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Integration between oxidative stress parameters to evaluate insects' tissues response as a result of normal levels exposure of environmental pollutants

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Abstract
Monitoring of possible impact of environmental pollutants concentration can be assessed using oxidative stress parameters such as protein carbonyls amount, levels of and antioxidant enzymatic system. The potential assessment ensures the impacts of environmental contamination on living organisms. The concentration of protein carbonyls in the brain of *Coccinella undecimpuctata* and *Physiophora alceae* were significantly difference between each other with the value of 48.77, and 28.66 respectively. Also, the activities of antioxidant enzymes such as CAT, and APOX were significantly difference. Specific pollution resulting from the normal levels of the environmental pollutants caused comparable adverse effects in the brain of flies and ladybird beetle when compared with other tissues. Therefore, the success of using of insect tissue sensitivity, inform of oxidative stress parameters, as a result of exposure to normal levels of environmental pollutants was discussed.

Keywords: Tissue sensitivity, oxidative stress, protein carbonyls, antioxidant enzymes, environmental pollutants, insect

Introduction

Biological tissues require oxygen to meet their energetic demands. However, the consumption of oxygen also results in the generation of free radicals that may have damaging effects on cells. The brain is particularly vulnerable to the effects of reactive oxygen species (ROS) due to its high demand for oxygen, and its abundance of highly peroxidizable substrates. Oxidative stress which is defined as the potential of oxygen free radicals and other reactive oxygen species (ROS) to damage tissues and cellular components. Oxidative stress is caused by an imbalance in the redox state of the cell (Hermes-Lima, 2004) [21], either by overproduction of reactive oxygen species, or by dysfunction of the antioxidant systems. (Migula *et al* , 2004; Azam *et al* , 2015; Nassar *et al* . 2020; Abdelfattah *et al* ., 2021) [32, 7, 33,].

Environmental pollution is considered as a serious problem, both in developed and developing countries even its levels within normal range, so environmental pollution has become an issue of serious international concern (vij, 2015). Diverse protective systems must exist to enable adaptation to oxidative environments. Oxidative stress (OS) results when production of (ROS) exceeds the capacity of cellular antioxidant defenses to remove these toxic species. There are three major ROS that are of physiological significance are superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) (Gilbert, 2000; Abdelfattah, 2016; Abdelfattah *et al* , 2017; Abdelfattah, 2020; Abdelfattah *et al* ., 2021) [1-4, 41].

Epidemiological and clinical studies have linked environmental factors such as diet and lifestyle to cancer, diabetes, atherosclerosis, and neurodegenerative disorders. All of these conditions, as well as the aging process, are associated with OS due to elevation of ROS or insufficient ROS detoxification. Many environmental pollutants engage signaling pathway that is activated in response to OS. The same sequences of events are also associated with the etiology and early pathology of many chronic diseases. Investigations of oxidative responses in different *in vivo* models suggest that, in complex organisms such as mammals, organs and tissues contain distinct antioxidant systems, and this may form the basis for differential susceptibility to environmental toxic agents. Thus, understanding the pathways leading to the

induction of antioxidant responses will enable development of strategies to protect against oxidative damage. (Schmidt and Ibrahim, 1994; Yousef, *et al* ., 2017; Renault *et al* ., 2016) [36, 40, 35].

The balance between pro-oxidant endogenous and exogenous factors (environmental pollutants) and antioxidant defenses (enzymatic and non-enzymatic) in biological systems can be used to assess toxic effects under stressful environmental conditions, especially damage induced by different classes of chemical pollutants.(Yousef *et al* ., 2019) [41]. Toxic metals (lead, cadmium, mercury and arsenic) are widely found in our environment. Humans are exposed to these metals from numerous sources, including contaminated air, water, soil and food. (Kassir *et al* ., 2012) [23]. Highly reactive molecules called free radicals can cause tissue damage by reacting with polyunsaturated fatty acids in cellular membranes, nucleotides in DNA, and critical sulfhydryl bonds in proteins. Free radicals can originate endogenously from normal metabolic reactions or exogenously as components of tobacco smoke and air pollutants and indirectly through the metabolism of certain solvents, drugs, and pesticides as well as through exposure to radiation (Yousef *et al* ., 2019; Abdelfattah *et al* ., 2021) [4, 41].

Defenses against free radical damage include tocopherol (vitamin E), ascorbic acid (vitamin C), beta-carotene, glutathione, uric acid, bilirubin, and several metalloenzymes including glutathione peroxidase (selenium), catalase (iron), and superoxide dismutase (copper, zinc, manganese) and proteins such as ceruloplasmin (copper). The extent of tissue damage is the result of the inbalance between the free radicals generated and the antioxidant protective defense system. Several dietary micronutrients contribute greatly to the protective system. (Livingstone, 2001; Abdelfattah *et al* ., 2020) [28, 2]. Insects such as lady beetles and flies, can be seen, as sensitive to environmental changes. They can be considered as an interesting subject of Eco toxicological research, and a bio monitor of environmental pollutant, including heavy metals (Chen *et al* ., 2005; Abdelfattah *et al* ., 2021) [12, 4].

In line with the 2030 Agenda (Egypt's vision 2030), the Egyptian Government has launched a working plan called Egypt's Vision 2030 especially governance and sustainable natural resource assets to support the economy and increase competitiveness and create new jobs and keeping the balance between ecological systems and biodiversity, also known as Sustainable Development Strategy (SDS), which encompasses the economic, social and environmental dimensions of development. SDS promotes economic flourishing based on justice, social integrity and participation. It is under the SDS that all development plans in Egypt are incorporated while at the same time being strongly guided by the SDGs. Also, at the United Nations Sustainable Development Summit on 25 September 2015, world leaders adopted the 2030 Agenda for Sustainable Development, which includes a set of 17 Sustainable Development Goals (SDGs) to end poverty, fight inequality and injustice, and tackle climate change by 2030. So, the present work was conducted to assess the sensitivity response of various insect tissues, in form of oxidative stress parameters, as a result of exposure to the normal levels of environmental pollutants on living organisms by a) evaluate macromolecules oxidative damage, protein carbonyls level, and lipid peroxides concentration. b) Evaluate antioxidant enzymatic level such as (APOX, and CAT) in the brain and cuticle tissues of the adult insects, *Coccinella undecimpuctata* and *Physiophora alceae*.

Materials and Methods

The samples collected from Hawd El-Akhmas, Cairo University Campus, (Latitude 30◦ 1 ′ 48.76″N) (longitude 31◦ 11′ 23.07″). the samples were collected in autumn using the sweep method, the killing glass jar used contain acetone or ethyl acetate can melt the plastic and insects can get stuck in it, the percentage of each collected insect order were visualized using pie chart. The selected experimental insects were dissected to isolate samples tissue like brain and gut for further analysis and were stored at -20◦C until using it in experimental analysis and compared with laboratory insect species.

Antioxidant enzymes assays

The activity of catalase (CAT) was assessed in compliance with the method of Aebi (1984) [5]. The reaction mixture contained 0.9 mL of a potassium phosphate buffer (50 mM, pH 7.0), 60 µL of the supernatant and 40 µL of freshly prepared H_2O_2 (10 mM). The change in absorbance was measured at 240 nm over a period of 1 min. The CAT activity was expressed as OD/µg protein/min. In addition to, the activity of ascorbate peroxidase (APOX) was determined according to Asada (1984) [6]. The reaction mixture containing 0.9 mL of phosphate buffer (100 mM, pH 7.0) containing 0.1 mM EDTA, 0.3 mM ascorbic acid, 0.06 mM H_2O_2 and 0.1 mL of the enzyme extract from each sample tissue. Distilled water instead of enzyme extract served as the controls. The change in absorbance was recorded using a spectrophotometer at 290 nm over a period of 30 s after the addition of H_2O_2. The APOX activity was expressed as OD/µg protein/min.

Oxidative stress damage assays

Protein carbonyls assay were assessed according to the procedure from Levine *et al* . (1990) [27], with the below-described modifications. After tissue isolation, samples were homogenized in 5 mL ice-cold phosphate buffer (60 mL of 50 mM phosphate buffer, 10 mL of 0.1% Triton X100, 5 mL of 0.05 mM $CaCl_2$; then completed to 100 mL with distilled water after adjusting pH to 7.0 with 2M HCl or NaOH). After homogenization (mortar, 10 strokes/ 30 seconds), the samples were centrifuged at 2000 ×g for 10 min at 4 °C. For each tissue extract, a 800 µL aliquot of the supernatant was transferred to a clean microtube with 200 µL of 10 mM 2, 4- dinitrophenyl hydrazine (DNPH) prepared in 2 M HCl. The samples were incubated for 30 minutes at room temperature, precipitated with 10% Tricholoroacetic acid (TCA), and left for 10 min at 4 °C. The samples were centrifuged at 5000 ×g for 7 min at 4°C. The pellet was washed four times with an ethanol/ethyl acetate (1:1) mixture, and redissolved in 1 mL of sodium phosphate buffer (60 mL of 150 mM phosphate buffer, 30 mL of 3% sodium dodecyl sulphate, adjusted to a final volume of 100 mL with distilled water after adjusting the pH to 13 6.8 with 2M HCl or NaOH). Insoluble material was removed by centrifugation at 2000 ×g for 1 min. Finally, the absorbance was measured at 366 nm, and the rate of protein carbonyls concentration was expressed as OD/mg protein. Blank was similarly prepared and treated as above except for DNPH that was not added to the sample.

The lipid peroxides concentration was measured according to Hermes-Lima *et al* . (1995) [22]. Midgut and thoracic muscles were isolated in phosphate buffer (pH 7.0), and homogenized in ice-cold methanol (1:5, w/v). After homogenization (mortar, 10 strokes/30 seconds), the samples were centrifuged at 2000 *g* for 10 min at 4 °C. A 5 mL aliquot of the supernatant was used for the assay. The following components were sequentially added to the samples (200 µL of supernatant): 400 µL of 1 mM $FeSO_4$, 200 µL of 0.25 M H_2SO_4, and 200 µL of 1 mM xylenol orange. Samples were then incubated under dark conditions at room temperature for 3 h. The absorbance was measured at 580 nm. Then, 10 µL of 0.5 mM cumene hydroperoxides (as an internal standard) was added to each sample, 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. Lipid peroxides concentration was expressed as mM cumene hydroperoxides/µg protein.

Total protein amount

Total protein concentration was quantified for each body sample with Bradford's method (Bradford 1976), using Coomassie Brilliant Blue (COBB). A volume of 0.9 mL of the dye reagent (10 mg COBB mixed with 5 mL methanol and 10 mL of 85% O-phosphoric acid, completed to 100 mL with distilled water) was mixed with 0.1 mL of body sample, then left for 2 min before measuring the optical density of the samples at 595 nm. The blank consisted in distilled water instead of the protein sample. Bovine serum albumin (BSA) fraction V (Sigma-Aldrich) dissolved in 0.15 M NaCl was used as drawing the calibration curve.

Statistical analysis

As a first step, a Generalized Estimating Equation (GEE) was computed to examine tissue samples, insect species, area samples, and the interaction of these terms affected significantly the oxidative stress parameters we measured (PC, LP, CAT, APOX). In a second step, the effect of the field collection and lab collection on the levels of PC, LP, CAT, APOX in the brain and cuticle of *C. undecimpuctata* and *P. alceae* were assessed by performing a one-way analysis of variance (ANOVA), followed by Tukey′s *post-hoc* tests when necessary. The comparison of the amounts of PC, LP, CAT, APOX among the different tissues was also conducted by performing a one-way ANOVA. All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 17.0. Armonk, NY: IBM Corp.).

Results

The number of insect species and orders which collected from field site, Hawd El-Akhmas, Cairo University Campus, (Latitude 30◦ 1 ′ 48.76″N) (longitude 31◦ 11′ 23.07″), were showed in Fig. (1). The pie chart showed that the percentage of dipteran species has the largest species number, then coleopteran species, however, the least occurred insect species in this area was lepidopteran species (Fig. 1). The level of CAT activity in adult insects collected from field sites revealed a significant incresed compared to individuals from the control site (Fig. 2 A and B). Compared to control and field insects, the median values in brain, were significantly increased than cuticle tissue homogenates ($p < 0.05$). (Fig. 2 A and B). Also, the results ensured this relation on the activity of APOX (Fig. 3 A and B). The results reveled that the relative levels of protein carbonyls and lipid peroxides in insects were shown in Fig. 4 and 5. The insects collected from the field site showed significant increase in protein carbonyls and lipid peroxide level compared to the control site insects (Fig. 4 and 5). In the field site, the variations in contents of carbonyls in respect to control site was higher in brain than cuticle (Fig. 4 A and B), with respect to control value. The lipid peroxides concentrations in tissues of insects collected from field sites were differed significantly. In the experimental tissues, the median values in insects from field sites were always significantly higher in relation to the control insects (Fig. 5 A and B). In *C. undecimpuctata* collected insect, the lipid peroxides increased in the filed site than *P. alceae* insect especially brain homogenates with the factor of 0.66 x-fold . The lipid peroxides concentrations were the highest in insects collected from field sites. The lowest values were recorded mainly in individuals collected from control sites (Fig. 5 A and B). The interaction analysis using Generalized Estimating Equation (GEE) showed significant influence of tissues, species, and collected sites on level of oxidative stress parameters (CAT, APOX, protein carbonyls and lipid peroxides) (Table 1), except for CAT in case of interaction of species with sites and species with tissues, also, interaction among species with tissues in protein carbonyls and finally tissues of protein carbonyls (Table 1).

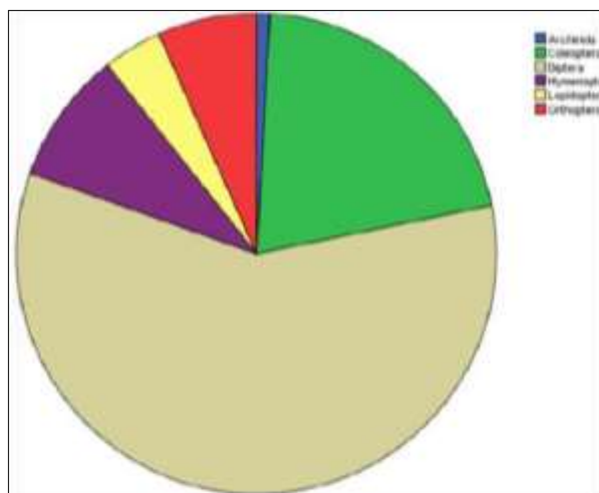

Fig 1: Number of insects, classified into orders, collected from field site, Hawd El-Akhmas, Cairo University Campus, (Latitude 30◦ 1 ′ 48.76″N) (longitude 31◦ 11′ 23.07″).

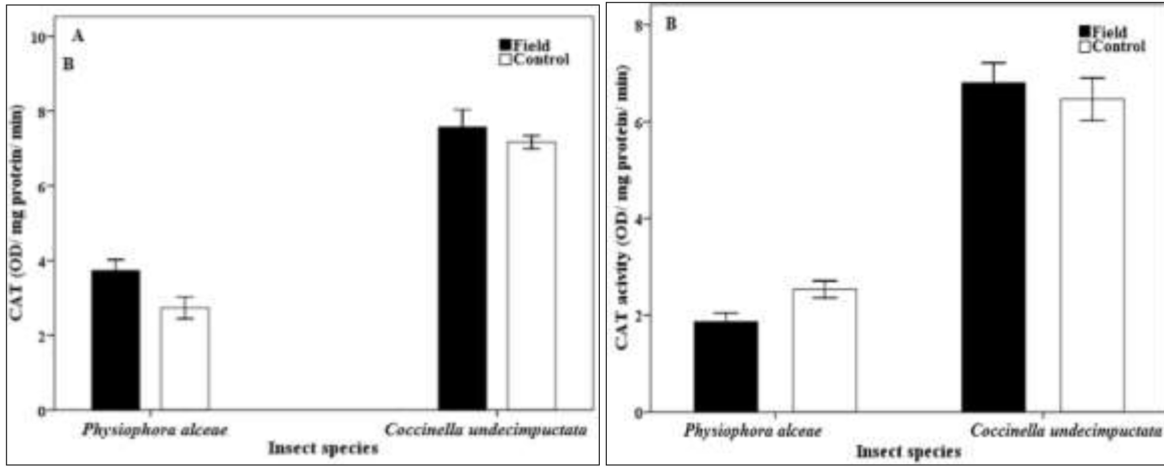

Fig 2: Activity of catalase (CAT) antioxidant enzymes, expressed as mean and SE of CAT activity obtained from brain (A), and cuticles (B) of *Physiophora alceae and Coccinella undecimpuctata* adult insect, which were collected from control and field area. Mean values marked with * are not significantly different among control and field sites (Tukey′s *post-hoc*, $p > 0.05$).

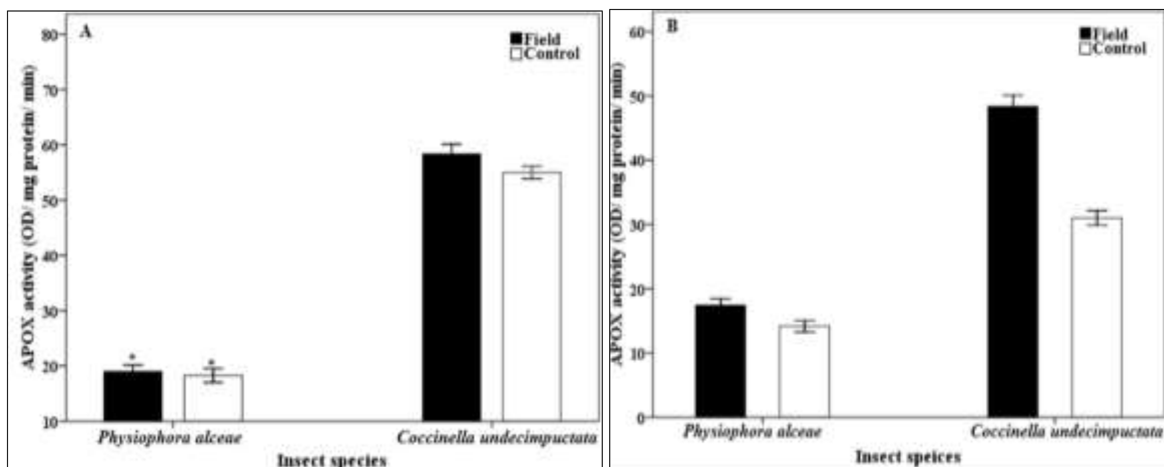

Fig 3: Activity of ascorbate peroxidase (APOX) antioxidant enzymes, expressed as mean and SE of APOX activity obtained from brain (A), and cuticles (B) of *Physiophora alceae and Coccinella undecimpuctata* adult insect, which were collected from control and field area. Mean values marked with * are not significantly different among control and field sites (Tukey′s *post-hoc*, $p > 0.05$).

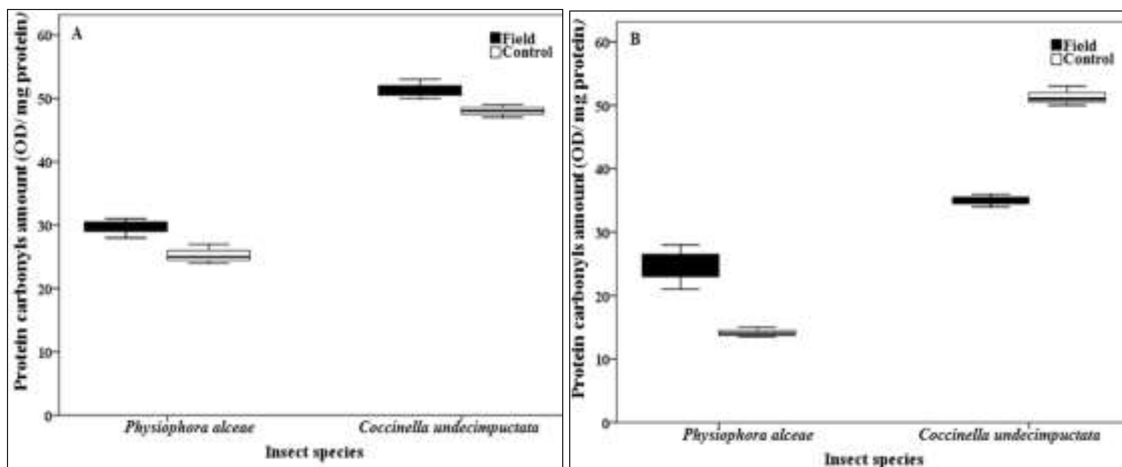

Fig 4: Protein carbonyls amounts expressed as mean and SE of protein carbonyls amount obtained from brain (A), and cuticles (B) of *Physiophora alceae and Coccinella undecimpuctata* adult insect, which were collected from control and field area mean values marked with * are not significantly different among control and field sites (Tukey′s *post-hoc*, $p > 0.05$).

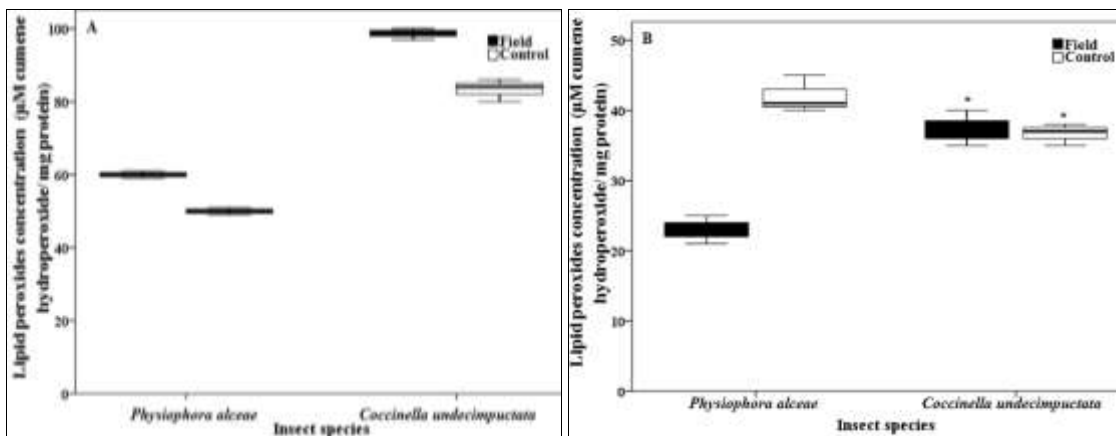

Fig 5: Lipid peroxides concentration, expressed as mean and SE of lipid peroxides concentration obtained from brain (A), and cuticles (B) of *Physiophora alceae and Coccinella undecimpuctata* adult insect, which were collected from control and field area. Mean values marked with * are not significantly different among control and field sites (Tukey′s *post-hoc*, $p > 0.05$).

Table 1: Generalized Estimating Equation to analyze the interactions among tissues, species, and sites on oxidative stress parameters (CAT, APOX, protein carbonyls and lipid peroxides).

Source	Chi-square (χ^2)	*df*	*p* value
Species			
CAT	1981.4	1	<0.0001
APOX	74516.0	1	<0.0001
Protein carbonyls	1833.8	1	<0.0001
Lipid peroxides	781.5	1	<0.0001
Sites			
CAT	8.4	1	<0.05
APOX	217.4	1	<0.0001
Protein carbonyls	0.5	1	<0.0001
Lipid peroxides	5.6	1	<0.0001
Tissues			
CAT	84.8	1	<0.0001
APOX	891.2	1	<0.0001
Protein carbonyls	175.5	1	<0.05
Lipid peroxides	3792.2	1	>0.01
Species × Sites			
CAT	1.1	1	>0.05
APOX	99.2	1	<0.0001
Protein carbonyls	130.5	1	<0.0001
Lipid peroxides	72.3	1	<0.0001
Species × Tissues			
CAT	2.4	1	>0.05
APOX	454.7	1	<0.0001
Protein carbonyls	2.1	1	>0.01
Lipid peroxides	642.9	1	<0.0001
Sites × Tissues			
CAT	22.5	1	<0.0001
APOX	109.1	1	<0.0001
Protein carbonyls	29.4	1	<0.0001
Lipid peroxides	297.0	1	<0.0001
Sites × Tissues ×Species			
CAT	19.2	1	<0.0001
APOX	52.4	1	<0.0001
Protein carbonyls	108.1	1	<0.0001
Lipid peroxides	32.1	1	<0.0001
Intercept			
CAT	10196.6	1	<0.0001
APOX	33255.5	1	<0.0001
Protein carbonyls	16987.2	1	<0.0001
Lipid peroxides	22127.4	1	<0.0001

Discussion

In the present work, part of assessment of biomonitoring program was designed to evaluate the biochemical changes, inform of oxidative stress parameters (CAT, APOX, protein carbonyls and lipid peroxides), in different insect species, *Physiophora alceae and Coccinella undecimpuctata*, collected from different sites field site, Hawd El-Akhmas, Cairo University Campus, (Latitude 30◦ 1′ 48.76′′N)

(longitude 31◦ 11′ 23.07″), with respect to control insect (Fig. 1-5). Also, the interaction between all the factors on the oxidative stress parameters were determined using generalized estimating equation model (Table 1). The biomonitoring programs involve bioaccumulation, biochemical alterations, morphological and behavior observation, population- and community-level approaches. The applications of biomonitoring programs are evaluation of metal concentration, toxicology prediction and researches on toxicological mechanism, toxicological prediction and bioremediation (Blackmore and Wang, 2004; Zhou *et al* ., 2008; Abdelfttah *et al* ., 2021) [9, 24]. Toxicological mechanism included detecting the action mechanism through interaction between pollutants and biological macromolecules such as proteins, enzymes and nucleic acid (Markert, *et al* ., 2003) [31]. The suitable bioindicators groups which capable of reflecting the pollutants in the ecosystem (Klein, 1999) [26]. Proper quality management system (QA) should be applied to make sure that biomonitoring studies are scientifically valid, comparable and robust to evaluate the relationship between environmental pollutants and its toxicological effect in living organism.

Environmental pollutants lead to imbalance between ROS levels and antioxidants of exposed organisms and cause oxidative stress such as hydrofluoric acid fumes HF, SO_2, NO_2, PM_{10}, waste products of fertilizer industry such as PO_4^{3-}, SO_4^{2-}, dust, and heavy metals can increase production of ROS in cells of exposed organisms and caused oxidative stress. The pollutants which used in industry and agriculture include metals, metalloids, and numerous other organic compounds, lead to increase the production of reactive oxygen species (ROS) in the cells of individuals exposed to them, and therefore caused oxidative stress with all the adverse consequences for organisms (Farahat *et al* ., 2010; Okamoto *et al* ., 2014; Zhu *et al* ., 2014; Shinkai *et al* ., 2015; Yousef *et al* ., 2017; Abdelfattah *et al* ., 2017; Abdelfattah 2020; Abdelfattah *et al* ., 2021) [40, 2, 3, 41]. This might explain the fluctuating results among the different insect source whatever from field site or control. In the present work, the level of stress was evaluated indirectly, by assessing oxidative damage of macromolecules: proteins (Fig. 4 A and B) and lipids (Fig. 5 A and B), as well as measurement of enzymatic antioxidant (CAT, and APOX,) (Fig. 2-3) response in the brain, and cuticle homogenates of *P. alceae and C. undecimpuctata*,. This was done in accordance with generally accepted knowledge (Fang *et al* ., 2002; Halliwell and Whiteman, 2004; Yousef *et al* ., 2019) [15, 20, 41].

The obtained results revealed the information that proteins are important targets of free radical attack in the cells (Lushchak, 2011; Renault *et al* ., 2016) [30, 35], and thus the antioxidant defense, cellular function, and finally organism survival can be impaired. ROS are known to convert amino groups of proteins and thereby, change protein structure and function. The oxidative stress increased the number of modified carbonyl groups correlates with protein damage (Hermes-Lima, 2004) [21]. Also, ROS can cause fragmentation of the peptide chain, alteration of electrical charge of proteins, cross-linking of proteins, and oxidation of specific amino acids and therefore lead to increase susceptibility to proteolysis by degradation of specific proteases (Kelly and Mudway, 2003) [24]. The oxidation of proteins leads physiologically to disruption of conformation and vital functions of protein molecules, including enzymes, and other regulatory functions of the cell (Abdelfattah *et al* ., 2021) [4]. In addition to, lipid peroxidation usually measured as a level of lipid peroxides has been used frequently to analyze the effect of environmental pollutants (Livingstone, 2001; Lushchak, 2011) [28, 30]. Lipid peroxidation products, such as isoprostanes and thiobarbituric acid reactive substances, were used as indirect biomarkers of oxidative stress (Birben *et al* ., 2012) [8]. As in the case of protein carbonyls, the highest concentration of lipid peroxides was observed in tissues of individuals from site A. Significant oxidative damage, including lipid peroxidation occurred if antioxidant defense systems are overwhelmed by ROS production (Halliwell and Gutteridge, 2015) [19].

High level of ROS lead to increases the activity of antioxidant enzymes, such as catalase (CAT), and ascorbic acid peroxides (Fig. 1 and 2). There are key antioxidant enzymes responsible for scavenging of oxygen radicals (Donahue *et al* ., 1997; Khaper *et al* ., 2003; Halliwell and Gutteridge, 2015; Dutta *et al* ., 2016; Yousef *et al* ., 2019; Abdelfattah *et al* ., 2021) [19, 13, 25, 4]. However, environmental pollutants such as heavy metals increase the production of reactive oxygen species (ROS), and, directly or indirectly, cause oxidative damage by inhibiting activity of antioxidant enzymes. Previous studies suggested that inhibition of antioxidant enzymes activity occurred due to decrease expression level of antioxidant enzymes (Chaitanya *et al* ., 2016) [11]. Catalase scavenges H_2O_2 at high concentrations, whereas ascorbate peroxidase scavenges H_2O_2 at low concentrations, the negative significant correlation between enzymes activity was revealed. The key role of CAT in ROS scavenging, also in plants, was studied (Sofo *et al* , 2015) [38]. Also, APOX, catalyzes the reduction of H_2O_2 with consumption of ascorbate as the reducing agent. Therefore, APOX activity depends exclusively on the availability of reduced ascorbate. Under normal conditions the cellular pool of ascorbate is kept in a reduced state by a set of enzymes, namely mono-dehydroascrobate reductase (MDAR) and dehydroascorbate reductase (DHAR) capable of using NAD(P)H to regenerate oxidized ascorbate (Farooqui and Farooqui, 2011) [17]. The present work confirmed the positive correlation between APOX activity in brain of both inset species, in addition to assess the sensitivity response of various insect tissues, in form of oxidative stress parameters, as a result of exposure to the normal levels of environmental pollutants on living organisms by evaluating macromolecules oxidative damage, protein carbonyls level, and lipid peroxides concentration. Also, evaluation the antioxidant enzymatic level such as (APOX, and CAT) in the brain and cuticle tissues of the adult insects, Coccinella undecimpuctata and Physiophora alceae.

Declarations

Ethical approval and consent to participate

This article does not contain any studies with human participants or animals that require ethical approval.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable

request.

Competing interests
The authors declare that they have no conflict of interest.

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