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RESEARCH ARTICLE

Assessment of oxidative stress and activities of antioxidant enzymes depicts the negative systemic effect of iron-containing fertilizers and plant phenolic compounds in the desert locust

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Abstract For herbivore insects, digesting can be somewhat challenging, as the defense mechanisms evolved by plants, including the release of phenolics like the non-protein amino acid L-3,4-dihydroxyphenylalanine (L-DOPA), can cause fitness costs. In addition, industrial and agricultural activities have elevated the amounts of iron that can be found in nature and more particularly $FeSO_4$ that is used as fertilizer. Traces of iron can enhance the auto-oxidation of L-DOPA, in turn, generating reactive oxygen species (ROS) and consequently oxidative stress in insects. We examined the effects of the ion Fe^{2+} (as $FeSO_4$) and L-DOPA on fifth instars of the desert locust *Schistocerca gregaria*. We measured the level of oxidative damage occurring to macromolecules (proteins and lipids) from midgut and thoracic tissues and assessed the activities of responsive antioxidant enzymes. Injected L-DOPA and redox-active metal iron generated ROS which caused oxidative damages to proteins and lipids to *S. gregaria*. The protein carbonyls and lipid peroxides present in tissue homogenates were elevated in treated insects. No synergism was observed when L-DOPA was co-injected with Fe^{2+}. K_m values of superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx) were 4.3, 2.6, and 4.0 mM in thoracic muscles and 5.00, 2.43, and 1.66 mM in whole midgut for SOD, GR, and GPx, respectively, and 8.3 and 3.43 M for catalase (CAT) in the two tissues, respectively. These results suggest higher affinities of GPx and CAT to H_2O_2 in midgut than in muscles. The time-course changes in activities of antioxidant enzymes and amounts of protein carbonyls and lipid peroxides showed fluctuating patterns, suggesting complex interactions among macromolecules, L-DOPA and $FeSO_4$, and their degradation products. Our results demonstrated the stressful effects of L-DOPA and $FeSO_4$, proving that iron-containing fertilizers are pollutants that can strongly affect *S. gregaria*.

Keywords Oxidative stress · Antioxidant enzymes · Insect · Protein carbonyls · Lipid peroxides · Thoracic muscles · Midgut

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Introduction

The life cycle of insect species is dictated by variations of biotic and abiotic conditions of their microhabitats. Because of their ectothermic nature, the effects of thermal environments on the fitness of insects have been investigated in several studies (Colinet et al. 2015; Deutsch et al. 2008). Nevertheless, several additional abiotic and biotic factors are conditional for the successful achievement of their life cycle, including access to water and nutritional resources (Chown and Gaston 2010; Chown and Terblanche 2006). The availability of trophic resources, both in qualitative and quantitative terms, represents a central point priming reproduction, and pilots the growth rate and conversion efficiency in several insect models. Food availability and reproductive effort are

highly intertwined (Stahlschmidt et al. 2013), suggesting that ad libitum nutritional resources should favor abundance of species (Wilder et al. 2011). However, in these conditions, several side effects can also arise, with herbivore animals being confronted, for instance, by plant defense mechanisms (see Mithöfer and Boland 2012). The release of phenolics, like L-3,4-dihydroxyphenylalanine (L-DOPA), has been shown to confer effective protection against herbivore attacks, for example, by increasing mortality levels in larvae of the southern armyworm *Spodoptera eridania* (Rehr et al. 1973), the black cutworm *Agrotis ipsilon* (Reese and Beck 1976), and the forest tent caterpillar *Malacosoma disstria* (Barbehenn and Martin 1994).

In a recent work, Zhang et al. (2016) demonstrated that pea aphids, *Acyrthosiphon pisum*, contain large amounts of sequestered L-DOPA when fed on broad beans (*Vicia faba* L.), which naturally contain large quantities of this compound. Results from these authors suggest that L-DOPA can cross biological barriers in some herbivores and attain digestive tract and digestive epithelium without significant alteration of its bioavailability. Meanwhile, even if naturally present in insects, L-DOPA can be harmful for many herbivorous pests. The metabolism and auto-oxidation of L-DOPA can induce cytotoxicity and apoptosis in insects and contribute to increasing the amount of reactive oxygen species (ROS), including the free radicals superoxide anion ($O_2^{\cdot-}$), hydroxyl (OH·), hydroperoxyl ($HO_2^{\cdot}$), and the non-radical hydrogen peroxide (H_2O_2) (Bolton et al. 2000; O'Brien 1991). These free radicals are generally unstable, highly reactive molecules (Gilbert 2000), and their metabolism can produce other ROS (Evans and Halliwell 2001; Halliwell and Whiteman 2004; Pardini 1995).

Anthropogenic-related perturbation of the habitats represents another constraint that insects must overcome (Raffa et al. 2008). Specifically, the increasing amount of pollutants associated with human activities has increased exposure to metal pollutants and xenobiotics in the form of pesticides, in turn, affecting biodiversity. Industrial and agricultural activities have elevated the amounts of iron that can be found in nature, with $FeSO_4$ being used, for instance, as iron fertilizer in order to increase its amounts in food crops (Aciksoz et al. 2011; Zuo and Zhang 2011). Even if this helps in combating human deficiency in iron, redox-active transition metals, particularly iron, are well-known inducers of oxidative stress in insects due to (i) their enhancing effects to redox-cycling reactions of *o*-diphenols and (ii) the formation of reactive iron-oxygen complexes which can oxidize macromolecules (Pattison et al. 2002; Schafer et al. 2000). Plant-feeding insects can incorporate these metal ions by different routes: direct, residual, or oral uptake through exposure to spray droplets, contaminated surfaces, or contaminated food, respectively.

Whatever their inducers, ROS production can be deleterious to body macromolecules, causing peroxidation of polyunsaturated fatty acids (Chaudière 1994; Wagner et al. 1994; Yu 1994), oxidative alterations of proteins, more particularly the thiol-dependent ones (Costa et al. 2007; Gutierrez-Correa and Stoppani 1997), and DNA oxidative damages (Birben et al. 2012; Kohen and Nyska 2002), in turn, affecting the overall biotic performance of organisms. Oxidative damages to macromolecules such as proteins, lipids, and DNA have been reported in a large range of insects (Barbehenn 2002; Buricova and Hodkova 2013; George and Gatehouse 2013; Zhang et al. 2014). To counteract these effects, ROS can be scavenged by non-enzymatic and enzymatic mechanisms. The latter involves four principal antioxidant enzymes, the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) (Kim et al. 2011; Krishnan and Kodrík, 2006; Landis and Tower 2005; Suh et al. 2010; Wang et al. 2001).

Widely cultivated legume beans like *V. faba*, seed sprouts, pods, and broad beans can contain significant amounts of iron and L-DOPA (Ramya and Thaakur 2007). In addition, velvet beans, which are commonly employed for silage and soil cover, can release for up to 450 kg ha^{-1} of L-DOPA into the soil (Soares et al. 2014). Insect herbivores can thus be exposed to high levels of plant phenolics, and in these circumstances, catalytic activity of prophenoloxidases may be exceeded, thus hampering the degradation of L-DOPA (Zhang et al. 2016). However, the mechanisms involved in the cytotoxic effect of L-DOPA and $FeSO_4$, and their interaction, in different tissues is not yet fully understood. In the present work, we were interested in determining if Fe^{2+} of $FeSO_4$ and L-DOPA generate a systemic response in insects when injected into the hemocoel of fifth instars of the desert locust *Schistocerca gregaria*. We used different experimental measurements to assess the system-wide amount of oxidative stress experienced by hoppers of *S. gregaria* exposed to these compounds, by focusing on two distinct tissues (thoracic muscles and midgut). Our specific hypotheses were the following: (i) injection of $FeSO_4$ or L-DOPA will elevate the amount of oxidative stress experienced by the organisms in all tissues, in turn, affecting macromolecules, (ii) an effective repair mechanism suggests that we should progressively be back to control levels after the insects were exposed to $FeSO_4$ or L-DOPA, (iii) the sensitivity to $FeSO_4$ or L-DOPA should be tissue specific, i.e., midgut tissues should be more able to cope with ROS production through elevating the levels of antioxidant enzymes as compared with muscle tissues, and (iv) similar defense mechanisms should be elicited in fifth instars of *S. gregaria* injected with $FeSO_4$ or L-DOPA, both stressors augmenting the level of generated oxidative stress.

Materials and methods

Insect-rearing conditions and tissue collection

Desert locusts *S. gregaria* (Forskål) were obtained from a laboratory colony established at the Entomology Department, Faculty of Science, Cairo University, Egypt. The insects were originally collected from Aswan, Egypt; the colony is periodically re-inoculated with wild specimens. Insects were reared into wooden cages (60 cm × 60 cm × 40 cm) at 30 ± 2 °C, 60 ± 5 % RH, and 16:8 h (light/darkness) photoperiod. Hoppers were fed ad libitum on fresh alfalfa, *Trifolium alexandrinum* (Fabaceae). A detailed description of the colony and rearing methodologies can be found in Hinks and Erlandson (1994).

For the experiments, fifth-instar hoppers (nymphs) aged of 6 days were starved for 24 h prior to being used for the experiments. This procedure assured gut lumen free from food remnants. Fifth-instar hoppers are commonly used for experimental studies in order to avoid transient physiological fluctuations that may be more variable in the adult stage. Then, fifth-instar hoppers were dissected in saline solution, and approximately 2.5 g of whole midgut and of thoracic muscles were isolated, homogenized, and used freshly prepared from a pool of five individuals ($N = 3$ replicates for each experiment).

Optimal stressor concentrations and time-course changes

A preliminary experiment was conducted to assess the effects of different concentrations of $FeSO_4$ and L-DOPA on fifth instars of *S. gregaria*. Three groups, each containing 15 individuals, were used: one as a naïve control, and two other groups injected with aqueous $FeSO_4$ or with L-DOPA. Individuals were injected with 15 μl of either of the stressors at various concentrations (see Table 1 for the concentrations). Injection was accomplished with the use of a Hamilton microsyringe (Hamilton Corp., NV, USA) into the dorsum between the second and third abdominal segments. Because protein carbonyl concentrations are considered as reliable indicators for measuring oxidative stress in all conditions (Levine et al. 1990), the stressor concentration that caused the highest amount of protein carbonyls was used for all experiments described below concerning oxidative damage of macromolecules, and also measurements of antioxidant enzyme activities.

Following the injection of $FeSO_4$ or L-DOPA, time-course changes of antioxidant enzyme activities were measured 1, 3, 6, 9, 18, 24, 48, and 72 h post-injection (PI). Further tests were conducted to assess the amount of oxidative damages (measured as the amount of protein carbonyls) when Fe^{2+} ($FeSO_4$) was replaced by Fe^{3+} ($FeCl_3$). This experimental test was conducted in order to assess the effect of each separate type of ion, and also the potential synergistic effect of co-injection of Fe^{2+} or Fe^{3+} with L-DOPA. Assays were also conducted with control (injected with 15 μL of a saline solution) and naïve insects over the same time series. For these insects, the various measured parameters did not much vary over time (Table S1). Thus, for the readability of the figures, only values measured 1 h PI (1 h after the start of the experiment for naïve insects) are presented in the results' section for saline-injected and naïve insects.

Oxidative damage assays

Protein carbonyls

The general oxidative damage resulting from the injection of $FeSO_4$ and L-DOPA was measured as the amount of protein carbonyls formed. We used the procedure from Levine et al. (1990) for these assays, with minor modifications. Specifically, midgut tissues and thoracic muscles were isolated and homogenized separately in 5 ml ice-cold phosphate buffer (60 ml of 50 mM sodium phosphate, 10 ml of 0.1 % Triton X100, 5 ml of 0.05 mM $CaCl_2$; then brought to 100 ml with distilled water after pH adjustment to 7.0). After homogenization (mortar, 30 strokes for 10 s), the samples were centrifuged at 2000*g* and 4 °C for 10 min. For each tissue extract, a 800 μl

Table 1 The effect of intrahemocoelic injection of different concentrations (μM) of $FeSO_4$ or L-DOPA on the protein carbonyl amount (OD_{366}/μg proteins) (as a recommended index for oxidative damage) in thoracic muscles and whole midgut homogenates of the fifth instar *S. gregaria*

Stressor	Concentration of the stressors (μM)						
	Tissue	Control	15	30	60	90	120
$FeSO_4$	Muscles	0.477 ± 0.013^{a}	0.930 ± 0.024^{a}	2.200 ± 0.060^{b}	2.515 ± 0.060^{bc}	2.380 ± 0.237^{bc}	2.733 ± 0.045^{c}
	Midgut	0.768 ± 0.015^{a}	0.057 ± 0.010^{b}	5.500 ± 0.075^{c}	4.832 ± 0.159^{d}	5.078 ± 0.078^{d}	5.418 ± 0.053^{cd}
L-DOPA	**Tissue**	**Control**	**100**	**200**	**400**	**600**	**800**
	Muscles	0.477 ± 0.013^{a}	0.163 ± 0.018^{a}	2.549 ± 0.091^{b}	0.120 ± 0.004^{a}	0.404 ± 0.292^{a}	0.129 ± 0.001^{a}
	Midgut	0.768 ± 0.015^{a}	0.695 ± 0.024^{a}	8.478 ± 0.465^{b}	0.698 ± 0.038^{a}	0.636 ± 0.026^{a}	0.726 ± 0.030^{a}

Each data point represents the mean ± SE of triplicate analyses. In the same line, mean values marked with different small superscript letters are significantly different ($p < 0.05$)

aliquot of the supernatant was transferred to a clean microtube with 200 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2 M HCl. The samples were incubated for 30 min at room temperature, then precipitated with 10 % TCA, left for another 10 min at room temperature before being centrifuged at 5000*g* and 4 °C for 7 min. The pellet was washed four times with an ethanol/ethyl acetate (1:1) mixture and re-dissolved in 3.5 ml of sodium phosphate buffer (60 ml of 150 mM sodium phosphate, 30 ml of 3 % sodium dodecyl sulfate, adjusted to a final volume of 100 ml with distilled water after pH adjustment to 7.0). Insoluble material was removed by centrifugation at 2000*g* for 10 min. The absorbance of the samples was measured immediately at 366 nm, and the rate of protein carbonyls concentration was expressed as optical density (OD_{366})/microgram proteins. An additional sample was similarly prepared and treated for the evaluation of background noise; DNPH was not added to this sample.

Lipid peroxides

The concentration of lipid peroxides was measured according to the ferrous oxidation in xylenol orange (FOX) method described in Hermes-Lima et al. (1995). Midgut tissues and thoracic muscles were isolated into phosphate buffer (pH 0.0) and homogenized in ice-cold methanol (1:5, *w*/*v*). After homogenization (mortar, 30 strokes for 10 s), the samples were centrifuged at 2000*g* and 4 °C for 10 min. An aliquot of 5 ml of the supernatant was used for the assay. The following components were sequentially added to the samples: 1750 μl of 1 mM $FeSO_4$, 700 μl of 0.25 M H_2SO_4, and 700 μl of 1 mM xylenol orange. Samples were then incubated under dark conditions at room temperature for 3 h. The initial absorbance of the developed color was measured at 580 nm. Then, volume of 10 μl of 0.5 mM cumene hydroperoxides was added to each sample in order to calibrate the FOX method, and the samples were maintained at room temperature for 1 h before the absorbance was re-measured at 580 nm. The change in absorbance due to addition of internal standard was calculated, and concentration of lipid peroxides was expressed as millimolar of cumene hydroperoxide equivalents/microgram lipids.

Antioxidant enzyme assays

Samples (midgut tissues, thoracic muscles) were homogenized (mortar, 30 strokes for 10 s) in ice-cold phosphate buffer (60 ml of 50 mM sodium phosphate, 10 ml of 0.1 % Triton-X 100, 5 ml of 0.05 mM $CaCl_2$; then completed to 100 ml with distilled water after pH adjustment with 2 M HCl). pH of the ice-cold phosphate buffer was 10.0 for SOD assays and 7.0 for CAT, GPx, and GR assays. The ratios of tissue mass and homogenization buffer were 1:1 for SOD, GPx, and GR and 1:4 for CAT. The homogenates were centrifuged at 10,000*g* and 4 °C for 30 min, and an aliquot from each supernatant was used for measuring total protein amount and enzyme activities.

Total proteins

The total protein concentration of samples was determined spectrophotometrically according to the method of Bradford (1976) with bovine serum albumin fraction V (Sigma-Aldrich) as a protein standard.

Superoxide dismutase

For SOD assays, we used the procedure from Misra and Fridovich (1972). The absorbance was measured at 480 nm with a UV/Vis Jenway-7305 spectrophotometer (Bibby Scientific Limited, Staffordshire, UK). SOD activity was expressed as OD_{480}/microgram proteins/minute.

Catalase

The activity of CAT was determined according to Aebi (1984). The decrease in absorbance was measured at 240 nm, and the CAT activity was expressed as OD_{240}/microgram proteins/minute.

Glutathione peroxidase

The activity of GPx was determined according to Hafeman et al. (1974) with the following minor modifications. The reaction mixture contained 32 μl of 10 mM EDTA, 20 μl of 100 mM sodium azide, 28 μl of 60 mM reduced glutathione (GSH), 10 μl of 50 mM H_2O_2, and 1600 μl of the tissue homogenate supernatant. The mixture was incubated for 10 min at 37 °C, and 1200 μl of 10 % trichloroacetic acid were added to the mixture which was subsequently centrifuged at 1730*g* for 5 min at room temperature. The supernatant was transferred to clean tubes with 1400 μl of 0.3 M Na_2HPO_4 (pH 7.5) and 350 μl of 0.4 % 5, 5′-diothio-bis-2-nitrobenzoic acid (DTNB) just before measuring the absorbance at 420 nm. The activity of GPx was expressed as OD_{420}/microgram proteins/minute.

Glutathione reductase

The activity of GR was determined according to Carlberg and Mannervik (1985) with the following minor modifications. The reaction mixture contained 1750 μl of 2 mM oxidized glutathione (GSSG), 175 μl potassium phosphate buffer (50 mM, pH 7.5), 875 μl of 3 mM DTNB, 175 μl of 2 mM NADPH,

and 350 μl supernatant of the tissue homogenate. The absorbance was measured at 420 nm, and the GR activity was expressed as OD_{420}/microgram proteins/minute.

Statistical analysis

The differences among the treated groups (injected with L-DOPA or $FeSO_4$) and saline-injected and naïve insects were compared by independent *t* test and one-way analysis of variance (ANOVA). Most often, values measured in saline-injected and naïve insects were comparable (Table S1), and it was thus hard to decipher if the slight differences observed for protein carbonyls resulted from (i) the injection itself, (ii) the augmentation of the volume of body fluids, or (iii) changes in hemolymph osmolality. Hence, we decided to refer to naïve insects as the controls in the text of results' section. Tukey's post-hoc test was applied for comparisons among data. All statistical analyses were performed with IBM SPSS Statistics for Windows (version 22.0. Armonk, NY: IBM Corp.). Data were expressed as mean ± SE. For the enzyme assays, K_m and V_{max} values were calculated by GraphPad Prism 6.0 software (GraphPad Software, Inc., USA).

Results

Oxidative damages

Protein carbonyls

In a preliminary test, we aimed at determining the concentration of $FeSO_4$ and L-DOPA that induced the highest quantity of protein carbonyls in the fifth instars of *S. gregaria*. Among the different tested concentrations, injection of 15 μl of 30 μM $FeSO_4$ and 200 μM L-DOPA per individual was the most effective (Table 1). One hour PI of $FeSO_4$, the amount of protein carbonyls was significantly increased in thoracic muscles (1.95-fold increase, $t = 14.38$, $p < 0.001$) and whole midgut (6.17-fold increase, $t = 53.51$, $p < 0.001$), as compared to control (Fig. 1a). The amount of protein carbonyls fluctuated over the course of the experiment, but did not return to control values in the whole midgut tissues 72 h after the injection. A similar pattern was observed with L-DOPA, with protein carbonyls being increased 4.70-fold ($t = 23.005$, $p < 0.001$) and 5.10-fold ($t = 13.658$, $p < 0.001$) in thoracic muscles and whole midgut, respectively, as compared to the controls (Fig. 1b). The amount of protein carbonyls declined over time, but did not return to control values in the midgut tissues. The effects of L-DOPA on protein carbonyls were significantly higher in midgut tissues than those in thoracic muscles ($t = 5.684$, $p < 0.001$).

Lipid peroxides

For $FeSO_4$, the levels of lipid peroxides, 1 h PI, were highly significantly increased in thoracic muscles and whole midgut, and were about 20.6-fold ($t = 23.156$, $p < 0.001$) and 59.4-fold ($t = 83.896$, $p < 0.001$) higher, respectively, than those of the controls (Fig. 2a).

For L-DOPA, the increase in lipid peroxides concentration 1 h PI was 157.6-fold ($t = 48.258$, $p < 0.001$) and 41.6-fold ($t = 14.993$, $p < 0.001$) higher than that of the

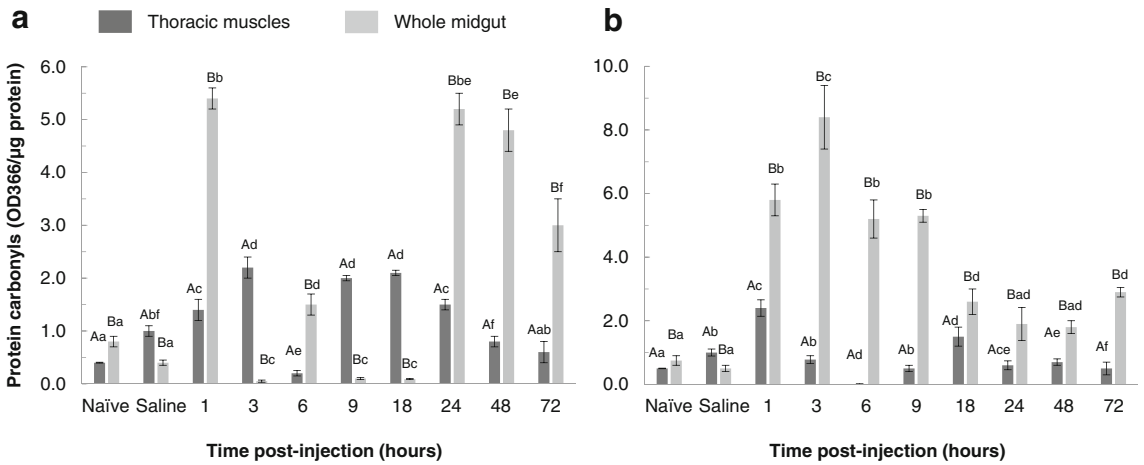

Fig. 1 Concentrations of protein carbonyls in thoracic muscles and midgut tissues of fifth instar *S. gregaria* treated with 15 μl of **a** 30 μM $FeSO_4$ or **b** 200 μM DOPA per individual. Assays were also conducted with control (injected with 15 μL of a saline solution) and naïve insects. Only values measured 1 h post-injection (1 h after the start of the experiment for naïve insects) are presented. Values are means ± SE ($N = 3$). Bars marked with different *capital letters* indicate statistical differences between thoracic muscle and midgut tissues (independent *t* test, $p < 0.05$); *small letters* indicate statistical significance among experimental sampling times (one-way ANOVA, $p < 0.05$)

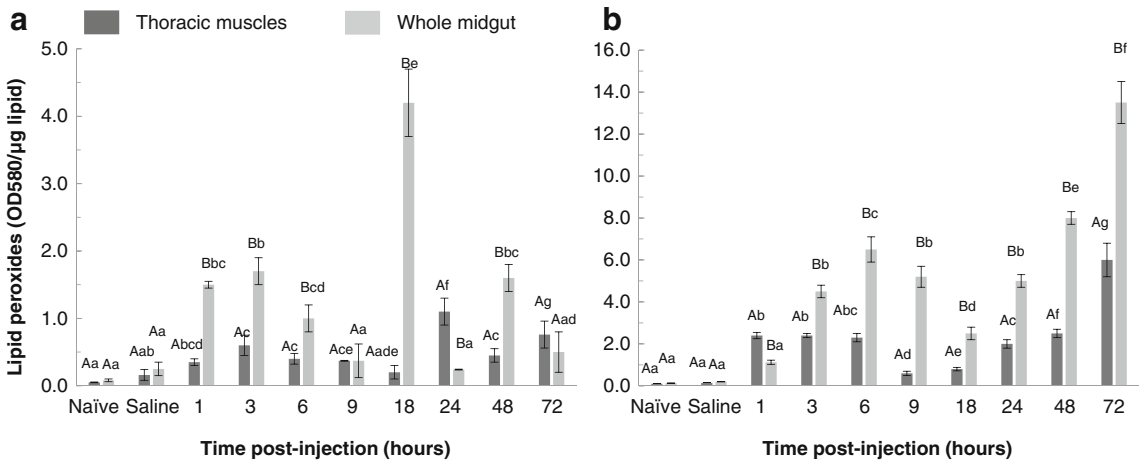

Fig. 2 Concentrations of lipid peroxides in thoracic muscles and whole midgut homogenates in fifth instar *S. gregaria* treated with 15 µl of **a** 30 µM $FeSO_4$ or **b** 200 µM L-DOPA per individual. Assays were also conducted with control (injected with 15 µL of a saline solution) and naïve insects. Only values measured 1 h post-injection (1 h after the start of the experiment for naïve insects) are presented. Values are means ± SE ($N = 3$). Bars marked with different *capital letters* indicate statistical significance between thoracic muscles and midgut tissues (independent *t* test, $p < 0.05$); *small letters* indicate statistical significance among experimental sampling times (one-way ANOVA, $p < 0.05$)

controls for both thoracic muscles and midgut tissues, respectively (Fig. 2b). The effects of $FeSO_4$ on lipid peroxides were significantly higher on thoracic muscles than those in midgut tissues ($t = 3.850$, $p < 0.05$).

Activity of antioxidant enzymes

The constitutive activity levels of the four principal antioxidant enzymes (SOD, CAT, GPx, and GR) was very

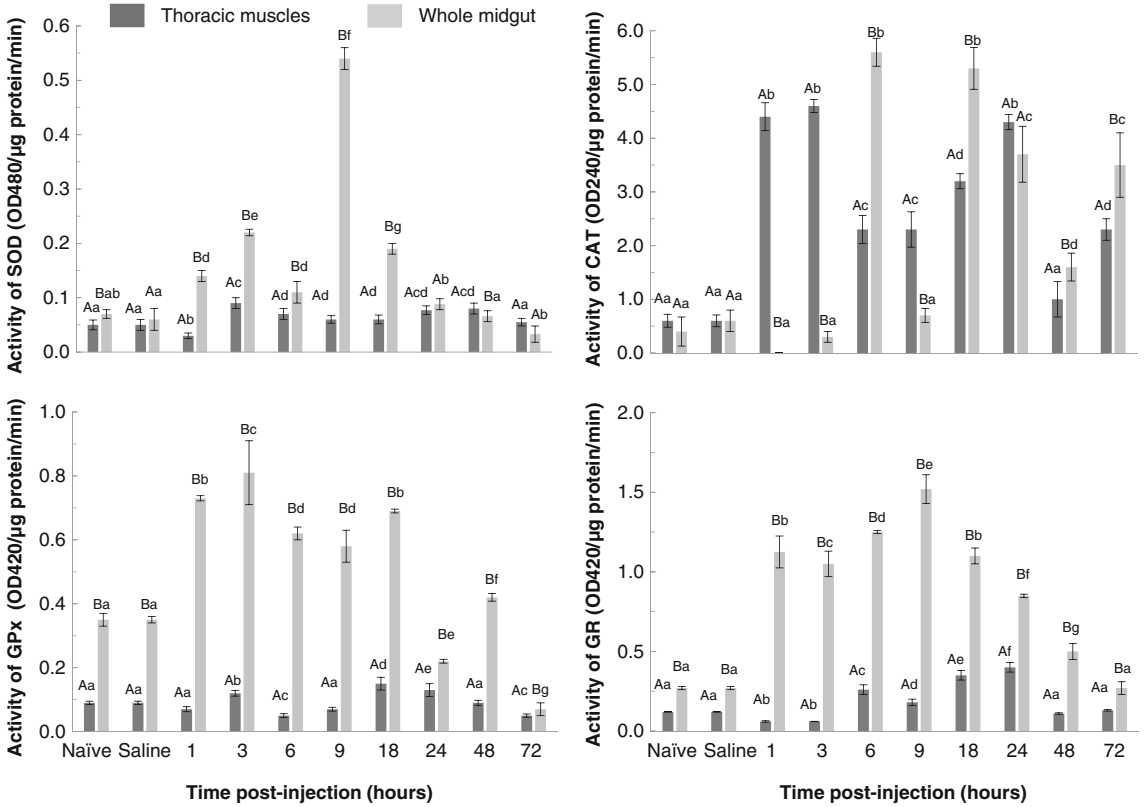

Fig. 3 Activity of the antioxidant enzymes SOD, CAT, GPx, and GR from the thoracic muscles and whole midgut homogenates of fifth instars of *S. gregaria* measured at different time intervals post-injection with 15 µl of 30 µM $FeSO_4$. Assays were also conducted with control (injected with 15 µL of a saline solution) and naïve insects. Only values measured 1 h post-injection (1 h after the start of the experiment for naïve insects) are presented. Values are means ± SE ($N = 3$). Bars with different *capital letters* indicate statistical significance between thoracic muscles and midgut tissues (independent *t* test, $p < 0.05$); *small letters* indicate statistical differences among experimental times (one-way ANOVA, $p < 0.05$)

low in non-stressed tissue homogenate extracts from thoracic muscles and whole midgut, in comparison to those from stressed fifth instars of *S. gregaria*. The data showed that the injection of $FeSO_4$ or L-DOPA generally induced large elevation of the activities of the four tested antioxidant enzymes, well above their constitutive levels (Figs. 3 and 4). The increased levels of the activity of the GPx and GR were always higher in the whole midgut than in thoracic muscles (Table 2) (GPx $t = 9.420$, $p < 0.05$ and $t = 8.896$, $p < 0.05$ after injection with Fe^{2+} or L-DOPA, respectively, and GR $t = 7.516$, $p < 0.05$ and $t = 7.175$, $p < 0.05$ after injection with Fe^{2+} or L-DOPA, respectively). The injection of $FeSO_4$ induced a pronounced increase in the activity of SOD, GPx, and GR in midgut tissues and of CAT in both of thoracic muscles and midgut tissues (Fig. 3). Except for CAT, the activity of the three other enzymes tended returning towards control values 72 h PI for both tissues. Similar patterns were observed for the L-DOPA-injected fifth instars (Fig. 4), except that the changes in the activities of antioxidant enzymes were more visible in thoracic muscles. Finally, the activities of both the constitutive and induced GPx and GR are always higher in whole midgut homogenates as compared to thoracic muscles; however, this difference is not always present in the case of SOD and CAT (Figs. 3 and 4).

The K_m values of the four enzymes are presented in Fig. 5. The enzyme characteristics were rather similar for GR as well as SOD from thoracic muscle and midgut tissues. For this latter enzyme, V_{max}, however, was lower in the thoracic muscle tissues. For CAT and GPx, both values, and more particularly K_m values, were higher in the thoracic muscle tissues (Fig. 5).

Discussion

Injection of $FeSO_4$ or L-DOPA generates oxidative stress and affects macromolecules

Protein carbonyls and lipid peroxides are common biomarkers of oxidative stress (Dalle-Donne et al. 2003). Both biomarkers had their levels augmented in thoracic muscles and midgut tissues from fifth instars of *S. gregaria* after the injection of $FeSO_4$ or L-DOPA. There exists a large range of radical species with different effects on biomolecules (Augustin and Partridge

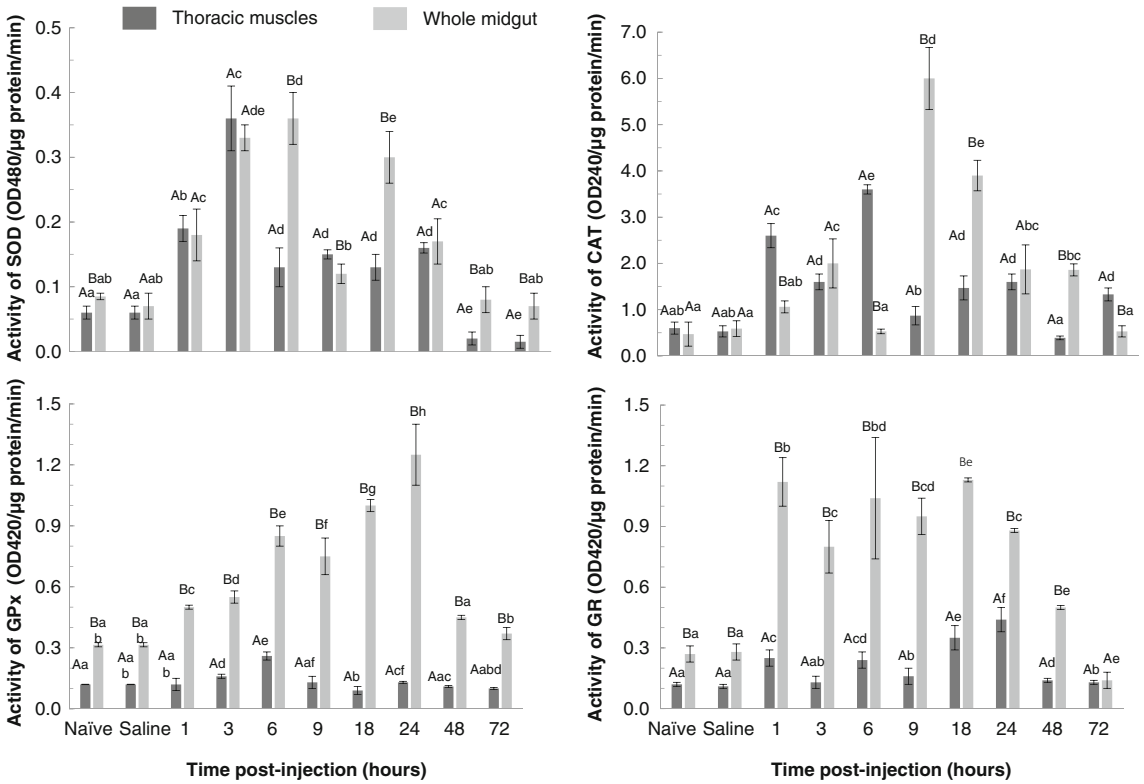

Fig. 4 Activity of the antioxidant enzymes SOD, CAT, GPx, and GR from thoracic muscles and whole midgut homogenates in fifth instars of *S. gregaria* at different time intervals post-injection with 15 µl of 200 µM L-DOPA. Values are means ± SE ($N = 3$). Bars marked with different *capital letters* indicate significant differences between thoracic muscles and midgut tissues (independent *t* test, $p < 0.05$); *small letters* indicate statistical significance among experimental times (one-way ANOVA, $p < 0.05$)

Table 2 The effect of intrahemocoelic injection of different concentrations of $FeSO_4$ or L-DOPA on the activity of antioxidant enzymes in thoracic muscles and whole midgut homogenates of the fifth instar *S. gregaria*. In the same line, mean values marked with different small superscript letters are significantly different ($p < 0.05$)

Stressor		SOD (OD_{480}/μg proteins/min)		CAT (OD_{240}/μg proteins/min)		GPx (OD_{420}/μg proteins/min)		GR (OD_{420}/μg proteins/min)	
		Muscles	Midgut	Muscles	Midgut	Muscles	Midgut	Muscles	Midgut
	Control	0.069 ± 0.001^{a}	0.086 ± 0.002^{a}	0.587 ± 0.002^{a}	0.423 ± 0.003^{a}	0.094 ± 0.006^{a}	0.373 ± 0.026^{a}	0.131 ± 0.006^{a}	0.264 ± 0.017^{a}
$FeSO_4$ (μM)	**15**	0.021 ± 0.002^{b}	0.019 ± 0.005^{b}	2.117 ± 0.132^{b}	0.940 ± 0.073^{a}	0.078 ± 0.004^{ab}	0.494 ± 0.010^{b}	0.148 ± 0.002^{a}	0.698 ± 0.026^{b}
	30	0.092 ± 0.002^{c}	0.546 ± 0.004^{c}	4.423 ± 0.139^{c}	5.593 ± 0.133^{b}	0.088 ± 0.002^{ac}	0.723 ± 0.002^{c}	0.458 ± 0.010^{b}	1.526 ± 0.023^{c}
	60	0.019 ± 0.002^{b}	0.034 ± 0.004^{b}	2.968 ± 0.248^{d}	3.404 ± 0.339^{cd}	0.073 ± 0.002^{bce}	0.487 ± 0.011^{b}	0.095 ± 0.001^{c}	0.783 ± 0.005^{bd}
	120	0.050 ± 0.003^{d}	0.070 ± 0.002^{a}	3.484 ± 0.034^{d}	3.940 ± 0.087^{d}	0.067 ± 0.004^{bf}	0.506 ± 0.007^{b}	0.098 ± 0.002^{c}	0.806 ± 0.001^{d}
DOPA (μM)	**100**	0.165 ± 0.010^{b}	0.041 ± 0.005^{b}	1.054 ± 0.007^{b}	0.696 ± 0.036^{a}	0.058 ± 0.001^{b}	0.454 ± 0.024^{ac}	0.309 ± 0.019^{b}	0.343 ± 0.014^{a}
	200	0.360 ± 0.014^{c}	0.353 ± 0.011^{c}	3.580 ± 0.046^{c}	6.017 ± 0.345^{b}	0.323 ± 0.005^{c}	1.296 ± 0.032^{e}	0.453 ± 0.011^{c}	1.096 ± 0.026^{b}
	400	0.021 ± 0.002^{d}	0.356 ± 0.003^{c}	1.907 ± 0.092^{d}	4.051 ± 0.128^{c}	0.117 ± 0.001^{d}	0.272 ± 0.005^{bf}	0.307 ± 0.011^{b}	0.345 ± 0.014^{a}
	800	0.033 ± 0.002^{ad}	0.406 ± 0.007^{d}	2.454 ± 0.102^{e}	5.215 ± 0.065^{b}	0.138 ± 0.005^{e}	0.281 ± 0.005^{df}	0.367 ± 0.004^{d}	0.527 ± 0.015^{c}

Each data point represents the mean ± SE of triplicate analyses

2009; Barbehenn 2002; Even et al. 2012). In the present study, we experimentally demonstrated that both $FeSO_4$ and L-DOPA inflicted damages to proteins and lipids of the targeted tissues of the hoppers. Time-series measurements revealed that damages were already measurable 1 h PI, which suggests that both $FeSO_4$ and L-DOPA quickly diffused into these tissues after injection. The effects were prolonged for up to 48 h, as depicted by the amounts of protein carbonyls and lipid peroxides that remained high, and by the elevated activities of the antioxidant enzymes SOD, CAT, GPx, and GR.

Interestingly, large fluctuations in the amounts of protein carbonyls were measured over the 72-h-monitoring period in the treated insects, but not in the saline-injected and naïve ones. Protein carbonyls are formed from oxidation of specific amino acids (arginine, histidine, lysine, and proline) and also polypeptide chains cleavage (at the amino acids aspartate, glutamate, and proline) (Costa et al. 2007; Levine 2002). Protein carbonyls can be recognized and degraded through a genetically controlled proteasomal- and lysosomal-mediated cellular proteolytic process (Bulteau et al. 2006; Chen et al. 2004; Kiffin et al. 2004; Thorpe et al. 2004; Yu 1994). Several proteins are catalysts of important biochemical reactions, suggesting that their alteration may result in damages to other biological systems greater than stoichiometric (Squier 2001; Stadtman and Levine 2000). As a result, the fluctuating pattern, which was fairly apparent for protein carbonyls and lipid peroxides but not for antioxidant enzymes, may reveal the progression of the stress in parallel to the repair of the inflicted damages. For instance, the removal of oxidized proteins may have unbalanced fluxes and homeostasis of some reactions/metabolic pathways, thus displacing the initial problem elsewhere. This speculative hypothesis will have to be assessed in future studies. In parallel, products of lipid peroxidation are capable of disrupting conformation of many cellular proteins, by forming cross links with these proteins, in turn, leading to inactivation of cell functions (Birben et al. 2012). Hence, this fluctuating pattern suggests complex interactions among macromolecules, L-DOPA and $FeSO_4$, and their degradation products.

Injection of Fe^{2+} (or Fe^{3+}) into the hemocoel of *S. gregaria* caused oxidative damages to the two macromolecules (proteins and lipids) that we assessed. It is likely that these ions formed perferryl and ferryl ions that are intermediate products of Fe^{2+} under aerobic conditions, as reported before (Qian and Buettner 1999; Schafer et al. 2000). Perferryl ion has high electron affinity that favors its role as an oxidant over $^{\cdot}OH$ in biological systems. Under normal physiological conditions, the steady-state ratio of $[O_2]/H_2O_2$ is very high (Boveris and Cadenas 1997; Jones 1986). Therefore, dioxygen can react readily with steady-state Fe^{2+} forming species that are the

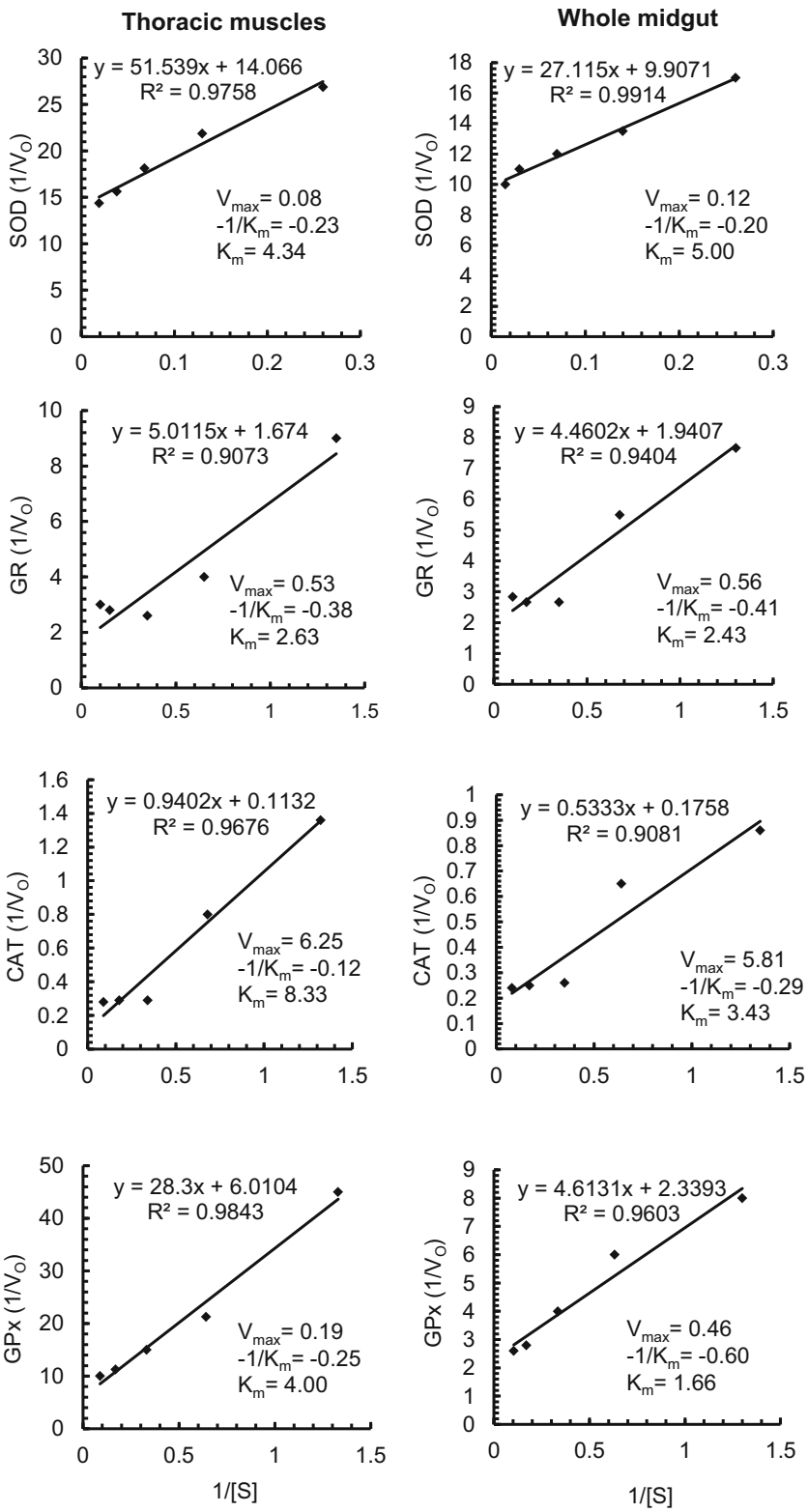

Fig. 5 Determination of Michaelis-Menten constant (K_m) of SOD (at pH 10.0), GR, CAT, and GPx (at pH 7.0) of the thoracic muscles and whole midgut homogenates of the fifth instar *S. gregaria*, as determined by the Lineweaver-Burk plot (S is expressed in M for CAT and in mM the three other enzymes), and measured as OD/μg protein/min. Each point represents the mean of three independent replicates ($N = 3$)

primary route to the initiation of biological free-radical oxidations (Qian and Buttner 1999; Schafer et al. 2000).

Finally, whole midgut tissues were greatly affected by the injection of L-DOPA or $FeSO_4$, as levels of protein carbonyls, lipid peroxides, and activities of antioxidant enzymes remained high even 72 h PI. Possible side effects to other biological functions will have to be investigated in future studies.

Similar antioxidant enzyme defense mechanisms are elicited, but sensitivity to $FeSO_4$ and L-DOPA differs between tissues

The redox homeostasis can be restored through integrated antioxidant systems involving both non-enzymatic and enzymatic mechanisms (Birben et al. 2012; Fang 2004; Fang et al. 2006; Felton and Summers 1995). In the present work, we focused on the activity of SOD, CAT, GR, and GPx. As expected, the injection of Fe^{2+} ($FeSO_4$) or L-DOPA led to elevated activity in the four antioxidant enzymes, starting from the first hour PI onwards. The increased activity of the antioxidant enzymes after the injection of $FeSO_4$ or L-DOPA occurred in concomitance with oxidative damages to the macromolecules proteins and lipids. This correlative response likely suggests that changes in enzyme activity directly results from the formation of ROS PI. Moreover, formed ROS were previously reported by Birben et al. (2012) to act as a signal affecting regulation of antioxidant genes.

Our results showed low but measurable levels of constitutive activity of the four principal antioxidant enzymes SOD, CAT, GPx, and GR in naïve specimens of *S. gregaria* whatever the tissue considered. Interestingly, identified K_m values were higher in CAT and GPx in thoracic muscles, with values more than two times higher than in midgut tissues. The lowest values of K_m for midgut enzymes as compared with thoracic muscles likely suggest higher affinity to the H_2O_2 substrate. This differential affinity may result from the presence of different enzyme isoforms in these two tissues. In general, most of the oxidative stress was reported to result from H_2O_2 (Perić-Mataruga et al. 1997). Therefore, the higher affinity to H_2O_2 substrate of CAT and GPx from the midgut and their elevated antioxidant activities are consistent with our assumption of the higher ability of midgut tissues to cope with ROS production. This is probably because the gut of this herbivore insect must frequently deal with the ingested pro-oxidants from the nutrients (Ahmad 1992; Felton and Summers 1995; Krishnan and Kodrík 2006). Consistently, activities of both GPx and GR were always higher in whole midgut homogenates than in thoracic muscles of *S. gregaria*; however, this difference was not always present in the case of SOD and CAT.

Finally, the K_m value of CAT for H_2O_2 is severalfold higher than that of GPx for this substrate. This finding indicates that GPx can catalyze degradation of H_2O_2 at very low concentrations more efficiently than CAT in *S. gregaria*. Therefore, the previously mentioned phenomenon that CAT acts at higher concentrations of H_2O_2 in comparison to GPx (Bouayed and Bohn 2010) may be due to their different affinities to the H_2O_2 substrate.

Co-injection of $FeSO_4$ or $FeCl_3$ with L-DOPA

L-DOPA is an *o*-diphenolic compound which occurs naturally in tissues of many organisms, including insects (Riley 1997). Depending on the cellular redox potential (Siraki and O'Brien 2002), this metabolite may behave as a pro-oxidant or as an antioxidant when present at high concentrations (Cadenas et al. 1989). Of note, a 1:1 ratio of Fe^{3+}:L-DOPA induces damages to lipids, but this deleterious effect was reduced when concentrations of L-DOPA were increased; this compound behaves as an antioxidant (Sotomatsu et al. 1990). The oxidative damages measured in tissues of the L-DOPA-injected *S. gregaria* suggest that the pro-oxidant behavior prevails under our experimental conditions. In this case, L-DOPA can undergo auto-oxidation (Riley 1997), a process which is enhanced in the presence of trace concentrations of Fe^{3+} (Pattison et al. 2002). In L-DOPA redox cycling, one electron transfer produces L-DOPA-$SQ^{\cdot-}$ that can act by itself as an oxidative radical. In addition, it can undergo auto-oxidation reactions leading to production of $O^{\cdot}{}_2^{-}$ and subsequently other ROS which can cause oxidative damage (Beyer 1992; Bolton et al. 2000; Pattison et al. 2002).

In a simple test to detect the level of oxidative damages (assessed as the amount of protein carbonyls produced) 3 h after the injection of Fe^{2+} ($FeSO_4$) or Fe^{3+} ($FeCl_3$) or co-injection with L-DOPA, unexpected results were encountered (Table 3). At equimolar injection amounts of Fe^{2+} or Fe^{3+}, a significantly higher amount of protein carbonyls was formed for Fe^{2+} as compared with Fe^{3+}. This finding suggests that the participation of Fe^{2+} to redox reactions is higher than that of Fe^{3+}, maybe because ferric ions can be stored within ferritin. Furthermore, when Fe^{2+} or Fe^{3+} was co-injected with L-DOPA, the amount of protein carbonyls was greatly reduced for both iron ions, to values lower than the summed values measured for each iron ion and L-DOPA (Table 3). In this respect, it was reported that chelation between L-DOPA-$SQ^{\cdot-}$

Table 3 Protein carbonyl amount (OD_{366}/μg protein, as a recommended index for oxidative damage) formed in thoracic muscles and whole midgut tissues under the effect of different stressors (15 μl of 30 μM $FeSO_4$ or $FeCl_3$, or 200 μM of L-DOPA) injected into the hemocoel of the fifth instar *S. gregaria*

Tissue	Control	Fe^{2+}	Fe^{3+}	DOPA	L-DOPA + Fe^{2+}	L-DOPA + Fe^{3+}
Thoracic muscles	0.423 ± 0.023^{Aa}	0.720 ± 0.017^{Ab}	0.489 ± 0.009^{Aac}	0.594 ± 0.016^{Ad}	0.517 ± 0.011^{Acde}	0.494 ± 0.017^{Aae}
Whole midgut	0.502 ± 0.015^{Ba}	1.012 ± 0.055^{Bb}	0.579 ± 0.014^{Ba}	0.653 ± 0.008^{Bc}	0.576 ± 0.017^{Bac}	0.592 ± 0.019^{Bac}

Each data point represents the mean ± SE of triplicate analyses. In the same line, mean values marked with different small superscript letters are significantly different ($p < 0.05$). In the same column, mean values marked with different capital superscript letters are significantly different ($p < 0.05$)

and metal ions stabilizes this radical, i.e., it becomes a less reactive redox-cycling compound (Felix and Sealy 1981). Also, L-DOPA can act as high affinity chelator of some metal ions. Hence, it affects the redox properties of these ions and subsequently their ability to catalyze or participate in radical formation (Maskos et al. 1992; Lloyd 1995; Paris et al. 2007).

Conclusion

In the present study, we demonstrated that both $FeSO_4$ and L-DOPA generated oxidative stress to *S. gregaria*, which manifested by damages to macromolecules of tissues and elevated activities in their antioxidant enzymes. Our results are consistent with the previous works reporting that herbivores are defended against allelochemicals by increased activities of antioxidant enzymes (constitutive or inducible) in their gut, as compared with their non-herbivore relatives. Defense includes a group of enzymes represented by SOD, CAT, GR, and GPx that are crucial for obviating the free-radical cascade of oxygen and semiquinones. As herbivores, *S. gregaria* specimens have great abilities to deal with oxidative stress. This capacity has been developed in response to *o*-diphenols, such as L-DOPA, produced by plants. We also first demonstrated using in vivo injections that iron-containing fertilizers can disrupt the physiology of desert locusts, by provoking an intense oxidative stress. In future studies, residual and oral exposure of *S. gregaria* to L-DOPA and $FeSO_4$ should be conducted to assess if these different routes of uptake would conduct to similar conclusions.

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