

Exposure to Mercury at Trace Concentrations Leads to Collapse of the Hepato-Nephrotoxic System in Two Neotropical Species of Bumblebee

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Abstract: This research aimed to investigate the effects of exposure to safe concentrations of mercury on the morpho-physiology of cells of the hepato-nephrotoxic system of two species of neotropical bees. Workers of the neotropical bumblebee species *Bombus morio* and *B. atratus* were exposed to 0.2 ppb mercury for 48 h by ingestion. Bioassays were performed according some direction of OECD (2017) for bioassays in *B. terrestris*. The mercury concentration used is allowed for all types of water bodies for the Brazilian Environmental Council standards. The results showed that exposure severely impacted the fat body cells (oenocytes and trophocytes) and pericardial cells, either morphology and HSP70 expression, of both species and may represent a threat for these neotropical bee species. We observed that *B. atratus* is more sensitive to mercury exposure, although the two species have a very close phylogenetic relationship. To the best of our knowledge, this is the first study to compare the expression pattern of HSP70 in the fat body and pericardial cells of neotropical bumblebees under normal and mercury-exposure conditions. Both species expressed the protein in all studied cells under normal and exposed conditions; however, mercury exposure led to overexpression of HSP70 in *B. morio* workers only, with reduced expression observed in *B. atratus* following exposure. We conclude that 0.2 ppb of mercury severely impacts both species, being *B. atratus* more susceptible.

Keywords: *Bombus*, Mercury, Pericardial Cells, Fat Body, HSP70

Introduction

The worldwide decline and loss of several species of bees of the *Bombus* genus has been recorded annually for the last decade (Gallai *et al.*, 2009; Grixti *et al.*, 2009; Carswell, 2015; Goulson *et al.*, 2015; Woodcock *et al.*, 2016; Rhoades *et al.*, 2016). Populations of *Bombus affinis* have decreased drastically in the USA and the species is now considered critically endangered by the U.S. Fish and Wildlife Service (2017). In the state of Paraná and in some parts of the states of Santa Catarina, Brazil, the decline and subsequent disappearance of *B. bellicosus* has also been reported (Martins and Melo, 2010).

In recent decades, the presence of metals such mercury has been noted in the combs and honey pots of bees which inhabit industrial areas (Roman, 2010; Carrero *et al.*, 2013; Pablo *et al.*, 2013). Therefore, the concentration of mercury in honey and pollen is a strong indicator of environmental pollution and an indicator of potential threats to the bee communities (Toporcák *et al.*, 1992; Bogdanov *et al.*, 2007). Mercury can contaminate the raw materials of bee products (nectar and pollen) via the air, water and soil during plant cultivation. Contaminants are transported into the colonies along with the raw materials (Buldini *et al.*, 2001; Bogdanov *et al.*, 2007; Bratu and Georgescu, 2005; Perna *et al.*, 2012; Mbiri *et al.*, 2011 Carrero *et al.*, 2013; Pablo *et al.*, 2013), with serious detrimental effects, as

mercury exposure can be lethal to bees (Roman, 2010; Tchounwou *et al.*, 2012; Martín *et al.*, 2015).

The impact of trace metals on terrestrial organisms is a growing area of research in Brazil and methods of environmental assessment should be developed in case of environmental disaster such as that which occurred in the state of Minas Gerais recently. The collapse of a dam containing millions of litres of mining waste led to many trace metals including mercury, arsenic, chromium and manganese being released into the Rio Doce river at levels which exceeded safe limits for human consumption (Massarini, 2015). The contamination reached the coast of the state of Espírito Santo, with potential impacts for the wider marine ecosystem (Massarini, 2015). Despite the magnitude of this ecological disaster, few is known about the effect of these metals on the impacted native fauna and flora.

Mercury is not an essential trace metal and is highly toxic for all living beings at any concentration. Environmental levels of mercury have markedly increased in the past few decades (Martín *et al.*, 2015). Besides the well-known neurotoxic effects, cationic trace metals bind to DNA, proteins, enzymes and COO- groups of fatty acids; which can disturb the entire metabolic system of the affected organism. This can cause general metabolic disturbance, endocrine disruption, mutagenesis, cell death and cyto- and genotoxicity (Duruiibe *et al.*, 2007; Tchounwou *et al.*, 2012, Abdalla *et al.*, 2015; Abdalla *et al.*, 2018).

Mercury salts such as mercury chloride, ethylmercury, methylmercury and bacterial metabolic residues have strong affinities for organic sulphhydryl thiols. Thiol groups contain sulphur, carbon and hydrogen, which are important constituents of all biological macromolecules and the arrangement of these elements determines the structure and function of such molecules. Mercury can bind to the amino acids of sulphhydryl groups, such as cysteine, glycine, methionine and taurine; forming mercaptides (methyl thiolates). When this reaction occurs in the amino acids of enzymes, the catalytic function can be disrupted or completely eliminated (Jordan and Bhatnagar, 1990; Zimmermann *et al.*, 2013).

The most common reaction of mercury is the interaction with sulphur atoms of cysteine residues to form relatively stable mercury–cysteine complexes. Such complexes can alter the tertiary structure of the protein, with consequent disruption of protein function (Bernhoft, 2012; Caterino *et al.*, 2014). In this way, mercury can lead the inactivation of Heat Shock Proteins (HSPs) which are ubiquitous ATP-dependent molecular chaperones essential for the maintenance of cellular function and homeostasis in almost all living beings (Silva-Zacarin *et al.*, 2006; Pratt *et al.*, 2011). These proteins are classified according to their molecular weight and the most well studied are members of the

HSP70 family (Pratt *et al.*, 2011). Constitutively present in the cytoplasm and nucleus of all eukaryotic cells, HSP70 is involved in ensuring correct protein folding. When eukaryotic cells are under conditions of stress; HSP70 is overexpressed to prevent protein misfolding, mitochondrial damage and nuclear fragmentation (Pratt *et al.*, 2011). In the salivary glands of honeybees under conditions of stress, HSP70 functions as an effective antiapoptotic protein (Silva-Zacarin *et al.*, 2006), leading it to be considered an effective molecular biomarker to study cells under stress in ecotoxicology.

The Hepato-Nephrocytic System (HNS) is a system of fat body and pericardial cells that surround the dorsal vessel, along with haemocytes. The HNS was first described by Abdalla and Domingues (2015) in workers of the neotropical bumblebee *B. morio* that had been exposed to a “safe” concentration of cadmium. The HNS is involved in immune cell responses via the activity of the haemocytes. Through the pericardial cells, oenocytes and trophocytes the HNS filters, detoxifies and/or alters the bioavailability of xenobiotics for excretion by the Malpighian tubules. Thereby, the survival and mortality rates of bees that are exposed to xenobiotics may not be representative, due to detoxification of the toxins through the HNS (Domingues *et al.*, 2017).

This study aimed to evaluate the morphological, histochemical and HSP70 localisation effects of mercury exposure in the oenocytes, trophocytes and pericardial cells of the HNS (Abdalla and Domingues, 2015) of *B. morio* and *B. atratus* workers. A concentration of mercury was used that is considered environmentally safe (0.2 ppb) according to the Brazilian Environmental Council (CONAMA, 2005).

Materials and Methods

Bee Collection

Workers of *Bombus morio* (Swederus, 1787) and *B. atratus* (Franklin, 1913) 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") in the State of São Paulo, Brazil. All workers were collected from flowers of *Cassia Linnaeus Sp.* (1753), 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 stress on bees.

Ecotoxicological Procedures

The workers were kept individually in plastic boxes of 10×14×10 cm with two feeders glued to the bottom of the box (close to the wall), one for food and one for either fresh water or contaminated solution. The boxes were kept inside an incubator (26°C, relative humidity [RH] 70%, in the dark). They were fed *ad libitum* with a

solid mixture of honey, dehydrated pollen and organic soy flour. Bioassays were conducted in replicate 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 mercury solution (mercury chloride; Sigma-Aldrich, 99.5+%) at a concentration of 0.2 ppb (0.2 ng·L⁻¹) mercury. After 48 hours of exposure, all bees were sacrificed and the dorsal vessels were dissected directly in fixative solution of 4% paraformaldehyde. The OECD (2017) guidelines for the testing of chemicals in *B. terrestris* were consulted.

Morphological and Histochemical Techniques

The dorsal vessels of the bees were fixed in 4% paraformaldehyde for 24h. After fixation, the material was embedded in JB-4 resin (Polysciences, Leica Biosystems Nussloch GmbH, Heidelberg, Germany) according to manufacturer's recommendations, or in paraffin for Acridine Orange and HSP70 immunohistochemistry. Histological sections of 2 µm were cut using a Leica microtome (RM2255). The material was stained with haematoxylin and eosin for routine morphological analysis. For fluorescence analysis of HSP70 expression and Acridine Orange analysis, paraffinised histological sections of 6 µm were used. For histochemical analysis, the material was stained with Acridine Orange to verify chromatin fragmentation and condensation level. Immunofluorescence was used for the in-situ detection of HSP70 (primary antibody: Goat Serum Normal, secondary antibody: Mouse Anti-Goat IgG-FITC, 1/500) according to the manufacturer's protocol (polyclonal antibody protocol; Santa Cruz Biotechnology Inc., USA). Imaging was carried out on a Leica photomicroscope (DM1000). Fluorescence imaging was carried out on a Leica Fluorescence photomicroscope (DM4000) with a B/G/R fluorescence optical system with ultraviolet (BP 465/20), blue (BP 530/30) and green (BP 640/40) excitation bands.

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

Analysis of Fluorescence Intensity

The automatic analysis function of the Leica LAS V4.5 software was used to quantify the intensity of the green fluorescence in pericardial and fat body cells for immunofluorescence analysis. Ten panoramic micrographs were analysed (pericardial and fat body cells at magnification of 10×) for each individual bee of both the control and experimental groups, resulting in a total of 110 measurements for each group of each species. The variation of the average green intensity was compared within each group (control and experimental) using the Mann-Whitney U test. Tukey's post-hoc multiple comparisons test and one-way analysis of variance (ANOVA) for non-parametric samples (GraphPad Prism

V5.04 software) were carried out for each species in isolation and between the two species.

Results

Morphology of the Hepato-Nephrotic System

Trophocyte nuclei from typical branches of the control groups are shown in Figs. 1A and 2A. Following exposure to mercury, the nuclei of trophocytes of both species were decreased in number and showed a rounded morphology (Figs. 3A and 4A). In the exposed group, trophocytes and oenocytes were identified as collapsed structures; an effect that was especially pronounced in *B. atratus* (Figs. 3A and 3B). The oenocytes of the exposed groups lost their characteristic shape (Figs. 3B and 4B) and these cells of *B. morio* exhibited irregular contours and central pyknotic nuclei (Fig. 4B). In *B. atratus*, the oenocytes formed an indistinguishable cellular mass (Fig. 3B).

The pericardial cells of the control groups were observed to be in stage I for both species; with central nuclei in an epithelioid arrangement, homogeneous cytoplasm and an absence of pinocytosis (Figs. 1C and 2C). The pericardial cells of *B. atratus*, following exposure to mercury, presented pyknotic nuclei (Fig. 3C). In mercury-exposed *B. morio* workers, the pericardial cells were turgescient with obvious chromatin fragmentation and condensation, as well as peripherally-located lumps of chromatin (Fig. 4C).

Histochemistry and In-Situ Localisation of HSP70

Immunofluorescence analysis using acridine orange staining revealed that the nuclei of oenocytes, trophocytes and pericardial cells of the control group emitted green fluorescence (Figs. 1D-F, 2D-F). Oenocytes of *B. morio* showed red/orange staining in the cytoplasm (Fig. 2E), the trophocytes only showed green staining (Fig. 2D). Following exposure to mercury, the HNS cell nuclei of both species became red/orange (Figs. 3D-F, 4D-F).

In the control group of both species, HSP70 was localised to both the cytoplasm and nuclei of oenocytes (Fig. 1H and 2H). Examination of the trophocytes revealed that HSP70 was only present in the cytoplasm for both species (Fig. 1G and 2G). The pericardial cells of both species showed weak HSP70 fluorescence (Figs. 1I and 2I). When the exposed group of *B. atratus* was examined, HSP70 was not detected in the nuclei of trophocytes, oenocytes or pericardial cells (Figs. 3G-I). In the exposed group of *B. morio*, overexpression of HSP70 was observed in the cytoplasm of the trophocytes, oenocytes and pericardial cells (Figs. 4G-I).

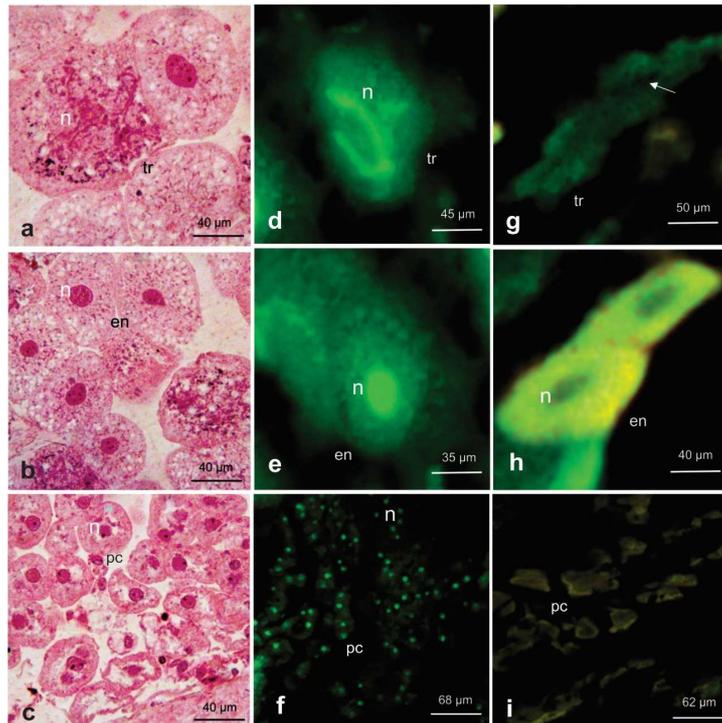


Fig. 1: Morphological and histochemical analysis of trophocytes, oenocytes and pericardial cells of the *Bombus atratus* control group. (A-C) stained with haematoxylin and eosin; (D-F) stained with Acridine Orange; (G-I) HSP70. **Key:** tr = trophocytes; en = oenocytes, n = nucleus; pc = pericardial cells, arrow = level of fluorescence in the nucleus.

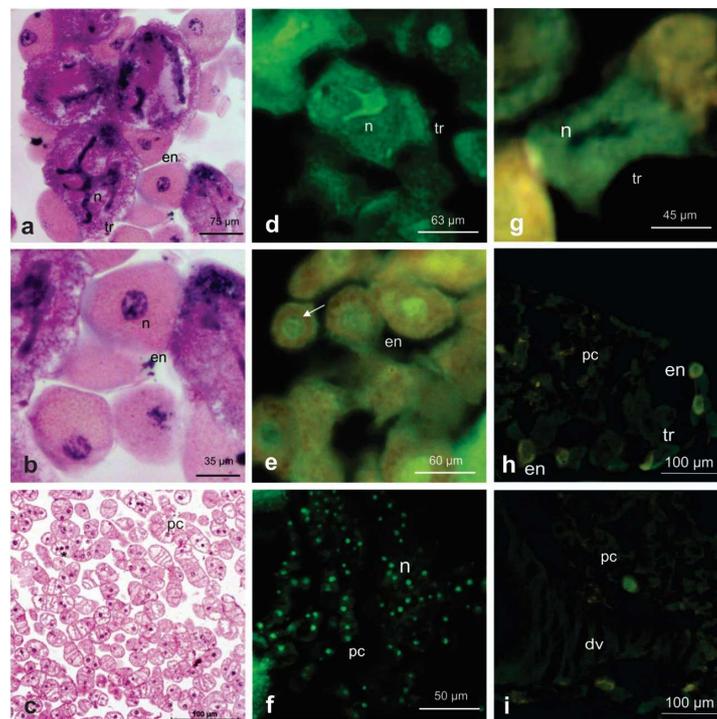


Fig. 2: Morphological and histochemical analysis of trophocytes, oenocytes and pericardial cells of the *Bombus morio* control group. (A-C) stained with haematoxylin and eosin; (D-F) stained with Acridine Orange; (G-I) HSP70. **Key:** tr = trophocytes; en = oenocytes; n = nucleus, arrows = RNA; pc = pericardial cells; dv = dorsal vessel; arrow = level of fluorescence in the nucleus.

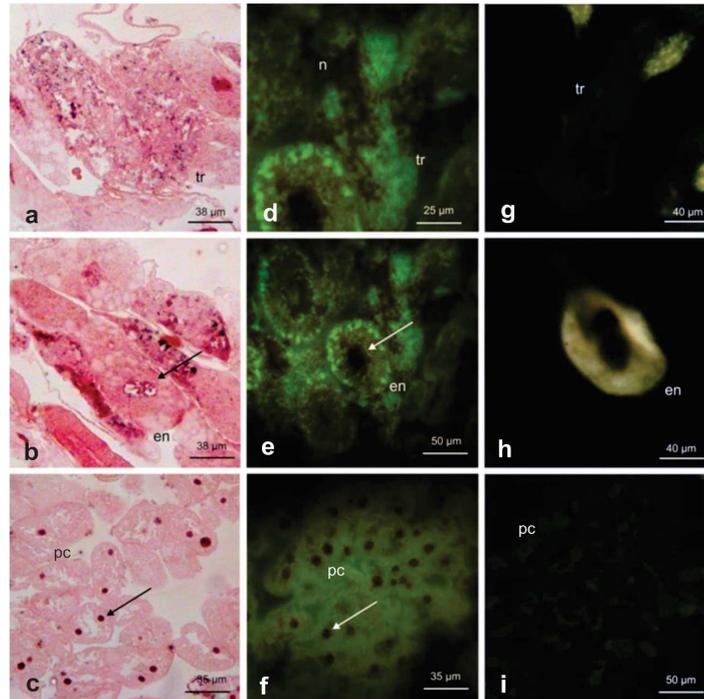


Fig. 3: Morphological and histochemical analysis of trophocytes, oenocytes and pericardial cells of the *Bombus atratus* exposure group. (A-C) stained with haematoxylin and eosin; (D-F) stained with Acridine Orange; (G-I) HSP70
Key: tr = trophocytes; en = oenocytes; n = nucleus; arrow = oenocytes and pericardial cell nuclei in (e) and (f), respectively, with chromatin compacted and fragmented; pc = pericardial cells; arrow = level of fluorescence in the nucleus.

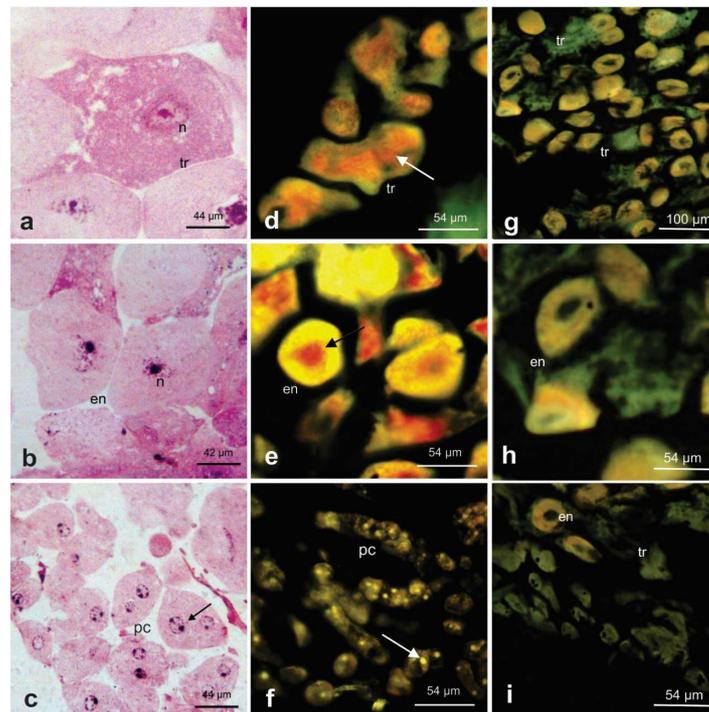


Fig. 4: Morphological and histochemical analysis of trophocytes, oenocytes and pericardial cells of the *Bombus morio* exposure group. (A-C) stained with haematoxylin and eosin; (D-F) stained with Acridine Orange; (G-I) HSP70.
Key: tr = trophocytes; en = oenocytes; n = nucleus; arrow = in (c) pericardial cells with chromatin fragmented and peripheral localisation; arrow = in (d), (e) and (f) fragmentation of the chromatin in the nuclei of the trophocytes, oenocytes and pericardial cells, respectively; pc = pericardial cells

Comparison of HSP70 Expression in *B. atratus* and *B. morio*

Fluorescence intensity is a tool which can be used to measure the expression level of fluorochrome-labelled proteins, providing insight that cannot be obtained through examination of the micrographs.

All cells of *B. morio* showed increased HSP70 expression following exposure to mercury (Fig. 5), whereas expression was reduced in *B. atratus* after exposure (Fig. 6). In *B. atratus*, the cells exposed to mercury showed significant damage (evident from the morphology and histochemistry results) and HSP70 was found to be under-expressed in the oenocytes and trophocytes ($P < 0.0001$). However, no significant difference in HSP70 expression was detected between the control and experimental pericardial cells of this species ($P = 0.4664$). In addition, the oenocytes of the control groups of both species had the highest levels of HSP70 expression, followed by trophocytes and pericardial cells (Figs. 5 and 6).

In worker bees who were not exposed to mercury, the expression of HSP70 was lower in the oenocytes, trophocytes (40/92) and pericardial cells (40/61) of *B. morio* compared with *B. atratus* (Figs. 5 and 6). The ratios of expression levels between the cell types was significantly different for the control and exposed groups and HSP70 expression increased significantly in all cell types following mercury exposure in *B. morio* (Fig. 5). However, this trend is almost identical in *B. morio*; that is, HSP70 expression is 230% higher in the oenocytes compared with trophocytes in the control group. Following mercury exposure, the fluorescence intensity in the trophocytes did not differ significantly from that of the pericardial cells in either species (Figs. 5 and 6).

Discussion

The results of the present study indicate that mercury has general systemic effects, as evidenced by the changes observed in all cell types of *B. atratus* and *B. morio* that were analysed (trophocytes, oenocytes and pericardial cells). This suggests that the effects of mercury exposure reach beyond the cells of the nervous system, as HNS cells were found to be in the process of cell death.

Workers of *B. morio* and *B. atratus* showed extensive morphological injury of the HNS following 48 h exposure to 0.2 ppb of mercury. Our morphological analysis provides clear evidence that the fat body cells of both species were damaged, despite the fact that the concentration of mercury that was used is considered safe. The collapse of trophocytes indicated that the exchange machinery between the nucleus and cytoplasm were deactivated, which can lead to disruption of the

intermediary metabolism (Roma *et al.*, 2010). The oenocytes also showed significant damage, which could suggest that all of the detoxication enzymes expressed by these cells—such as the P450 enzyme superfamily (Komagata *et al.*, 2010)—may be disabled as a result.

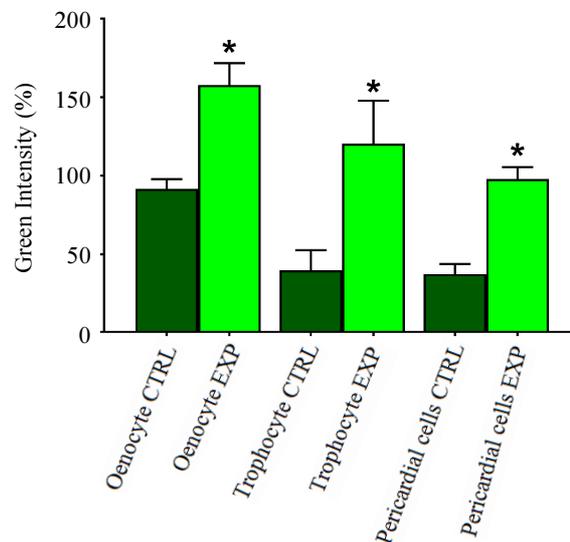


Fig. 5: Histogram of fluorescence intensity of labelled HSP70 in trophocytes, oenocytes and pericardial cells of the control and experimental groups of *Bombus morio*.

Key: * = HSP70 under-expression in oenocytes and trophocytes for the experimental groups ($P < 0.0001$), CTRL = control group, EXP = experimental group.

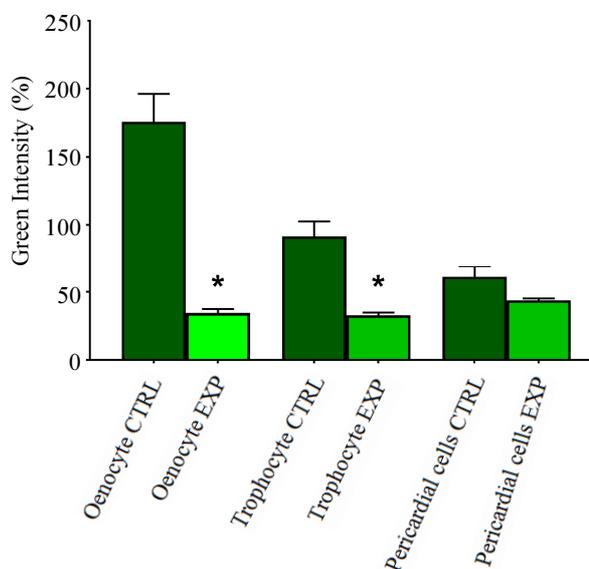


Fig. 6: Histogram of fluorescence intensity of labelled HSP70 in trophocytes, oenocytes and pericardial cells of the control and experimental groups of *Bombus atratus*

Key: * = HSP70 under-expression in oenocytes and trophocytes for the experimental groups ($P < 0.0001$), CTRL = control group, EXP = experimental group.

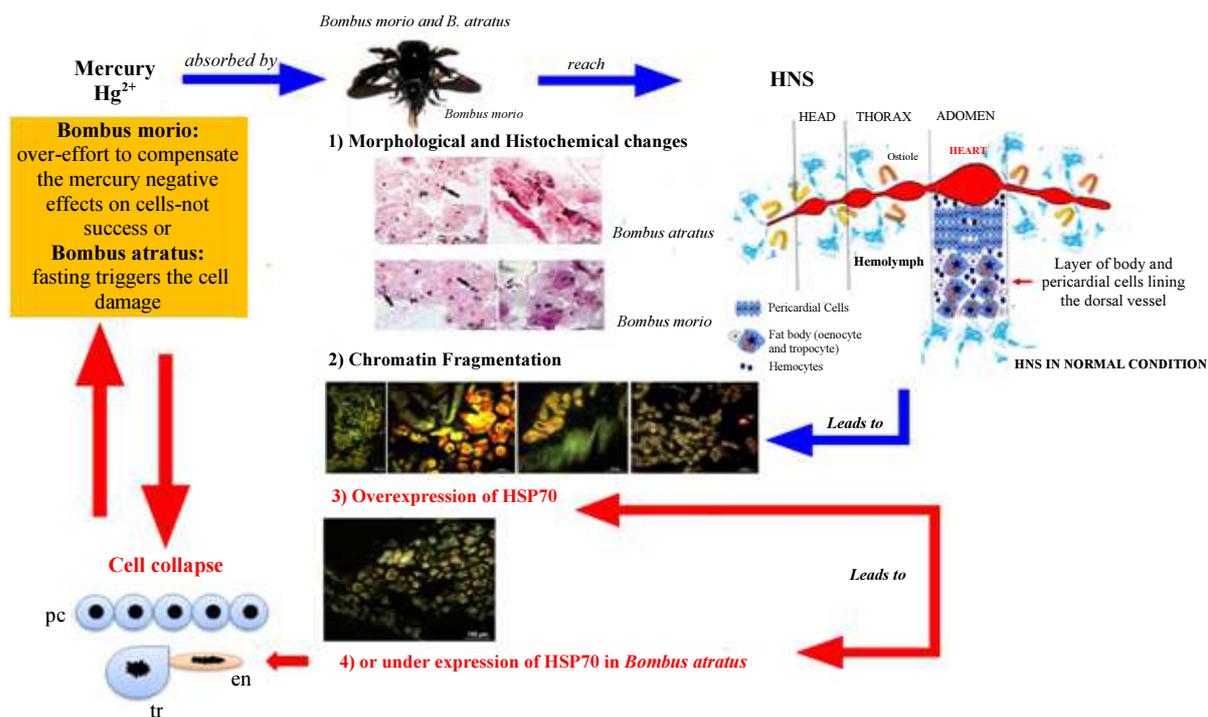


Fig. 7: Infographic summarizing the main the results

The oenocytes of *B. atratus* became an indistinguishable cell mass following mercury exposure and the detoxication system provided by these cells was clearly disabled. The same effects were observed in *B. morio* following exposure to 1 ppb cadmium (Abdalla and Domingues, 2015). In summary, morphological analysis revealed that the effects of mercury exposure on HNS cells were more dramatic in *B. atratus* (Fig. 7).

In healthy bees, the pericardial cells are composed of stage I cells, as was observed in the control groups of the present study. When the HNS is activated, the cells increase in size due to increased pinocytosis activity (Mills and King, 1965; Abdalla and Domingues, 2015; Domingues *et al.*, 2017). If the pericardial cells reach stage IV, pinocytosis has reached maximal activity, which may affect the distribution of nutrients and hormones amongst the internal organs of bees due to the associated decrease in haemolymph (Abdalla and Domingues, 2015; Domingues *et al.*, 2017). However, if the pericardial cells are disabled, haemolymph homeostasis is disrupted because xenobiotics in the haemolymph remain in contact with the internal organs for longer (Abdalla and Domingues, 2015; Domingues *et al.*, 2017).

Following exposure to 1 ppb cadmium, pericardial cells have been observed to enter stage IV (Abdalla and Domingues, 2015), whereas exposure to 0.2 ppb mercury resulted in the pericardial cells entering an atypical stage I for both species of the present study. However, *B.*

atratus appeared to be more sensitive to mercury than *B. morio*, as the pericardial cells showed signs of more significant damage. The same signs were observed for trophocytes and oenocytes.

The results of acridine orange staining indicated the extent of chromatin fragmentation and cell death in the HNS cells of mercury-exposed *B. atratus* and *B. morio* workers. Acridine orange is cell-permeable and nucleic acid selective, with metachromatic properties. When it binds to DNA it emits green fluorescence at 520 nm and red fluorescence at 650 nm when it binds to RNA in acidic conditions. The nuclei of non-damaged cells of paraffinised histological preparations which emitted green fluorescence therefore contain intact chromatin, whereas the condensed or fragmented chromatin of apoptotic or necrotic cells is stained orange. The cytoplasm may also be stained green or orange, depending on the absence or presence of cytoplasmic RNA, respectively. This dye is a rapid and inexpensive technique to study nucleic acid metabolism (Ribble *et al.*, 2005); and when used in combination with other dyes, provides a useful tool to investigate cell death and molecular chaperones such as heat shock proteins (HSPs), thus representing a good method to study cells under stress (Silva-Zacarin *et al.*, 2006).

Our results suggested that the oenocytes, trophocytes and pericardial cells of the control group did not suffer chromatin damage. The red/orange cytoplasm of *B.*

morio oenocytes was due to the presence of abundant cytoplasm RNA and correlated with the function of these cells in producing enzymes related to detoxication (Roma *et al.*, 2010). Oenocytes of *B. atratus* did not contain cytoplasmic RNA. For both species, the HNS cell nuclei of mercury-exposed bees became red/orange, which confirms that the metal caused chromatin compaction and fragmentation and consequent damage of the cell nuclei, which will hinder cell functions.

Under normal conditions, HSP70 is expressed in constitutive quantities and is an essential molecule for vital biological processes including folding of newly synthesised polypeptides or refolding of misfolded proteins, assembly of multiprotein complexes and transport of proteins across cellular membranes (Pratt *et al.*, 2011). Overexpression of HSP70 suppresses mitochondrial damage and nuclear fragmentation; therefore, the protein functions as a potent antiapoptotic agent (Silva-Zacarin *et al.*, 2006). Under conditions of stress, the synthesis of stress-induced HSP70 can inhibit cell death and therefore increase the survival of cells exposed to a wide range of lethal stimuli (Silva-Zacarin *et al.*, 2006; Pratt *et al.*, 2011). Overexpression of HSP70 can also cause activation of HSP70-dependent proteins to induce ubiquitin-degradation of abnormal proteins (Pratt *et al.*, 2011). For these reasons, HSP70 is considered to be a molecular biomarker of cellular stress. In the present study, weak HSP70 fluorescence was observed in the cytoplasm or nuclei of trophocytes, oenocytes and pericardial cells of mercury-exposed *B. atratus* bees, which indicates that the cells had lost their ability to express the protein due to mercury-induced cellular damage.

Analysis of fluorescence intensity provided quantitative data on the differences in HSP70 expression patterns between the control and exposed groups of both species. We measured the intensity of green fluorescence of the cytoplasm of oenocytes, trophocytes and pericardial cells of the control and experimental group of both species. Surprising and very informative results were obtained. The HSP70 expression patterns of the control and experimental groups were dramatically different between the species studied. Because the synthesis of stress-induced HSP70 can inhibit cell death and therefore increase the survival of cells exposed to a wide range of lethal stimuli (Pratt *et al.*, 2011), overexpression of the protein in the oenocytes of mercury-exposed *B. morio* indicates that this species is significantly more resistant to mercury compared with *B. atratus*. The morphological and histochemical results support this observation.

To the best of our knowledge, this study is the first to show the expression patterns of HSP70 in these three cell types under normal and experimental conditions.

Conclusion

The results of this study indicate that exposure to 0.2 ppb mercury severely impacts the fat body and pericardial cells of *B. atratus* and *B. morio* worker bees, even though it is considered a “safe concentration” by the Brazilian Environmental Council. Exposure impaired the fitness of individuals and may represent a threat for neotropical bee species, which confirms our initial hypothesis. This study also demonstrates that *B. atratus* is more sensitive to mercury exposure than *B. morio*, although the species are phylogenetically close. This study is the first to investigate the expression pattern of HSP70 in the fat body and pericardial cells in neotropical bumblebees under normal and mercury exposure conditions. Finally, we can conclude that the oenocytes are the primary cells involved in the HSP70 protective response.

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Author Contributions

The authors contributed equally to this work.

Conflict of Interest

The authors declare no conflict of interest.

Ethics

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

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Abbreviation and Units

- HNS: Hepato-Nephrotoxic System
Hg: mercury
ppb: parts per billion
NADPH: Dihydropyridine-adenine dinucleotide phosphate
RH: relative humidity
mL: milliliter
ng L⁻¹: nanogram per liter
µm: micrometer
h: hour
P450: cytochrome P450
nm: nanometer
RNA: ribonucleic acid
HSP: heat shock proteins
CONAMA: National Council for the Environment
tr: trophocytes
en: oenocytes
n: nucleus
pc: pericardial cells
dv: dorsal vessel
CTR: control group
EXP: experimental group