# Overexpression of an antioxidant enzyme improves male mating performance after stress in a lek-mating fruit fly

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**Abstract**

In many species, courtship displays are reliable signals of male quality, and current hypotheses suggest that these displays allow females to choose males with high cellular function. Environmental stressors generate excess reactive oxygen species (ROS) that impair cellular function, and thus antioxidant pathways that remove ROS are likely critical for preserving complex sexual behaviors. Here, we test the hypothesis that enhanced antioxidant activity in mitochondria preserves mating performance following oxidative stress. Using a transgenic approach, we directly manipulated mitochondrial antioxidant activity in the Caribbean fruit fly, *Anastrepha suspensa,* a lek-mating species with elaborate sexual displays and intense sexual selection that is also a model for Sterile Insect Technique programs. We generated seven transgenic lines that overexpress mitochondrial superoxide dismutase (MnSOD). Radiation is a severe oxidative stressor used to induce sterility for sterile insect programs. After radiation treatment, two lines with intermediate MnSOD overexpression showed enhanced mating performance relative to wild-type males. These improvements in mating corresponded with reduced oxidative damage to lipids, demonstrating that MnSOD overexpression protects flies from oxidative stress at the cellular level. For lines with improved mating performance, overexpression also preserved locomotor activity, as indicated by a laboratory climbing assay. Our results show a clear link between oxidative stress, antioxidant capacity, and male performance. Our work has implications for fundamentally understanding the role of antioxidants in sexual selection and shows promise for using transgenic approaches to enhance the field performance of insects released for area-wide pest management strategies and improving performance of biological control agents in general.

**KEYWORDS:** Oxidative stress, superoxide dismutase, mitochondrial function, transgenic insect, condition-dependent traits

## Introduction

The ability to accurately assess mate quality is a critical function for animals. Many species have evolved ornaments and other elaborate behaviors as indicators of male quality [1]. The current understanding of condition-dependent traits is that they provide an honest signal of physiological and cellular function [2]. Reactive oxygen species (ROS) are essential cell signaling molecules, but an excess of ROS compromises cellular function and causes oxidative stress [3]. Many environmental challenges (e.g., temperature, desiccation, hypoxia-reperfusion, etc.) lead to ROS accumulation [3, 4], which in turn causes oxidative damage and reduces male quality [5-7]. Mitochondria are the primary source of ROS production, and initial ROS release following stress can trigger a positive feedback loop of sustained ROS release, resulting in long-lasting effects on organismal function [8]. Thus, current hypotheses suggest mitochondrial function is a key mechanism that underscores phenotypic variation in sexual displays [9], and on evolutionary time scales mitonuclear interactions may be critical determinants of life history traits [10].

Oxidative stress is pervasive in nature, and antioxidant defenses play a critical role in maintaining the vitality of cells, ultimately mediating whole-organism performance [1, 5-7]. Organisms have evolved a suite of antioxidant defense systems to detoxify ROS and preserve cellular function in the face of an oxidative challenge [11]. In natural systems, current hypotheses about the role of antioxidants in mating performance mostly stem from a large body of work on the role of dietary carotenoids in sexual ornamentation [reviewed by 12, 13]. Carotenoids are the main pigments used in vertebrate sexual ornaments and have antioxidant

activity, so long-standing hypotheses suggest these carotenoids provide a direct link between antioxidant function and mate choice. However, carotenoids represent a minor component of an animal's total antioxidant capacity [14], and there is conflicting evidence regarding a physiological role for carotenoids in defense against oxidative stress. For example, white canaries that carry a recessive mutation resulting in extremely low levels of tissue carotenoids are no more susceptible to oxidative challenges than wild-type canaries [15]. Thus, endogenous antioxidants that represent the majority of an organism's antioxidant capacity likely play a substantial role in mediating condition-dependent sexual traits through their roles in maintaining cellular vitality in stressful environments.

Here, we experimentally test the relationship between antioxidant function, oxidative stress, and mating performance in the Caribbean fruit fly (caribfly), *Anastrepha suspensa.* This species uses a lek mating system that involves an elaborate series of visual displays, courtship songs, male-male fighting, and pheromone release [16]. Male courtship in caribflies is energetically demanding and requires high levels of physiological function [17, 18]. Caribflies are amenable to laboratory rearing and genetic transformation [19], allowing for direct manipulation of antioxidant function to test hypotheses regarding the role of endogenous antioxidants in maintaining cellular function and sexual displays following stress. In this species, hormetic-conditioning treatments that improve male mating performance after stress are correlated with increased activity of two endogenous antioxidant enzymes, suggesting these pathways may be involved in maintaining courtship behavior by attenuating oxidative stress that reduces cellular vitality in stressful environments [20].

Beyond impacts on fundamental understanding of the role of antioxidants and mitochondrial function in the face of stress, our work also has implications for the practical

improvement of biological control agents. Sterile Insect Technique (SIT) is an environmentally friendly pest control strategy in which mass-reared males are sterilized with ionizing radiation and released to suppress wild populations [21]. Caribflies were once controlled with field SIT releases, and although those programs have ended, *A. suspensa* is routinely used to evaluate strategies for improving SIT in other pests. Several of their close relatives (e.g., *A. ludens*) are used in active SIT programs, and caribflies are also a useful model for other economically important fruit flies as well [e.g., 20, 22, 23, 24]. The radiation used for sterilization in SIT causes oxidative stress that reduces the mating competitiveness and lifespan of sterilized males, thereby hindering the performance of these biological control agents and increasing economic costs associated with SIT [20, 25-28]. Numerous transgenic strategies have been developed to improve SIT, for example conditional lethality strains that obviate the need for radiation sterilization and genetic sexing strains that eliminate unwanted females [29, 30]. Our work stands out from these previous studies because we are specifically focused on transgenic approaches that improve male performance. Overexpression of antioxidants may have additional benefits for SIT and other types of biological control where ionizing radiation is not used, for example by improving longevity [31] and/or preventing declines in muscular performance [32] in insects used for augmentative biological control. Even if genetic sterilization eventually replaces the need for radiation as a sterilizing agent, any insect released for population control requires successful field performance, and our work demonstrates the potential of using transgenic strategies to target specific deficits in the performance of beneficial released insects.

## 2. Methods and materials

### (a) Insect husbandry and germline transformation

Wild type and transgenic colonies of the Caribbean fruit fly, *Anastrepha suspensa,* were maintained under standard laboratory conditions [19]. Larvae were reared on an artificial diet containing, in g/l, 4 agar, 75 dextrose, 75 torula yeast, 75 wheat germ, 0.6 cholesterol, 1.3 sodium benzoate, 1.3 methyl 4-hydroxybenzoate, and 1.0 butyl 4-hydroxybenzoate at 27°C, 14:10 L:D, and allowed to pupariate on vermiculite. Adults were fed 3:1 sugar:yeast hydrolysate and water *ad lib*, and eggs were laid onto red fabric cloths placed on top of the cage.

PiggyBac transformation vectors for MnSOD (GenBank: MK840869) and Cu/ZnSOD (GenBank: MK840870) were generated using restriction cloning (figure *1a;* see details in Electronic Supplementary Material). The MnSOD transformation vector (500 ng/ul) and a piggyBac transposase helper plasmid (200 ng/ul) were co-injected into wild type embryos as described previously [19]. Additional details on the generation of transgenic lines are found in the Electronic Supplementary Material. qPCR was used to estimate the number of genomic insertion events for each transgenic line. The quantity of *DsRed* sequence was measured relative to a known single copy gene, *Pros26* [33] using primers DsRed-F, DsRed-R, Pros26-F, and Pros26-R (table S1).

**(b) SOD transcript expression**

qPCR was used to measure the transcript expression of both mitochondrial superoxide dismutase (*MnSOD*) and cytosolic superoxide dismutase (*Cu/ZnSOD*) in each line. Expression of *Cu/ZnSOD* was measured as a control to indicate the specificity of MnSOD overexpression. A single *Cu/ZnSOD* overexpressing line was also included as a control in the gene expression and enzyme activity experiments to further evaluate the specificity of the transgene. All flies used for transcript abundance and enzyme activity were snap frozen in liquid nitrogen, and each experiment consisted of at least four independent replicates. In all lines, gene expression was

144 measured in both 3 d old adult males and pharate adult males. In a subsequent experiment, we
145 measured expression in a single line (line 3.9) with robust overexpression to assess the
146 developmental trajectory of overexpression and the functionality of the exogenous *Drosophila*
147 heat shock promoter. While this line was not one of our focal lines in later experiments (see
148 sections 3 e and f), we elected to use it in these early evaluations of transgene function because it
149 had consistently high mRNA expression and enzyme activity in preliminary experiments. Flies
150 from line 3.9 were sampled as eggs, wandering larvae, pupae, pharate adults, and 3 d old adult
151 males and females. Adult were also heat shocked for 2 h at 37°C, and flies were sampled
152 immediately after the heat shock and after 2 h recovery at 25°C to test the extent to which heat
153 shock could further increase MnSOD expression.

154 RNA was extracted from pools of four flies using the Direct-zol RNA MiniPrep kit
155 (Zymo) according to manufacturer's protocols. RNA purity and concentration were measured
156 using a Nanodrop, and the iScript cDNA Synthesis Kit (Bio-Rad) was used to generate cDNA
157 from 1 µg RNA from each sample. Each qPCR reaction contained 2 µl cDNA, 2 µl of each
158 primer (at 250 nM), 10 µl iTaq Universal SYBR Green Mastermix, and 4 µl water. The relative
159 quantity of *MnSOD* and *Cu/ZnSOD* transcripts was calculated using the 2-ΔCt method, as in
160 [34], with *RP49* (GenBank: MK847891) as the reference gene. Primer sequences for *MnSOD,*
161 *Cu/ZnSOD,* and *RP49* can be found in table S1.

162 **(c) SOD enzyme activity**

163 Enzyme activity of MnSOD and Cu/ZnSOD was measured using the Superoxide
164 Dismutase Assay Kit (Cayman Chemical), according to the manufacturer's protocol. Total SOD
165 activity was measured in protein extracts, and MnSOD activity was measured by adding 3 mM
166 KCN to inhibit Cu/ZnSOD. Additional details for the SOD assay can be found in the Electronic

Supplementary Material. As with gene expression, enzyme activity was measured in adult and pharate adult males of all lines, as well as across development and following heat shock in line SOD2-3.9 (see details above).

**(d) Mate choice tests**

We evaluated the precopulatory mating success of transgenic and wild type males in mate choice tests under laboratory conditions [20]. Copulation success of males was assessed by exposing one wild type virgin female to two virgin males. Males, 12 days after eclosion, were marked with a small dot of water-based paint on the thorax and released into plastic cages the day prior to the experiments. On the following day, females were released into cages with two marked males and observed for up to 4 h during the afternoon. At least 50 pairs spread across at least 3 cohorts were measured for each of the four combinations tested: 1) untreated transgenic males paired with untreated wild-type males, 2) irradiated transgenic males paired with irradiated wild type males, 3) irradiated wild type and transgenic males paired with untreated wild type males, and 4) untreated wild type and transgenic males paired with irradiated wild type males.

**(e) Biochemical measurement of oxidative stress**

To test the extent to which MnSOD overexpression reduces irradiation-induced oxidative damage, we measured two indices of oxidative damage in males. The thiobarbituric acid reactive substances (TBARS) assay measures the abundance of malondialdehyde (MDA), a product of lipid peroxidation, while the protein carbonyls assay measures oxidative damage to proteins. Assays were conducted according to [20]; see Electronic Supplementary Material for additional details.

**(f) Climbing assay**

We used a climbing assay as a proxy for muscle performance in the two high-performing lines (SOD2-5.2 and SOD2-6.1). Irradiated flies were treated at 70 Gy as pharate adults, two days prior to emergence. For the climbing assay, male flies were placed in the bottom of a 33 cm long by 1.9 cm diameter clear acrylic tube, and flies were observed until they either reached the 33 cm mark on the tube or five minutes had expired. For each fly, we scored whether it made it to the top of the tube, and the number of times it fell while crawling. For each group, we measured 10 individual flies, and each fly was tested three times. For each group, we calculated the average number of falls per fly and the proportion of the total of the 30 trials for each line in which a fly made it to the top.

**(g) Longevity measurements**

The life span of irradiated and untreated males was monitored daily to determine the effect of MnSOD overexpression on transgenic flies' longevity. Briefly, 10 newly emerged transgenic or wild type males were transferred into plastic cages containing 5 wild type females, and the number of deaths was recorded daily until all died. During the experiments, flies had free access to hydrolyzed protein diet (3 parts of sugar: 1 part of protein) and water. A randomized block design was applied, and at least 4 blocks with 4 cages each were carried out to evaluate the longevity of irradiated and untreated males.

**(h) Statistical analyses**

Statistical analyses were conducted in JMP 12 (SAS), SAS, or R. For all experiments, we used $\alpha = 0.05$. To analyze qPCR data, we conducted ANOVAs on the $\Delta$Ct values, and pairwise comparisons were made using t-tests, with a Tukey's multiple comparisons adjustment. Enzyme activity data were analyzed with a least squares regression model with genotype as a fixed effect

and experimental block as a random effect. For the heat shock experiment, we fit a linear mixed model with genotype, treatment, and their interaction as main effects, with block as a random effect. The developmental enzyme activity data were analyzed by fitting a linear model with genotype, developmental stage, and their interaction as main effects, with block as a random effect, where a block included all samples analyzed together on the same microplate. Mate choice tests were analyzed with logistic regression, fitting the proportion mating success of each pairwise comparison as a function of genotype, with block as a random effect. Cox regression was used to compare the longevity of transgenic and wild type males. To analyze oxidative stress data (TBARS and protein carbonyls), we fit TBARS or carbonyls levels as a function of irradiation status, genotype, sampling time, and their interactions, with block as a random effect. Linear contrasts were used to compare levels of each variable.

## Results

### (a) Generating and characterizing MnSOD overexpression lines

Using piggyBac germline transformation, we initially created 12 transgenic lines, and segregation analysis indicated that six lines carried a single autosomal insertion while one line carried a Y-linked, male-only insertion (table S2). These seven lines were retained for subsequent experiments. After breeding the autosomal lines to homozygosity, real-time quantitative PCR (qPCR) analysis of genomic copy number confirmed the segregation analysis, and all lines showed good viability compared to wild type (figure S1).

MnSOD was significantly overexpressed relative to wild type in adult males of all transgenic lines (figure 1*b*), resulting in significantly elevated MnSOD enzyme activity in five of the lines (figure 1*c*). *MnSOD* transcript abundance in transgenic lines ranged from 1.8 to 4.5-fold

higher than wild type. MnSOD transcript abundance and enzyme activity were highly correlated (figure 1*d*), indicating that mRNA derived from the transgene was translated into a functional protein. MnSOD is also significantly overexpressed at the pharate adult stage, the developmental stage that is irradiated for SIT sterilization (figure S2*a,b*). As in adults, MnSOD transcript abundance and activity were highly correlated in pharate adults (figure S2*c*), and lines that had high expression and activity as pharate adults also had high expression and enzyme activity as adults (figure S2*d,e*). Overexpression was specific for the mitochondrial isoform of SOD, as the transgene did not elevate transcript abundance or enzyme activity of the cytosolic Cu/ZnSOD in both adults and pharate adults (figure S2*f-i*). In line SOD2-3.9, MnSOD was significantly overexpressed at the pupal stage and beyond (figure S3*a,b*), and overexpression was specific to the mitochondrial version of SOD (figure S3*c*). While heat shock treatment caused a slight increase in MnSOD mRNA levels (figure S3*d*), heat shock failed to drive an increase in MnSOD activity (figure S3*e*), indicating the heat shock response element of the *Drosophila* hsp70 promoter was not capable of driving higher levels of MnSOD activity in these lines despite high baseline activity. Overall, these experiments indicate that we successfully created lines that overexpress MnSOD, and transgene activity is highest at the times required to protect against damage from sterilizing irradiation even in the absence of additional stimulation of the transgenic promoter by heat shock.

**MnSOD Overexpression Enhances Mating Performance of Irradiated Flies**

To test the extent to which male mating performance was enhanced by MnSOD overexpression, we conducted a series of female mate choice assays in the lab. When both wild-type and transgenic males were irradiated at 70 Gy, the recommended sterilization dose for tephritid SIT, and competed against each other in head-to-head assays with a single female, two

of the lines (SOD2-5.2 and SOD2-6.1) outperformed wild-type males, with transgenic males 1.5 times more likely to be chosen by the female ($X^2 = 6.246$, df = 1, p = 0.012 for SOD2-5.2; $X^2=4.499$, df =1, p=0.033 for SOD2-6.1,). The mating success of the other five transgenic lines was indistinguishable from wild type after irradiation (figure 2a). However, when both males were unirradiated, transgenic males underperformed relative to their wild-type counterparts (figure 2b*;* $X^2 = 14.143$, df = 1, p = 0.0002), indicating that a cost to being transgenic under non-stressful conditions. Taken as a whole, the relative mating competitiveness of transgenic males improved after irradiation (Nominal Logistic Model, $X^2=13.270$, df=1, p=0.0003), pointing to a specific, protective effect of MnSOD overexpression only when flies were stressed. Mate choice results for other combinations of males (irradiated transgenic males vs. unirradiated wild type and unirradiated transgenic males vs. irradiated wild type) are shown in figure S4. While MnSOD overexpression improved mating performance, irradiation at 70 Gy still resulted in sterility. For each of the eight lines (wild type + seven transgenic), a minimum of 50 eggs were collected from each of 10 crosses. In these 80 crosses, no viable eggs were observed after irradiation. In contrast, control crosses consisting of an untreated wild-type male with an untreated female yielded viable offspring in 7/10 crosses.

**MnSOD Overexpression Protects Against Oxidative Stress at the Cellular Level**

Our initial screens above indicated that lines 5.2 and 6.1 experienced the largest improvements in mating performance, so the remainder of the experiments focused on these two lines. To confirm that MnSOD overexpression is protecting against oxidative damage as expected if oxidative damage mediates cellular vitality and thus male performance, lipid peroxidation and protein carbonylation were measured in males at four time points following irradiation: 1) immediately after irradiation as pharate adults, 2) 24 h after irradiation, 3) as 3 d

old adult males, and 4) as 12 d old male adults (the time at which mating performance peaks). In response to irradiation, MDA levels, an indicator of lipid peroxidation [e.g., 35], were significantly elevated in wild-type flies across the four measured time points, particularly in 3 d old adult males that were 5 days post-irradiation (figure 3*a;* Linear Model, $F_{1,69}$=25.91, p=3E-6). In unirradiated males, there were no significant differences in MDA levels across all four time points (figure S5*;* Linear Model, $F_{1,69}$=0.5312, p=0.47), although there was a slight increase in SOD2-5.2 males as 3 day-old adults. In contrast, there was a significant trend towards lower MDA levels in irradiated transgenic flies across the four time points (figure 3*b,c;* Linear Model, $F_{1,69}$=25.92, p=2.9E-6). Sterilized SOD2-5.2 males had significantly reduced MDA levels immediately after irradiation and 24 h later, while SOD2-6.1 males had lower MDA levels at the middle time points. Our other measure of oxidative damage, protein carbonylation, did not change in response to sterilizing irradiation, and there were no differences between wild-type and transgenic flies (figure S6).

**MnSOD Overexpression Improves Muscle Performance of Sterilized Flies**

For lines SOD2-5.2 and SOD2-6.1, we measured the extent to which MnSOD overexpression preserved muscle performance in males following oxidative stress. Muscle performance was assessed with a negative geotaxis assay, routinely used to measure deficits in neuromuscular performance in flies [e.g., 36]. Flies were tested 2 weeks after adult emergence, the time when mating performance peaks. When flies were irradiated, both transgenic lines were nearly twice as likely to reach the top of the arena as wild-type flies (figure 4*a*), and transgenic flies were only half as likely to fall while climbing (figure 4*b*). Together, these results indicate that MnSOD overexpression improves locomotor ability of irradiated flies.

**MnSOD Overexpression Fails to Increase Lifespan**

According to the free radical theory of aging [31], gradual accumulation of damage from excess reactive oxygen species contributes to age-related declines. We hypothesized that MnSOD overexpression would enhance longevity of caribflies and thus increase their opportunities for mating in the field. In unirradiated flies, transgenic flies had reduced longevity, with median longevity being 3-14 days shorter than wild-type flies (figure 4*c;* Cox Regression, $p<0.05$). Irradiation reduced longevity by 11 days in wild-type flies (figure 4*c,d;* Cox Regression, $p<0.05$), which is consistent with the free radical theory of aging. The longevity of irradiated transgenic flies was still significantly shorter than that of irradiated wild type flies (figure 4*d*), although the differences were less pronounced than in unirradiated flies.

## DISCUSSION

Here we show that increasing the activity of a mitochondrial antioxidant decreases oxidative damage and increases male mating success and muscular performance after exposure to a substantial oxidative stressor. We generated seven transgenic lines of caribflies that overexpress MnSOD, a key mitochondrial enzyme that is one of the cell's first lines of defense against reactive oxygen species [37, 38]. After irradiation, which causes substantial oxidative stress, two lines with intermediate MnSOD overexpression levels showed enhanced mating performance relative to sterilized wild-type flies (figure 2*a*). When flies were not irradiated, transgenic males were less competitive for mates than their wild-type counterparts (figure 2*b*). Yet, the overall competitiveness of all transgenic lines was higher following irradiation. This observation reflects the presumed costs of transgenesis [39], which include energetic costs associated with synthesizing the transgene and marker proteins, as well as costs of insertional mutagenesis. Additionally, ROS are important signaling molecules for cell growth and survival

[40], so excess antioxidant capacity in transgenic flies may reduce performance in non-stressful environments.

As opposed to previous data on the role of antioxidants in mating performance, which have been based largely on correlational observations [12, 13], we provide direct evidence that endogenous antioxidants are critical for maintaining physiological function and sexual performance under stressful conditions. These results are in line with current models suggesting that sexual displays allow females to discern males with higher relative cellular vitality [2]. While we predicted that lines with the highest expression of MnSOD would perform best after stress, we found that our two best performing lines had intermediate levels of increased antioxidant activity. Similar effects of MnSOD transgene dosage have been observed in *Drosophila,* where moderate overexpression improved lifespan while strong overexpression had negative effects on survival [41]. It is also possible that lines differed in their tissue-specific expression of MnSOD, which could explain variation in mate performance across lines. However, identifying the precise reasons why certain transgenic lines outperformed others is beyond the scope of this study.

Biochemical measures of oxidative damage confirmed that MnSOD overexpression reduced oxidative damage caused by irradiation. In wild-type flies, sterilizing radiation resulted in a substantial increase in lipid peroxidation levels, and the effects of irradiation were long lasting. Levels of the oxidized lipid MDA were nearly 60% higher in irradiated wild-type flies 24 h post-irradiation and nearly 80% higher five days post-irradiation than those in unirradiated wild-type flies (figure 3*a*). These same radiation treatments significantly lowered mating performance (figure 2), consistent with previous work on birds [42] and fish [43] demonstrating that accumulation of oxidative damage reduces male mating success. MnSOD overexpression

attenuated radiation-induced lipid peroxidation, with levels of MDA significantly lower in transgenic flies following irradiation compared to wild-type flies (figure 3*b,c*). A similar reduction in lipid peroxidation was observed when we previously used anoxic treatments to boost antioxidant capacity prior to irradiation [20], indicating that our transgenic strategy recapitulated the phenotypic effects of anoxic hormesis.

A recent synthesis of the biomedical literature by Koch and Hill [9] proposes that sexually selected behavioral displays are dependent on mitochondrial function, and our experiments provide experimental evidence in support of this hypothesis. Our results are consistent with previous work that oxidative stress and condition-dependent sexual traits are tightly linked [5-7]. This previous work, mostly in birds, primarily consists of correlational analyses of dietary carotenoids, antioxidant capacity and sexual selection [14, 44]. Our work extends these findings by developing a tractable insect system in which antioxidant function can be directly manipulated through genetic means. Furthermore, to our knowledge, this study is the first direct demonstration of a critical role for endogenous antioxidants in maintaining sexually selected behaviors after stress. Previous work from our group has demonstrated that low oxygen treatments boost antioxidant capacity and increase performance after stress [20, 25, 26], and here we show a precise genetic mechanism using targeted overexpression of MnSOD. The elaborate sexual displays of tephritid fruit flies [16, 45], coupled with their genetic tractability, make this system an excellent model for rigorously testing the links between cellular redox balance, courtship displays, and male performance.

MnSOD overexpression also improved locomotor ability of male flies (figure 4*a,b*). Neuromuscular performance is critical for elaborate courtship displays [9], and for SIT to be successful, males must disperse from a release point and locate mates. Oxidative stress is known

to reduce muscle force production and contribute to atrophy [32, 46], and thus the rearing conditions and radiation doses used in SIT often reduce muscle performance, thereby limiting the efficacy of released males [27, 47, 48]. In addition to improving male performance, enhanced antioxidant capacity is predicted to increase longevity under the free radical hypothesis for aging [31]. However, our results were not consistent with the free radical hypothesis for aging. MnSOD overexpression failed to improve longevity in both fertile and sterile flies (figure 4*c,d*), and most transgenic lines in fact had reduced lifespans. While the free radical theory of aging is widely touted, results in the literature are mixed. In *Drosophila,* SOD overexpression increases lifespan in some genotypes [e.g., 41] but not others [e.g., 49], while in *C. elegans* overexpression of Cu/ZnSOD enhances lifespan independent of oxidative damage [50], and deletion of MnSOD actually increases lifespan [51]. In our system, additional experiments with controlled genetic backgrounds are needed to fully test the link between antioxidant capacity and longevity, but our experiments do strongly suggest that aging and MnSOD activity are not coupled in caribflies.

The results of this study also have implications for biological control programs, especially SIT, an environmentally friendly control strategy that relies on sterilized males to suppress and/or eradicate pest populations. Global demands for food production and sustainable agriculture practices are increasing [52], and thus strategies like SIT that reduce dependence on chemical pesticides are likely to be relied on more heavily in the future. While SIT is effective at reducing pest populations, and in some cases is more cost effective than chemical control [53], the poor performance of sterilized males limits the potential economic efficiency of SIT. Poor male performance necessitates releasing large numbers of males (in some cases 100:1 ratio of released to wild males), which increases the cost of current programs and can make it challenging to initiate and sustain new programs. Here, we primarily targeted stress associated

with irradiation, but oxidative stress may be a facet of many steps in the rearing, sorting, and shipping of live biological control agents, and insect quality is still a challenge in programs using alternative sterilization strategies such as genetic sterilization [39, 48]. Our experiments demonstrate the potential of using transgenic approaches to enhance male quality and drive down the costs associated with SIT. Clearly, additional work is needed to further increase the positive performance benefits of antioxidant overexpression beyond current modest levels of improvement we have shown here to make a substantial impact on performance of biological control agents in the field. However, our current proof of concept suggests that with further optimization of parameters like recombination site, transgene copy number, tissue specificity of expression, etc., such improvement of male sexual performance in sterile insect programs is indeed possible. We also provide a proof of concept that similar transgenic strategies to improve physiological performance could also benefit other insect release programs, such as augmentative biological control, managed pollinators, and even gene-drive systems.

## Data accessibility

Data are available from the Dryad Digital Repository: https://doi:10.5061/dryad.nk58h0m [61].


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## References

[1] Hill, G.E. 2013 The evolution of ornaments and armaments. In *Animal Behavior: Function and Evolution* (ed. K. Yasukawa), pp. 145-172. New York, Prager.

[2] Hill, G.E. 2011 Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol. Lett.* **14**, 625-634. (doi:10.1111/j.1461-0248.2011.01622.x).

[3] Schieber, M. & Chandel, N.S. 2014 ROS function in redox signaling and oxidative stress. *Curr. Biol.* **24**, R453-R462. (doi:10.1016/j.cub.2014.03.034).

[4] Apel, K. & Hirt, H. 2004 Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**, 373-399.

[5] Dowling, D.K. & Simmons, L.W. 2009 Reactive oxygen species as universal constraints in life-history evolution. *Proc. R. Soc. B-Biol. Sci.* **276**, 1737-1745. (doi:10.1098/rspb.2008.1791).

[6] Monaghan, P., Metcalfe, N.B. & Torres, R. 2009 Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75-92. (doi:10.1111/j.1461-0248.2008.01258.x).

[7] von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D. & Wittzell, H. 1999 Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. B-Biol. Sci.* **266**, 1-12.

446 [8] Zorov, D.B., Juhaszova, M. & Sollott, S.J. 2014 Mitochondrial reactive oxygen species
447 (ROS) and ROS-induced ROS release. *Physiol. Rev.* **94**, 909-950.
448 (doi:10.1152/physrev.00026.2013).

449
450 [9] Koch, R.E. & Hill, G.E. 2018 Behavioural mating displays depend on mitochondrial
451 function: a potential mechanism for linking behaviour to individual condition. *Biol. Rev.* **93**,
452 1387-1398. (doi:10.1111/brv.12400).

453
454 [10] Wolff, J.N., Ladoukakis, E.D., Enriquez, J.A. & Dowling, D.K. 2014 Mitonuclear
455 interactions: evolutionary consequences over multiple biological scales. *Philosophical*
456 *Transactions of the Royal Society B-Biological Sciences* **369**. (doi:10.1098/rstb.2013.0443).

457
458 [11] Espinosa-Diez, C., Miguel, V., Mennerich, D., Kietzmann, T., Sanchez-Perez, P., Cadenas,
459 S. & Lamas, S. 2015 Antioxidant responses and cellular adjustments to oxidative stress. *Redox*
460 *Biol.* **6**, 183-197. (doi:10.1016/j.redox.2015.07.008).

461
462 [12] Catoni, C., Peters, A. & Schaefer, H.M. 2008 Life history trade-offs are influenced by the
463 diversity, availability and interactions of dietary antioxidants. *Anim. Behav.* **76**, 1107-1119.
464 (doi:10.1016/j.anbehav.2008.05.027).

465
466 [13] Blount, J.D. 2004 Carotenolds and life-history evolution in animals. *Arch. Biochem.*
467 *Biophys.* **430**, 10-15. (doi:10.1016/j.abb.2004.03.039).

468
469 [14] Costantini, D. & Moller, A.P. 2008 Carotenoids are minor antioxidants for birds. *Funct.*
470 *Ecol.* **22**, 367-370. (doi:10.1111/j.1365-2435.2007.01366.x).

471
472 [15] Koch, R.E., Kavazis, A.N., Hasselquist, D., Hood, W.R., Zhang, Y.F., Toomey, M.B. &
473 Hill, G.E. 2018 No evidence that carotenoid pigments boost either immune or antioxidant
474 defenses in a songbird. *Nat. Commun.* **9**. (doi:10.1038/s41467-018-02974-x).

475
476 [16] Burk, T. 1983 Behavioral ecology of mating in the Caribbean fruit fly, *Anastrepha suspensa*
477 (Loew) (Diptera:Tephritidae). *Fla. Entomol.* **66**, 330-344. (doi:10.2307/3494128).

478
479 [17] Kaspi, R., Taylor, P.W. & Yuval, B. 2000 Diet and size influence sexual advertisement and
480 copulatory success of males in Mediterranean fruit fly leks. *Ecol. Entomol.* **25**, 279-284.
481 (doi:10.1046/j.1365-2311.2000.00266.x).

482
483 [18] Yuval, B., Kaspi, R., Shloush, S. & Warburg, M.S. 1998 Nutritional reserves regulate male
484 participation in Mediterranean fruit fly leks. *Ecol. Entomol.* **23**, 211-215. (doi:10.1046/j.1365-
485 2311.1998.00118.x).

[19] Handler, A.M. & Harrell, R.A. 2001 Transformation of the Caribbean fruit fly, *Anastrepha suspensa*, with a piggyBac vector marked with polyubiquitin-regulated GFP. *Insect Biochem. Mol. Biol.* **31**, 199-205. (doi:10.1016/s0965-1748(00)00119-3).

[20] Lopez-Martinez, G. & Hahn, D.A. 2012 Short-term anoxic conditioning hormesis boosts antioxidant defenses, lowers oxidative damage following irradiation and enhances male sexual performance in the Caribbean fruit fly, *Anastrepha suspensa*. *J. Exp. Biol.* **215**, 2150-2161. (doi:10.1242/jeb.065631).

[21] Klassen, W. & Curtis, C.F. 2005 History of the sterile insect technique. In *Sterile Insect Technique: Principles and Practice in Area-wide Integrated Pest Management* (eds. V.A. Dyck, J. Henrichs & A.S. Robinson), pp. 3-36. Dordrecht, Springer.

[22] Schetelig, M.F. & Handler, A.M. 2012 A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). *Insect Biochem. Mol. Biol.* **42**, 790-795. (doi:10.1016/j.ibmb.2012.07.007).

[23] Schetelig, M.F. & Handler, A.M. 2012 Strategy for enhanced transgenic strain development for embryonic conditional lethality in *Anastrepha suspensa*. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 9348-9353. (doi:10.1073/pnas.1203352109).

[24] Zimowska, G.J., Nirmala, X. & Handler, A.M. 2009 The beta 2-tubulin gene from three tephritid fruit fly species and use of its promoter for sperm marking. *Insect Biochem. Mol. Biol.* **39**, 508-515. (doi:10.1016/j.ibmb.2009.05.004).

[25] Lopez-Martinez, G., Meagher, R.L., Jeffers, L.A., Bailey, W.D. & Hahn, D.A. 2016 Low oxygen atmosphere enhances post-irradiation survival of *Trichoplusia ni* (Lepidoptera: Noctuidae). *Fla. Entomol.* **99**, 24-33.

[26] Lopez-Martinez, G., Carpenter, J.E., Hight, S.D. & Hahn, D.A. 2014 Low-oxygen atmospheric treatment improves the performance of irradiation-sterilized male cactus moths used in SIT. *J. Econ. Entomol.* **107**, 185-197. (doi:10.1603/ec13370).

[27] Calkins, C.O. & Parker, A.G. 2005 Sterile insect quality. In *Sterile Insect Technique: Principles and Practice in Area-wide Integrated Pest Management* (eds. V.A. Dyck, J. Henrichs & A.S. Robinson), pp. 269-296. Dordrecht, Springer.

[28] Gallardo-Ortiz, U., Perez-Staples, D., Liedo, P. & Toledo, J. 2018 Sexual Competitiveness, Field Survival, and Dispersal of Anastrepha obliqua (Diptera: Tephritidae) Fruit Flies Irradiated at Different Doses. *J. Econ. Entomol.* **111**, 761-769. (doi:10.1093/jee/tox326).

[29] Handler, A.M. & Schetelig, M.F. 2014 Tephritid fruit fly transgenesis and applications. In *Transgenic Insects: Techniques and Applications* (ed. M.Q. Benedict), pp. 117-137. Wallingford, UK, CAB International.

[30] Schetelig, M.F. & Wimmer, E.A. 2011 Insect Transgenesis and the Sterile Insect Technique. In *Insect Biotechnology* (ed. A. Vilcinskas), pp. 169-194. London, UK, Springer.

[31] Balaban, R.S., Nemoto, S. & Finkel, T. 2005 Mitochondria, oxidants, and aging. *Cell* **120**, 483-495. (doi:10.1016/j.cell.2005.02.001).

[32] Powers, S.K. & Jackson, M.J. 2008 Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. *Physiol. Rev.* **88**, 1243-1276. (doi:10.1152/physrev.00031.2007).

[33] Nirmala, X., Zimowska, G.J. & Handler, A.M. 2009 Characterization of the proteasome beta 2 subunit gene and its mutant allele in the tephritid fruit fly pest, *Anastrepha suspensa*. *Insect Mol. Biol.* **18**, 333-340. (doi:10.1111/j.1365-2583.2009.00875.x).

[34] Teets, N.M., Kawarasaki, Y., Lee, R.E. & Denlinger, D.L. 2013 Expression of genes involved in energy mobilization and osmoprotectant synthesis during thermal and dehydration stress in the Antarctic midge, *Belgica antarctica*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **183**, 189-201. (doi:10.1007/s00360-012-0707-2).

[35] Hermes-Lima, M. & Zenteno-Savin, T. 2002 Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **133**, 537-556.

[36] Gargano, J.W., Martin, I., Bhandari, P. & Grotewiel, M.S. 2005 Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in *Drosophila*. *Exp. Gerontol.* **40**, 386-395. (doi:10.1016/j.exger.2005.02.005).

[37] Fridovich, I. 1995 Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem.* **64**, 97-112.

[38] Finkel, T. & Holbrook, N.J. 2000 Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247. (doi:10.1038/35041687).

[39] Marrelli, M.T., Moreira, C.K., Kelly, D., Alphey, L. & Jacobs-Lorena, M. 2006 Mosquito transgenesis: what is the fitness cost? *Trends Parasitol.* **22**, 197-202. (doi:10.1016/j.pt.2006.03.004).

[40] Ray, P.D., Huang, B.W. & Tsuji, Y. 2012 Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **24**, 981-990. (doi:10.1016/j.cellsig.2012.01.008).

[41] Ford, D., Hoe, N., Landis, G.N., Tozer, K., Luu, A., Bhole, D., Badrinath, A. & Tower, J. 2007 Alteration of *Drosophila* life span using conditional, tissue-specific expression of transgenes triggered by doxycyline or RU486/Mifepristone. *Exp. Gerontol.* **42**, 483-497. (doi:10.1016/j.exger.2007.01.004).

[42] Torres, R. & Velando, A. 2007 Male reproductive senescence: the price of immune-induced oxidative damage on sexual attractiveness in the blue-footed booby. *J. Anim. Ecol.* **76**, 1161-1168. (doi:10.1111/j.1365-2656.2007.01282.x).

[43] Pike, T.W., Blount, J.D., Bjerkeng, B., Lindstrom, J. & Metcalfe, N.B. 2007 Carotenoids, oxidative stress and female mating preference for longer lived males. *Proc. R. Soc. B-Biol. Sci.* **274**, 1591-1596. (doi:10.1098/rspb.2007.0317).

[44] Costantini, D. 2008 Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol. Lett.* **11**, 1238-1251. (doi:10.1111/j.1461-0248.2008.01246.x).

[45] Sivinski, J., Aluja, M., Dodson, G.N., Freidberg, A., Headrick, D.H., Kaneshiro, K.Y. & Landolt, P. 2000 Topics in the evolution of sexual behavior in the Tephritidiae. In *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (eds. M. Aluja & A. Norrbom), pp. 751-792. Boca Raton, Florida, CRC Press.

[46] Powers, S.K., Kavazis, A.N. & McClung, J.M. 2007 Oxidative stress and disuse muscle atrophy. *J. Appl. Physiol.* **102**, 2389-2397. (doi:10.1152/japplphysiol.01202.2006).

[47] Calkins, C.O. & Ashley, T.R. 1989 The impact of poor quality of mass-reared Mediterranean fruit flies on the Sterile Insect Technique used for eradication. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie* **108**, 401-408.

[48] Bargielowski, I., Kaufmann, C., Alphey, L., Reiter, P. & Koella, J. 2012 Flight performance and teneral energy reserves of two genetically-modified and one wild-type strain of the yellow fever mosquito *Aedes aegypti*. *Vector Borne Zoonotic Dis.* **12**, 1053-1058. (doi:10.1089/vbz.2012.0994).

[49] Seto, N.O.L., Hayashi, S. & Tener, G.M. 1990 Overexpression of Cu-Zn superoxide dismutase in *Drosophila* does not affect life-span. *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4270-4274. (doi:10.1073/pnas.87.11.4270).

[50] Cabreiro, F., Ackerman, D., Doonan, R., Araiz, C., Back, P., Papp, D., Braeckman, B.P. & Gems, D. 2011 Increased life span from overexpression of superoxide dismutase in *Caenorhabditis elegans* is not caused by decreased oxidative damage. *Free Radic. Biol. Med.* **51**, 1575-1582. (doi:10.1016/j.freeradbiomed.2011.07.020).

[51] Van Raamsdonk, J.M. & Hekimi, S. 2009 Deletion of the mitochondrial superoxide sismutase sod-2 extends lifespan in *Caenorhabditis elegans*. *PLoS Genet.* **5**. (doi:10.1371/journal.pgen.1000361).

[52] Popp, J., Peto, K. & Nagy, J. 2013 Pesticide productivity and food security. A review. *Agron. Sustain. Dev.* **33**, 243-255. (doi:10.1007/s13593-012-0105-x).

[53] Enkerlin, W. & Mumford, J. 1997 Economic evaluation of three alternative methods for control of the Mediterranean fruit fly (Diptera: Tephritidae) in Israel, Palestinian territories, and Jordan. *J. Econ. Entomol.* **90**, 1066-1072. (doi:10.1093/jee/90.5.1066).

[54] Teets, N.M., Dias, V.S., Pierce, B., Schetelig, M.F., Handler, A.M. & Hahn, D.A. 2019 Data from: Overexpression of an antioxidant enzyme improves male mating performance after stress in a lek-mating fly. *Dryad Digital Repository*. (https//doi:10.5061/dryad.nk58h0m).

## Figure Legends

**Figure 1**. **Construct design and molecular characterization of transgenic lines.** (A) Design of transgenic construct for overexpressing MnSOD. (B) transcript abundance of MnSOD in wild type (WT) and the seven transgenic lines overexpressing MnSOD. The SOD1 line is an additional control line transformed with a vector that contains Cu/ZnSOD in lieu of MnSOD, to demonstrate that overexpression is specific to MnSOD. (C) Enzyme activity of MnSOD in wild-type and transgenic flies. (D) Correlation between enzyme activity and mRNA expression in wild-type and transgenic lines. In B-D, bars and symbols represent mean expression or activity while whisker bars represent standard error of the mean, N=4-5 pools of males for each genotype. In B-C, groups arranged from lowest to highest expression/activity, and different

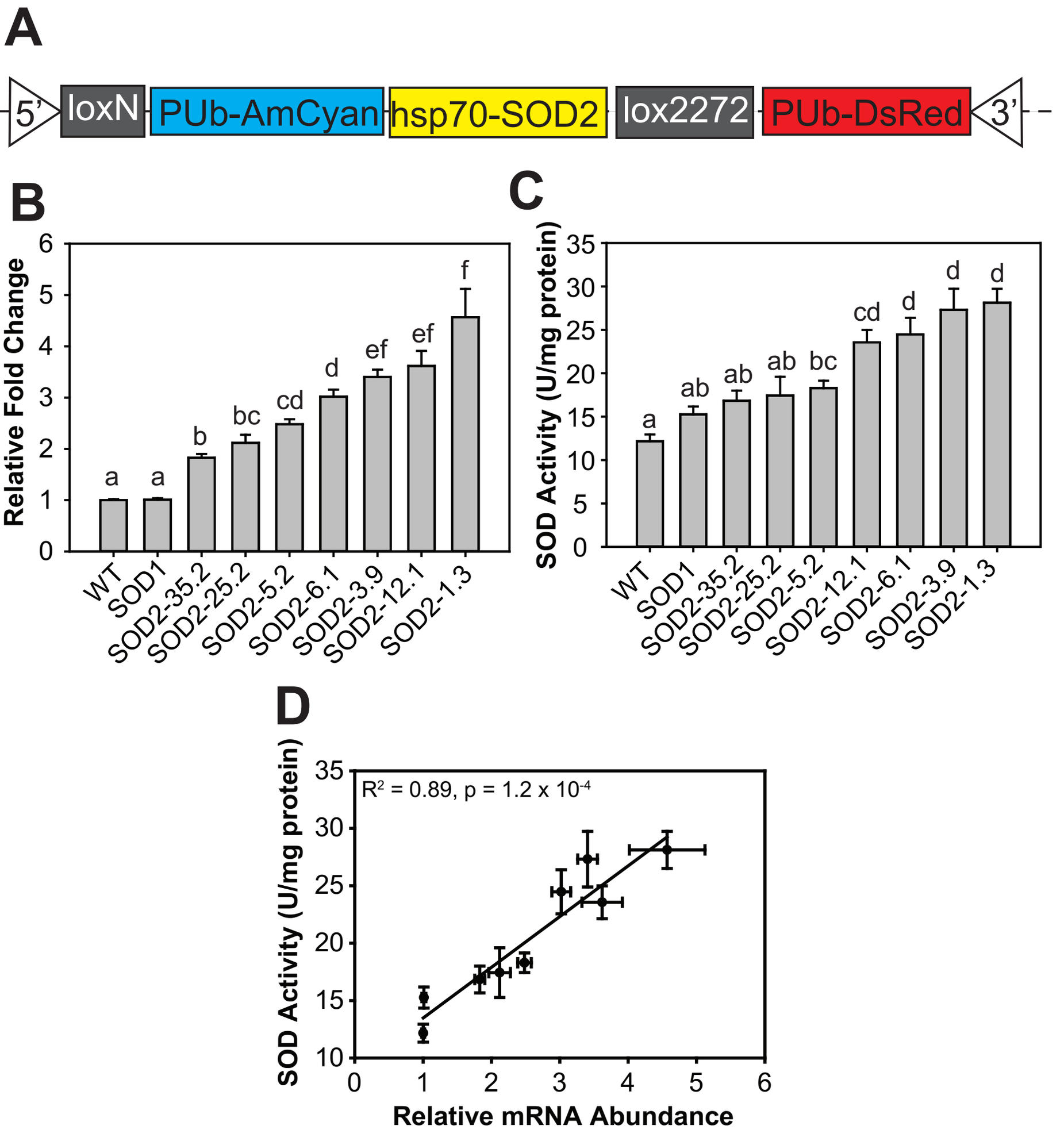
639 letters represent significant differences between groups following ANOVA and Tukey's HSD,
640 $p<0.05$.

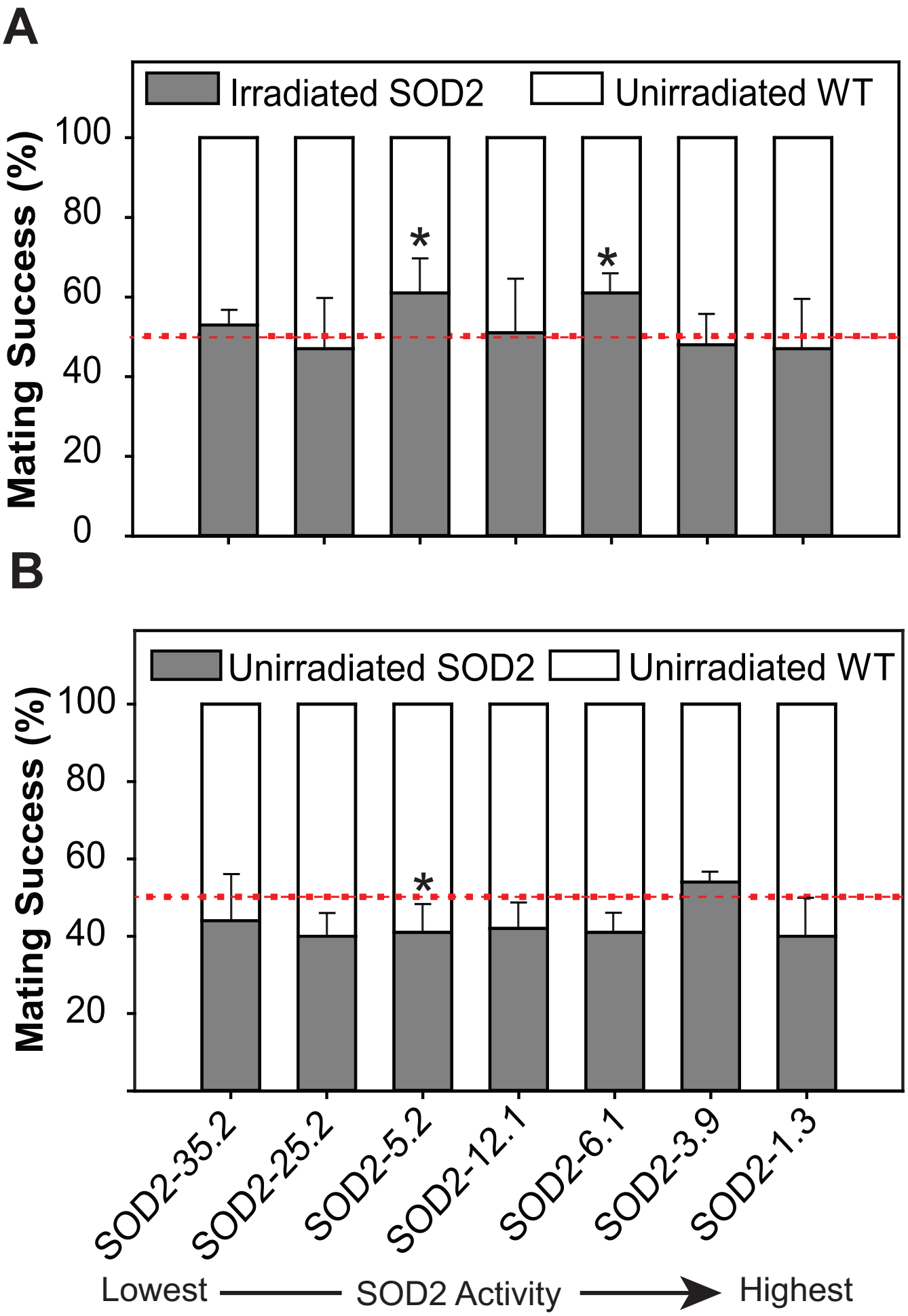
641 **Figure 2. MnSOD overexpression improves mating performance in irradiated flies.** Males
642 from the indicated genotypes were paired up with wild-type males and introduced to virgin wild-
643 type females. (A) Irradiated transgenic males vs. irradiated wild-type males. (B) Unirradiated
644 transgenic males vs. unirradiated wild-type males. In each panel, the grey portion of the stacked
645 bar indicates the mating success of transgenic males, while the white portion indicates the mating
646 success of wild type. Bars represent the mean mating probability of each group relative to the
647 wild-type male used in that particular experiment (i.e., untreated males in A, irradiated males in
648 B). Whisker bars represent SEM, N = 3 to 7 separate trials per comparison, with each trial
649 consisting of at least 5 mate choice assays. * indicates a significant difference from 50% mating
650 success.

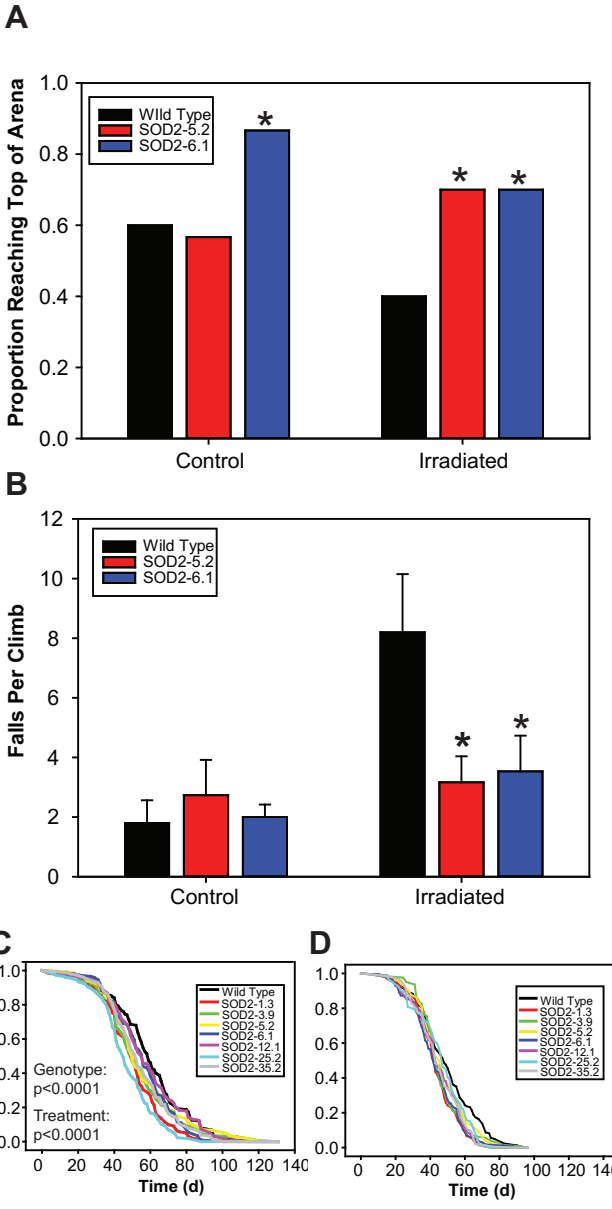
651 **Figure 3**. **MnSOD overexpression reduces oxidative damage following irradiation.** (A)
652 Irradiation significantly increases lipid peroxidation, as indicated by the TBARS assay. (B)
653 TBARS levels in irradiated flies from wild type and line SOD2-5.2. (C) TBARS levels in
654 irradiated flies from wild type and line SOD2-6.1. In all graphs, symbols represent mean and
655 whisker bars represent SEM, N=4 per group. The p-values indicate the p-values from a linear
656 contrast across all four time points between wild type and the indicated group, while an *
657 indicates a significant difference between a group and its respective control at a particular time
658 point.

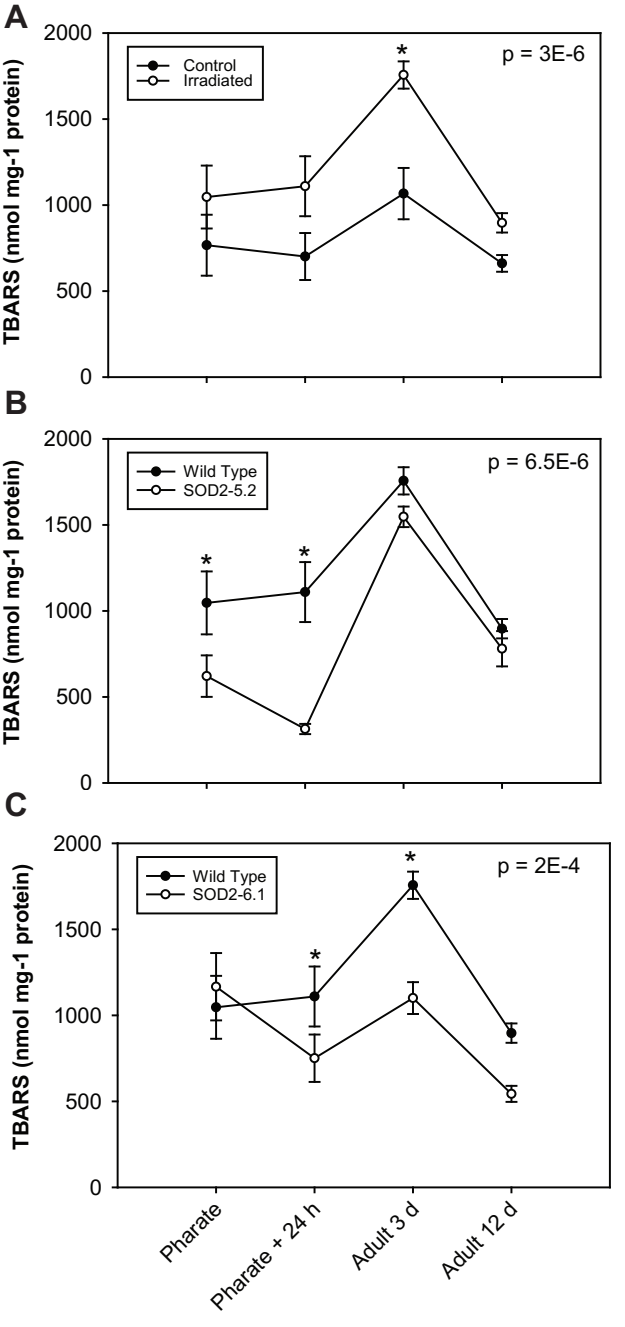
659 **Figure 4. MnSOD overexpression improves climbing performance of irradiated flies, but**
660 **fails to improve longevity.** In *A* and *B* groups of males were placed into a 33 cm long acrylic
661 tube and tapped to the bottom of the tube to start the trial. (A) The proportion of males reaching

the top of the tube. (B) The number of times a fly fell during each climbing trial. (C-D) Longevity curves for unirradiated (C) and irradiated (D) males from each genotype. In (B), bars represent the mean and whisker bars represent SEM. In A and B, an * indicates a significant different between transgenic and wild type, based on a Chi-Square test (A) or ANOVA with Tukey's HSD post-hoc comparisons (B). In C-D, the p-values indicate the effects of genotype and treatment on longevity from a Cox Regression analysis.


A
5'
loxN
PUb-AmCyan
hsp70-SOD2
lox2272
PUb-DsRed
3'
B
Relative Fold Change
0
1
2
3
4
5
6
a
a
b
bc
cd
d
ef
ef
f
WT
SOD1
SOD2-35.2
SOD2-25.2
SOD2-5.2
SOD2-6.1
SOD2-3.9
SOD2-12.1
SOD2-1.3
C
SOD Activity (U/mg protein)
0
5
10
15
20
25
30
35
a
ab
ab
ab
bc
cd
d
d
d
WT
SOD1
SOD2-35.2
SOD2-25.2
SOD2-5.2
SOD2-12.1
SOD2-6.1
SOD2-3.9
SOD2-1.3
D
SOD Activity (U/mg protein)
10
15
20
25
30
35
R2 = 0.89, p = 1.2 x 10-4
0
1
2
3
4
5
6
Relative mRNA Abundance


A
Irradiated SOD2
Unirradiated WT
Mating Success (%)
100
80
60
40
20
0
*
*
B
Unirradiated SOD2
Unirradiated WT
Mating Success (%)
100
80
60
40
20
*
SOD2-35.2
SOD2-25.2
SOD2-5.2
SOD2-12.1
SOD2-6.1
SOD2-3.9
SOD2-1.3
Lowest
SOD2 Activity
Highest


A
Proportion Reaching Top of Arena
1.0
0.8
0.6
0.4
0.2
0.0
WIld Type
SOD2-5.2
SOD2-6.1
*
*
*
Control
Irradiated
B
Falls Per Climb
12
10
8
6
4
2
0
Wild Type
SOD2-5.2
SOD2-6.1
*
*
Control
Irradiated
C
Proportion Survival
1.0
0.8
0.6
0.4
0.2
0.0
Wild Type
SOD2-1.3
SOD2-3.9
SOD2-5.2
SOD2-6.1
SOD2-12.1
SOD2-25.2
SOD2-35.2
Genotype:
p<0.0001
Treatment:
p<0.0001
0
20
40
60
80
100
120
140
Time (d)
D
1.0
0.8
0.6
0.4
0.2
0.0
Wild Type
SOD2-1.3
SOD2-3.9
SOD2-5.2
SOD2-6.1
SOD2-12.1
SOD2-25.2
SOD2-35.2
0
20
40
60
80
100
120
140
Time (d)


A
TBARS (nmol mg-1 protein)
2000
1500
1000
500
0
Control
Irradiated
p = 3E-6
*
B
TBARS (nmol mg-1 protein)
2000
1500
1000
500
0
Wild Type
SOD2-5.2
p = 6.5E-6
*
*
C
TBARS (nmol mg-1 protein)
2000
1500
1000
500
0
Wild Type
SOD2-6.1
p = 2E-4
*
*
Pharate
Pharate + 24 h
Adult 3 d
Adult 12 d