American Journal of Agricultural and Biological Sciences

Original Research Paper

Effect of Cadmium on Worker Ovary Morphology of *Bombus morio* (Hymenoptera: Bombini)

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Article history
Received: 16-12-2017
Revised: 26-03-2018
Accepted: 09-04-2018

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Abstract: Bees are valuable bioindicators, providing information regarding environmental conditions through different kinds of analyses. Therefore, the effect of cadmium on the morphology of ovaries of *Bombus morio* workers was used to study a concentration considered environmentally safe (1 ppb) in Brazil. Workers of the same estimated age exposed for 48 h to 1 ppb cadmium showed extensive morphological changes in the germarium and vitellarium compared with the control group. Bees exposed to cadmium showed death of or damage to entire vitellogenic follicles, with chromatin condensation and fragmentation of nurse cell nuclei. In the germarium, the intercellular bridge connecting the primary oocyte to the developing nurse cells was broken. The nurse cells formed a cluster of cells with compacted chromatin, detached from an isolated primary oocyte. In some cases, the cytoblasts were absent from the germarium, leaving an empty space surrounded by collapsed peritoneal sheaths. We further propose that such a hazardous impact of 1 ppb cadmium on the ovaries of *B. morio* workers is not only mainly due to cadmium itself as an endocrine disruptor, indirect oxidative stress promoter, cytoskeleton destabilizer and mutagenic trace metal, but by disruption of the trophocytes, where perivitelline space filled with eosinophil material in the control group, was totally empty in exposed bees, suggesting interruption of trophocyte vitellogenin production.

Keywords: Biomarker, *Bombus Morio*, Ecotoxicology, Morphology, Ovary

Introduction

In addition to the invaluable pollination services of bees, these insects are also successful bioindicators. Thus, they can provide information regarding environmental risk assessment via several methods of analysis. Hence, bees have been the subject of investigations ranging from the study of their mortality rate and the presence of residual contaminants in their nests to physiological, morphological and molecular studies on their tissues and cells (Celli and Maccagnani, 2003).

Regarding the genus *Bombus*, a sudden decline and loss of populations of several species have been recorded annually worldwide (Celli and Maccagnani, 2003; Martins and Melo, 2010; Cameron *et al*., 2011; Gradish *et al*., 2011; Carswell, 2015; Rhoades *et al*., 2016). The causes of such dramatic changes in the populations of these species are multifactorial and they act together to pose a serious threat to bees (Martins and Melo, 2010). Some causes of the mass death of bees are well defined, such as global warming, intensive agricultural activity, mining and industrialization (Gradish *et al*., 2011; Martín *et al*., 2015; Dicks *et al*., 2016; Potts *et al*., 2016).

Anthropogenic interference with the environment has resulted in bees being exposed to high levels of xenobiotics, disturbing and contaminating the natural balance among bees and their biomes and unbalancing the relationships between bees and natural parasites and predators (Celli and Maccagnani, 2003; Pywell *et al*., 2006; Gallai *et al*., 2009; Grixti *et al*., 2009; Martins and Melo, 2010; Cameron *et al*., 2011; Gradish *et al*., 2011; Abdalla *et al*., 2014; Goulson *et al*., 2015; Martín *et al*., 2015; Dicks *et al*., 2016; Potts *et al*., 2016; Woodcock *et al*., 2016; Domingues *et al*., 2017).

The increased need for the extraction of trace metals and metalloids used in the pharmaceutical, industrial and commercial technological production chains does not only destroy entire biomes, it can also release and disperse numerous types of highly hazardous trace metals into the environment through several types of biological and geological processes (Duruibe *et al*., 2007; Sims *et al*., 2013). Notably, the fraction of solid waste consisting of metals and metalloids, such as mercury, has increased markedly in the past few decades (Bernardes *et al*., 2004; Martín *et al*., 2015).

Numerous trace metals, such as mercury, copper, selenium, lead and cadmium, have been found in the nectar, resin, pollen and nests of *Apis* spp. and stingless bees (Roman, 2010; Carrero *et al*., 2013; Johnson, 2015). Trace metals are very harmful and their concentrations in the environment have been increasing, making them an important risk factor for bees (Celli and Maccagnani, 2003; Duruibe *et al*., 2007; Roman, 2010; Carrero *et al*., 2013). These metals are highly cationic elements in organic systems, given that they are converted into a stable oxidation state in acidic pH, a condition that exists in the stomach of all animals. The highly reactive cationic trace metals bind to DNA, proteins and enzymes, disturbing the whole metabolic system of the organism. The trace metals usually bond to sulfhydryl groups (-SH) of cysteine and sulfur atoms of methionine ($-SCH_3$), inhibiting and/or interfering with the enzymatic and DNA functions, causing a general metabolic disturbance, endocrine disruption, mutagenesis, cytotoxicity and genotoxicity (Duruibe *et al*., 2007; Ruttkay-Nedecky *et al*., 2013).

Cadmium (Cd), a by-product of zinc mining exploration, is one of the most dangerous trace metals and causes all sorts of damage, as mentioned above (Godt *et al*., 2006; Abdalla and Domingues, 2015; Wallace, 2015). Moreover, Cd can also be found as a component of agrochemicals, enhancing the harmful effects of such toxicants on the non-target entomofauna, especially bees (WHO, 2011; Wallace, 2015).

Studies associate Cd with a wide spectrum of deleterious effects on the reproductive tissues, including ovaries (Soares *et al*., 2013). The oviposition rate, hatchability and fecundity of female adults are impaired by exposure to cadmium. Cadmium exposure also delays ovarian maturation, inhibits vitellogenesis and changes the ultrastructure of cells in ovary tissues (Cervera *et al*., 2005; 2006; Abdalla and Domingues, 2015; Płachetka-Bożek *et al*., 2018). In addition, Cd can induce apoptosis in chicken ovarian tissue and decrease activities of SOD and GPx (Yang *et al*., 2012; Wan *et al*., 2017). When Cd exposure occurs in rats from weaning to maturity, Cd decreases ovarian wet weight and ovarian/body weight ratios and increases follicle apoptosis (Weng *et al*., 2014). Cadmium can also decrease male fertilization rate and sperm mobility (Zhao *et al*., 2017). In tadpoles of *Lithobates catesbeianus* (Shaw, 1802), Cd exposure provoked important anatomical changes on gonads, from total reabsorption to polygonadism and sex reversal (Abdalla *et al.*, 2013).

The internal organs of bees are sensitive biomarkers for monitoring the effects of environmental stress, especially of those stresses that are directly related to reproduction and general metabolic homeostasis (Abdalla and Domingues, 2015). It is imperative to study the internal organs of bees to understand how and why bees die when exposed to xenobiotics and trace metals, however, the internal organs are frequently overlooked in ecotoxicological studies of bees (Landa *et al*., 1983; Forkpah *et al*., 2014; Skaldina and Sorvari, 2017).

According to the World Health Organization (WHO, 2011), the cadmium limit for drinking water is 3 ppb. In Brazil, the trace metal concentration in the water is regulated by the Brazilian Environmental Council (CONAMA) (2005), which considers a concentration of 1 ppb cadmium to be environmentally safe for class 1 and class 2 water. Such water is used in the domestic supply after simplified (class 1) or conventional (class 2) treatment, it is also used for recreation, the irrigation of vegetables and fruits and the protection of aquatic communities (CONAMA, 2005). Both in the countryside and in urban areas, bees have access to these types of water. Therefore, the presence of trace metals in the water an important route for poisoning, possibly contributing to the disappearance of bees (WHO, 2011; Johnson, 2015).

We investigated the effect of 1 ppb cadmium on the ovaries of *Bombus morio* to verify if the cadmium concentration considered environmentally safe impacts the cells of the reproductive biology of the females. As the colony biological cycle of *B. morio* begins with one fecundated solitary queen, or solitary phase, these bees are predisposed to be more susceptible to environmental impacts.

Materials and Methods

Bee Collection

Workers of *Bombus morio* (Swederus, 1787) were collected from the remaining fragments of the semi-deciduous forest and Cerrado in the municipality of Sorocaba (23° 34 53.1 S 47° 31 29.5 W), state of São Paulo, Brazil. All workers were collected from *Cassia* sp. Linnaeus (1753) flowers between 9 am and 11:30 am. Bees were collected individually with an entomological net and immediately transferred to a 50 mL Falcon tube (one bee per tube). All Falcon tubes containing bees were kept in a thermic box in the dark to avoid stressing the bees.

Ecotoxicological Bioassay

The workers were kept individually in plastic boxes that were 10×14×10 cm with two feeders glued to the bottom of the box (close to the wall), including one for food and another for water or the contaminated solution and the boxes were kept inside an incubator (31°C, RH 70%, in the dark). The bees were fed *ad libitum* with a solid mixture of honey, dehydrated pollen and organic soy flour. Bioassays were conducted with replicates for both the control and experimental groups (n = 11 for each). The control group was offered 2 mL of water and the experimental group was offered 2 mL of cadmium solution (cadmium chloride; Sigma-Aldrich, ≥99.5% purity) at a concentration of 1 ppb or 1 ng L^{-1}. The dilution calculations were made only with the cadmium molecular weight. After 48 h of exposure, all bees were sacrificed by cryo-anesthesia (bees were hibernated at 4°C for 30 minutes before being dissected) and the ovaries of the bees were dissected directly in a fixative solution.

No formal permission or ethical form submission was required for the areas where the bees were collected.

Preparation of the Material for Light Microscopy

The ovaries were dissected and fixed in 4% paraformaldehyde in 0.2 M sodium phosphate buffer (pH 7.4), following preparation by slow dehydration in increasing ethanol solutions according to the methodology described by Silva-Zacarin *et al*. (2012). Upon fixation, the material was embedded in JB-4 resin (Leica Biosystems Nussloch GmbH, Heidelberg, Germany), according to the manufacturer's recommendations. Histological sections of 1.5 μm thickness were cut with a Leica microtome (RM 2255) and stained with hematoxylin and eosin (Merck). The material was analyzed using a Leica light microscope (DM 1000).

Results and Discussion

Morphology of the Worker Ovary of the Control Group

The ovarian cycle and general morphology of the ovarioles of non-exposed bees were typical of polytrophic meroistic ovaries, as described for other bee species (Martins and Serrão, 2004; Tanaka and Hartfelder, 2004; Tanaka *et al*., 2009; Chapman, 2012), i.e., each ovariole was comprised of a terminal filament, germarium and vitellarium (Fig. 1A, Fig. 1E). The terminal filament was short and continuous with the anterior end of the germarium (Fig. 1A). The germarium: was in the proximal region of the ovarioles; was below and continuous with the terminal filament (Fig. 1A); contained the germ cells, mainly cytoblasts and clusters of cystocytes at different stages of development (Fig. 1B-C); and was permeated by small somatic cells or prefollicular cells (Fig. 1C). Also in the germarium, the nurse cells, oocyte and primordial pre-vitellogenic follicles had begun to differentiate (Fig. 1B-C). The initial development of the follicles was marked by a larger cell, the primary oocyte, with an aggregation of smaller cells, the early nurse cells, closely associated with the primary oocyte (Fig. 1C). In contrast, the vitellarium occupied the distal region of the ovariole and contained vitellogenic ovarian follicles at various stages of development (Fig. 1D-E).

Mature ovary follicles were divided into two distinguishable chambers that wereare covered by mature follicular cells (Fig. 1E). Two distinct chambers, the nurse chamber and the oocyte chamber, formed an ovarian follicle (Fig. 1E). They wereare arranged such that the follicles of the proximal region differed from the distal follicles. The most developed follicles were in the distal portion of the ovariole and the newly developed follicles, with small, early vitellogenic oocytes and large nurse chambers, were in the proximal portion of the ovariole, near the least differentiated portion of the germarium. Each chamber contained an oocyte and many nurse cells (Fig. 1E) that were connected through communication via intercellular bridges (Snodgrass, 1956; Chapman, 2012).

The main function of the nurse cells is to provide the oocyte with macromolecules, such as mRNA, rDNA, lipids and organelles (e.g., mitochondria); the yolk is provided indirectly by the follicular cells (Telfer *et al*., 1982). The yolk is produced by trophocytes as a protein precursor, which is released into the hemolymph and subsequently taken up by follicular cells. During vitellogenesis, the vitellogenin is exocytosed into the space between the follicular epithelium (Fig. 1E) and the vitellogenin and from that space, is endocytosed by the oocyte (Telfer *et al*., 1982; Fleig, 1995; Zelazowska, 2005).

The entire ovariole was surrounded outside by a cellular peritoneal sheath and by an inner non-cellular tunica propria (Fig. 1A-E). Although not shown in the present work, follicular abortion and the *corpus luteum* were also observed, which is a normal condition in the ovarian development of this and other species of bees; this morphology is found mainly in queens but can also be observed in egg-laying workers (Berger and Abdalla, 2005; Berger *et al*., 2005; Patrício and Cruz-Landim, 2008; Tanaka *et al*., 2009).

Morphology of the Worker Ovary when Exposed to Cadmium

In the germaria of cadmium-exposed bees, the follicular epithelium was unstructured, with the follicular cells detached from each other, or absent, or even organized in clusters of cells with very fragmented condensed chromatin remaining inside the lumen by the tunica propria and peritoneal sheath

(Fig. 2A-C, Fig. 3B-C). In a more advanced stage of early oogenesis, the intercellular connection between the oocyte and the nurse cells was broken (Fig. 2B) and the nurse cells became isolated (Fig. 2B-C). In addition, in this case, the follicular layer was incomplete and the isolated oocyte presented signs of nucleus disruption, with loss of the shape of the nucleus, which ranged in shape from spherical to very sinuous (Fig. 2B-C). The pre-vitellogenic follicles showed disorganization of the nurse cells or of entire pre-follicles (Fig. 2B-D). Both in the germarium and in the vitellarium, the peritoneal sheath was quite thick (Fig. 2A, Fig. 2D).

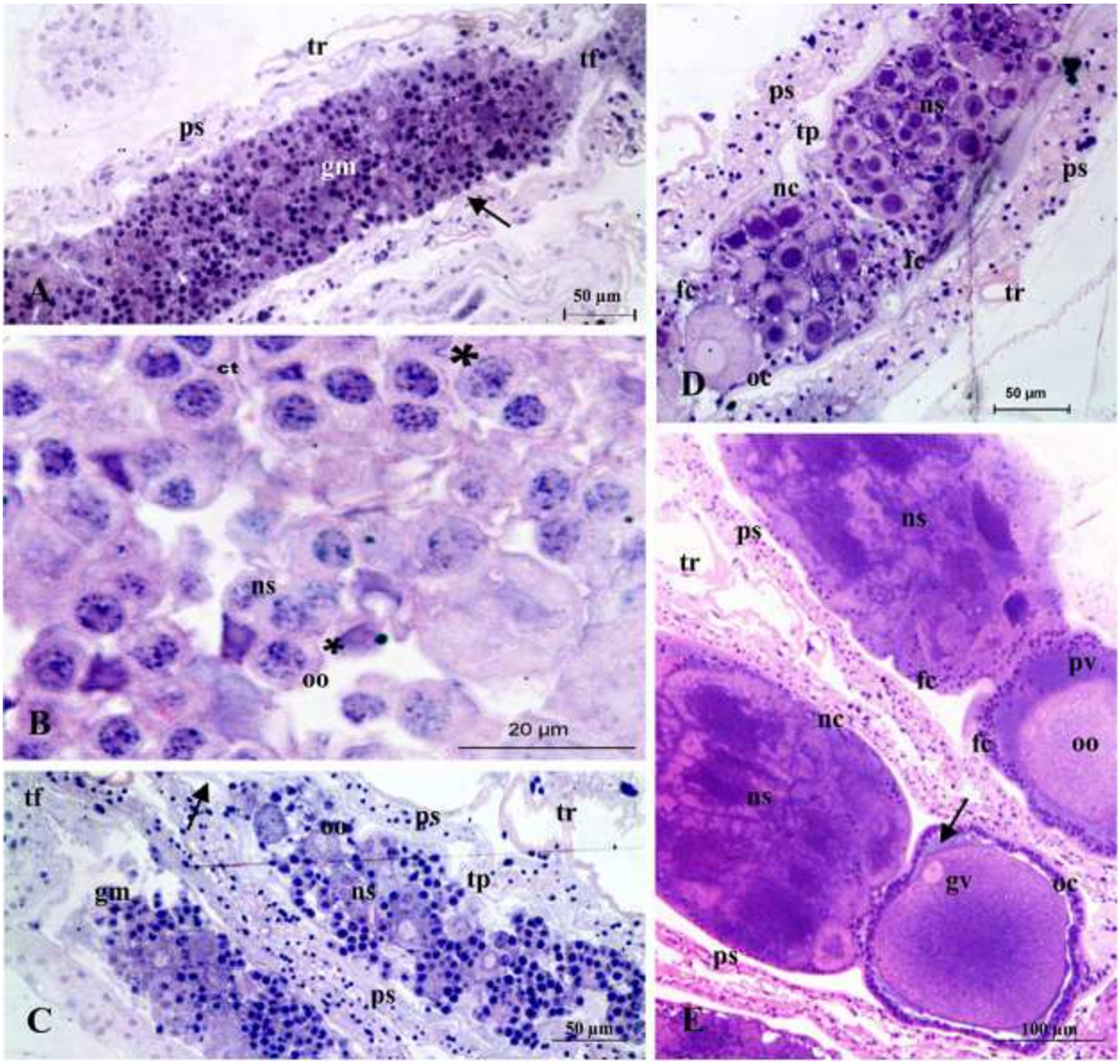

Fig. 1: Morphology of the ovaries of *B. morio* workers not exposed to cadmium. (A) A germarium (gm) enclosed by an outer peritoneal sheath (ps) and tracheoles (tr) and an inner non-cellular tunica propria (arrow). The germarium is continuous with the terminal filament (tf). (B) Detail of the germarium with cystocytes (ct) undergoing differentiation (asterisk). Some groups of cystocytes present a developing oocyte (oo) connected to the smaller nurse cells (ns). (C) Detail of the germarium (gm) with cysts differentiated into an oocyte (oo) that is linked to smaller, differentiated nurse cells (ns). (D) Vitellarium with differentiating pre-vitellogenic follicles, showing developing nurse (nc) and oocyte (oc) chambers and follicular cells (fc). (E) Vitellogenic follicles with defined nurse chambers: surrounded by somatic cells (sc), where nurse cells (nc) and an oocyte (oo) developed; and surrounded by the follicular cells (fc), which form the nurse (nc) and oocyte (oc) chamber. Notice the oocyte (oo) with yolk and germinal vesicle (gv). The perivitelline space (pv) contains basophilic material inside (arrow)

DOI: 10.3844/ajabssp.2018.28.37

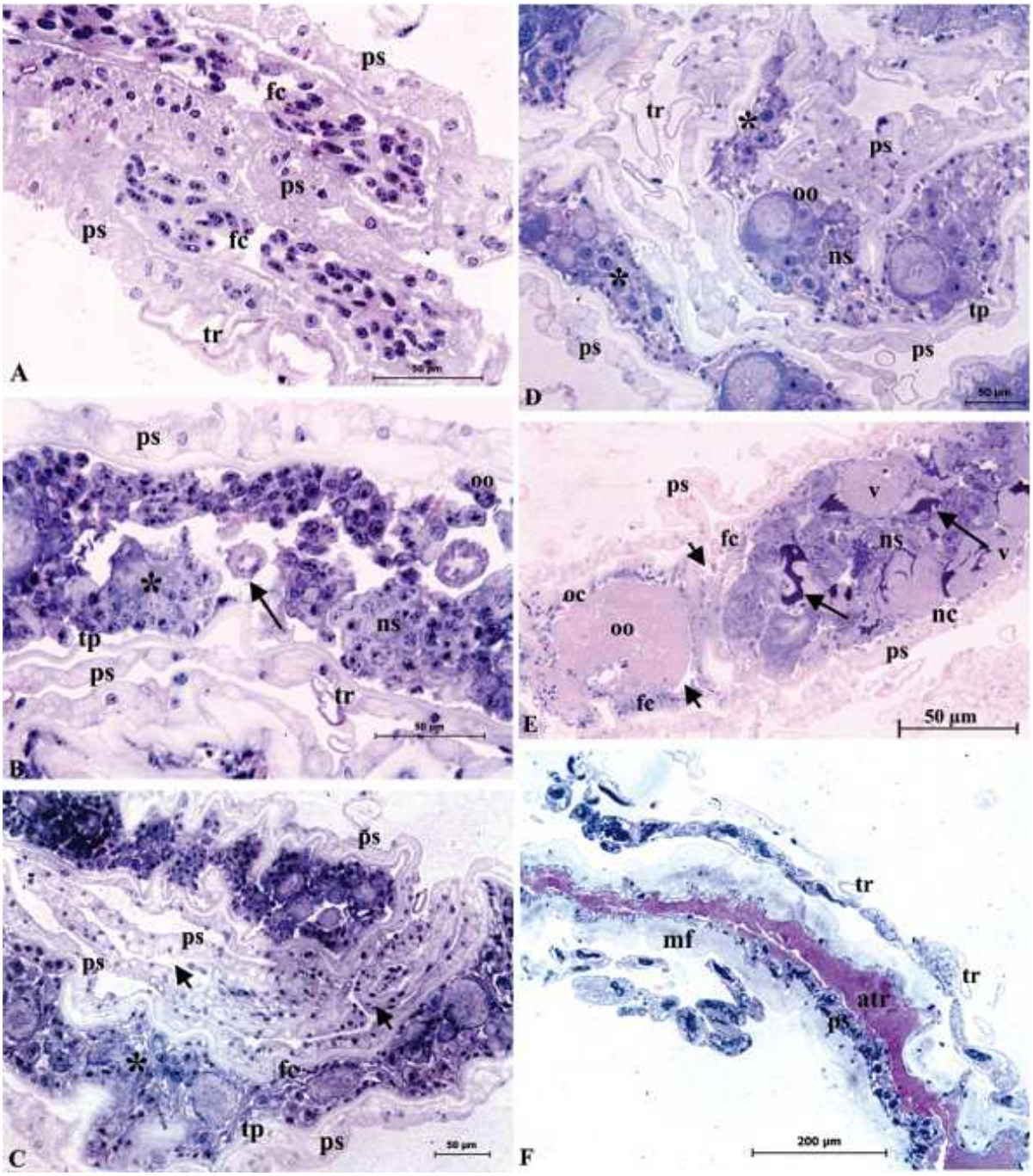

Fig. 2: Morphology of the ovaries of *B. morio* workers that have been exposed to cadmium. (A) Ovarioles of a germarium enclosed by a thick peritoneal sheath (ps) filled with follicular cells (fc) and tracheoles (tr). (B) Ovarioles of a germarium with disorganized pre-follicles (asterisk), detached (arrow) oocytes (oo) and a mass of dying nurse cells (ns). Note the thick peritoneal sheath (ps) and tunica propria (tp). (C) The germarium with the ovarioles described in panels (A) and (B) showing collapsed peritoneal membranes with empty lumens (arrowheads). (D) A subset of pre-vitellogenic ovarioles show degeneration of the nurse cells (asterisk). (E) A vitellogenic follicle showing nurse cells (ns) with large vacuoles (v) and compacted, fragmented chromatin (arrow). Note the oocyte (oo) with signs of degeneration. Notice in the nurse (nc) and oocyte chambers (oc), disrupted follicular cells (fc) and emptiness of the perivitelline space (arrowheads) (F) The posterior portion of the vitellarium showing atresia (atr) of an entire follicle. Follicle atresia is characterized by destruction of the follicular cells and both nurse and oocyte chamber, forming a mass of basophilic nature covered by a thick and disrupted peritoneal sheath (ps) and muscle fiber (mf)

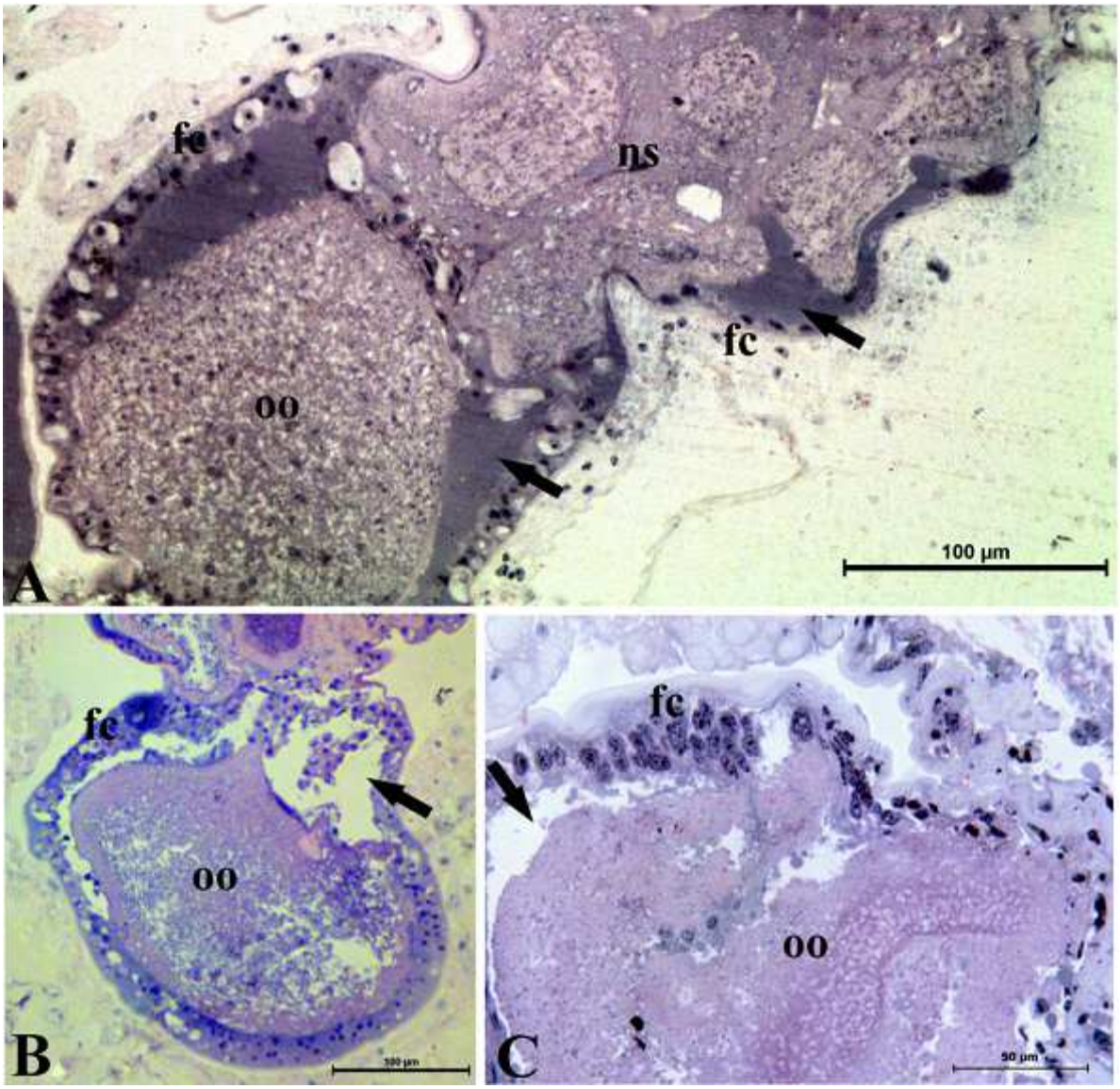

Fig. 3: Detail of the follicular cells and perivitelline space in ovaries of *B. morio* workers control and exposed to 1 ppb cadmium. (A) Follicle of a non-exposed worker, showing the follicular cells (fc) surrounded by a well-developed perivitelline space with basophilic material inside (arrow). Notice the nurse cells with decondensed chromatin (ns) and a typical initial oocyte (oo). (B) Detail of the oocyte chamber of an exposed worker, showing a vacuolated, unshaped oocyte (oo) and the emptiness of the perivitelline space (arrow). (C) Detail of the follicular epithelium disruption of the oocyte chamber. Notice the disruption of the epithelium of follicular cells (fc) and the emptiness of the perivitelline space (arrow)

All these ovarian alterations occurred in the pre-vitellogenic phase or with the initial development of the ovarian follicles. This ovarian phase does not depend on any exogenous stimuli and other than the fact that the fat body may supply the primary oocyte with some compounds for initial development, its participation in ovarian development begins in the vitellogenic phase, which depends on exogenous stimuli, such as mating (Tanaka and Hartfelder, 2004; Berger *et al*., 2005).

The death of nurse cells culminates in the full development of an oocyte (Reginato and Cruz-Landim, 2003; Tanaka *et al*., 2009), but what was observed in the ovaries of cadmium-exposed bees is the death of or damage to entire follicles (Fig. 2F), with chromatin condensation of the nurse cells in the initial (Fig. 2B-C) and advanced vitellogenic follicles (Fig. 2D). In addition, extreme fragmentation and compaction of the nurse cell chromatin were observed in vitellogenic follicles, which contained severe vacuolization of the cytoplasm (Fig. 2E, Fig. 3C). The most remarkable result concerning the perivitelline space was that it was filled with eosinophil material in the control group (Fig. 3A),

whereas in exposed bees, it was totally empty (Fig. 3B). The follicular epithelium had clearly broken down (Fig. 3B-C) and the oocyte appeared vacuolated and misshapen (Fig. 3B), providing strong evidence that the vitellogenin transfer from the follicular cells (Telfer *et al*., 1982) was blocked stopped in cadmium-exposed bees. In an extreme case, the oocyte and nurse chambers were totally degenerated, forming an acidophilic homogenous mass with a very thick and atypical peritoneal sheath (Fig. 2F). The peritoneal sheath cells showed signs of cell death, with cytoplasm vacuolization and very condensed chromatin (Fig. 2F), a condition called follicle atresia (Uchida *et al*., 2004).

From a broader perspective, the pronounced effect of cadmium exposure on the ovaries of workers of *B. morio* can affect several aspects of the biology of this species. Cadmium may have a synergetic effect on the ovaries of *B. morio* workers: it may act directly on the entire ovary but may also affect the cells of the HNS (Abdalla and Domingues, 2015), primarily by affecting trophocytes, which supply all the vitellogenin to the follicular cells, which then release the vitellogenin for the oocyte (Telfer *et al*., 1982; Fleig, 1995; Zelazowska, 2005). Cadmium also severely compromised the availability of nutrients in the hemolymph because, as previously described by Abdalla and Domingues (2015), all the pericardial cells exposed to 1 ppb cadmium in *B. morio* workers were at stage IV, i.e., maximum capacity for hemolymph absorption. This leads to a decrease in the availability of not only vitellogenin but also other nutrients in the hemolymph; hemolymph is intensively sequestered by the pericardial cells, dramatically reducing the cycling of hemolymph into the hemocoel. The emptiness of the perivitelline space and the disruption of the follicular cells in cadmium-exposed bees are in accordance with the total disruption of trophocytes of *B. morio* exposed to cadmium (Abdalla and Domingues, 2015).

At the molecular and biochemical levels, cadmium exerts its toxic effects by replacing the co-factor of the metalloenzymes and metalloestrogens, such as the metallothioneins, a group of enzymes involved in protecting the natural and essential metal ion homeostasis and also in controlling oxidative stress (Margoshes and Vallee, 1957; Ruttkay-Nedecky *et al*., 2013; Wallace, 2015). The enzyme co-factor can be substituted for a metal ion of similar size (e.g., zinc for cadmium) and this replacement prevents the enzymes from performing their functions. In most cases, the enzymes are destroyed, disturbing all metabolic homeostasis of the organism (Margoshes and Vallee, 1957; Duruibe *et al*., 2007). Cadmium can also associate with non-metalloenzymes, interacting with the sulfhydryl radicals of cysteine-rich proteins (Margoshes and Vallee, 1957; Duruibe *et al*., 2007), such as vitellogenin (Piulachs *et al*., 2003). Thus, cadmium itself is a potent endocrine disruptor, cytoskeleton destabilizer and mutagenic agent (Godt *et al*., 2006; Wallace, 2015).

The ovary collapsed in the workers exposed to 1 ppb cadmium and this could also have further implications on the whole biological cycle of the colony of *B. morio*. As in *Bombus terrestris* (Garófalo, 1978a; 1978b; Duchateau and Velthuis, 1988), the colony biological cycle of *B. morio* begins with one fecundated solitary queen and at the end of the colony cycle the workers contribute by laying eggs that will develop into males. In this way, the collapse of the cadmium-exposed ovary could affect the foundation of new colonies and the cadmium exposure could also severely impair the fitness and success of new colonies by damaging the ovaries of the workers. Although there is clear evidence of the morphological impacts in bees exposed to cadmium, further studies are required to corroborate some of the aforementioned postulations.

Conclusion

Although 1 ppb cadmium is considered environmentally safe by the Brazilian Environmental Council (CONAMA) and by the World Health Organization (WHO), this trace metal acted negatively on the ovary morphology of *B. morio*, leading to the collapse of the ovary in the workers. As a consequence, this is likely to have implications on their reproductive capability and this negative impact may also be extended to include the queens, as well as egg-laying workers.

Acknowledgement

We thank the Department of Biology (DBio) and Laboratory of Structural and Functional Biology (LABEF) for the use of their facilities.

Funding Information

Special thanks to the São Paulo Research Foundation, FAPESP (grants 2017/0605-09, 2016/15743-7, 2014/04697-9), for the fellowship in productivity of research (level 2), the Brazilian National Council for Scientific and Technological Development, CNPq (grant 302812/2016-4) and the Coordination Improvement of Higher Education Personnel, CAPES.

Author's Contributions

Fábio Camargo Abdalla: Supervision and analyses.

Marina Pedrosa: Material processing and analyses.

Caio Eduardo da Costa Domingues: Analyses, diagramming and review.

Paulo José Balsamo: Analyses and review.

Ethics

The authors have declared that there are no conflicts of interest.

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