Molecular and ultrastructure abnormalities in ovaries of *Calosoma olivieri* collected from Kafr El Zayat, Egypt triggered by heavy metal exposure

Lamia M. El-Samad¹*, Hala M Mashaal¹, Hussein K. Hussein¹, Eman H. Radwan², Khalid H. Radwan³, Mohamed A. Hassan⁴, Nessrin Kheirallah¹

¹Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt, ²Zoology department, Faculty of Science, Damanhour University, Damanhour, Egypt, ³Agriculture Genetic Engineering Research Institute, Cairo, Egypt. ⁴Protein Research Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technological Applications (SRTA-City), New Borg El-Arab City, 21934 Alexandria, Egypt.

*Corresponding authors lamya.moustafa@alex.edu.eg

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**ABSTRACT**

Heavy metal pollution is one of the main causes of environmental changes. *C. olivieri* is used as a biomonitoring beetle in a polluted site in the city of Kafr El-Zayat in the province of Al Gharbia in Egypt which houses pesticides and chemicals factories. After analyzing our data, we noticed a significant variation in mRNA expression of MT and HSp70 in the polluted group compared with the control group which indicates the effect of exposure to heavy metals. On the level of ultrastructural examination, the data revealed several abnormalities in semithin and transmission sections as the trophocytes and oocytes in the polluted group undergo degeneration. The ooplasm were clearly seen to vacuolate and dissociate. There were also deteriorated yolk granules seen. The trophocytes in the control group looked like spherical cells with normal nuclear envelopes and agglomerated chromatin in the nucleus. On the other hand, there were some cellular deteriorations in the polluted group. Occasionally, aberrant nuclei with a defined or intended nuclear envelope were observed in the FECs.

**KEYWORDS:** Insects, MT, HSP 70, Histological alternation, Ultrastructural abnormalities, Heavy metals, Industrial pollution.

**INTRODUCTION**

Anthropogenic heavy metal (HM) pollution from mining, smelting, electroplating, and sewage irrigation is a major global environmental problem (1-3). Excessive levels of HMs pose a hazard to the ecological security and health of natural habitats (4, 5). Heavy metals (HMs) including arsenic, cadmium, chromium, lead, mercury, and zinc have been categorized as priority pollutants by the US Environmental Protection Agency (6). Prolonged exposure to heavy metals (HMs) can seriously harm cells and alter how living things work. As a result, the biotoxicity of HMs lead has attracted a lot of interest.

Heavy metal combination pollution is currently a major global environmental health hazard. Owing to the extensive usage of lead (Pb), manganese (Mn), and chromium (Cr) in anthropogenic (particularly...
industrial) activities, there is a chance that wastewater will be poured into rivers, the heavy metal exhaust will be released into the atmosphere, and soil surfaces will become covered in particle residue (1). Multiple heavy metal leaks into the environment over time lead to heavy metal combination pollution (7).

Pesticides cover a broad spectrum of chemical and organic combinations, including plant growth regulators, fungicides, insecticides, and more. The primary sources of pesticides in ecosystems are forestry and agriculture. It is well known that as the world's population grows, so too must the amount of food produced. A pesticide should ideally be fatal to the target organisms rather than catastrophic to people and the environment to control the influence of weed species (8).

The greater parts of heavy metals are dangerous and toxic at low concentrations and can enter the food chain, where they amass and perpetrate harm to living beings. All metals can possibly show destructive impacts at higher concentrations and the toxicity of each metal relies on the amount accessible to life forms, the consumed dose, the route, and the length of exposure (9). Heavy metals are brought into aquatic systems, rivers, lakes, or seas through atmospheric fallout, dumping wastes, and overflow of terrestrial systems (industrial and domestic effluents) (10).

Since xenobiotics can enter organisms through the air, water, or soil, this means that they can respond to environmental perturbations more quickly, accurately, and adaptably. According to Anna (11). A novel method for identifying many types of environmental mismanagement, including contamination, high-input farming that undermines soil health, incorrect waste disposal, and pollution, is the use of bioindicators.

There are many possible representatives of the class Insecta that can be used as environmental bioindicators because of their higher degree of structural and functional organization, highly complex morphology, physiology, well-developed sense organs, complex behavior, and greater species diversity (12).

Many beetles are used in bioindication because; a) they are polyphagous predators and important for biological control; b) pitfall traps make collecting easy; and c) captures are usually large enough to allow quantifiable investigation (13).

In addition to being able to survive harsh environmental conditions (14) producing secondary sclerotization in the event of elytra damage (13) responding to environmental stimuli and activity rhythms to maintain themselves in favorable microhabitats (15), being able to withstand temperatures above those that would kill most other arthropods (16). Beetles are secured by different structural adjustments and types of behaviors at each formative stage. Thus, beetles are employed as bio-indicators of pollutants in the environment.

Metal ions can infiltrate the nucleus in certain situations and disrupt cellular metabolism by penetrating within the cell. Metal cations can reversibly bind to DNA through coordinated and ionic interactions, although they are not able to cause all of the chromatin lesions seen in cells. Therefore, it is necessary to give greater weight to both the direct and, for the most part, indirect effects of metals on nuclear chromatin when discussing DNA damage. Over the past ten years, research has demonstrated that the production of reactive free radicals, particularly HO• and ROS, is the mechanism by which metals cause cancer (17). As the molecular harmful consequences of important environmental pollutants, genotoxic biomarkers are routinely assessed in ecotoxicology (18).

According to several studies (19, 20), certain proteins can be employed as biomarkers of environmental contaminants. Since proteins make up a significant portion of the cell membrane, they play a crucial role in the continuous interaction
between intra- and extracellular fluids (21). Stress facilitates the growth of energy requirements and modifies the metabolic functions of living things (22). When an organism is exposed to a stressor, its physiological reaction sets off a process that results in the creation of particular proteins to potentially repair any damage produced by the exposure. According to various studies (23), these proteins were dubbed heat shock proteins or molecular chaperones.

Based on their molecular weights, sequence similarities, and homologies, the six families of HSPs (small HSPs (sHSPs), HSP40, HSP60, HSP70, HSP90, and HSP 100) are separated from each other as a supergene family (24, 25). They help with appropriate protein folding, synthesis, transcription, cell signaling, and metabolism (26). They also have a function in encountering stressed cells (6). HSP70 and HSP90 were the most susceptible to stress stimuli in the organism cells among these HSPs, and they were also the most conservative (27). HSP60 is more synthesized in unfavorable environmental conditions and renatures damaged proteins to restore their physiological activity (28).

When stressors are present in insects, the rate at which most proteins are synthesized decreases, but the expression of heat shock proteins (HSPs) rises (29). Heat shock proteins (HSPs) have been used in a number of studies to assess the potentially toxic effects of various stressors, particularly heavy metals in insects, (30).

The synthesis of RNA that codes for metallothioneins can be accelerated by specific metals entering the nucleus. MT are tiny molecular weight peptides that are predominantly present in the cytosol, lysosomes, and nucleus. They are rich in the amino acid cysteine, which has a thiol group (-SH). Heavy metals can be bound by MTs because of the thiol (31). Metals have been shown to up-regulate the expression of the MT gene in some taxa, and in insects and other species, this gene has been developed as a biomarker of metal contamination (32). D. melanogaster's metallothioneins have been thoroughly investigated by (33). In response to Cd and Pb, at least one of the two mosquito MT exhibits changes in expression (34). Additionally, the expression of MT proteins has been linked to the induction of OS rather than its suppression; this may be due to the release of MT-bound metals under oxidizing physiological conditions, which then interact with the SOD system (35). This counterintuitive association of a metal-tolerance gene family with induction of ROS further highlights the complexity of these stress response elements.

Environmental pathology was identified through histopathological analysis. Even at the sublethal effects of contaminants, ultrastructure is an effective approach that can indicate selectivity and sensitivity. The ultrastructural changes brought on by contamination are generally poorly understood, particularly with regard to the gonads of insects (36).

The ultrastructure analysis, which sheds light on nuclear changes and the disruption of subcellular organelles, is another method for assessing intracellular damage (37). Proliferation causes apoptosis because reproductive cells might accumulate poisons that can change the viability of the cell (38). HMs are more prone to accumulate in females, according to numerous research. Consequently, HMs can penetrate the female reproductive system and harm the cells and organs of the female reproductive system, impairing fetal development and fertility (39). Because HMs deposited after blood circulation, they harm the uterus and ovaries. Numerous investigators noted abnormalities in the ultrastructure of ovarian cells exposed to heavy
metals. Trophocytes with disintegrating mitochondria and pyknotic nuclei were visible (40).

Materials and Methods
Collection and identification of beetles

320 beetles weighing an average of 1.96 g were collected from two sites: the garden of the Faculty of Science, Alexandria University, Alexandria, Egypt, which was formerly considered an uncontaminated area, and the area surrounding the KZ chemical and pesticide factory (41). The beetles were identified as Calosoma olivieri, Family: Tenebrionidae, as soon as they were brought to the laboratory. C. olivieri is the predominant adult species in the researched sites, according to preliminary collections of coleopteran insects that live in the chosen sites. At the Department of Entomology, Faculty of Agriculture, Alexandria University, the specimen was determined to be C. olivieri. The species under study is a member of the Carabidae family.

Study Areas

To sample the studied insect, two locations were selected. These locations were (A) the El Shatby Garden of the Faculty of Science at Alexandria University in Alexandria, Egypt, which is regarded as a reference site (36) and (B) a densely populated urban area. The KZ Company for Chemicals and Pesticides is situated in Kafr EL- Zayat, Al Gharbiya Governorate, Egypt.

Fig (1): The polluted location (Zafr El- Zyat)
Sampling procedure

Live *C. olivieri* specimens were selected at random from a number of sampling sites in July 2022. The sampling regions in Kafr El-Zayat, the polluted site, were chosen because of the chemical and fertilizer firms. The mean air temperature varied between 27 and 35 °C, and the relative humidity was 76%. A total of 320 insects were collected from each site. The samples were sexually assaulted. They brought 160 males and 160 females into the lab. After being rendered unconscious with 95% ethanol, beetles were placed in a drop of Ringer's physiological solution to be dissected under a dissecting microscope. When the abdominal cavity was accessible, the testes and ovaries were removed. The treatment of the insects was done in compliance with moral guidelines for the usage of and the maintenance of laboratory animals. Concurrent with the beetle collection, soil samples were collected at a depth of 25 cm from the previously stated locations.

MOLECULAR ANALYSES

Assessment of HSP70 mRNA Expressions & MT

Three ovarian tissues of *C. olivieri* were randomly selected for total RNA isolation utilizing the TRIzol™ Plus RNA Purification Kit (Invitrogen, USA) following the manufacturer’s instructions. The integrity and purity of the isolated RNA were evaluated using agarose gel electrophoresis and a spectrophotometer at 260/280 nm, respectively.

The relative expression levels of HSP70 in the ovarian tissues of beetles were analyzed using a one-step RT-PCR reaction. The primers used in the RT-qPCR reactions were (Forward) 5'-AAA ATG AAA GAA ACG GCA GAG G-3' and (Reverse) 5'-TAA TAC GCA GCA CAT TGA GAC C-3' for HSP70. The RT-qPCR reactions were conducted using the Qiagen Rotor-Gene SYBR Green PCR Kit (QIAGEN, Hilden, Germany) in a 25 μL mixture containing 1 μg of RNA, 12.5 μL of SYBR Green, 2.5 μL of each primer and 9 μL of H2O. The RT-qPCR program consisted of an initial step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 10 s. The assays were performed using the Rotor-Gene Q using Rotor-Gene Q-Pure Detection version 2.1.0 (Qiagen, Montgomery, MD, USA). The quantification of the transcript levels of HSP70 and HSP90 mRNA was accomplished following the comparative 2-ΔΔCT method (42).

Histological and ultrastructure preparation

Ovaries were fixed immediately in 4% formaldehyde and 1% glutaraldehyde (4F1G) in a 0.1 M phosphate buffer solution (pH 7.2) at 4 °C for 3 h. Postfixation occurred using 2% osmium tetroxide (OsO 4) in the same buffer for 2 h. The samples were washed in the buffer and dehydrated at 4 °C through a graded series of ethanol and then embedded in an Epon–Araldite mixture in labeled beam capsules. The semi-thin sections (1 mm thick) were stained with toluidine blue. Ultra-thin sections (0.06–0.07 mm thick) were picked up using copper grids and were stained with uranyl acetate for half an hour and lead citrate for 20–30 min (43). Electron micrographs were taken at several magnifications. Scoping and photographing the grids were achieved using the JEOL 100 CX TEM at the Electron Microscope Unit described previously.

STATISTICAL ANALYSIS

All measurements were performed in 10 replicates. The normality of the data and homogeneity of variance were checked for all of the parameters before statistical analysis. To evaluate the significance of differences between sites for all parameters, a t-test was used (p < 0.05).

RESULTS
Fig (2): Evaluation of mRNA expression of HSP70 in the ovarian tissues of *C. olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and ***p < 0.001.

Fig (3): Evaluation of mRNA expression of MT1 in the ovarian tissues of *C. olivieri* collected from the clean site (site A) and the polluted site with industrial pesticides (site B). Values are presented as mean ± SD and ***p < 0.001.
Histological and Ultrastructure Patterns of ovaries of *C. olivieri*

At different stages of mitotic division, the trophocytes in the control group displayed a spherical nucleus and a polyploid appearance (Fig. 4a). Indicators of yolk deposition were also observed in the spaces between the follicular cells (Figs. 4b, c). The trophocytes and oocytes in the polluted group underwent degeneration. The ooplasm was clearly seen to vacuolate and dissociate (Figs 6 a, b, c). There were also deteriorated yolk granules seen (Fig 6c).

The trophocytes in the control group looked like spherical cells with normal nuclear envelopes and agglomerated chromatin in the nucleus, as seen by the electron micrographs of the ovarian cells. Normal cytoplasmic organelles were visible in the cytoplasm (fig 5 a-c).

The control group's follicular epithelial cells (FECs), which encircle the egg cell (oocyte), showed a typical structure (Figs. 5b), complete with regular nuclear envelopes, heterochromatic nuclei, and normal cytoplasmic organelles. In addition, the oocyte had the typical ooplasm, which is composed of mitochondria, lipid droplets, and yolk granules.

On the other hand, there were some cellular deteriorations in the polluted group. Occasionally, aberrant nuclei with a defined or intended nuclear envelope were observed in the FECs (Fig 7a). Degenerated yolk granules disintegrated and expanded mitochondria, and vacuolated cytoplasm were all seen in (Fig 7b-c).

Fig (4): Semithin section micrographs of the ovarian tissues obtained from the control beetles. Fig. (a) shows normal oocyte (OC), normal follicular cells (FC) and yolk granules (YG), Fig. (b) is a magnified section of Fig. (a), exhibiting yolk granules (YG), Ooplas (arrow), Follicular Epithelial cells (FEC) and Fig. (c) is a magnified section Fig. (b), illustrating Trophocytes cells (TC) with different stages of mitotic division. N: nucleus, follicular epithelial cells (arrow).
Fig (5): Transmission electron micrograph of ovarian tissues of *C. Olivieri* from the control group portrays in Fig. (a) normal oocytes of adult *C. olivieri* showing normal nucleus (N), mitochondrion (M) follicular layer with follicular epithelial cells (FEC), lipid droplets (LD) Yolk granules (YG) and a thin layer of microvilli (MV). Fig. (b) shows nuclei (N), regular nuclear envelope (Ne), heterochromatin (HC), lipid interstitial cells (IC), and yolk granules (YG). Fig. (c) presents showing normal nucleus (N), nuclear envelope (Ne), and a thin layer of microvilli (MV).

Fig (6): Semithin section micrographs of the ovarian tissues obtained from the polluted beetles group. Fig. (a) shows abnormal oocyte (OC), Vacuolation in the ooplasm (V), yolk granules (YG), Fig. (b) is a magnified section of Fig. (a), manifesting showing trophocytes (TC), malformed trophocytes with abnormal chromatin condensation in the nucleus (N) follicular epithelial cells (FEC), yolk granules (YG), degenerated yolk granules (DYG) and Vacuoles (V). Fig. (c) are magnified sections of Fig. (b), demonstrating Malformed trophocytes (TC) with lipid droplets (LD), malformed cells (head arrow), and Vacuoles (V).
Fig (7): Transmission electron micrograph of ovarian tissues of *C. Olivieri* from the polluted group; fig (a) showing abnormal follicular epithelium cells (FEC), Nucleus (N), Vacuoles (V) distorted microvilli (MV), dense bodies (arrow) and Yolk granules (YG), fig (b) abnormal trophocyte Nucleus (N), heterochromatin (HC), pyknotic nucleus (curved arrow) and apoptotic interstitial cells (head arrow), lipid droplets (LD), dense vesicles (DV) and Mitochondria (M) and fig (c) showing degenerated nucleus (N), heterochromatin (CH), disintegrated mitochondria (M) pyknotic nuclei (arrow), dense bodies (head arrow).

**DISCUSSION**

The results of stress protein expression (MT and HSP70) become very interesting. We see a significant increase of these parameters in insects from the polluted site in comparison to the reference site. Balali (44) showed that in cells under normal conditions, HSPs are expressed at low levels; while under stress caused by factors such as temperature, pathogenic infections, or metals, their expression is increased for cellular protection. Indeed, several studies, by Ajitha (45) showed that HSPs play an important role in overcoming adverse conditions. In particular, HSP70 has been suggested as a potential biomarker of heavy metal pollution in invertebrates (46).

MT could also protect cells against the toxic effects of ROS (47). Others pointed to its role in Zn and Cu homeostasis, alongside the detoxification of Cd contaminations in certain vertebrates and invertebrates due to its high cysteine levels (48). In insects, induction of MT is considered to provide tissue protection against metal stress (49). An increase in stress protein expression observed in our research most probably implies an increased degradation and synthesis of protein in cells, which
can be an effect of both direct and indirect influence of metals, through increased ROS production.

Stress protein expression results (MT and HSP70) become quite fascinating when viewed in the light of the previous data. When comparing insects from the polluted site to the reference site, we find a statistically significant increase in these parameters. Several studies demonstrated that HSPs are expressed at low levels in cells under normal circumstances, but their expression is elevated for cellular protection during stress brought on by elements like temperature, pathogenic infections, or metals, (50). Sharifi-Rad (51) demonstrated how crucial HSPs are for conquering challenging circumstances. Specifically, it has been proposed that HSP70 may serve as a biomarker for heavy metal contamination in invertebrates (52).

Additionally, MT may shield cells from ROS’s harmful effects (53). Many researchers highlighted its function in maintaining equilibrium in zinc and copper levels, as well as its high cysteine content, which aids in the detoxification of mercury pollution in some vertebrates and invertebrates (54). It is thought that inducing MT in insects protects tissue against metal stress (23).

According to the current findings, cDNA libraries revealed significantly higher transcript levels of the three Heat Shock Proteins (Hsps) under test in the polluted group when compared to the control group in response to heavy metal pollution. This increase in the expression of stress proteins in our study most likely indicates enhanced protein synthesis and breakdown in cells, which can result from increased ROS generation as a direct or indirect consequence of metals. As previously noted by a number of researchers, it was discovered that there was a large induction when these molecular chaperones’ transcriptional activity increased by 1.5–4 fold (16).

According to Cheng (55), transcripts for Hsp60, Hsp70, and Hsp90 were expressed throughout all phases of insect development, indicating a potential role for these proteins in the control of development. Environmental stressors include chemicals (56), metals (57), damage or adaptation (58), and other factors that cause an increase in Hsps mRNA or protein in insects. HSP60, HSP70, and HSP90 induction may offer defense against various kinds of environmental stressors (58).

The trophocytes and oocytes appear normal. The trophocytes had regularly dispersed cytoplasmic organelles and heterochromatic nuclei with regular nuclear envelopes (59). The last step of oogenesis produces mature oocytes, which are larger than before. At different phases of mitotic division, the reference group’s trophocytes had a spherical nucleus and appeared polyploid (60).

Microvilli, finger-like projections from the follicular cells’ plasma membrane, are connected to the microvilli of the oocyte’s plasma membrane to form channels. The rounded nucleus of follicular epithelial cells is accompanied by well-developed nuclear envelopes and organelles that seem normal in the cytoplasm. Numerous yolk granules, mitochondria, and lipid droplets are present in the ooplasm (61).

The insects belonging to the polluted group still had immature ovaries with atrophied ovarioles. Their oocytes withered and lost their contents as a result of follicular epithelial rupture. There are documented further follicular epithelial damages, such as the external sheath’s separation and shrinking. Within the ovarioles, there were varying degrees of vacuolation and spaces (23). Within the trophocytes of the contaminated group, there was some degradation. Often seen were apoptotic trophocytes. Pyknotic nucleus, as in the lytic regions (62).

The oocytes' follicular wall displayed cuboidal cells with regular, spherical nuclei. Additionally, gaps between the follicular cells were observed, which is a sign of yolk deposition. The germinal vesicle and yolk granules are found in the ooplasm. In comparison to the control group, the contaminated...
group showed clear signs of ooplasm separation and vacuolation. Degenerated yolk granules were additionally detected (63).

The polluted group’s vitellogenic oocyte had deteriorated yolk granules and vacuolated ooplasm when it first emerged. The follicular epithelial cells have lytic regions in the cytoplasm and pyknotic nuclei. Additionally, malformed microvilli were seen (64).

CONCLUSION

In summary, this research explores the harmful influences of heavy metals in the ovarian tissues of the ground beetle (Colembus olivieri), our data revealed significant variation in MT & HSP 70 mRNA expressions in the two groups. Moreover, the exposure of beetles to heavy metals triggered perceptible histopathological and ultrastructural.

Conflict of interest

All authors declared that there were no conflicts of interest.

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REFERENCES


