

RESEARCH ARTICLE

Biochemical and histological biomarkers in the midgut of *Apis mellifera* from polluted environment at Beheira Governorate, Egypt

Ahmed M. Abu El-Saad^{1,2} · Dalia A. Kheirallah¹ · Lamia M. El-Samad¹

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Abstract The aim of this study was to analyze the impact of organophosphorus (OP) pollutants on oxidative stress and ultrastructural biomarkers in the midgut of the honeybee *Apis mellifera* collected from three locations that differ in their extent of spraying load with OP insecticides: a weakly anthropised rural site, Bolin which is considered as a reference site; moderately spraying site, El Kaza; and a strongly anthropised urban site, Tiba with a long history of pesticide use. Results showed that high concentrations of chlorpyrifos, malathion, diazinon, chlorpyrifos-methyl, and pirimiphos-methyl were detected in midgut at locations with extensive pesticide spraying. Reduced glutathione content, superoxide dismutase, catalase, and glutathione peroxidase displayed lowest activities in the heavily sprayed location (Tiba). Lipid peroxidation level in the midgut of honeybees in the sprayed locations was found to be significantly higher compared to the reference values. Meanwhile, various ultrastructural abnormalities were observed in the epithelial cells of midgut of honeybees collected from El Kaza and Tiba, included confluent and disorganized microvilli and destruction of their brush border, the cytoplasm with large vacuoles and alteration of cytoplasmic organelles including the presence of swollen mitochondria with lysis of matrices, disruption of limiting membranes, and disintegration of cristae. The nuclei with indented nuclear envelope and disorganized chromatin were observed.

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✉ Ahmed M. Abu El-Saad
amhafez@uod.edu.sa; ahmedmokhtar8@yahoo.com

¹ Department of Zoology, Faculty of Science, Alexandria University, Alexandria 21511, Egypt

² Department of Biology, Faculty of Medicine, Dammam University, Dammam 34212, Saudi Arabia

These investigated biomarkers indicated that the surveyed honeybees are being under stressful environmental conditions. So, we suggest using those biomarkers in the assessment of environmental quality using honeybees in future monitoring of ecotoxicological studies.

Keywords Biomarker · Honeybees · Biomonitoring · Oxidative stress · Midgut

Introduction

The indiscriminate use of pesticides for the eradication of pests causes tremendous changes in the environment and also in other nontarget organisms (Ambika and Selvisabhanayakam 2012; Hladik et al. 2016). Egypt is one of the developing countries which heavily uses pesticides in agriculture and is sharing most of the environmental problems. The quantity of pesticides used in Egypt and Africa since the mid-1960s has been greatly increased and large quantity has been injected into the Egyptian environment (Amr 1999). Latest Egyptian Agriculture Ministry reports put the total amount of pesticides imported last year at about 7 billion tons (Khaled 2014). Consequently, Egypt has unique circumstances of public health problems making it an ideal setting for increased attention to environmental pollution research. In view of the important and often irreversible impacts of human activity on the ecosystem, there is an increasing need to develop tools to monitor the impacts of pollution. However, characterization of the physiological integrity and functionality of individuals requires tools to act as biomarkers of exposure to environmental stressors. Biomarkers can be defined as observable or measurable modifications at the molecular, cellular, physiological, or behavioral levels, which reveal the exposure of an organism to xenobiotics (Lionetto et al. 2003). Biomonitoring programs are usually based on studying a set

of biomarkers in sentinel species of interest (Damiens et al. 2007; Lambert et al. 2012).

The honeybees are particularly pertinent model for the development of biomarkers in order to assess environmental contamination by anthropogenic chemicals (Wu et al. 2011a, 2011b). They can constitute reliable indicators of environmental quality because their intense foraging activity brings them into contact with a large number of pollutants within a radius that generally ranges from 1.5 to 3 km around the hive, depending on food abundance (Chauzat et al. 2009). Honeybees are good biological indicators because they give information about the chemical impairment of the surroundings they live in through two signals: the high mortality and the residues present in their bodies or in beehive products (Krupke et al. 2012; Hladik et al. 2016). A decline in honeybee populations is currently being seen in many parts of the world, resulting in an active strategy for the monitoring and diagnosis of population health (Nguyen et al. 2009; Schneider et al. 2012; Fairbrother et al. 2014; Codling et al. 2016). The honeybee is therefore a species of particular interest in terrestrial ecotoxicology because its physiology, behavior, and ecology have been extensively studied (Hardstone and Scott 2010; Alaux et al. 2010; Henry et al. 2012; Chakrabarti et al. 2015; Gauthier et al. 2016). It may also be worth noting that several ethological and morphological characteristics make the honeybee a reliable ecological detector: it is easy to breed, almost ubiquitous organism, with modest food requirements; its body is covered with hairs, which make it particularly suitable to hold the pollutants it comes into contact with; its very high rate of reproduction and relatively short average lifespan cause the colony to undergo rapid and continuous regeneration; and its great mobility and wide-flying range allow a vast area to be monitors (Bénéteau et al. 2013; Boily et al. 2013).

Biochemical studies are increasingly used in ecological risk assessments to identify the incidence of exposure to xenobiotics (Renzi et al. 2016). Terrestrial organisms, including herbivorous insects, are subjected to oxidative stress resulting from overproduction of reactive oxygen species (ROS) under exposure to insecticides including organophosphates, carbamates, and pyrethroids (Ranjbar et al. 2002). Because of widespread use and easy accessibility, poisoning with organophosphorus (OPs) has become a global health problem in both developing and developed countries (Abhilash and Singh 2009). In chronic and subchronic exposures to OPs, induction of oxidative stress has been reported as the main mechanism of toxicity in many studies (Tuzmen et al. 2008). Various experimental studies have shown that oxidative stress in biological systems originates as a result of imbalance between the generation of oxidizing species and cellular antioxidant defenses. The most important antioxidant defense systems include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) as well as nonenzymatic

antioxidants (e.g., glutathione(GSH)), which have been extensively used as biomarkers of oxidative stress (Mamidala et al. 2011; Abdel-Moneim et al. 2013). Oxidative stress has become an important subject for terrestrial and aquatic toxicology (Abdollahi et al. 2004). Recent studies have implicated the oxidative stress as a possible causative mechanism for the nontarget toxicity of pesticides (Osborne 2012; Wang et al. 2014). The study of enzymatic modulation induced by pesticides in honeybees is increasingly inspected (Bénéteau et al. 2012; Carvalho et al. 2013; Badawy et al. 2015).

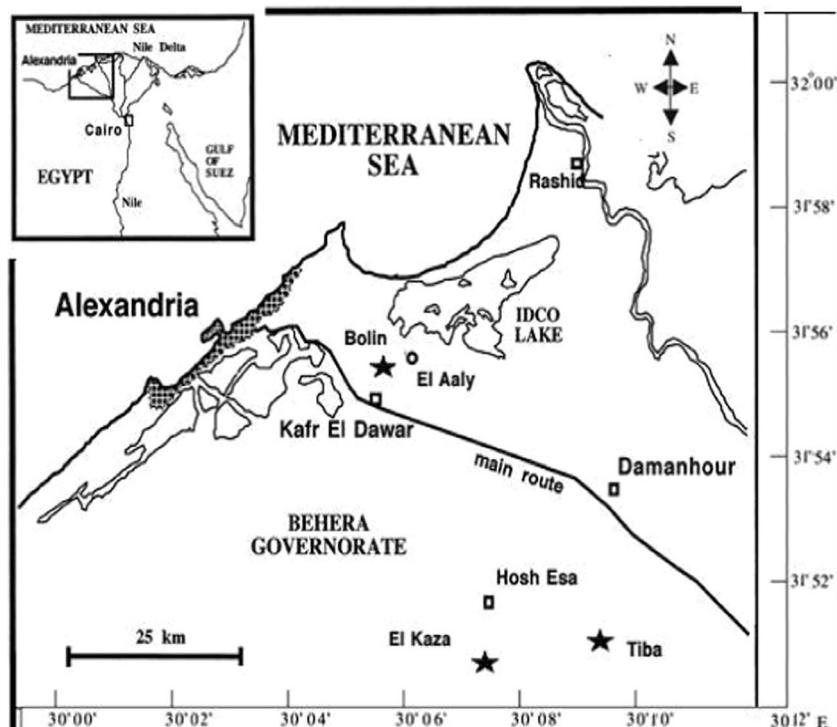
The study of abnormalities affecting cellular and tissue levels is a sensitive tool for environmental quality monitoring (Au 2004). The exposure of insects to pesticides is likely to induce a number of lesions in several organs. As the principal metabolic organ, insect midgut is responsible for food digestion and nutrient absorption (Cristofolletti et al. 2001). The epithelium of the midgut in insects is the main target of pollutants as it represents an effective physical and chemical barrier against the potentially toxic materials that are ingested with the feeding (Sayed et al. 2011; Decio et al. 2013). Hence, the present study focused on the effect of pollution on the midgut of the studied insects. The purpose of the present work was therefore to validate honeybee biomarkers under field conditions. Heavily sprayed (Tiba), moderately sprayed (El Kaza), and seminatural unsprayed (Bolin) locations in Beheira, the north-central part of Egypt, were compared in order to evaluate the impacts of OP insecticides as a common type of local pollution using the discriminating potential of biomarkers. The biomarkers of pollutant toxicity chosen in the present study included lipid peroxidation and antioxidant profiles as well as the identification of the main cellular changes in midgut of workers of *Apis mellifera* bees inhabiting these degraded habitats. Moreover, an attempt was made to render a more representative picture of honeybees exposed to OPs. Therefore, some OP insecticides were also quantified in the midgut of honeybees in order to determine the residue concentrations to which they had been exposed.

Materials and methods

Sampling locations and tested insects

The three study sites were located in Beheira, the north-central part of Egypt ($30^{\circ} 58' 60''$ N and $30^{\circ} 12' 0''$ E; Fig. 1). The three study sites were chosen along an altitudinal and climatic gradient and displayed contrasting degrees of anthropisation as indicated by the variation of the load of OP insecticides spraying: a weakly anthropised rural site, Bolin (in the Kafir El Dawar district) which is relatively free of pesticide applications and was thus considered as a reference site; a moderately spraying site, El Kaza (in Hosh Esa district) which receives two sprays of OP insecticides/year; and a strongly

Fig. 1 Location map showing the location of the three study sites (Bolin in the Kafr El Dawar district, El Kaza in the Hosh Esa district, and Tiba in the Delengate district). Bolin was chosen as the relative reference. Study sites are indicated by stars



anthropised urban site, Tiba (in the suburbs of Delengate district) with a long history of pesticide use, which receives four to six sprays of OP insecticides/year. Selected sprayed locations were considered highly active agricultural areas for many crops. The most common plant communities in the study areas are trees of orange and clovers. There are little available data in the literature regarding the existence of other contaminants in these areas. In addition to OP contamination in these locations, several classes of pesticides including carbamates and pyrethroids (Mansour 2004) are used. In addition, heavy metals (Cu, Ni, Cd, and Pb) are important factors of pollution in the soils of Beheira (Ragab et al. 2007). However, there was no detection of herbicide residues under conventional spraying treatments in soils of agricultural sites at Beheira Governorate (Abdel-Aziz 2006).

To reduce any variations due to geographical factors (microclimates prevailing on Beheira Governorate), the sampling sites were situated within the same ecoregion, separated by a distance lower than 5 km. It was assumed that the foraging zones of the bees were relatively independent and restricted to their respective sites because (i) food resources were deemed to be sufficient in the area surrounding the hives, based on the amount of honey produced, and (ii) a relatively broad and deep ravine separates the sites, dissuading the bees from crossing it (Bénéteau et al. 2013). The relative humidity exhibits little fluctuations as it ranges from 70 to 85 %.

Four *A. mellifera* (Hymenoptera: Apidae) honeybee colonies were placed at each location and sampled during September 2014. These colonies came from low-

anthropised apiary located at Ezbat Haggag, El-Beheira Governorate. They are local hybrid derived from Italian bee *A. mellifera ligustica* and Carniolan bee *A. mellifera carnica* and Egyptian bee. Honeybees were captured at the hive entrance, from morning until early afternoon (8 am to 1 pm) and were free of obvious diseases. Sampling for analysis was carried out simultaneously in the colony of each site, with approximately 350 honeybees workers collected in each site.

Fresh specimens of *A. mellifera* from the studied sites were put in clean plastic bags and transported to the laboratory. The workers of *A. mellifera* were washed with distilled water and sacrificed after anesthesia. The midguts were rapidly dissected out. Half of the sample size of the midgut tissue was stored in vials and kept at -80 °C until used in biochemical and OP residue analyses and the others were used for ultrastructural studies. The technique for collecting the whole midgut tissue is based on the method of Liu (1984).

Determination of organophosphorus (OPs) pollutant residues

Twenty grams of midgut tissue was mixed with 10 g of anhydrous sodium sulfate and homogenized in 100 ml of chloroform + acetone. The homogenate was acidified with 10 drops of concentrated acetic acid and filtered. The filtrate was evaporated to dryness at 35 °C. The residue was redissolved in 1 ml of *n*-hexane + acetone and cleaned up using 10 g of 1 % deactivated Florisil (Diaz et al. 1997). The samples were concentrated using a rotary evaporator to 5 ml and completely

evaporated to dryness under a stream of N_2 . The residues were dissolved in 0.5 ml of *n*-hexane. Recovery experiments of OP pesticides were carried out by using fortified samples through addition of 25 μ g to specimens of tissue samples. The samples were extracted, cleaned up, and determined as described before. Then, concentrations were corrected for 100 % recovery and procedural recovery trials were undertaken which were above 85 %. The analysis was performed on Hewlett-Packard HP-6890 series Gas Chromatograph. The injection port and flame photometric detector, which is very sensitive to halogenated OP insecticides, were operated at 245 and 250 °C, respectively. The data were calculated to final values in parts per billion. The detection limits for the examined compounds were determined. The compound identities were accomplished by comparing retention times against pure multistandards of OP pesticides at the same conditions. The retention times for diazinon, chlorpyrifos-methyl, pirimiphos-methyl, malathion, and chlorpyrifos were 4.80, 5.10, 5.42, 5.63, and 5.78 min, respectively.

Biochemical assay

The midgut of *A. mellifera* collected from different sites was rinsed with distilled water and blotted using a filter paper. The tissues from each location were weighed and homogenized with 10 volumes (*w/v*) of ice-cold saline solution (0.9 %) and 40 mM sodium phosphate using a homogenizer (*Tekmar tissumizer*) for 30 s. The homogenates were centrifuged at 6500 rpm for 30 min at 4 °C using IEC-CRU5000 centrifuge. The resulting supernatants were split and frozen at -20 °C for subsequent analysis. GSH level was assayed spectrophotometrically using the methods followed by Anderson (1989). The activity of SOD was measured according to the method described by Nebot et al. (1993). CAT activity was measured following the decrease in hydrogen peroxide (H_2O_2) concentration at 240 nm (Aebi 1984). To detect the activity of GPx, continuous decrease in NADPH concentration was measured following the method described by Chu et al. (1993). As a marker of LPO, the concentrations of malonaldehyde (MDA) were determined according to the method of Nair and Turner (1984). The protein content of the samples was determined according to the method of Lowry et al. (1951) using crystalline serum albumin as standard.

Transmission electron microscopic study

Specimens of midgut tissues from each study location ($N = 8$ bees/location) were cut into small pieces (1 mm thick) and fixed for 24 h in formalin-glutaraldehyde (4F₁G) then rinsed in phosphate buffer solution (pH 7.2) at 4 °C for 3 h. Specimens were then postfixed for 2 h in 2 % osmium tetroxide (OsO_4), and then the specimens were washed with phosphate buffer several times for 10 min. Specimens were

dehydrated in a graded of ethanol series, followed by propylene oxide, and embedded in epon-araldite mixture in labeled beam capsules. Ultrathin sections (50 nm thick) were cut using glass knives, collected on naked copper mesh grids, and stained with uranyl acetate for 20 min and lead citrate for 20–30 min. The sections were examined and viewed using Jeol 100CX TEM.

Statistical analysis

All the data presented as mean \pm SD were subjected to analysis of variance (ANOVA), and the least square means were compared for significant differences by the Student-Newman-Keuls test at the probability of 0.05. All statistical analyses were conducted using the software package Statistical Package for the Social Sciences (SPSS), version 16.0 (SPSS Inc., Chicago, IL, USA). Normality was tested with the Shapiro-Wilk test, and Levene's test was used to verify homogeneity of variances. Data that displayed heterogeneity of variances were log transformed and analyzed with one-way ANOVA.

Results

Bioaccumulation of organophosphorus pesticides in the midgut

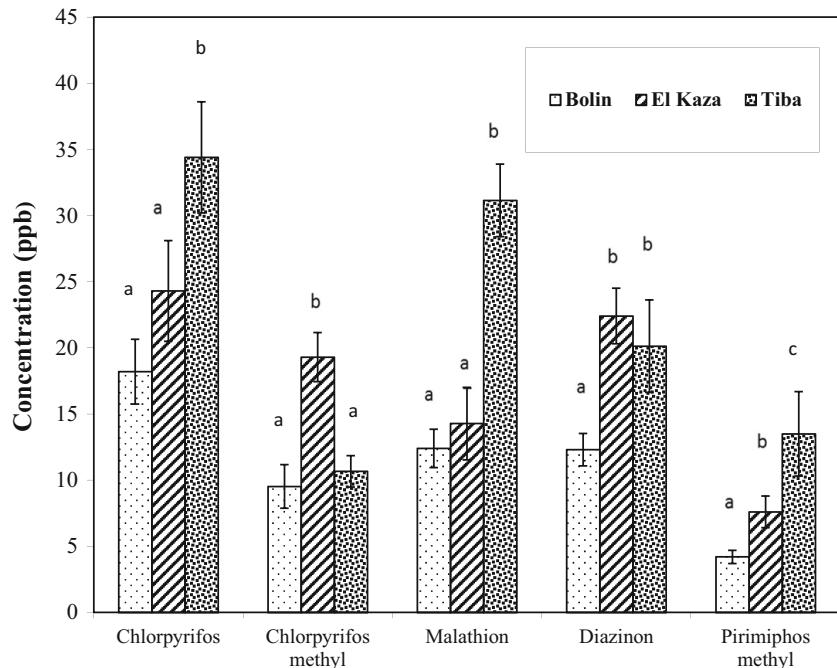
Figure 2 shows residue levels of sprayed OP (chlorpyrifos, chlorpyrifos-methyl, malathion, diazinon, and pirimiphos-methyl) in the midgut of the bees collected from the selected sampling locations. It is apparent that bees collected from El Kaza and Tiba have higher residue levels of OP pesticides in midgut compared to bees collected from Bolin. Although bees of El Kaza contained higher residue levels of chlorpyrifos-methyl and diazinon, the highest values of chlorpyrifos, malathion, and pirimiphos-methyl were reported in bees of Tiba. In addition, bees of Bolin exhibited the lowest levels of OP pesticide residues confirming the selection of Bolin as a reference.

Profiles of biomarker response

Reduced glutathione content

GSH content differed significantly between Bolin (reference site) and Tiba ($p < 0.01$), while El Kaza exhibited an intermediate value which did not differ significantly from Bolin and Tiba ($p > 0.05$). In addition, lower midgut GSH content was observed in bees collected from Tiba when compared to those collected from Bolin (ANOVA, $F = 38.47$; $df = 2$) (Fig. 3a).

Fig. 2 Organophosphorus pollutant residues (ppb) in the midgut of *A. mellifera* collected from the three sampling locations. Values are expressed as means \pm SD ($n = 12$ pools of five animals each). Vertical bars indicate standard deviation. Different letters identify significant differences (LSD, $p < 0.05$) among locations



Superoxide dismutase

The lowest SOD activity in midgut was observed at Tiba, followed by El Kaza (Fig. 3b). Midgut SOD activity differed significantly ($p < 0.05$) in bees collected from Tiba when compared to those from Bolin. No significant ($p > 0.05$) variation in the SOD activity between Bolin and El Kaza was found (ANOVA, $F = 75.80$; $df = 2$).

Catalase

CAT activity in midgut tissue showed no significant ($p > 0.05$) difference between Bolin and El Kaza. The lowest mean CAT value was recorded at Tiba ($p < 0.001$) when compared with the reference site (ANOVA, $F = 51.71$; $df = 2$) (Fig. 3c).

Glutathione peroxidase

A significant variation in GPX activity was seen in the midgut of *A. mellifera* at the three different locations, with the lowest value at Tiba ($p < 0.001$), followed by El Kaza ($p < 0.01$) and Bolin (ANOVA, $F = 85.45$; $df = 2$) (Fig. 3d).

Malondialdehyde concentration

The highest MDA level in midgut was observed at Tiba ($p < 0.001$), followed by El Kaza ($p < 0.01$) when compared to those from Bolin (Fig. 3e). Moreover, ANOVA revealed significant variation in the MDA levels among all studied locations (ANOVA, $F = 108.75$; $df = 2$).

Total protein content

Figure 3f shows the changes in total protein content in midgut of bees in the selected sites. Statistical analysis showed that the total protein content differed significantly ($p < 0.01$) in polluted insects as compared with those of the reference one (ANOVA, $F = 17.04$; $df = 2$).

Electron microscopic observations

Ultrastructure of the midgut epithelial cells of *A. mellifera* collected from Bolin location shows that the midgut epithelial cells are lined along the entire luminal surface with a brush border of regularly arranged microvilli (Fig. 4a). The microvilli are clearly visible with parallel core of microfilaments and covered with fine coat of minute fibrils and extended downwards with the filament-core rootlets. The basement lamina of these cells shows a number of narrow infoldings which appear deep in the cytoplasm. An impressive number of elongated and rounded mitochondria with electron dense matrix is observed adjacent to the basal infoldings as well as in the cytoplasmic region underlying the brush border. In addition, the cytoplasm of these cells contains rough endoplasmic reticulum, some smooth endoplasmic reticulum, and free ribosomes. The nucleus seems to have several large nucleoli. The heterochromatin patches are evenly distributed without any particular localization (Fig. 4b).

However, ultrastructural examination of eight random samples from the midgut epithelial cells of *A. mellifera* collected from sprayed locations revealed various degrees of pathological changes compared to the midgut cells of a reference. The

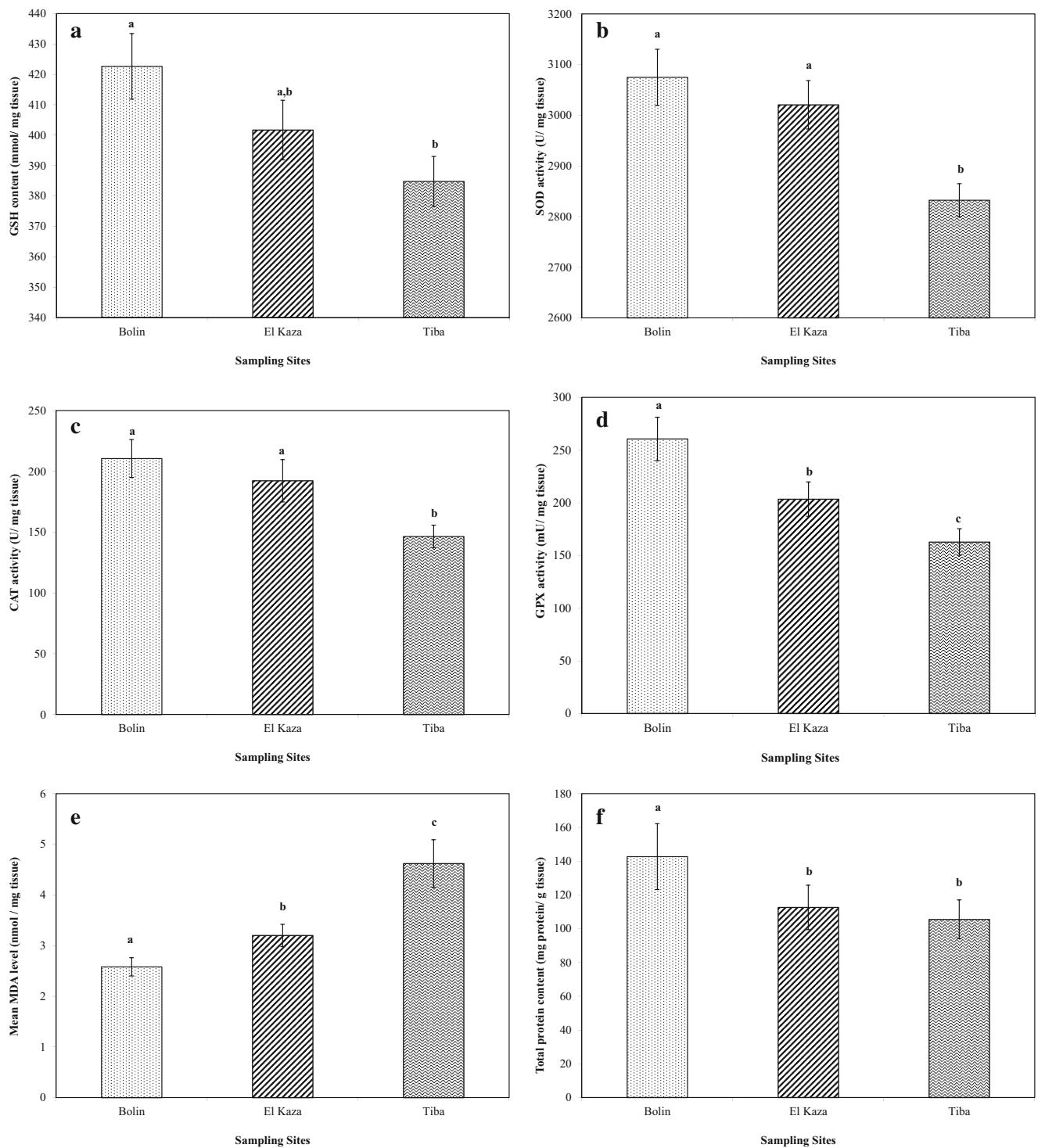


Fig. 3 Reduced glutathione content (a), superoxide dismutase activity (b), catalase activity (c), glutathione peroxidase activity (d), lipid peroxidation level (e), and total protein content (f) in the midgut of *A. mellifera* collected from the three sampling locations. Reference site (Bolin). Values are expressed as means \pm SD ($n = 10$ pools of five animals each). Vertical bars indicate standard deviation. Different letters identify significant differences (LSD, $p < 0.05$) among locations

(Bolin). Values are expressed as means \pm SD ($n = 10$ pools of five animals each). Vertical bars indicate standard deviation. Different letters identify significant differences (LSD, $p < 0.05$) among locations

midgut epithelial cells showed cytoplasmic alterations as well as nuclear degeneration. The prominent changes are diffusion and vacuolization of the cytoplasm almost in all examined cells (Figs. 5a–g and 6b, d, e, g). Most cytoplasmic organelles

are clearly affected. The brush border seems to be deformed in some cells. Such changes include a decrease in size and number of distorted microvilli (Fig. 5a). In addition, disorganized basement membrane was found (Fig. 5d). In these cells, the

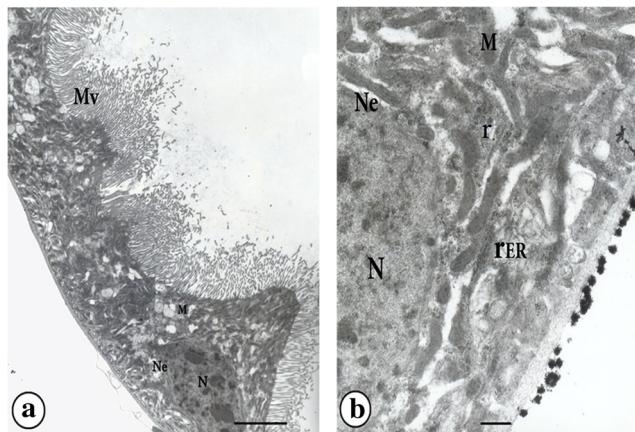


Fig. 4 a, b Transmission electron micrograph of midgut epithelial cells of *A. mellifera* collected from Bolin location showing luminal border lined with large number of a long densely packed non-altered microvilli (*Mv*), the cytoplasm shows a large number of electron dense granules and pleomorphic mitochondria (*M*), rough endoplasmic reticulum (*rER*), and free ribosomes (*r*). Also, the nucleus (*N*) with distinct nuclear envelope (*Ne*). [Scale bar = 0.5 μm in **a**. Scale bar = 0.25 μm in **b**]

cytoplasm is electron-lucent and devoid of any organelles in some areas (Fig. 5b–g). In addition, marked reduction in the number of mitochondria can also be observed (Fig. 5b–g). Other mitochondria are swollen and have fragmented cristae and electron-lucent matrix (Fig. 5e, f). Apart from the deformed mitochondria, numerous altered ones are scattered in the cytoplasm among numerous normal-looking mitochondria (Fig. 6f). Some of them appeared strongly distended, with breakage in their walls (Fig. 5e, g). However, giant mitochondria are observed in some cells (Figs. 5f, h and 6b). The enlargement of mitochondria is accompanied with degeneration and disintegration of internal architecture (Figs. 5e, f and 6c). Other mitochondria appeared disfigured with completely destroyed cristae (Fig. 5e). The cisternae of the rough endoplasmic reticulum seem to be vesiculated in most cells (Fig. 5e, f). Parts of the rough endoplasmic membranes showed degranulation in some cells (Figs. 5g and 6c, g). As a result of dissociation, a large number of free ribosome particles are dispersed randomly within the cytoplasm (Fig. 5e). Secretory vesicles were observed (Fig. 5c). Beside these cy-

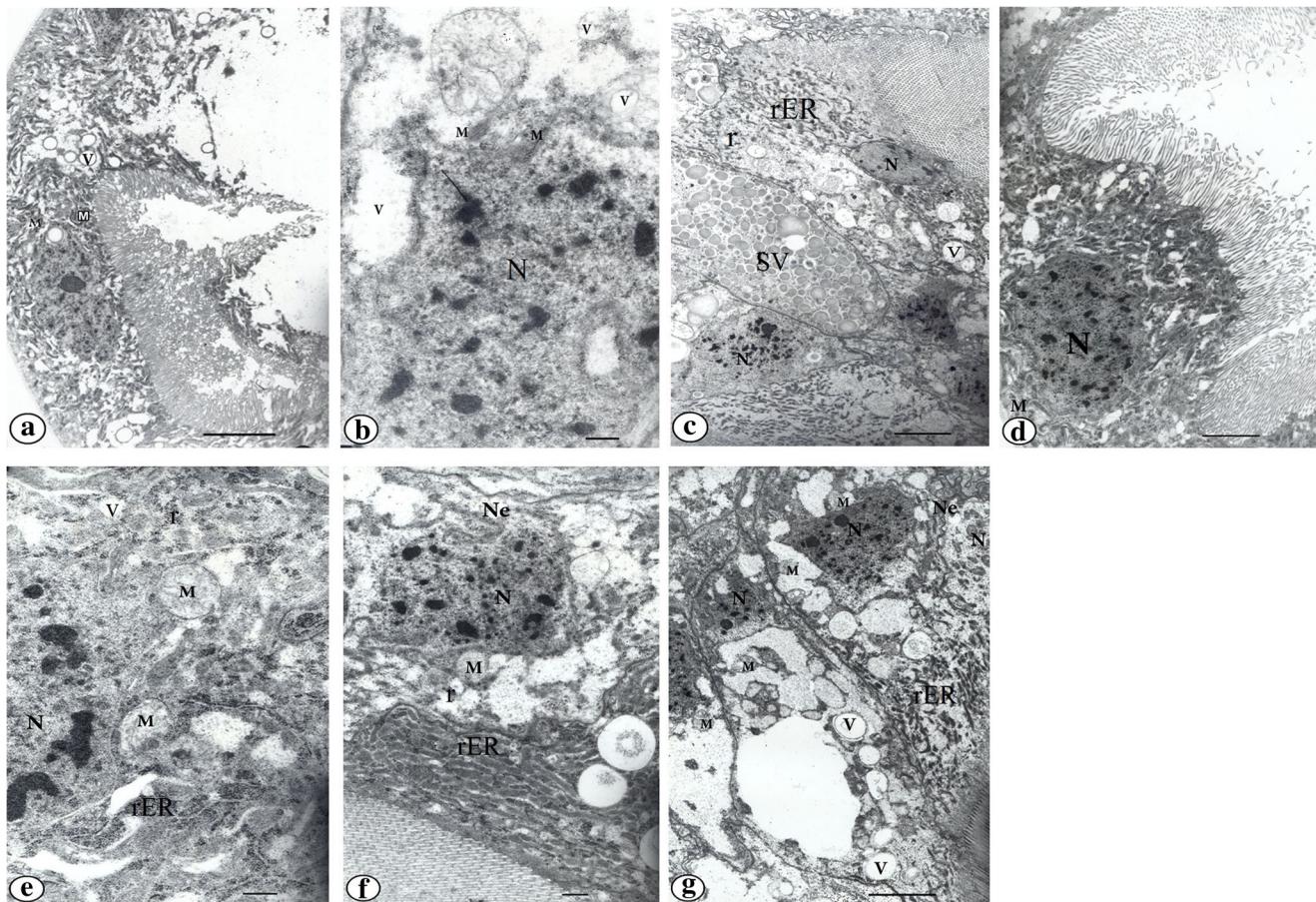


Fig. 5 a–g Transmission electron micrograph of midgut epithelial cells of *A. mellifera* collected from El Kaza location exhibiting partially nonaltered brush borders. Strongly altered cytoplasm of epithelial cells. Vacuoles (*V*) containing lamellar membranes, elongated and spherical

mitochondria (*M*), secretory vesicles (*SV*), rough endoplasmic reticulum (*rER*), and free ribosomes (*r*). Nucleus (*N*) displays severe chromatolysis. [Scale bar = 0.5 μm in **a**, **c**, **g**. Scale bar = 0.25 μm in **b**, **d**, **e**, **f**, **h**]

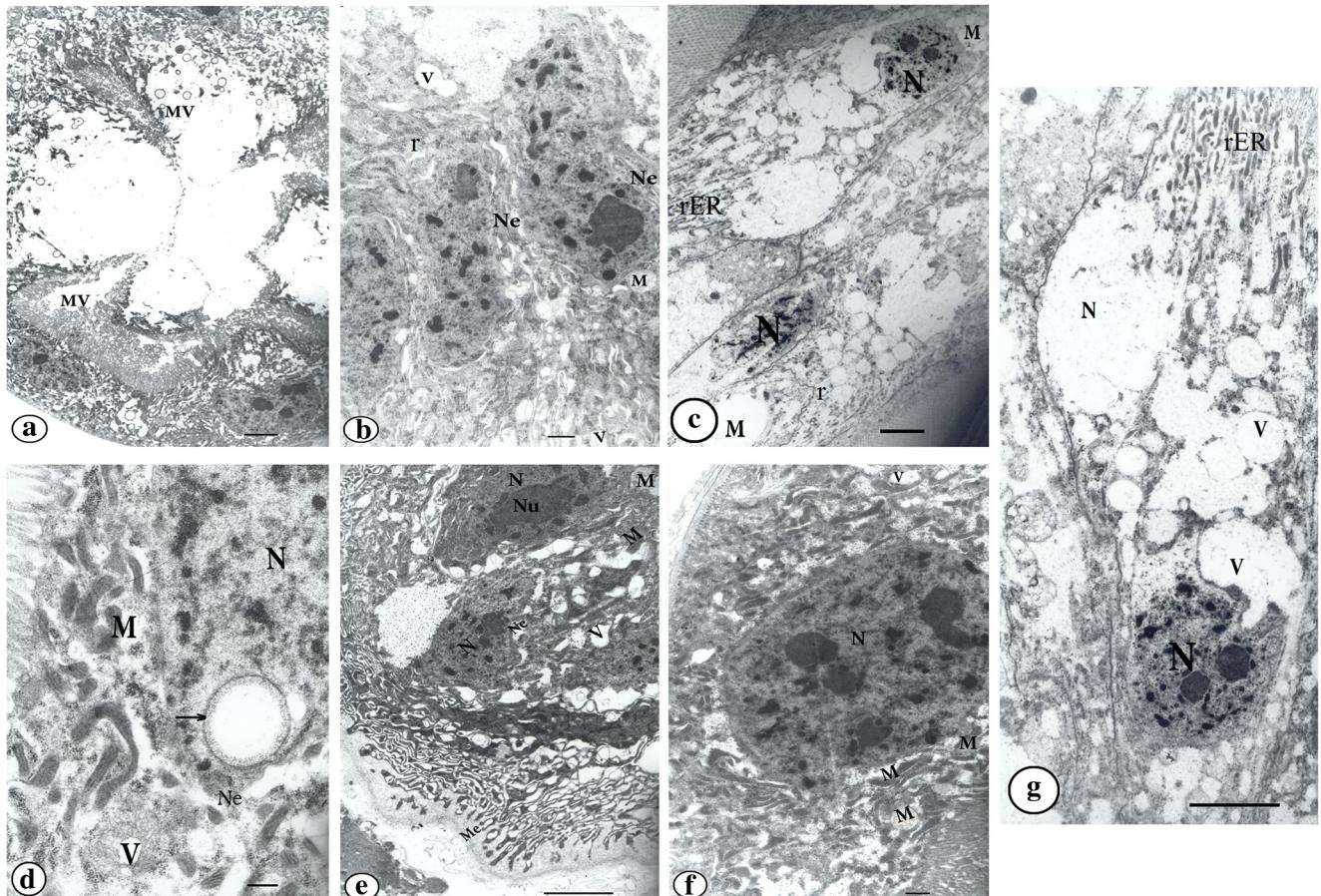


Fig. 6 a–g Transmission electron micrograph of midgut epithelial cells of *A. mellifera* collected from Tiba location showing confluent and disorganized altered microvilli (*Mv*) and destruction of their brush border. Note that all cells are strongly modified and the cytoplasm possess large vacuoles (*V*) and alteration of cytoplasmic organelles. Strong alterations of cytoplasmic structures and organelles are evident. Note altered swollen mitochondria (*M*) displayed lysis of matrices with

disruption of limiting membranes and disintegration of cristae, rough endoplasmic reticulum (*rER*), and free ribosomes (*r*). Note the fragmented chromatin inside the nucleus (*N*) and vacuolization in the nucleus (arrow). Note indented nuclear envelope (*Ne*) and disorganized chromatin. Nucleolus (*Nu*). [Scale bar = 0.5 μm in a, c, e, g, h. Scale bar = 0.25 μm in b, d, f]

toplasmic alterations in these epithelial cells, the nuclei exhibited also abnormal features in some cells (Figs. 5b–d, f, g and 6b–e, g). They become indented and irregular in form (Figs. 5b–d, f, g and 6b–e, g). In other cells, the nuclei show clumping of chromatin in several of their regions (Fig. 6f), while others display the signs of advanced degradation and the nuclear envelopes are damaged (Fig. 6d, g).

Discussion

Our results highlighted high accumulation of OP pollutant residue levels in midgut of the *A. mellifera* collected from El Kaza and Tiba indicating the presence of specific pollution with OPs in the investigated areas. These results showed a variability of OP load among these areas and the usefulness of bees as environmental quality and pollution sentinels for these tested OPs. Continuous exposure of all organisms to environmental stressors leads to greater risk of harmful health

outcomes. The stressors can trigger higher order of biological responses at the organismal level only after initiating biochemical and cellular events (Gauthier et al. 2016). Chemical analyses alone may not suffice to describe the adverse effects of these pollutants present at contaminated locations. Therefore, the use of a set of biomarkers for assessment of environmental quality has been recommended by many researchers (Fernandes et al. 2007; Linde-Arias et al. 2008). Since 2001, over 80 % of all insecticides used in delta soils from Beheira Governorate were OPs (Mansour 2004). So the present study was intended to assess the oxidative stress and ultrastructural alterations in midgut epithelium of *A. mellifera* under the influence of OP contamination.

The bee midgut is responsible for food digestion and nutrient absorption (Billingsley and Lehane 1996; Cristofolletti et al. 2001). The undigested material goes straight to the hind-gut where water absorption and elimination of feces occur (Adel and Sammour 2012). The digestive tract of bees is considered as an effective physical and chemical barrier against

the potentially toxic materials that are ingested with the feeding (Sayed et al. 2011). Midgut in insects has extreme importance in ecotoxicological studies. Several studies showed that xenobiotics included OPs were selectively concentrated in only one or few organs or in specific regions of the tissues in most of herbivorous insects, and typically these organs are parts of the digestive tract (Dallinger 1993).

GSH is involved in both enzymatic and nonenzymatic anti-oxidative processes. The present results indicated a significant decrease in GSH content in midgut of the studied insects in response to pollution in Tiba location. The reduction of GSH content may be due to their consumption in the scavenging free radicals probably generated by pesticides (Ben Amara et al. 2011). GSH plays a central role in maintaining cellular redox status and protecting cells from oxidative injury (Dickinson and Forman 2002). Dimethoate-exposed grasshopper *Chorthippus brunneus* showed considerable depletion of GSH to lower levels (Augustyniak et al. 2005). Consistent with this finding, a decrease in GSH concentration was recorded in the digestive gland of *Helix aspersa* collected from different sites polluted with heavy metals (Abdel-Halim et al. 2013) and hepatopancreas of *Porcellio leavis* collected from polluted sites in Egypt (Abu El-Saad 2010). Generally, it was found that direct utilization of GSH as an antioxidant in terminating free radical reactions initiated by pesticides or utilization of GSH for the detoxification of pesticide metabolism by GST leads to the reduction of GSH content (Banerjee et al. 1999).

Insects possess a suite of antioxidant enzyme systems like other eukaryotes which protect their cells from the damaging effects of oxidative radicals (Ahmad 1992). The obtained results pointed out a decrease in SOD activity in midgut of *A. mellifera* collected from Tiba location. Confirming the data in the current study, Dimitrova et al. (1994) suggested that the superoxide radicals by themselves or after their transformation to H₂O₂ cause an oxidation of the cysteine in the enzyme and decrease SOD activity. Consequently, the decreased SOD activities might have reflected a cellular oxidative stress due to OP exposure. There have been reported that the long-term intoxication with OPs leads to a gradual exhaustion of SOD in rats (Kalender et al. 2007). Consistent with this finding, SOD activity was significantly lower in the midgut of red palm weevil *Rhynchophorus ferrugineus* that were fed with 10 ppm bioinsecticide spinosad (Abdelsalam et al. 2016). The decreased activity of SOD might be due to the consumption of this enzyme in converting the O₂⁻ to H₂O₂ (Gultekin et al. 2000; Ben Amara et al. 2011; Chandran et al. 2005).

When compared with low-intensity OP spraying location in Bolin, samples collected from location with high OP use in Tiba showed a significant reduction in CAT activity in midgut of the collected bees. The present data agreed with Augustyniak et al. (2009) who concluded that pollution reduced the activity of CAT in *C. brunneus*. Felton and Summers (1995) estimated that there is a certain relationship

between CAT and SOD as SOD can convert the free O₂⁻ to H₂O₂, which is then eliminated by CAT. Hence, it could be concluded that the decline in the CAT activity may be due to reducing the conversion of O₂⁻ to H₂O₂. Also, the present results highlighted appreciable decrease in GPx activity in midgut of the insects from polluted areas (El Kaza and Tiba) as reported by other researchers (Augustyniak et al. 2009; Wu et al. 2011a, 2011b). Decrease of the GPx activity could result in a reduced capacity to scavenge H₂O₂ (Akbar et al. 2012). GPx, responsible for enzymatic defense against hydrogen peroxide, is strictly linked with the concentration of GSH because it catalyzes the reaction between glutathione and hydrogen peroxide, resulting in the formation of glutathione disulfide (Salvemini et al. 1999). The inhibition of enzymes involved in free radical removal leads to the accumulation of H₂O₂, which promotes lipid peroxidation and modulation of DNA, altered gene expression, and cell death (Stohs et al. 2000). Furthermore, a strong inhibition of GPx activity after insecticide application may be partly caused by the lack of substrate GSH used for conjugation with xenobiotics in other enzymatic reactions (Augustyniak et al. 2009).

The current investigation indicated that OP residues in polluted locations increased MDA concentration in midgut of the investigated bees especially in Tiba. Several investigators showed that pollutant exposure to pyrethroids, permethrin, and fenvalerate increases MDA concentration in *Helicoverpa armigera* (Akbar et al. 2012). Also the same trend was observed by Aslaneturk et al. (2011) on methidathion in midgut tissues of *Lymantria dispar* (Lepidoptera) larvae. The increase of MDA can be explained by the increase of ROS, especially HO[·], which acts on polyunsaturated fatty acids of membranes (Mansour and Mossa 2009) which is one of the consequences of the impairment of both antioxidant enzymes (such SOD and CAT) and antioxidant agents (such as GSH).

The present results pointed out that there was a decrease in total protein content in midgut of the insects collected from polluted areas. It has been shown that pollution stress can decrease the amount of total protein content of many insects (Bream 2003; Wu et al. 2006; El-barky et al. 2008). Consequently, the decrease in total protein content may reflect the decrease in the activities of some enzymes (Abd El-Aziz et al. 2007) that confirm our results concerning the reduction in the activities of GPx, SOD, and CAT. Moreover, the decrease in protein content could be due to the breakdown of protein into amino acids and with the entrance of these amino acids to Krebs cycle as keto acid to supply energy for the insect (Bizhannia et al. 2005). The depletion of protein content in tissues may constitute a physiological mechanism and might play a role in compensatory mechanisms under insecticidal stress to provide intermediates to the Krebs cycle by retaining free amino acid content in hemolymph which is in agreement with Zibaee et al. (2008).

There is evidence in the literature that structural changes in midgut of insects could have diagnostic magnitude in assessing sublethal effects of pollutants. Numerous studies were conducted in insects exposed to different classes of pollutants (Khan et al. 2011; Rawi et al. 2011; Decio et al. 2013). Few studies however were carried out in insects collected from natural systems receiving contaminants. It is worth mentioning that in Egypt this is the first report describing changes in midgut cells of *A. mellifera* collected from sites exposed to OP pollutants. The present study showed that the midgut epithelial cells of *A. mellifera* collected from the Bolin are lined along the entire luminal surface with a brush border of regularly arranged microvilli. These cells possessed numerous mitochondria, strands of endoplasmic reticulum, evenly distributed ribosomes, and lipid inclusions. These results are in agreement with the results of Liu (1984) on the ultrastructure of midgut epithelial cells of the worker honeybee *A. mellifera*. Conversely, the present results showed several ultrastructural modifications in the midgut of the polluted bees collected from El Kaza and Tiba locations including disruption of the epithelial cells as observed in *Spodoptera littoralis* larvae (Rawi et al. 2011) and appearance of vacuoles in cotton leafworm, *S. littoralis*, as reported by Adel et al. (2010). Rupture of peritrophic membrane was also observed in addition to separation of the muscular layer and rupture of microvilli of the columnar cells, which are in agreement with Khan et al. (2011) in midgut of *Periplaneta americana* after treatment with *Datura alba* leaf extract. Cavados et al. (2004) estimated that there is a definite synchronization between the external toxicity symptoms and the pathological effect upon the midgut epithelia. These pathological changes in the cells may cause disturbance in the normal physiology of the vital important system of insects (Rawi et al. 2011). It has been observed that possible damage to the gut cells and their secretions could be attributed to the ROS action in other systems (Krishnan et al. 2009). The overproduction of ROS decreases the absorption of ingested nutrient causing oxidative damage to the midgut cells and subsequently leads to non-functional digestive tract.

From this study, it is obvious that several cell organelles in the midgut of *A. mellifera* are affected by OPs which suggest that these pollutants disturb cell function at sprayed locations. Ultrastructurally, the midgut epithelial cells of bees collected from El Kaza showed cytoplasmic alterations as well as nuclear degeneration. The presence of vacuolated, lytic areas in the cytoplasm is evidence of cytotoxicity; the injuries might be consequence of the release of lysosomal hydrolases into the cytoplasm (Vandenbulcke et al. 1998) or may be proceeded from enlarged rough endoplasmic cisterns that had lost their ribosomes (Percy and Fast 1983). These results are in accordance with the results of Ranjini and Nambiar (2015) who observed a large number of vacuoles and signs of increased secretory activity accompanied with extensive lysis within the

midgut epithelial cells of the *Orthaga exvinacea* treated with leaf extracts of *Clerodendrum infortunatum* and with Cavados et al. (2004) who reported vacuolization in the midgut cells of dipteran larvae *Simulium pertinax* exposed to endotoxins of *Bacillus thuringiensis*.

The cell damages due to OP pollutants in *A. mellifera* midgut cells in this study were also related to brush border microvilli degeneration. The dissolution of cytoskeleton structures inside and at the basis of the microvilli was responsible for its decrease in size and further disappearance, when bubbles of cytoplasmic substances protrude into the midgut lumen (Seidman et al. 1986). Electron micrographs also showed pronounced ultrastructural changes in cytoplasmic organelles. In some areas, the cytoplasm is electron lucent and devoid from any organelles may be due to the enhanced endocytotic activity as described by Cavados et al. (2004). Mitochondria were very sensitive to environmental contamination. The occurrence of swollen mitochondria is a well-documented feature of OP intoxication (Abu El-Saad and Elgerbed 2010). Mitochondrial swelling reflects a deregulation of mitochondrial membrane transport (Meyer et al. 2013). Pathological reactions of mitochondria to toxins were similarly observed in insects intoxicated cells including distortion of mitochondrial membranes and cristae (Braeckman et al. 1999). Moreover, it was found that the cisternae of the RER seems to be vesiculated and a large number of free ribosomes particles are dispersed in midgut of bees collected from sprayed locations especially Tiba location. These results are in agreement with Roel et al. (2010) who observed aggregated ribosomes in midgut epithelial of *Spodoptera frugiperda* (Lepidoptera) treated with sublethal doses of Meliaceae oil. The filling of the cisternae together with the increased amount of ribosomes seemed to be an indication of an initial increase of stress proteins or the expansion of the lysosomal system (Braeckman et al. 1999). Degenerative nuclear changes such as chromatin clumping, deformation of some nuclei, and nuclear envelope damage are unambiguous indicators of impairment of nucleic acid and protein metabolism (Krishnan and Kodrik 2006). Nuclear degeneration was mentioned by Braeckman et al. (1999) in their study on pesticide pathology in an insect cell line. Also the appearance of distorted nuclei is noteworthy referring to the damage of RNA (Khan et al. 2011).

Consistently, our data highlight the interest of measuring biochemical markers of oxidative stress in the honeybee midgut to detect an OP insecticide exposure. In this paper, it was shown that OP residues found in the agricultural areas altered the honeybee's components involved in antioxidative responses. These results suggest that *A. mellifera* could be used as bioindicator species for OP pesticide-contaminated Egyptian agroecosystems. However, further studies are crucial to corroborate the presence of other environmental toxics affecting *A. mellifera* within the studied locations. On the other hand, there is a strong necessity for monitoring the effects of xenobiotics on honeybees in other parts of Egypt.

Compliance with ethical standards

Conflict of interest The authors declared no conflicts of interest.

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