Impact of Dopamine and Hydrogen Peroxide on Physiological Stress Parameters of *Sarcophaga surcoufi* (Diptera: Sarcophagidae)[1]

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Abstract Natural phenolics and reactive oxygen chemical species can have metabolic and fitness costs in insects and thus represent potential insecticidal agents. The response levels of total protein, phenoloxidase, proteases, chitinases, and total antioxidant activities to dopamine and hydrogen peroxide (H_2O_2) injected into third-instar flesh fly, *Sarcophaga surcoufi* Villeneuve (Diptera: Sarcophagidae), larvae were measured. In comparison to control groups, total protein initially increased following treatment with H_2O_2, but decreased at later sampling intervals. Treatment with dopamine did not affect total protein or protease activity for up to 12 h after treatment, but a slight decrease was observed in both at 24 and 36 h after treatment. Treatment with H_2O_2 decreased protease activity at all sampling intervals. H_2O_2 treatment increased phenoloxidase activity at each sampling interval, while treatment with dopamine decreased the activity at each interval. Total antioxidant activity increased substantially 12 h after treatment with H_2O_2 and increased only slightly 24 h after treatment before decreasing at 36 h, while dopamine increased antioxidant activity at all sampling intervals. Chitinase activity increased 12 h after H_2O_2 treatment, but then decreased at 24 and 36 h, but dopamine had no effect on chitinase activity. Antioxidants, phenoloxidase, and chitinase are important metabolic defense components in insects, and increases or decreases in each impacts the physiological integrity of the insect exposed to stressors that increase or decrease their quantity and activity.

Key Words Sarcophagidae, phenol oxidase, ROS, chitinase, physiological stress

The synanthropic filth-breeding flesh fly *Sarcophaga surcoufi* Villeneuve (Diptera: Sarcophagidae) is a nosocomial myiasis agent with a Palearctic distribution (Sawaby et al. 2018). Adults are associated with the transmission of enteric disease-causing agents such as bacteria, tapeworms, and poliovirus (Thompson et al. 2013, Nayduch 2017).

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Oxidizing agents such as hydrogen peroxide (H_2O_2, a normal by-product of cellular metabolism in aerobic cells) are used as biocides in medical, food, and industrial applications due to their broad-spectrum activity, which includes efficacy against bacterial endospores, their lack of environmental toxicity, and their complete degradation over time (Linley et al. 2012). These agents react with macromolecules, such as DNA, RNA, proteins, and lipids, causing alterations to their structures (Ahmad et al. 1991). In insects, as in other animals, lipid peroxidation is potentially very harmful because lipids are components of cell membranes and play an important role in developmental and reproductive physiology (Canavoso et al. 2001, Renault et al. 2016). High concentrations of reactive oxygen species damage the absorption of ingested nutrients and can cause oxidative damage to the midgut cells and impair the absorption of ingested nutrients (Bi and Felton 1995). However, only fragmentary reports are available on their overall physiological impacts on flies.

In biological systems, oxidative stress occurs when the balance between reactive oxygen species and antioxidants is disrupted because of excess reactive oxygen species, depletion of antioxidants, or both (Summers and Felton 1994, Waris and Ahsan 2006). To protect against the oxidative damage of reactive oxygen species, insects possess specific defense mechanisms in the form of a network of antioxidant enzymes associated with the protection against oxidative stress (Lukasik et al. 2009, Renault et al. 2016, 2018).

Dopamine is a neurotransmitter involved in the transmission of signals in the brain and other vital areas and is found in both vertebrates and invertebrates (Blenau and Baumann 2001, Yamamoto and Vernier 2011). In insects, dopamine plays transmitter and modulatory roles (Duch et al. 1999). In peripheral tissues, decarboxylase enzyme forms dopamine required for the sclerotization of the cuticle, which is under hormonal control (Sugumaran 1998). Environmental stress can lead to fluctuation of dopamine, norepinephrine, and serotonin concentrations in insect hemolymph leading to cell death (Péqueux et al. 2002).

Chitinase enzymes, glycosyl hydrolases with sizes ranging from 20 kDa to about 90 kDa, degrade chitin to low-molecular-weight chitooligosaccharides (Bhattachrya et al. 2007). They can play an important role in the biocontrol of insects (Narayanan 2004).

Digestive proteinases are divided into four classes: serine, cysteine, aspartic, and metalloproteases. All four classes occur in insects (Reeck et al. 1999). Digestive enzymes are targets for pest control, wherein proteolytic enzymes are responsible for protein digestion and, consequently, for the supply of amino acids needed for development (Milne and Kaplan 1993).

Oxidation of phenols catalyzed by polyphenol oxidase and peroxidase is a potential defense mechanism. Phenols play an important role in cyclic reduction of reactive oxygen species such as H_2O_2, which, in turn, activate a cascade of reactions leading to the activation of defensive enzymes (Renault et al. 2016).

The focus of the present study was on the effects of dopamine and H_2O_2 on some biochemical parameters of third-instar *S. surcoufi* larvae. The physiological parameters studied were the quantity and activity of phenoloxidase, chitinase, protease, and total antioxidant enzymes, as well as total protein content of the larvae following injection of either dopamine or H_2O_2.

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Materials and Methods

Sarcophaga surcoufi larvae used in the study were from a colony that originated from field-collected flies and was maintained in the laboratory at 27 ± 2°C and fluctuating relative humidity (Mohamed et al. 2018). Adult flies were confined in rearing cages (40 × 40 × 40 cm^3), each cage supplied with a 250-g block of fresh meat for larviposition, larval feeding, and as a rearing medium. A solution of sucrose (5%, w/v) was offered as food supplement to adults. Third-instar larvae were used for all treatments and physiological determinations.

We followed an injection-based approach (Renault et al. 2016, 2018), with modifications, to bioassay the effects of H_2O_2 and dopamine on *S. surcoufi* third instars (a dose-mortality response study). Larvae were treated with injections to standardize the amount of stressor given to each individual insect. Briefly, cohort batches of third instars were washed with distilled H_2O and surface-sterilized by swabbing with 70% ethanol. Then a Hamilton 7636-01 microsyringe (Hamilton Corp., Reno, NV) was used for injections. Nine different concentrations of stressor solutions were freshly prepared and 15 μL of stressor concentration was injected into each larva (aqueous dopamine [Sigma-Aldrich, St. Louis, MO] prepared in 0.7% NaCl [Allen et al. 2011]: 25, 50, 75, 100, 125, 150, 175, 200, 225 mmol/L/larva; H_2O_2: 0.25, 0.50, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25% [v/v] per larva as per Grover et al. [2009]). The dose-mortality response of the flies (each experimental group started with an initial number of 100 flies) was then measured. Control flies were flies pricked with the syringe (naïve or no treatment). All injections were made in the hemocoel, by inserting the syringe needle beneath the last abdominal segment, close to the posterior abdominal spiracle. All experiments were conducted under the aforementioned rearing conditions. Percentage of mortality was determined after 24 h and corrected using Abbott's formula (Abbott 1925). Data were subjected to probit analysis for fitting multiple regression using SAS 9.2 (SAS Institute Inc., Cary, NC) to estimate 50% mortality (LC_{50}) and compare the different stressor doses.

For the remainder of the experiments, that is, for the characterization of physiological intracellular actions of dopamine and H_2O_2 treatments, the applied concentrations were those giving optimum effect, pilot testing, and LC_{50}.

Three groups of third instars, each containing 15 individuals, were used: one as a naïve control, and two other groups injected with either 15 μL 1.0% H_2O_2 or with aqueous dopamine (100 mmol/L in 15-μL volumes in 0.7% NaCl). Larvae were injected in the posterior end using a Hamilton microsyringe. At each sampling interval, whole-insect body tissues were dissected in saline solution. Approximately 2.5 g of tissue homogenates were isolated, homogenized, and combined with tissue from four other insects with three replicates for each treatment and sampling interval. These were processed and prepared as described by Renault et al. (2016). Briefly, larval tissues were homogenized (mortar, 30 strokes for 10 s) 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 H_2O (pH 7.0). Samples were then centrifuged at 5,000 × *g* and 4°C for 10 min. One-milliliter aliquots of the supernatant, supplied with a few crystals of N-phenylthiourea ([Sigma-Aldrich], except for phenoloxidase assay) to prevent melanization, were transferred to a clean microtube and stored at −70°C for further use.

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Phenoloxidase activity in the larvae was determined according to a modified protocol of Ishaaya (1971) in a reaction mixture consisting of 0.5 mL phosphate buffer (0.1 M, pH 7), 200 μL enzyme solution, and 200 μL catechol solution (2%, v/v). Prior to the initiation of the reaction, the substrate and other ingredients of the reaction mixture were separately incubated at the optimum temperature of the reaction (25°C). Enzyme reaction was initiated by adding the catechol solution. After 1 min, the optical density was determined. Zero adjustment was against a sample blank at 405 nm in a double-beam ultraviolet/visible spectrophotometer (Spectronic 1201, Milton Roy Co., Ivyland, PA).

Chitinase activity was determined by preparing colloidal chitin according to the methods of Bade and Stinson (1981). Briefly, chitinase was colorimetrically assayed by measuring the liberated N-acetylglucosamine (reaction product). The reaction mixture (1.0 mL total volume) contained 50 mM phosphate-acetate buffer (pH 6.5), 2.5 g chitin substrate, and 1 mM CaCl. The enzymatic reaction was initiated by addition of 0.1 mL chitin–chitinase (sample solution). Incubations were conducted at 37°C with constant stirring. In each assay, 0.1 mL was sampled into a mixture of 0.3 mL phosphate-acetate buffer (0.1 M, pH 7.0) and 0.2 mL potassium tetraborate (0.8 M in boric acid, pH 9.1). The reaction was stopped by adding 0.5 mL 20% trichloracetic acid. The optical density was read against the buffer blank at 540 nm. The enzyme activity was expressed as μg N-acetylglucosamine $\times 10^3$/min/g body weight (b. wt.) (Ishaaya and Casida 1974).

Antioxidant capacity was measured with Biodiagnostic Kit No. TA 2513 (Biodiagnostic, Dokki, Giza, Egypt). The assay measures the capacity of the biological fluids to inhibit the production of thiobarbituric acid reactive substances from sodium benzoate under the influence of the free oxygen radicals derived from Fenton's reaction (Koracevic et al. 2001). Briefly, antioxidants in the sample react with a known quantity of exogenous H_2O_2. The antioxidants in the sample eliminate a certain amount of the provided H_2O_2. The residual H_2O_2 is determined colorimetrically by an enzymatic reaction which involves the conversion of 3,5-dichloro-2-benzene-sulphonate to a colored product read at 505 nm in a Spectronic 1201 (Milton Roy Co.).

Proteolytic activity was measured as per methods described by Tatchell et al. (1972). Amino acids were colorimetrically assayed by ninhydrin reagent according to the method described by Lee and Takabashi (1966). L-Alanine was used as the standard, and amino acids were expressed as μg alanine/min/g b. wt.

Total protein content of larvae was determined by the method of Bradford (1976). The absorbance at 595 nm was measured after 2 min against a blank (1 mL of phosphate buffer plus 5 mL protein reagent).

Statistical analysis. Results are presented as means ± standard deviation, unless otherwise is stated. Data were subjected to statistical analysis by two-way analysis of variance and separated by the least significant difference test. The probability level $P < 0.01$ was considered statistically significant, unless otherwise indicated (SSPS Statistics, version 13.0).

Results

Toxicity analysis. We measured the impact of dopamine and H_2O_2 on the mortality of the third instars of *S. surcoufi* in response to injection with different

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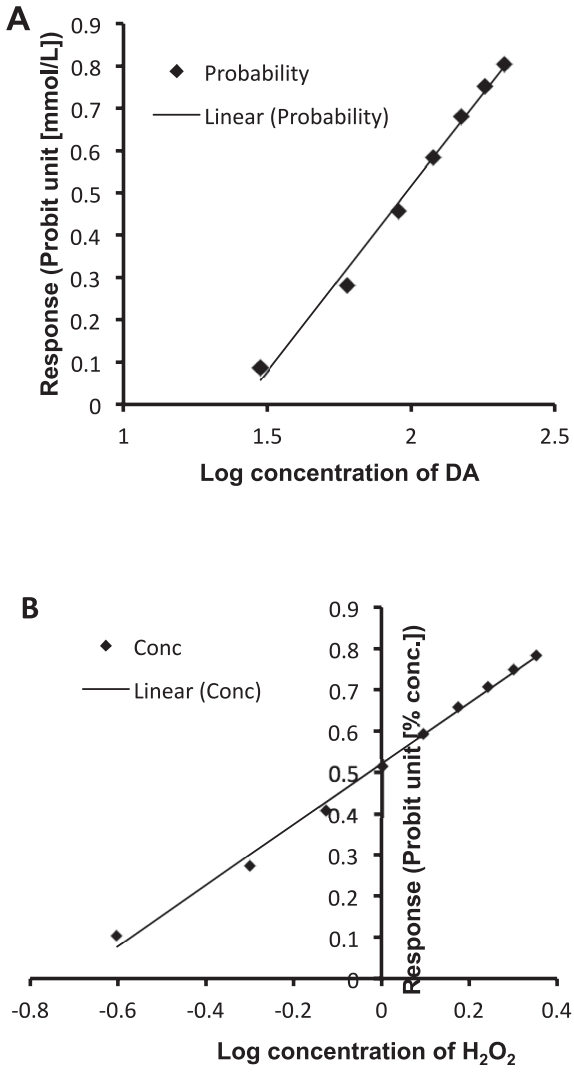

Fig. 1. Dose-response curves of the tested stressors against *Sarcophaga surcoufi* third instars. A. Dopamine (DA): LC_{50} = 99.24 mmol/L, slope = 2.11 ± 0.16 (SE), intercept = 0.79 ± 0.34, total χ^2 = 74.08, df = 7 at $P > 0.001$. B. H_2O_2: LC_{50} = 0.97% (v/v), slope = 2.11± 0.16, intercept = 5.03 ± 4.50E-02, total χ^2 = 74.08, df = 7 at $P > 0.001$.

doses of these two stressors. Data are presented as a dose-mortality response analysis (Fig. 1). Toxicologically, both H_2O_2 and dopamine showed varying potencies. The LC_{50} of dopamine and H_2O_2 were approximately 100 mmol/L and 1%, respectively. Subsequent to stressor application, compared to relevant controls, experimental larvae exhibited significant physiological changes in

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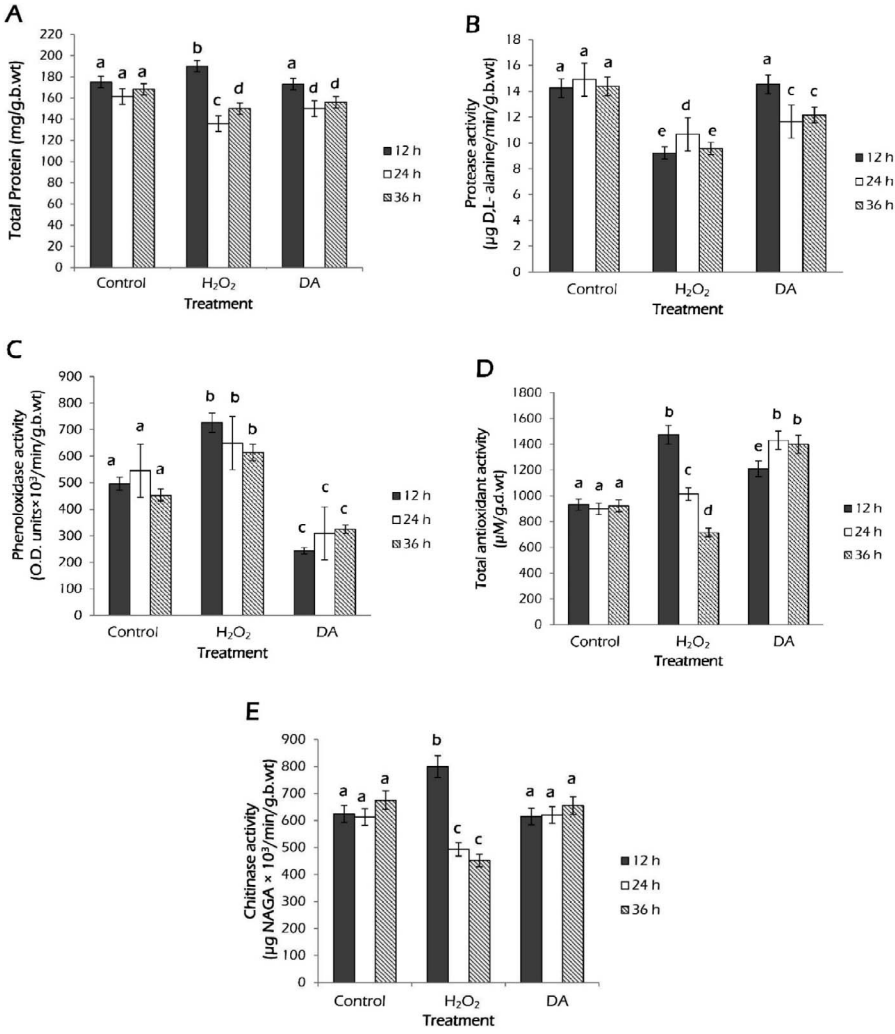

Fig. 2. Impact of dopamine (DA) and H_2O_2 and treatments on the different tested physiological stress parameters of *Sarcophaga surcoufi* third instars. A. Total protein content. B. Protease activity. C. Phenoloxidase activity. D. Total antioxidant enzymes activity. E. Chitinase activity. Different letters above the bars (SD) indicate significant differences ($P < 0.01$) based on Fisher's least significant difference test.

activities of the tested parameters in response to stressor challenge, as can be seen in Fig. 2, a subject of subtle interpretations, detailed later.

Total proteins. Treatment with dopamine had no effect on the total protein content of the *S. surcoufi* third instars 12 h after injection, but at 24 and 36 h after injection, total protein decreased to 150 ± 3.21 and 155.98 ± 5.41 mg/g b. wt.,

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respectively ($F = 61.4$; df $= 2$; $P < 0.001$), and was significantly lower than in the control treatments. Treatment with H_2O_2 increased total protein to a level significantly higher than the controls 12 h after injection (190.20 ± 5.51 mg/g b. wt.), while at 24 and 36 h, total protein was significantly lower than at 135.65 ± 6.01 and 149.97 ± 4.55 mg/g b. wt., respectively ($F = 175.8$; df $= 2$; $P < 0.001$) (Fig. 2A).

Protease activity. Treatment with dopamine had no effect on protease activity 12 h after injection (14.54 ± 0.38 μg), while protease activity was significantly lower than the controls, having decreased to 11.65 ± 0.19 μg at 24 h after injection and 12.18 ± 0.51 μg at 36 h after injection ($F = 41.2$; df $= 2$; $P < 0.001$). In comparison to control treatments, H_2O_2 significantly reduced protease activity at 12, 24, and 36 h after injection (Fig. 2B).

Phenoloxidase activity. In comparison to the control treatments, dopamine-treated insects exhibited significantly lower phenoloxidase activity, with activity levels of 244.33 ± 17.01 O.D. unit$\times 10^3$/min/g b. wt. at 12 h, 309 ± 25.05 O.D. unit$\times 10^3$/min/g b. wt. at 24 h, and 365 ±19.29 O.D. unit$\times 10^3$/min/g b. wt. at 36 h ($P < 0.001$) (Fig. 2C). Phenoloxidase activity was significantly higher in treated than in control insects at 12, 24, and 36 h after treatment with H_2O_2 (726.23 ± 23.59, 649 ± 30. 01, and 649 ± 25.78 O.D. unit $\times 10^3$/min/g b. wt., respectively) ($P < 0.001$).

Total antioxidant activity. Treatment of larvae with H_2O_2 yielded a significant higher total antioxidant activity at 12 h (1472.23 ± 18.77 μM/g b. wt.) ($F = 453.1$; df $= 2$; $P < 0.001$) in the treated group versus controls. There was only a slightly lower level 24 h, but activity was significantly lower at 36 h (715.14 ± 14.97 μM/g b. wt.) in comparison to the controls. Treatment with dopamine resulted in an increase ($P < 0.001$) in total antioxidant activity at all time intervals compared to control (Fig. 2D).

Chitinase activity. Chitinase activity was significantly higher ($F = 1029.1$; df $= 2$; $P < 0.001$) 12 h after treatment of larvae with H_2O_2 (295 mg/L) than in the controls. At 24 and 36 h after treatment, enzyme activity was significantly lower than in the controls ($P < 0.001$) (Fig. 2E). No differences between treated and control groups were observed at 12, 24, or 36 h after injection with dopamine ($P < 0.05$).

Discussion

When injected into *S. surcoufi* third-instar larvae, dopamine significantly depressed total protein content and the activity of proteases and phenoloxidases within 12 to 24 h after injection of the larvae. Chitinase activity was not significantly affected by injection of dopamine, while the level of total antioxidants was significantly higher in larvae injected with dopamine than in control larvae.

These findings appear to be consistent with those of Luqing et al. (2011) who found injection of dopamine into the Pacific whiteleg shrimp, *Litopenaeus vannames* Boone, significantly depressed protease and phenoloxidase activity within 6 to 9 h posttreatment. They postulated that the increased levels of dopamine interfered with normal hemocyte activity and the pro-phenoloxidase system. Matsumoto et al. (2003) found that increases in dopamine in *Mythima* (=*Pseudaletia*) *separata* Walker larvae increased metalloprotease activity with a concomitant decrease in total protein content. They determined that increases in dopamine concentration in the hemolymph resulted in an influx of dopamine into the brain through the externally damaged sheath, followed by apoptosis of brain cells.

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Biogenic amines, including dopamine, are widely distributed in the central nervous system and peripheral organs of insects where they may act as neuroregulatory chemicals (Duch et al. 1999). Péqueux et al. (2002) found that environmental stress leads to fluctuations in dopamine concentrations in insect hemolymph. Disrupting the mechanics of dopamine eventually leads to the insect death (Nuss et al. 2015).

With the exception of phenoloxidases, H_2O_2 dramatically reduced enzyme activity over time following injection of H_2O_2 into third-instar *S. surcoufi*. Protease activity was significantly depressed at 12, 24, and 36 h posttreatment. Total protein content was elevated at 12 h posttreatment, but was significantly lower at the 24- and 36-h sampling intervals, which could have been a delayed response to the protease activity observed. The activity levels of antioxidants and chitinase were similar with elevated levels at 12 h posttreatment followed by decreases in activity by 24 h (chitinase) and 36 h (antioxidants). Phenoloxidase activity, however, was significantly elevated at all three sampling intervals. Phenoloxidase is a marker enzyme for physiological stress (Dorrah et al. 2019).

These observed physiological responses to H_2O_2 may be the result of several factors. Because oxidants such as H_2O_2 release toxic by-products, such as free radicals, they can act by inducing oxidative stress, leading to death of target insects (Kodrík et al. 2015, Sohal and Dubey 1994). It may deactivate specific enzymes by oxidation of cofactors. Protease plays an important role in the removal of oxidatively damaged products in living systems (Cadenas and Davies 2000) and, thus, reduction in protease activity can have a deleterious physiological impact on insects.

H_2O_2 causes cell membrane damage due to its oxidation effect on the protein cell membrane content (protein oxidation) (Bi and Felton 1995, Shacter 2000). Breakdown of protein into free amino acids, due to the treatment stress, could result in the observed decrease in total protein. Protein depletion might play a role in the compensatory mechanisms under stress (Ali et al. 2014). While total protein was significantly lower in injected versus noninjected *S. surcoufi* larvae 36 h posttreatment, the content was only 10.8% lower than that of the controls and may possibly reflect the insect trying to physiologically overcome the effect of the H_2O_2 with production of defense proteins (Arakane and Muthukrishnan 2010). Phenols, which were elevated at all sampling intervals, also have an important role in activation of defensive enzymes (Renault et al. 2016, Shelby and Popham 2006).

In insects, antioxidants are an important component of defense mechanisms against both exogenous and endogenous oxidative radicals (Krishnan and Kodrík 2006), and alterations in the activities of antioxidant and metabolic enzymes correlate with physiological and metabolic activities (Jovanovic-Galovic et al. 2004, Renault et al. 2018). Increased oxidative stress leads to up-regulation of antioxidant enzymes in order to detoxify their effects, as has been reported by Korayem et al. (2012) with *Apis mellifera* L., Ahmad and Pardini (1990) with *Trichoplusia ni* (Hübner) larvae, Micheal and Subramanyam (2014) with *Bombyx mori* L., Renault et al. (2016) with *Schistocerca gregaria*, Forsskål and Renault et al. (2018) with *Chrysomya albiceps* (Wiedemann) larvae. Lukasik et al. (2009) also reported that increased oxidative stress resulted in a significant depletion of the primary nonenzymatic antioxidant, ascorbate, which together with ascorbate peroxidase eliminates toxic H_2O_2 in aphids.

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While the response in chitinase activity in third-instar *S. surcoufi* larvae injected with H_2O_2 is unclear, it is known that H_2O_2 enhances chitinase gene expression (Ma et al. 2009). The cuticle, constituting chitin primarily, is the first line of passive defense in insects, and chitinases or lysozymes with chitinase activity are involved in immune response (Elmogy et al. 2015, Mohamed et al. 2016, Narayanan 2004). Any interference with chitinase activity may limit formation of competent cuticle barriers.

In conclusion, the present study addressed the use of natural xenobiotics in insect control. H_2O_2 is a compound that occurs in nature associated with anthropogenic activities and is known for its toxic effect on different organisms (Watt et al. 2004). It is currently used as a bioinsecticide (Gašić and Tanović 2013). Both dopamine and H_2O_2 can affect *S. surcoufi* third-instar larvae by targeting different biochemical and regulatory molecules in different metabolic pathways. Our results demonstrate that the toxic effects of dopamine and H_2O_2 were evidenced by significant changes in the activity of defensive enzymes. Further studies on the pharmacological characterization of insect dopamine receptors may help us to develop new specific insecticides for pest management.

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