed greater preputial gland mass after the 3-wk exposure period than control animals. This finding is in accordance with a previous study that demonstrated wild-derived mice increase the masses of these glands when exposed to competitors and potential mates (25). This alteration presumably allows males to produce a greater concentration of volatile signals in their urine, and also occurs when males become dominant territory owners (26), facilitating their increased attractiveness to females. However, *Sod1*$^{-/-}$ males showed no increase in preputial gland mass when exposed to competitors and potential mates, resulting in *Sod1*$^{-/-}$ males in the territorial environment having smaller preputial glands than either *Sod1*$^{+/+}$ or *Sod1*$^{+/-}$ males (Fig. 1*C*) ($F_{2,\ 20}$ = 4.41, $P = 0.026$). The same relationship is observed when preputial gland mass is expressed as a percentage of body mass (Fig. S1).

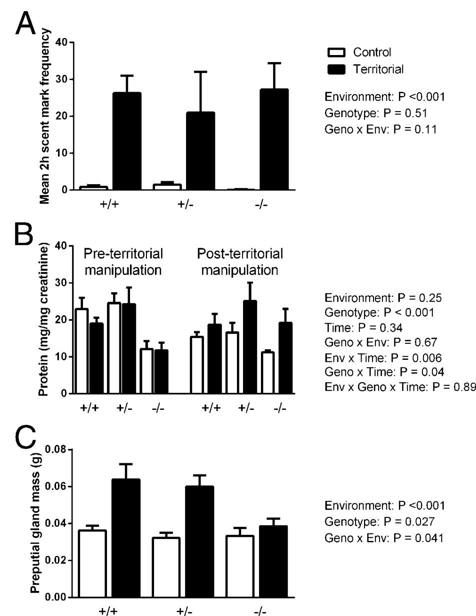

Fig. 1. SOD1-deficiency and olfactory signaling. All males, regardless of *Sod1* expression, increased their scent-marking rates when housed in a territorial environment (*A*). *Sod1*$^{-/-}$ males had a lower urinary protein concentration before territorial manipulations, although the difference in urinary protein between genotypes was diminished in the territorial environment when urine was collected after 2 wk of manipulations (*B*). The difference between genotypes in preputial gland mass was dependent on the environment males were housed, with *Sod1*$^{-/-}$ males only having smaller preputial gland masses when housed in the terrtitorial environment (*C*).

Our results reveal a direct link between oxidative stress and sexual signaling in a mammal. Because the only known role of SOD1 is the dismutation of the superoxide radical, the primary free radical produced from the electron transport chains (3), these changes in urinary MUP concentration and preputial gland weight are likely to have resulted from increased oxidative stress or a downstream consequence thereof. The effects of Sod1 deficiency on sexual signaling at the end of the experiment were also dependent on housing environment: differences in MUP concentration between genotypes were visible in the standard environment, but differences in preputial gland mass were only apparent in the territorial environment. Hence, housing mice under territorial conditions revealed patterns of sexual signaling investment that were not apparent under standard conditions resembling those usually used for experiments on genetically modified mice.

Condition-Dependent Oxidative Stress. At the end of the experiment we examined oxidative stress in each genotype of mouse in the two different environments. If oxidative damage is a particularly strong cost of metabolically demanding sexual signaling for males with preexisting oxidative stress, we would expect oxidative damage to increase most in *Sod1*$^{-/-}$ males that have increased investment in scent-marking (i.e., those in the territorial environment). As expected, males with complete Sod1 deficiency differed from the other genotypes in oxidative stress and antioxidant defense, as evidenced by the genotype effect in a multivariate analysis including all markers of oxidative stress ($F_{14,\ 46}$ = 3.85, $P < 0.001$) (Fig. 2 and Tables S1 and S2). Our results in the standard environment are consistent with previous assessments of oxidative stress in this mouse model. SOD1 activity is decreased in line with

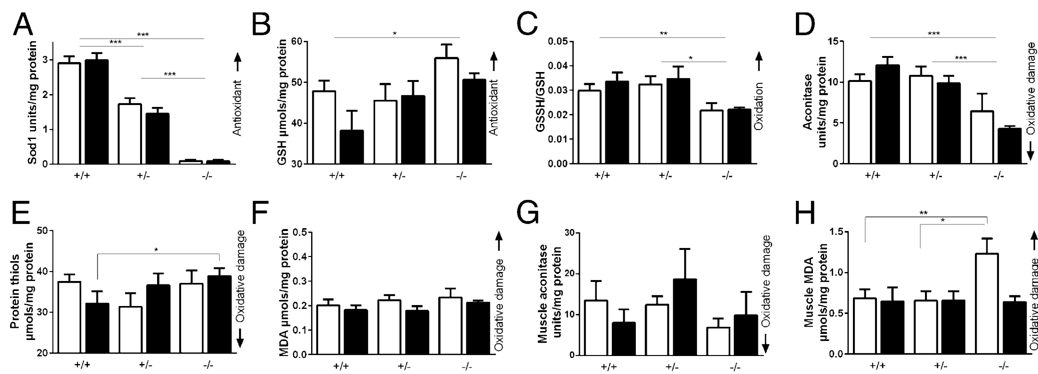

Fig. 2. SOD1 deficiency, territory defense and oxidative stress. Differences between groups for liver SOD1 activity (*A*) (not included in the multivariate analysis), liver glutathione concentration (GSH) (*B*), Liver glutathione oxidation (GSSH/GSH) (*C*), liver aconitase activity (*D*), liver protein thiol concentration (*E*), liver MDA (*F*), muscle aconitase activity (*G*), and muscle MDA (*H*). Black bars denote males in the territorial environment, white bars males in the control environment. Arrows on the right indicate the type of marker and direction of change. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

the gene knockout (Fig. 2*A*) (18); *Sod1*$^{-/-}$ males show a compensatory increase in the concentration of the antioxidant glutathione (Fig. 2*B*) (27) and a decrease in its oxidation (Fig. 2*C*) (18); aconitase activity is greatly decreased in the liver (Fig. 2*D*) (28), indicating increased oxidative damage; and oxidative damage to lipids is elevated in muscle (Fig. 2*H*) (17).

There was an overall effect of the environment in which males were housed ($F_{7,\ 23} = 2.93$, $P = 0.024$), although the effects for each individual oxidative stress marker were weak and tended to indicate a reduction in oxidative stress with reproductive investment, as previously reported in mice (25). There was also a nonsignificant genotype by environment interaction ($F_{14,\ 46} = 1.80$, $P = 0.068$), although this tended to indicate a further reduction in oxidative stress in *Sod1*$^{-/-}$ males that were investing in sexual signaling. *Sod1*$^{-/-}$ males tended to increase liver protein thiol concentration (Fig. 2*E*), indicating reduced oxidative damage, whereas *Sod1*$^{+/+}$ males showed a trend for the opposite response. The increased lipid peroxidation in muscles of *Sod1*$^{-/-}$ males was abolished in the territorial environment (Fig. 2*H*). Our results do not support the hypothesis that males with preexisting oxidative stress pay a particularly strong cost of sexual signaling in terms of oxidative damage. Instead the results show that housing mice in this more natural environment can actually reduce some of the oxidative damage previously reported in these animals.

SOD1 Deficiency and Whole-Body Metabolism. We next assessed several aspects of mouse energy metabolism. This process allowed us to explore the energetic costs of territory defense and probe for any energy impairments in knockout animals that may reduce energy allocation to sexual signaling. It is first notable that body weight is typically reduced in *Sod1*$^{-/-}$ animals because of a general reduction in lean body mass (17). Interestingly, in this experiment, although such a relationship was apparent in the standard environment (effect of genotype: $F_{2,\ 19.5} = 6.92$, $P = 0.005$), in the territorial environment this difference in weight between genotypes was abolished ($F_{2,\ 20} = 0.80$, $P = 0.46$), as a consequence of *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males losing weight and transitioning to the same weight as *Sod1*$^{-/-}$ males (Fig. 3*A*). *Sod1*$^{+/-}$ and *Sod1*$^{+/+}$ males also differed in body mass in the standard environment, but not in the territorial environment. These changes in body mass in the territorial environment highlight the metabolically demanding nature of sexual signaling. In males with normal *Sod1* expression (i.e., *Sod1*$^{+/+}$), investment in territory defense generates a negative energy balance and causes these males to lose weight. *Sod1*$^{-/-}$ males—which are already much smaller, despite similar levels of food consumption (17)—did not lose weight, and thus might have been limited to some degree in energy mobilization in this environment. Alternatively the males may have increased food consumption. Total metabolic rate (Fig. 3*B*) and energy expenditure (Fig. 3*C* and Table S3) (i.e., the production of heat) were lower in both *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ males, regardless of environment. This finding suggests that Sod1 deficiency, even by 50%, can impair energy expenditure and respiration. In relation to sexual signaling, however, the changes across genotypes in energy expenditure do not fully correlate with the changes we observed across genotypes in olfactory signaling: both *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ show reduced energy expenditure, whereas only *Sod1*$^{-/-}$ males suffer a noticeable reduction in olfactory signaling investment. This finding highlights that a simple reduction in energy production is unlikely to be the complete cause of reduced investment in sexual signaling.

SOD1 Deficiency and Bioenergetic Function. To explore the underlying causes of these energetic alterations, and to further probe bioenergetic adaptations to territory defense, we investigated mechanisms involved in energy production, by measuring mitochondrial respiration at different steps of the electron transport

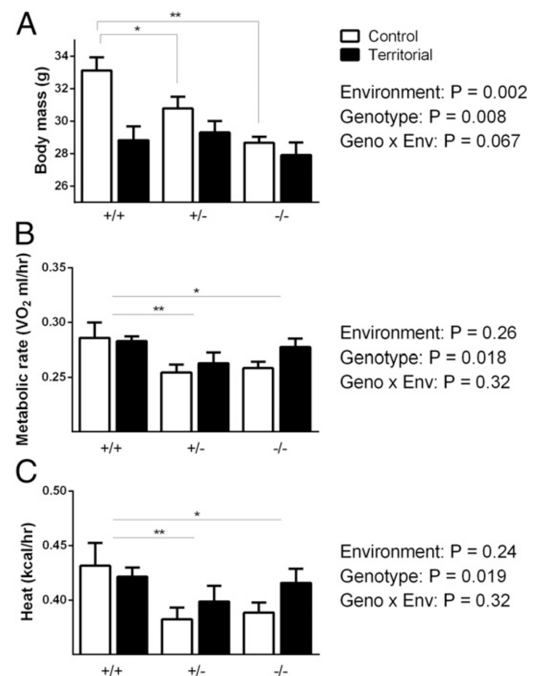

Fig. 3. Metabolic impacts of SOD1 deficiency and territory defense. Impact of genotype and environment on body mass (*A*), metabolic rate (*B*), and heat production (*C*). Results are mean values of the last 6 h of the dark period. $*P < 0.05$; $**P < 0.01$.

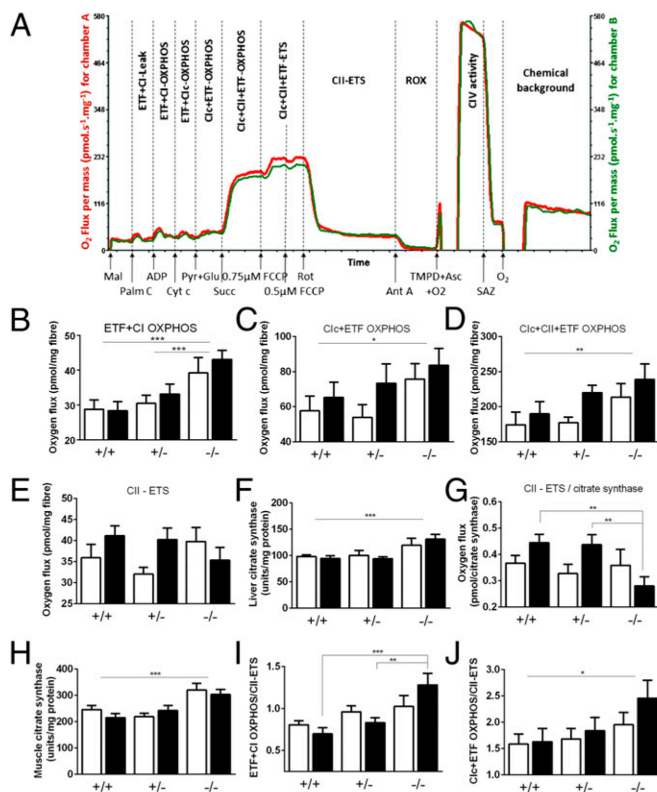

Fig. 4. Impact of SOD1 deficiency and territory defense on bioenergetics. (*A*) The methodological technique, measuring oxygen flux in duplicate (chamber A in red and chamber B in green) at 37 °C (see *SI Materials and Methods*). Mal (malate) and Palm C (palmitoylcarnitine) induced the Leak state (ETF+CI-Leak) with contributions of the ETF and of complex I (CI); ADP allowed the measurement of the OXPHOS state (ETF+CI-OXPHOS; *B*); Pyruvate (Pyr) and glutamate (Glu) were used to further stimulate respiration at the level of CI (CIc+ETF-OXPHOS; *C*); succinate (Succ) activated complex II (CII), and allowed the measurement of convergent electron flow from CI, CII, and ETF (CIc+CII+ETF-OXPHOS) (*D*); rotenone (Rot) was used to inhibit CI and therefore to measure the uncoupling respiration considering only the entry of electrons from CII (CII-ETS) (*E*). Citrate synthase was measured to correct respiration rates for mitochondrial density (*F*). When uncoupled complex II-mediated respiration rates are corrected for citrate synthase, strong differences between the genotypes are apparent in the territory defense environment (*G*). Strong differences in citrate synthase activity were also apparent in muscle (*H*). The ratios between ETF+CI OXPHOS/CII-ETF (*I*) and CIc + ETF OXPHOS/CII-ETF (*J*). Black bars denote males from the territorial environment, white bars denote males from the control environment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

system (ETS) (Fig. 4*A* and Table S4). Despite both *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ males showing reductions in heat production relative to *Sod1*$^{+/+}$, only *Sod1*$^{-/-}$ males differed to wild-type males in bioenergetic function. *Sod1*$^{-/-}$ males showed highly increased levels of oxygen consumption when the substrate supply is limited to electron-transferring flavoprotein (ETF) and complex I and coupled to the production of ATP (Fig. 4 *B* and *C*). This result indicates that males with deficiency in Sod1 show alterations in the functional capacity of their mitochondria that enhance the utilization of fatty acids for fuel. When succinate was added to allow convergent flow of electrons from complex I and complex II, *Sod1*$^{-/-}$ males also showed enhanced oxygen consumption, highlighting a surprising higher oxidative phosphorylation capacity in these males (Fig. 4*D*). Males housed in the territorial environment also showed increased respiration rates at the level of complexes I and II, revealing that bioenergetic capacity is altered when males increase investment in scent-marking and territory defense. Next, we assessed uncoupled respiratory capacity of complex II only. Although there was no overall group effect, a nonsignificant genotype by environment interaction was noted (Table S4), with *Sod1*$^{-/-}$ males tending to show decreased oxygen consumption with territorial defense, whereas males of the other two genotypes showed the opposite effect (Fig. 4*E*).

The results above, describing mass-specific flux per milligram of permeabilized liver tissue, provide an integrated measure of mitochondrial quality and quantity (29). To understand the quality of individual mitochondria, results are expressed in relation to a marker of mitochondrial density, such as citrate synthase activity (29). Interestingly, *Sod1*$^{-/-}$ males had greatly increased liver citrate synthase activity (Fig. 4*F*), indicating a greater mitochondrial density. When corrected for citrate synthase activity, the increased mitochondrial respiration rates of *Sod1*$^{-/-}$ males disappeared (Table S4), indicating that the reason these individuals had a higher mass-specific flux for complex I and complexes I and II combined was because of their greater mitochondrial density. Perhaps more importantly, when corrected for citrate synthase, uncoupled respiration at the level of complex II was in fact reduced in *Sod1*$^{-/-}$ males. This effect was extenuated for individuals in the territorial environment (Fig. 4*G*): both *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males in this environment increased uncoupling respiration at the level of complex II, whereas *Sod1*$^{-/-}$ males tended to show a reduction in this parameter.

We predict that increased mitochondrial density may be a compensatory mechanism that *Sod1*$^{-/-}$ males use in an attempt to maintain basal cellular energy demands. Citrate synthase activity was also greatly increased in gastrocnemius muscle (Fig. 4*H*), highlighting that this effect is not restricted to the liver. *Sod1*$^{-/-}$ males also failed to increase uncoupled respiratory capacity of complex II when increasing investment in territory defense, even though this response was evident in both *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males. *Sod1*$^{-/-}$ males, therefore, seem to show a greater reliance on electron influx from complex I, particularly with contributions from the ETF, which is mainly involved in the oxidation of fatty acids. These effects are additionally illustrated when considering the ratios for oxygen consumption between complexes (Fig. 4 *I* and *J* and Table S4). We predict that this impaired capacity for complex II-mediated respiration may limit the ability to produce energy efficiently and might also be related to the slower growth and reduced body mass of *Sod1*$^{-/-}$ males, a hypothesis that deserves further investigation. Importantly, this effect was only observable in the territorial environment, highlighting that the standard laboratory environment can mask phenotypic changes in traits of interest.

Discussion

In this experiment, direct genetic manipulation of *Sod1* expression allowed us to reveal a causal effect of oxidative stress on sexual signaling. Previous tests of this hypothesis have manipulated dietary antioxidants or activated components of the immune or detoxification systems (5), all of which have other effects on animal health in addition to increasing oxidative stress. It is also notable that the vast majority of previous research on sexual signaling and oxidative stress has focused on species that produce visual sexually selected traits, such as the colorful plumage and ornaments in birds, fish, and lizards (5). Our results suggest that oxidative stress might limit the expression of olfactory signals, which are used widely in mammals and also across a variety of nonmammalian species.

Our results suggest that investment in territory defense is energetically demanding, eliciting an increase in the total respiration capacity of liver mitochondria and causing *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males to lose weight. However, several lines of evidence suggest that impaired bioenergetic function, and a consequent reduction in energy production, is not the cause of reduced sexual signaling with oxidative stress in this study, as hypothesized (15), even though these traits are all affected by SOD1 deficiency. First, *Sod1*$^{+/-}$ males also suffered a reduced metabolic rate but they showed no detectable difference in mitochondrial function or investment in sexual signaling. This finding suggests that the reduced energy expenditure with SOD1 deficiency is not purely

a consequence of the altered mitochondrial respiration we detected, because $Sod1^{+/-}$ males had apparently normal mitochondrial function. If decreased whole-body metabolism necessitates a reduction in sexual signaling, we would also expect to see this in $Sod1^{+/-}$ males. However, $Sod1^{+/-}$ males seemed able to maintain urinary MUP concentration and increase preputial gland mass despite their reduced energy expenditure.

Second, the sexual signals that we found to be reduced with oxidative stress (MUPs and preputial gland mass)—although known to influence sexual attractiveness—may not entail substantial energetic costs. It has been suggested that the production of MUPs may be energetically costly (30), but the synthesis of these proteins actually makes up a small amount of a mouse's total energy budget. Estimates of the energy costs of protein synthesis range between 3 and 20 kj/g protein (31). Mice produce ~10 mg of MUP per day, equating to ~0.025 kj of energy per day spent on urinary MUP synthesis. Compared with total energy expenditure (Fig. 3*C*), this is a very small amount of total energy demand. In male mammals, reproductive behaviors that increase activity levels, such as mate guarding and scramble competition, often have substantial energetic costs, often more than doubling resting energy demands and sometimes exceeding the costs of lactation for females (32). It seems more likely that the elevated behavioral investment in scent-marking and patrolling of their home area for territorial males in this experiment may have been energetically costly, facilitating the reduction in body mass observed in this experiment and previously reported (30). We observed no differences between genotypes for this behavior, although we cannot exclude the possibility that SOD1 deficiency may influence scent-marking in a more subtle manner which was undetectable in our assay.

Our results do not support the hypothesis that oxidative stress is a condition-dependent cost of sexual signaling, or that males with poor antioxidant defense suffer these costs more than normal, healthy males. Elevated investment in territory defense actually tended to reduce some signs of oxidative damage in liver and gastrocnemius muscle of $Sod1^{-/-}$ males, which is counter to the direction of the previous prediction. It is possible that this change may be connected to the increased oxidative phosphorylation capacity of liver mitochondria (per weight of wet tissue) seen across territory defense males, as an increased respiration capacity can sometimes reduce the production of ROS (33). Von Schantz et al. (14), the original proponents of the oxidative stress–sexual signaling hypothesis, highlighted that some organs or tissues may be particularly sensitive to oxidative stress, and if these areas are involved in sexual signaling then those signals may be likely candidates to reveal oxidative stress. Reduced olfactory signaling in this study may simply be a consequence of oxidative damage to signal-producing tissues.

The liver, the site of urinary MUP production, is particularly sensitive to oxidative stress in the $Sod1^{-/-}$ mouse (16). Oxidative damage in the liver, or any pathological changes it induces, may have limited the ability of $Sod1^{-/-}$ mice to invest in this protein synthesis, physically impairing MUP production rather than making investment in these sexual signals more costly. It should be noted that the liver is also a site of pathology in old $Sod1^{-/-}$ mice, generating hepatocellular carcinoma, a leading cause of death in these animals late in life (16). However, these cancers do not develop until late age, and when young (as used in this study), $Sod1^{-/-}$ animals appear phenotypically normal apart from increased oxidative stress and smaller body mass (34). Therefore, it is unlikely that carcinogenesis itself is the cause of reduced MUP output. Similar to the liver, oxidative damage to the preputial glands might also be responsible for the smaller size of this tissue. Unfortunately we did not assess oxidative stress in these glands, and to our knowledge oxidative damage has not been examined in this tissue previously, either in wild-type mice or knockout mice with induced oxidative stress. A further investigation of damage to this tissue, and alterations in the composition of the volatile pheromones it produces, may help to reveal the physiological causes of reduced sexual signaling with oxidative stress.

We also reveal that the environment in which genetically modified mice are housed can have a strong effect on observed phenotypes, a result that has wide-ranging implications outside of the fields of sexual selection and life history evolution. $Sod1^{-/-}$ mice in the control environment of this study show a phenotype that parallels those previously reported: small body mass (17), high oxidative stress (17), and little difference in mitochondrial function when results are expressed per mitochondrion (28). However, for those animals housed in a more demanding territorial environment, the difference in body mass was abolished, as were some of the differences in oxidative damage markers, and impairments in mitochondrial function became apparent. As a consequence, we strongly urge further investigation of genetically modified mice and disease strains in these more demanding conditions, which are probably more similar to natural conditions than the standard laboratory environment. These changes with environment may well extend further than specific alterations in physiology and may influence mortality rates, disease onset, and reproductive capacity. With such an approach, important and previously undiscovered functions of a wide range of genes could be revealed.

Materials and Methods

Animals. This research was approved by the University of New South Wales (UNSW) Animal Care and Ethics Committee, approval 11/68A. *Sod1* mice were maintained on a C57BL/6 background (17). This line was imported from the Jackson Laboratories and maintained as a specific pathogen-free breeding colony at the Australian BioResource Center (Mossvale, NSW, Australia). When 6–8 wk old, experimental *Sod1* mice were transported and housed in conventional facilities at the UNSW. Mice were maintained at 22 ± 2 °C on a 12:12-h light/dark cycle. All experimental procedures were conducted in the dark period under dim red light. Two- to three-mo-old CBA males and females (CBA/CaHAusb) were used to stimulate experimental animals to invest in territory defense and were purchased from the Australian BioResource Center, transported to the UNSW, then housed in groups of two or three until experiments began.

Experimental Procedures. After a 2-wk habituation period in experimental cages (53 × 35 × 18 cm), during which males were singly housed, males of each genotype were randomly allocated to two experimental groups that had their social environment manipulated in one of two ways over a 3-wk period: one group of males were exposed to the presence and odor of male and female con-specifics to induce investment in territory defense (termed "territorial"; $Sod1^{-/-}$ $n = 6$; $Sod1^{+/-}$ $n = 8$; $Sod1^{+/+}$ $n = 8$), and the other were not (controls; $Sod1^{-/-}$ $n = 7$; $Sod1^{+/-}$ $n = 10$; $Sod1^{+/+}$ $n = 8$). These manipulations were based on a previously published approach (25). Briefly, territory defense males were exposed to the presence of a male or female four times a week; they also received a small handful of CBA female bedding twice a week and intruder scent-marks (on tiles) from a CBA male three times a week. See *SI Materials and Methods* for further information.

Investment in Olfactory Signaling. Urine samples were collected from males at the end of the habituation period and in the final week of the experiment. Total urinary protein levels were assessed with the Coomassie assay, which reflects total urinary MUP concentration, and were corrected for urinary dilution by measuring urinary creatinine levels (20). Scent-marking rates were assessed at the same periods, but on 2 separate days that did not coincide with urine collection, by placing a piece of benchkote (30 × 8 cm) in each male's cage for a 2-h period. Scent-marks deposited on this are visible under UV light and were counted manually by an experimenter. Preputial gland mass was assessed at the end of the experiment.

Oxidative Stress and Bioenergetic Assays. At the end of the experiment, males were culled via cervical dislocation, quickly dissected, a small sample of liver was taken for bioenergetic analysis, and relevant organs snap-frozen in liquid nitrogen and then kept at –80 °C before biochemical analysis. Total and oxidized glutathione content were measured using the automated glutathione recycling assay (35); glutathione is a ubiquitous antioxidant with an established antioxidant role in vivo, and the proportion of glutathione in the oxidized form is a common marker of oxidative stress and redox status (3). Protein thiols, which are organosulfide groups on proteins essential for stability but susceptible to oxidation (3), were measured as in ref. 35. Malonaldehyde (MDA), a secondary

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product of lipid peroxidation (4), was measured using the HPLC method of Fukunaga et al. (36). Aconitase, which is an enzyme of the tricarboxylic acid cycle that is very susceptible to deactivation by ROS, was measured according to ref. 37. With the exception of the proportion of glutathione in the oxidized form, markers of oxidative damage in tissues are reported as a concentration per milligram of protein. Mitochondrial bioenergetic assays were run as previously described (38). A brief outline of the method is given in Fig. 4*A* and is described in further detail in *SI Materials and Methods*.

Whole-Body Metabolism. Indirect calorimetry was measured using the metabolic cage system from Columbus Instruments. Measurements began at 10:00 AM, in the first hour of the dark period, and continued for 12 h. The first 6 h of the measurement period was an acclimatization period and the last 6 h were used for analysis. The calorimeter was calibrated before the experiment with a standard span gas (0.504% CO_2, 20.43% O_2 balanced with N_2) and cross-calibrated with room air. Data were collected every 13 min over this 12-h period. The metabolic measurements included the volume of carbon dioxide produced (VCO_2), the volume of oxygen consumed (VO_2), and the caloric (heat) value: $(3.815 + 1.232 \times RER) \times VO_2$. The data are represented as the mean values over each 6-h period.

Statistics. Statistics were conducted in SPSS v21. General linear mixed models (GLMM) were used to assess differences between genotypes and environment for sexual signaling, metabolic parameters, and individual markers of oxidative stress. Both genotype and environment and the interaction term between these parameters were added as fixed effects. Using these models also allowed us to add a "batch" variable as a random effect, because the experiment was staggered in two parts (with experimental groups evenly represented in both batches). Variables were log-transformed where necessary to conform to assumptions of normality. To assess overall oxidative stress we conducted a multivariate analysis of variance, with each oxidative damage parameter added as a dependent variable, allowing us to test whether there was an overall effect of either genotype or environment on this aspect of physiology. Batch in this model was added as a fixed effect. Because sample sizes differed slightly depending on the assay, we then conducted individual GLMMs for each separate oxidative damage variable.

The raw data used in this report are in Dataset S1.

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