University of South Bohemia

Faculty of Science

Effect of low doses of herbicide paraquat on antioxidant defense in *Drosophila*

RNDr. Thesis

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České Budějovice

2016

Krůček T., Korandová M., Szakosová K., Šerý M., Frydrychová R. (2015) Effect of low doses of herbicide paraquat on antioxidant defense in Drosophila. *Archives of Insect Biochemistry and Physiology* **88**: 235-248. DOI: 10.1002/arch.21222

IF 1,021

Annotation:

Disruption of cell equilibrium between production of free radicals and antioxidant defence is named oxidative stress. The main component of antioxidant mechanism is activity of antioxidant enzymes, which include superoxide dismutase (SOD) and catalase. In this study we evaluated the effect of herbicide paraquat on response of SOD and catalase, their transcription level and locomotion activity in *Drosophila melanogaster*. We exposed the flies to a wide range of paraquat concentration. Our results revealed that transcript and enzymatic levels of both SOD and catalase have a similar biphasic dose response with the peaks at 2,5 μM paraquat concentration, resembling hormetic effect. Then, males were more sensitive than females. However, females had an increased locomotion activity. We found that paraquat susceptibility is increased in males and mating flies. Therefore, this study supports hypothesis of stress sensitivity elevation as a physiological cost of reproduction.

Declaration [in Czech]

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Krůček T., **Korandová M.**, Szakosová K., Šerý M., Frydrychová R. (2015) Effect of low doses of herbicide paraquat on antioxidant defense in Drosophila. *Archives of Insect Biochemistry and Physiology* **88**: 235-248. DOI: 10.1002/arch.21222

Prohlašuji, že Mgr. Michala Korandová se na výše uvedené publikaci podílela významným způsobem, a to především v průběhu experimentálního testování pohybové aktivity, statistickém vyhodnocení všech experimentů a rovněž při textovém zpracování práce.

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Article

EFFECT OF LOW DOSES OF HERBICIDE PARAQUAT ON ANTIOXIDANT DEFENSE IN *Drosophila*

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Despite a high toxicity, paraquat is one of the most widely used herbicides in the world. Our study evaluated the effect of paraquat exposure on antioxidant response and locomotion activity in Drosophila melanogaster. *We examined the enzymatic activity of superoxide dismutase (SOD) and catalase, and the transcript levels of both enzymes. Flies were exposed to a wide range of paraquat concentrations (0.25 μM to 25 mM) for 12 h. SOD, at both transcript and enzymatic levels, revealed a biphasic dose–response curve with the peak at 2.5 μM paraquat. A similar dose–response curve was observed at transcript levels of catalase. Males revealed higher susceptibility to paraquat exposure, displaying higher lethality, increased levels of SOD activity, and increased peroxide levels than in females. We found that the exposure of females to 2.5 μM paraquat leads to an increase in locomotion activity. Because susceptibility to paraquat was enhanced by mating, the study supports the hypothesis of elevation of stress sensitivity as a physiological cost of reproduction.* © *2015 Wiley Periodicals, Inc.*

Grant sponsor: Grant Agency of the Czech Republic; Grant number: 14-07172S; Grant sponsor: Grant Agency of the University of South Bohemia; Grant sponsor: Grant Agency of the University of South Bohemia; Grant number: 038/2014/P; Grant number: 052/2013/P; Grant sponsor: Grant Agency of the University of South Bohemia; Grant sponsor: European Union Seventh Framework Programme; Grant number: 316304.
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ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY, Vol. 88, No. 4, 235–248 (2015)
Published online in Wiley Online Library (wileyonlinelibrary.com).
 DOI: 10.1002/arch.21222

Keywords: *Drosophila*; oxidative stress; paraquat; mating; superoxide dismutase; catalase

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) is widely used as nonselective herbicide. Paraquat is a strong redox agent that yields paraquat monocation radicals, which then react with molecular oxygen to produce superoxide anions. Paraquat toxicity via oxidative stress mechanisms is also clearly indicated by counteracting its effect through treatment of exogenous superoxide dismutase (SOD), one of the key enzymes in diminishing oxidative stress, or by SOD mimetics (Mollace et al., 2003; Choi et al., 2006). SOD converts two superoxide anions into one molecule of oxygen and one molecule of hydrogen peroxide. Catalase, another enzyme of antioxidant defense, reacts with the hydrogen peroxide to catalyze the formation of water and oxygen (Chelikani et al., 2004; Miller, 2012). There are numerous other antioxidants, such as vitamin (A), enzyme cofactors (Q10), or nitrogen compounds (uric acid), that diminish oxidative stress (Carocho and Ferreira, 2013). In insects oxidative stress may be counteracted by adipokinetic hormone (AKH) (Večeřa et al., 2007; Velki et al., 2011; Bednářová et al., 2013). Although there is no specific treatment for paraquat poisoning, several studies suggest an antioxidant therapy as the alternative treatment against paraquat toxicity, such as administration of antioxidants quercetin, selenium, vitamin C, naringin, etc. (reviewed in Blanco-Ayala et al., 2014).

For research purposes, paraquat is commonly used to generate oxidative stress and works as a model neurotoxicant (Fukushima et al., 2002; Cochemé and Murphy, 2008). Epidemiological studies have indicated paraquat exposure as a risk factor in Parkinson's disease (Berry et al., 2010), and when administrated to experimental organisms, it induces cardinal symptoms of parkinsonism (Chaudhuri et al., 2007; Fernagut et al., 2007; Jiao et al., 2012). For studying of molecular mechanisms of neurodegenerative diseases and oxidative stress associated neurodegeneration, the important model system is *Drosophila* (Whitworth, 2011; Jaiswal et al., 2012).

As a potent oxidative stress inducer, paraquat has been frequently used in various studies. Commonly, studies are performed with high doses of paraquat, in *Drosophila* usually between 10 and 25 mM. Flies treated with such paraquat doses revealed an antioxidant response (Rzezniczak et al., 2011; Hosamani, 2013) and also locomotor and behavior dysfunctions (Chaudhuri et al., 2007; Inamdar et al., 2012). However, little information is known about how flies respond to low and more tolerable paraquat concentrations. Therefore, the main idea of this study was to determine a range of effective concentrations of paraquat that are able to provoke a measurable antioxidant reaction and locomotion impairment after an acute, single exposure.

MATERIALS AND METHODS

Fly Culture Conditions

Drosophila melanogaster stock of the wild-type Oregon R strain (provided by Bloomington Drosophila stock center) was reared under 12 h light:12 h dark cycles at 25°C on cornmeal-molasses medium with dry yeast added to the surface.

Paraquat Exposure

Before the paraquat treatment, adult flies were collected within 2 h after eclosion, sexes were separated, and kept separately before the treatment. Three days later, the flies were transferred for 12 h into vials containing 2.5 cm Whatman Paper soaked with 400 μl of 1% (w/v) sucrose containing paraquat (1,1-dimethyl-4,4-bipyridinium dichloride hydrate, Sigma-Aldrich, St. Louis, MO) of various concentrations (0.25 μM, 2.5 μM, 25 μM, 250 μM, 2.5mM, and 25 mM) or 400 μl of 1% (w/v) sucrose as a control. A total of 20 flies were placed in each vial. Paraquat-treated samples were always tested concurrently with the control.

To determine the median lethal dose (LD_{50}), flies were treated with various concentrations of paraquat (ranged from 5 to 100 mM) for 24 h, and then the number of dead flies was recorded. The data were subjected to Probit analysis.

Preparation of Protein Extracts

Extracts were prepared by homogenizing 20 flies in 400 μl of ice-cold 50 mM potassium phosphate buffer, pH 7.8, and centrifuged for 10 min at 10,000 × *g* at 4°C. The supernatant was collected and recentrifuged for 10 min, at 20,000 × *g* at 4°C. Aliquots of the final supernatant were frozen in liquid nitrogen and stored at –80 °C before further use.

Enzymatic Activity Assays

Total SOD activity was determined using a commercially available kit (Sigma–Aldrich, St. Louis, MO). Briefly, the hypoxanthine and xanthine oxidase generated superoxide radicals that were detected through reaction with a tetrazolium salt resulting in formation of formazan dye that absorbs at 440 nm. The absorbance was measured using SpectraMax 360. The presence of SOD resulted in a decrease in formazan formation. One unit of SOD was defined as the amount of SOD needed to clear 50% of the superoxide.

Catalase was measured using Amplex® Red Catalase Assay Kit (Invitrogen, Eugene, OR). In the assay, catalase reacted with H_2O_2 to produce oxygen and water. Unreacted H_2O_2 reacted with Amplex Red reagent in the presence of horseradish peroxidase to produce a highly fluorescent oxidation product, resorufin. The absorbance was taken at 571 nm. The amount of catalase units was calculated using catalase standards. The specific activities of SOD and catalase were reported in units per microgram of soluble proteins. The protein content was determined by the bicinchoninic acid assay using BCA Protein Macro Assay Kit (Serva, Heidelberg, Germany).

Determination of Aqueous Peroxides

Aqueous peroxides were measured using Peroxi Detect kit (Sigma–Aldrich). The assay was based on the fact that peroxides oxidize Fe^{2+} to Fe^{3+} ions that form a colored adduct with xylenol orange. The reaction was enhanced by addition of sorbitol to the reaction mixture. The colored product was observed at 560 nm. The standard curve was prepared by performing reactions with serial dilutions of 100 μM H_2O_2 solution, and nanomoles of peroxide were calculated in the test samples.

Table 1. PCR Primers Used in This Study

Primer	*Sequence (5′-3′)*
Catalase (forward)	5′- AAGCAAAATGGCTGGACGCG-3′
Catalase (reverse)	5′- CAGGATAGGTCCTCGCGGA-3′
SOD (forward)	5′- TCGAAATGGTGGTTAAAGCTG-3′
SOD (reverse)	5′- AACTCGTGCACGTGGAATCC-3′
AKH (reverse)	5′- CTCAGAATGAATCCCAAGAGC-3′
AKH (forward)	5′- CCCTGCTGTGTCTCGAAAAA-3′
RpL32 (forward)	5′- GGACAGTATCTGATGCCCAAC-3′
RpL32 (reverse)	5′- ATCTCGCCGCAGTAAACGC-3′
Actin 42A (forward)	5′-AAGAGGTTGCAGCTTTAGTGG-3′
Actin 42A (reverse)	5′- GCCGACATAAGAGTCCTTTTG-3′

RNA Isolation and cDNA Synthesis

Total RNA was prepared using a Nucleospin RNA II kit including DNase I treatment (Macherey-Nagel, cat. no. 740955.50). cDNA synthesis was performed using 1 μg total RNA primed with oligo(dT), and Superscript II reverse transcriptase (Invitrogen).

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed using a Light Cycler CFX96 Bio-Rad real-time PCR system with SYBR Premix Ex Taq^{TM} II (Takara). Threshold cycle values (Ct) were normalized against *RpL32* or *Actin 42A* as internal controls (Ponton et al., 2011), and ΔΔCt method with correction for amplification efficiency (Pfaffl, 2001) was used to calculate levels of target transcripts. Quantification was performed in five independent samples for each strain set up in triplicate. Sequences of primers are shown in Table 1.

Negative Geotaxis Test

The negative geotaxis test was performed as described previously (Kiss et al., 2013) with some modifications. Briefly, 10 males or 10 females were placed into vertically oriented glass tubes (160 mm long with 14 mm internal diameter). After a 10-min recovery period, the flies were shaken down to the bottom of the tubes. After 10 sec, we recorded numbers of flies able to climb near the top of the column (10–14 cm), numbers of flies able to climb 2–10 cm, and numbers of flies remaining at the bottom or near the bottom (0–2 cm) of the column. The tests were repeated three times at 1-min intervals. Five independent replications were done for each data set.

Repetitive Startle-Induced Hyperactivity (ReSH)

The ReSH test was used as described previously (Lebestky et al., 2009; Kiss et al., 2013). Briefly, the experimental apparatus contained eight glass tubes (160 mm long with 14 mm internal diameter), each connected to a pressurized air container. The system was placed under a Web-Cam Pro 9000 video camera (Logitech, Lausanne, Switzerland). Into each tube, we placed 10 males or 10 females and allowed them to calm down for 10 min. Then the flies were pushed to the rear end of the tubes by two sudden emissions of air released in 1-sec intervals. The video recording started 1 min before the first air emission and lasted 4 min afterwards with a speed of 15 frames/sec. The experiment

was repeated after 10 min, with each group of flies tested in three cycles. The test was performed in three independent experiments. Based on the video records, the average velocity (mm/sec) of the flies was calculated by the Drosana custom computer program in 0.1- to 0.2-sec intervals and used to construct curves representing the changing mean velocity of movement in time.

Statistical Analysis

Statistical analyses were done by two-sample paired *t*-test, one-way ANOVA, or two-way ANOVA using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA); the type of the test used in individual analysis is determined in the legends of Figures 1–7. The results in the graphs represent the mean of measurement ± SE. Details of analyses are described in the legends of Figures 1–7.

RESULTS

The LD_{50} of Paraquat and Mortality Rate

We determined the median lethal dose (LD_{50}) of paraquat in mated and virgin flies. In virgin individuals, the LD_{50} of paraquat was estimated to be 25 mM for males and 40 mM for females. In mated individuals, LD_{50} of paraquat was 6 mM for males and 10 mM for females. The mortality rate was tested at 25 mM paraquat at 24, 48, and 72 h. The mortality increased in a dose- and time-dependent manner (Fig. 1). The lethal effect of paraquat was increased in males compared to females ($^{***}P < 0.0001$) and also, mortality was enhanced by mating ($^{***}P < 0.0001$). A range of paraquat concentrations that we used for further experiments varied from 0.25 μM to 25 mM.

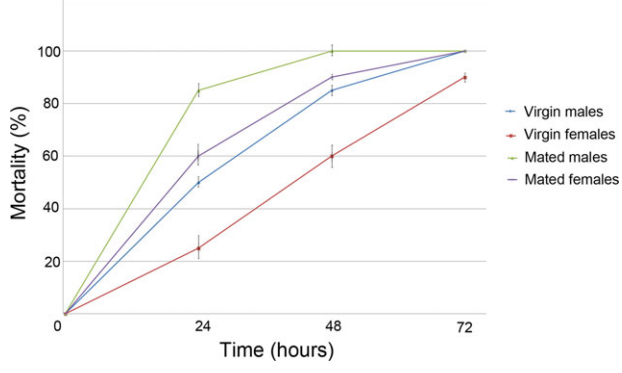

Figure 1. The lethal effect of 25 mM paraquat. The mortality of virgin and mated flies was measured at paraquat concentration of 25 mM. Data were analyzed by two-way ANOVA. The lethal effect of paraquat was increased in males compared to females ($P < 0.0001$) and also, mortality was enhanced by mating ($P < 0.0001$). Data are representative of five independent experiments.

Transcript Levels and Enzymatic Activities of SOD and Catalase

We have analyzed transcript levels of SOD and catalase in virgin males and females. In our pilot experiments, we used two internal controls (*RpL32* and *actin 42A*) to normalize

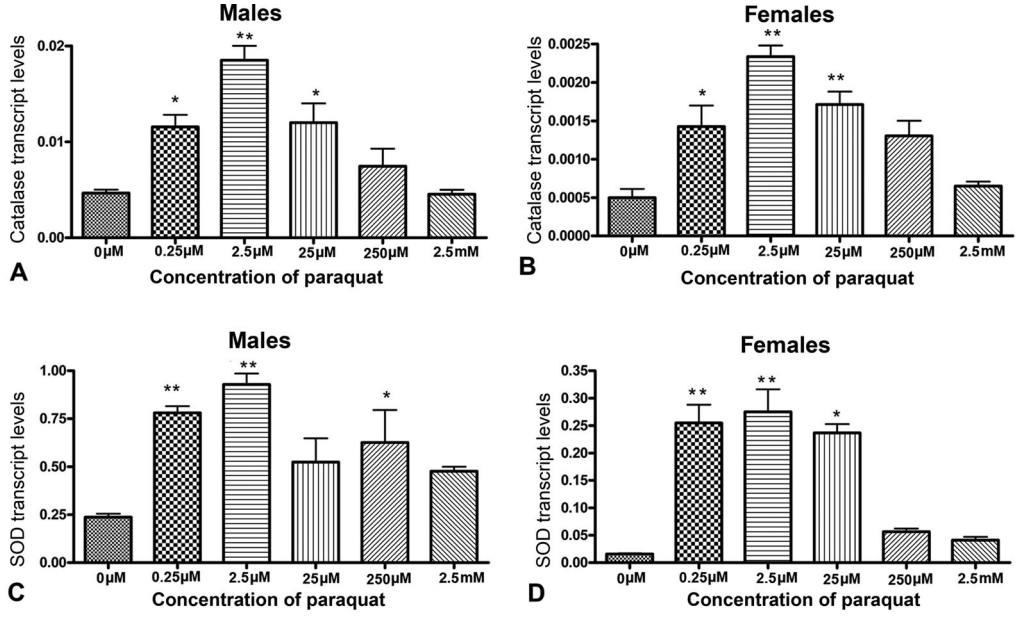

Figure 2. Effect of paraquat at different concentrations on transcript levels of SOD and catalase in virgin flies. The levels of catalase transcript (A, B) and SOD transcript (C, D) were measured relative to *RpL 32* transcript. Data are representative of five independent experiments. Transcript levels in correlation with paraquat concentration were statistically analyzed by one-way ANOVA ($P < 0.001$) followed by post hoc Dunnet's test. Significance was determined by making comparisons between control (no paraquat treatment) and paraquat-fed flies ($^{*}P < 0.05$, $^{**}P < 0.01$).

transcript levels. As no statistical differences were found between the data resulting from normalization against both internal controls (data not shown), in further experiments normalization was performed using *RpL32* only. In both controls and paraquat-treated flies, the transcript levels were substantially higher in males compared to females (Fig. 2). The difference between males and females was 10-fold ($P < 0.001$) and >2-fold ($P < 0.01$) at catalase and SOD, respectively. Transcript levels correlated with paraquat concentrations ($P < 0.05$). In both males and females, peaks of the transcript levels were seen at 2.5 µM paraquat.

Then, we asked whether the changes observed in transcript levels are followed by the same or similar trend in the enzymatic activity. Surprisingly, no substantial differences between paraquat-treated flies and controls were found in the activity of catalase (data not shown). For SOD enzymatic activity, however, the exposure to increasing doses of paraquat resulted in the similar pattern as was observed on transcript levels, that is, we observed the peak of activity at 2.5 µM paraquat (Fig. 3). SOD enzymatic activity correlated with paraquat concentrations ($P < 0.0001$). Also, females displayed higher activities of the enzyme compared to males ($P < 0.001$).

After exposure to 0.25 µM, 2.5 µM, and 25 mM paraquat, we measured transcript levels of AKH, but no differences compared to control flies were observed (data not shown).

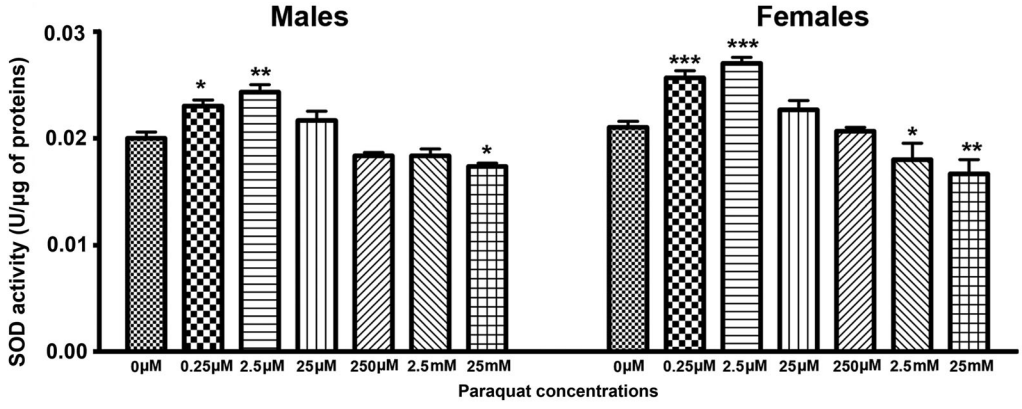

Figure 3. Effect of paraquat at different concentrations on SOD activity in virgin flies. Data were analyzed by two-way ANOVA followed by a Bonferroni posttest. Significance was determined by making comparisons between males and females (Df = 1, $P < 0.001$), in dependence on paraquat concentration (Df = 6, $P < 0.0001$), and by making comparisons between controls (no paraquat treatment) and paraquat-fed flies ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Data are representative of five independent experiments.

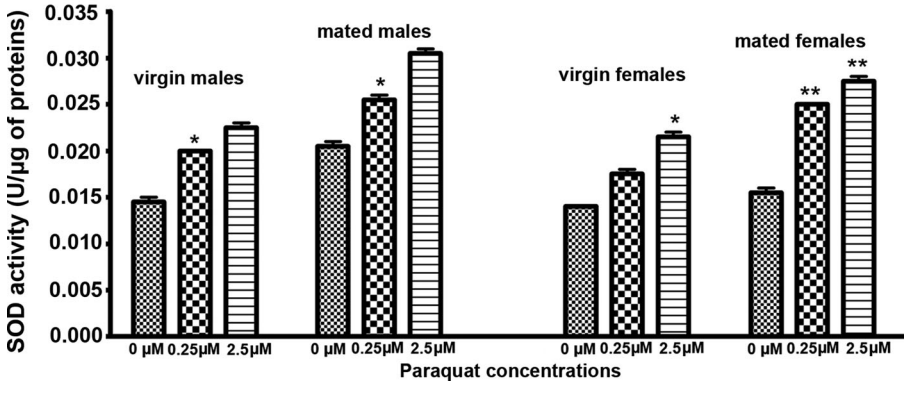

Figure 4. Comparison of paraquat on SOD activity in mated and virgin individuals. Data are representative of five independent experiments. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni's test. Significance was determined by making comparisons between mated and virgin individuals (Df = 3, $P < 0.0001$), in dependence on paraquat concentration (Df = 2, $P < 0.001$), and by making comparisons between controls (no paraquat treatment) versus paraquat-fed flies ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

The SOD Activity Increased With Mating

We speculated that the rate of oxidative stress caused by paraquat may be affected by various physiological conditions, and one of these conditions could be mating. Therefore, shortly after eclosion, we separated males from females. Three days later, we put the males and females together or separately on filter paper soaked with paraquat. The concentrations of paraquat were 0, 0.25, and 2.5 µM. In all the tested groups, SOD activity showed a correlation with paraquat concentration ($P < 0.001$). Both males and females displayed an elevated SOD activity when they got mated ($P < 0.0001$) (Fig. 4).

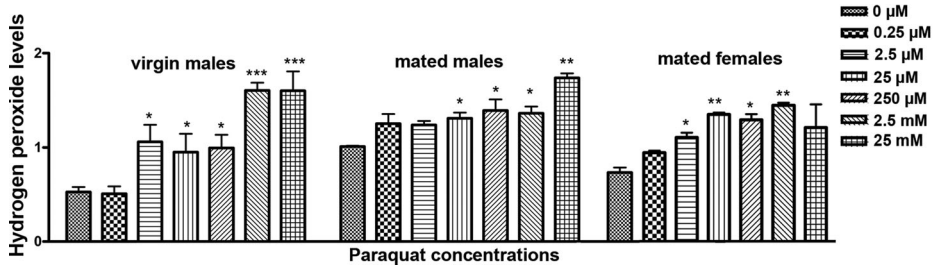

Figure 5. Effect of paraquat treatment on levels of hydrogen peroxide (nmoles/µg of proteins). Data are representative of five independent experiments. We used two-way ANOVA followed by Bonferroni's test. Significance was determined by making comparisons between virgin and mated males (Df = 1, $P < 0.001$), between groups treated with different paraquat concentrations (Df = 6, $P < 0.0001$), and in dependence on paraquat concentration (Df = 6, $P < 0.0001$). Also, significant differences were found by making comparisons between controls (no paraquat treatment) and paraquat-fed flies ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Hydrogen Peroxide Levels

The production of reactive oxygen species (ROS) induced in paraquat-treated flies and in controls was assessed by measurement of hydrogen peroxide levels. The test was performed in virgin and mated flies. However, in virgin females levels of hydrogen peroxides significantly varied between different experiments, and therefore the data were not included into statistical analysis (data not shown). In males and mated females, hydrogen peroxide level showed a gradual increase in a dose-dependent manner ($P < 0.0001$). With exception of flies exposed to 2.5 mM paraquat, the mated males revealed higher levels of hydrogen peroxide than their virgin counterparts ($P < 0.001$) (Fig. 5).

Effect of Paraquat on Locomotor Activity

To test an effect of paraquat treatment on locomotion activity, we performed negative geotaxis assay. In control groups of virgin individuals, 75% of males and 85% of females reached the top of a column in 10 sec. In control groups of mated individuals, the top of column was reached by 92% of males and 85% of females. Compared to the controls, no statistically significant differences were observed in flies treated with 0.025, 0.25, or 2.5 µM paraquat (data not shown), whereas flies exposed to 25 mM paraquat exhibited a decrease in climbing ability ($P < 0.01$). In paraquat-treated groups of virgin individuals, 50% of males and 45% of females reached the top of column in 10 sec. In paraquat-treated groups of mated individuals, the top of column was reached by 62% of males and 65% of females (Fig. 6).

Flies exposed to 2.5 µM paraquat were subjected to the ReSH test. Movement of paraquat-treated males did not reveal significant differences compared to their controls (not shown). In contrast, results showed that paraquat of this concentration influences the locomotion activity of females. Compared to the controls, paraquat-exposed females (both virgin and mated) had a higher activity before and after the air puffs ($P < 0.05$). Also, the puff-induced activity was higher in the paraquat-treated flies than in the control flies (Fig. 7).

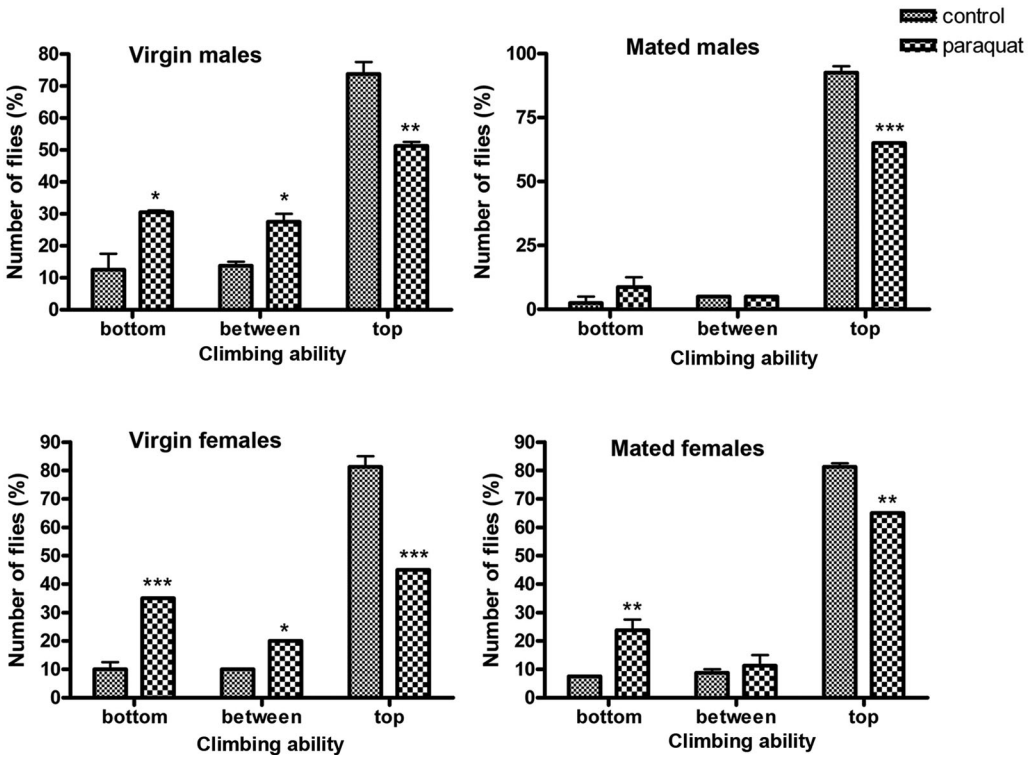

Figure 6. Negative geotaxis assay of flies treated with 25 mM paraquat. The effect of paraquat on mobility of males and females was determined by the percentage of flies able to climb to the top of the column (10–14 cm), of flies able to climb 2–10 cm, and the number of flies remaining at the bottom or near the bottom (0–2 cm) of the column. Locomotor activity in correlation with paraquat treatment was statistically analyzed by one-way ANOVA ($P < 0.01$) followed by post hoc Dunnet's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ are significant differences between control (no paraquat treatment) and paraquat-fed flies. Data are representative of five independent experiments.

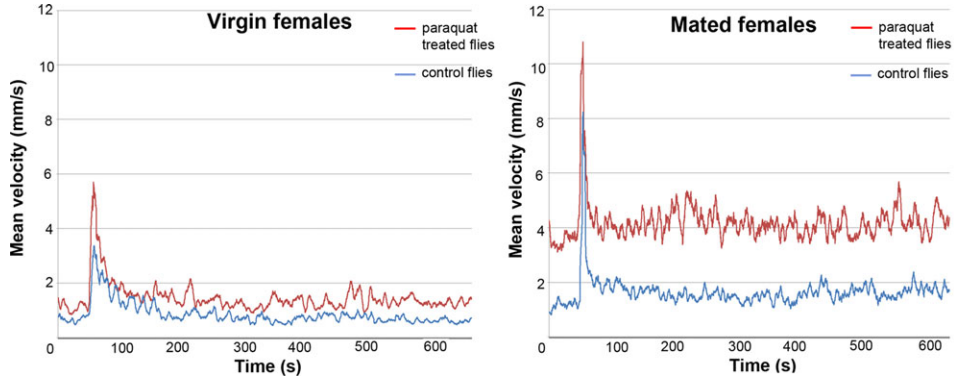

Figure 7. Paraquat treatment (2.5 μM) stimulates locomotor activity in females. The curves represent the changes in time of the mean velocity of movement before and after the air puffs in virgin and mated females. The peaks in the curves present the velocity of the movement during the puffs. The curves in red represent the activity of paraquat- treated flies; the curves in blue show the activity of control flies. Statistical analysis was performed by two-sample paired *t*-test using velocity data at 330 sec after the second air puff ($P < 0.05$). Data are representative of three independent experiments.

DISCUSSION

The mechanism of toxic action of paraquat involves cyclic reduction–oxidation reactions. The paraquat dication accepts an electron from a reductant to generate the paraquat monocation radical, which then reacts with O_2 resulting in superoxide production. Finally, the paraquat dication is regenerated and able to catalyze further superoxide production (Hassan et al., 1978; Hassan, 1984; Fukushima et al., 2002). Paraquat exposure is connected to various physiological and biochemical effects. Numerous studies have used paraquat as oxidative stress inducer to model cell and organismal aging (Cui et al., 2012; Wiemer and Osiewacz, 2014), activity of antioxidant defense (Rzezniczak et al., 2011), or Parkinson's disease (Lee et al., 2008; Varçin et al., 2012). The in vivo effect of paraquat exposure was particularly assessed in *Drosophila* (Zou et al., 2000; Landis et al., 2004; Haddadi et al., 2014). Most of these studies have been performed using shock experiments with high paraquat concentrations (usually around 20 mM). In spite of frequent use of paraquat, a physiological/genetic response to lower, more tolerable concentrations of paraquat has been poorly described. Therefore, we exposed flies to the wide range of paraquat concentrations.

Our study revealed that males had higher susceptibility to paraquat exposure, displaying a higher lethality, increased levels of SOD, and increased peroxide levels than females did. Also, a lower resistance to oxidative stress was observed when flies were mated. After application of a wide range of paraquat concentrations (0.25 μM to 25 mM), we recorded a biphasic curve rather than linear dose–response relationship between the paraquat treatment and level of antioxidant reaction. We found that activity of SOD and its transcript level were enhanced by low paraquat concentrations (0.25–2.5 μM), but further increase in paraquat dose (≥2.5 μM) caused a decrease in levels of both markers, presumably because antioxidant defense was impaired by the high concentrations of active oxygen species. Such biphasic dose–response of SOD activity has been found in various organisms when treated by wide range of paraquat concentrations (Casano et al., 1999; Abrashev et al., 2011; Zhang et al., 2013), and it seems to be consistent with a concept of a hormetic effect. Transcript levels of both catalase and SOD were substantially higher in males than those observed in females. This observation may reflect sexually dimorphic transcriptom. Highly significant differences in transcript abundance between males and females were reported previously (Jin et al., 2001; Ranz et al., 2003; Harbison et al., 2005). For instance, Harbison et al. (2005) found differences in transcript abundance for nearly half of *Drosophila* genome, the differences were not confined to stereotypical sex-specific biological processes.

Some previous studies reported an increase in SOD activity in *Drosophila* after exposure to high paraquat concentrations (around 20 mM) (Rzezniczak et al., 2011; Hosamani, 2013). On the other hand, our data seem to be consistent with a report by Mehdi and Qamar (2013) in which a decrease in SOD was observed in flies treated with 50–500 μM paraquat. We can hypothesize that the different response of antioxidant enzymes might be caused by different susceptibility of flies to oxidative stress based on different genetic background or different physiological conditions that are caused by slight differences in *Drosophila* rearing or differences in experimental designs.

Considering the biphasic curves of antioxidant activities, we speculated whether the low paraquat concentrations may have a somewhat boosting effect on antioxidant defense, manifested in diminishing of overall ROS production. Usually, if oxidative stress is evoked for research purposes, high doses of ROS-generating agents are used, and ROS production is increased in the dose-dependent manner (Cristovao et al., 2009; Hosamani, 2013). We,

therefore, asked if the same dose-dependent trend in ROS production could be seen even in the low paraquat concentrations despite the observed increase in antioxidant activity. Indeed, the levels of hydrogen peroxide we found revealed more or less linear increase in a dose-dependent manner.

Previous studies revealed a role of AKH in counteracting oxidative stress in insects (Večeřa et al., 2007; Velki et al., 2011; Bednářová et al., 2013). After exposure to paraquat, we measured transcript levels of AKH, but no differences compared to control flies were observed. However, the lack of response might be consistent with a fact that AKH is stored in a large supply in *corpora cardiaca* and there is no acute need for new AKH synthesis.

The rate of oxidative stress may be affected by various physiological conditions, such as the conditions associated with sex differences or mating. Although sex-specific differences in response to oxidative stress in *Drosophila* have not been fully explored yet, there are studies indicating that *Drosophila* males show higher susceptibility to paraquat treatment than females do (Chaudhuri et al., 2007; Minois et al., 2012), as well as males display a higher catalase activity (Ballard et al., 2007; Lushchak et al., 2011). These findings are consistent with the data presented here. It is well known that mating induces complex physiological changes. The mating-induced increase in oxidative stress sensitivity we observed is consistent with a previous reports in which resistance to oxidative stress was lower in mated females compared to virgin ones when exposed to 30% hydrogen peroxide or hyperoxia (Rush et al., 2007) or *Drosophila* mating resulted in a higher ROS production (Ballard et al., 2007). It is widely accepted that physical activity increases oxidative stress (Di Meo and Venditti, 2001; Radak et al., 2013), as well as it increases oxygen consumption, which exacerbates paraquat poisoning. Elevation of oxidative markers we observed in the mated flies might be simply a result of their higher physical activity connected with the mating that leads to a higher metabolic rate and higher oxygen flux. The higher susceptibility of mated flies to paraquat treatment seems to be consistent with a phenomenon known as a cost of reproduction (Reznick, 1985). The cost of reproduction, an increase in mortality or reduced fertility if reproductive rate is increased, has been demonstrated in a wide range of animals (Harshman and Zera, 2007; Speakman, 2008; Blazkova et al., 2011). Although the cost of reproduction is considered one of the most fundamental life-history trade-offs, the underlying physiological mechanisms are still poorly understood. Despite some ambiguities, however, there are numerous studies supporting the idea that oxidative stress is the important contributor to the cost of reproduction (Dowling and Simmons, 2009; Speakman and Garratt, 2014), and is supported by the presented study.

ACKNOWLEDGMENTS

We are grateful to James Mason and Dalibor Kodrík for critical reviews of the manuscript. This work was supported by the grant no. 14-07172S from the Grant Agency of the Czech Republic, by the grant no. 052/2013/P and the grant no. 038/2014/P from the Grant Agency of the University of South Bohemia, and by additional grants from the Grant Agency of the University of South Bohemia. We acknowledge the use of research infrastructure that has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 316304.

Authors confirm that there are no conflicts of interest. The authors declare that all experiments performed in this study comply with the current laws of the Czech Republic. All institutional and national guidelines for the care and use of laboratory animals were followed.

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