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Morphological abnormalities in *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) treated with eight toxic compounds

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Abstract

Toxicity testing is essential for preventing chemical pollution of the environment. *Tribolium castaneum* can be used as an alternative model for preliminary toxicity screening, eight chemical compounds having known toxicity were evaluated. For example, mercury (II) chloride is considered toxic at concentrations higher than 0.1 mg/L in drinking water, phenol exposure to concentrations greater than 5 mg/L in drinking water can be dangerous, in the case of toluene exposure to concentrations greater than 200 ppm (parts per million) in the air can be harmful to health, hydrazine can be dangerous at concentrations higher than 1 mg/L in drinking water, and caffeine can cause adverse effects such as nervousness, insomnia, tachycardia, and tremors from exposure to doses higher than 500 - 600 mg in adults. In this case, adult insects were fed a diet of oatmeal supplemented with each separately toxic. The number of offspring insects, size, weight, and observation of abnormalities was evaluated. The eight chemicals assessed compounds only five had a visible effect on insect development, BPA and mercury (II) chloride induced abnormalities in the larval and pupal stages, while the phenol, toluene, and metronidazole, in the pupal stage only. Major anomalies were observed necrosis in larvae appendices, in pupae, papillae for sex differentiation are sclerotic or absent, and abnormality in the formation of head, limbs, wings, and appendages. Mercury (II) chloride was the most toxic due to the affected growth, development and reproduction of the insect.

Keywords: *Invertebrate; Toxic effects; Ecotoxicology; Reproduction.*

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Anomalías morfológicas en *Tribolium castaneum* herbst tratado con ocho compuestos tóxicos

Resumen

Las pruebas de toxicidad son esenciales para prevenir la contaminación química del medio ambiente. *Tribolium castaneum* se puede utilizar como modelo alternativo para la detección preliminar de toxicidad; se evaluaron ocho compuestos químicos con toxicidad conocida. Por ejemplo, el cloruro de mercurio II se considera tóxico a concentraciones superiores a 0,1 mg/L en agua potable, el fenol a concentraciones superiores a 5 mg/L en agua potable puede ser peligroso, para el caso del tolueno la exposición a concentraciones superiores a 200 ppm (partes por millón) en el aire puede ser perjudicial para la salud, la hidrazina puede ser peligrosa a concentraciones superiores a 1 mg/L en agua potable, y la cafeína puede causar efectos adversos como nerviosismo, insomnio, taquicardia y temblores por exposición a dosis superiores a 500 - 600 mg en adultos. Los insectos adultos fueron alimentados con una dieta de avena complementada con cada tóxico por separado. Se evaluaron el número de crías, tamaño, peso y anormalidades morfológicas. De los ocho compuestos químicos evaluados, sólo cinco tuvieron un efecto visible sobre el desarrollo de los insectos: el BPA (bisfenol A) y el cloruro de mercurio (II) indujeron anomalías en los estadios larvarios y pupales, mientras que el fenol, el tolueno y el metronidazol sólo en el estadio pupal. Se observaron anomalías importantes: necrosis en los apéndices de las larvas, en las pupas, papilas para la diferenciación de sexos escleróticas o ausentes y anomalias en la formación de la cabeza, extremidades, alas y apéndices. El cloruro de mercurio (II) fue el más tóxico debido a que afectó el crecimiento, desarrollo y reproducción del insecto.

Palabras clave: *Invertebrado; Efectos tóxicos; Ecotoxicología; Reproducción.*

Anormalidades morfológicas em *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) tratado com oito compostos tóxicos

Resumo

Os testes de toxicidade são essenciais para prevenir a poluição química do meio ambiente. *Tribolium castaneum* pode ser usado como modelo alternativo para triagem preliminar de toxicidade, foram avaliados oito compostos químicos com toxicidade conhecida. Por exemplo, o cloreto de mercúrio II é considerado tóxico em concentrações superiores a 0,1 mg/L na água potável, o fenol pode ser perigoso em exposições a concentrações superiores a 5 mg/L na água potável, no caso do tolueno, a exposição a concentrações superiores a 200 ppm (partes por milhão) no ar pode ser prejudicial para a saúde, a hidrazina pode ser perigosa em concentrações superiores a 1 mg/L na água potável e a cafeína pode causar efeitos adversos como nervosismo, insônia, taquicardia e tremores em exposições a doses superiores a 500 - 600 mg em adultos. Neste caso, os insetos adultos foram alimentados com uma dieta de aveia suplementada com cada um deles separadamente tóxico. Foram avaliados o número de insetos descendentes, tamanho, peso e observação de anormalidades. Dos oito produtos químicos avaliados, apenas cinco tiveram um efeito visível no desenvolvimento do inseto, o BPA e o cloreto de mercúrio (II) induziram anormalidades nos estágios larval e pupal, enquanto o fenol, o tolueno e o metronidazol, apenas no estágio pupal. Foram observadas principais anomalias: necrose nos apêndices das larvas, nas pupas, papilas para diferenciação sexual são escleróticas ou ausentes, e anormalidade na formação da cabeça, membros, asas e apêndices. O cloreto de mercúrio (II) foi o mais tóxico devido ao crescimento, desenvolvimento e reprodução afetados do inseto.

Palavras-chave: *Invertebrados; Efeitos tóxicos; Ecotoxicologia; Reprodução.*

Introduction

Toxicological studies have reached enormous social significance due to the vast number of chemical substances on the market and their possible impact on public and environmental health [1]. In fact, in the last 150 years, chemists have synthesized about 70 million substances. More than 100,000 of these are found in consumer products of daily use, in drugs, in cosmetics, in detergents, in our food, our clothes and – last but not least – as contaminants of our natural environment [2]. This has led to the development of risk assessment strategies for regulatory purposes [3]. Toxicity testing is essential to prevent chemical pollution of the environment and associated health hazards. However, very little information is available about their effects on living and non-living components of the environment [4] and many xenobiotics that are released into the environment pose a hazard to the organisms that are exposed to them [5]. In terms of animal use, the area of reproductive and developmental toxicity testing is especially demanding [3,4,6].

Several studies in the last decades suggest that assays with invertebrates may be used as first screening methods for the assessment of the lethality of new chemicals to mammals and humans [7-8]. The major advantage of using these organisms in bioassays as pre-screening methods is a reduction of the number of mammals required for toxicity testing. It is less expensive than tests with mammals and requires less space, brief life cycle, small size and simple anatomy, so that a large number of invertebrates can be studied in a single experiment within a short period with less ethical problems [9]. The main difficulty in the use of invertebrate tests as pre-screening tests is the difference of biological organization level relative to mammals. Although this difference should be considered, it should be remembered that the objective is using them as pre-screening methods. Therefore, a final bioassay with a small number of mammals should be carried out. Despite this requirement, the routine use of invertebrate tests will represent a significant reduction in the number of mammals used for regulatory purposes [4,7,10].

Tribolium castaneum is a globally significant pest in agriculture, food industry, and toxicology. As a model organism, its genetic and molecular studies contribute to understanding fundamental biological processes and developing more sustainable pest control methods. Research on Tribolium also provides insights into ecological interactions and adaptations to changing environments. Tribolium facilitates the study of pesticide resistance mechanisms, enabling the development of alternatives to overcome resistance. Overall, its study deepens our understanding of insect biology and its impact on agriculture and the environment [11]. The main objetive of this work was to evaluate three toxicological endpoints (development, growth, and reproduction) in the invertebrate *Tribolium castaneum* exposed to eight toxic chemicals as an alternative model in preliminary studies of the toxicological effects of xenobiotics.

Materials and Methods

Culture of experimental unit:

Tribolium castaneum adults were taken from a stock colony maintained in the laboratory. Insects were fed with a diet of ground oatmeal as well as oatmeal in flakes (70:30) at 26 ± 2 °C, 70 to 85 % relative humidity and a 10:14 light:dark photoperiod [12-14]. Two to four week old healthy adults were randomly selected for bioassays [8,15].

Reagents and chemicals:

Mercury (II) chloride (Merck, 99 %), phenol (Sigma-Aldrich, 99 %), toluene (Sigma-Aldrich, 99 %), metronidazole (Pfizer, 99 %), bisphenol A (BPA) (Sigma Aldrich, 99 %), hydroquinone (Sigma Aldrich, 99 %), hydrazine hydrochloride (Merck, 98 %), and caffeine (Sigma-Aldrich, 99 %) were donated by the Faculty of Pharmaceutical Sciences of the University of Cartagena, Colombia.

Effects on reproduction and development:

Adult insects were fed a diet of ground oatmeal and oat flakes 70:30, the food was supplemented with several toxic as follows: per 50 grams of feed were placed in a beaker, 1 mL of acetone containing the desired amount of toxic (final concentration: 200 ppm) was added and the suspension was mixed for ten minutes at room temperature. Then the mixture was left for 30 min to evaporate the acetone. Control diet was prepared according to what is described above, but using acetone without the inclusion of toxic substances [16-17]. Rearing of insects was performed in a standard insect glass jar with a maximum of 40 animals per 50 g of diet. Previously, the insects were sexed in the pupal stage, testing 20 adult males and 20 females. Pupal stages of *T. castaneum* show sexually dimorphic structures which make it easy to separate males

from females, unambiguously [18]. The crops from insects were reviewed every 4 days, evaluating the number of offspring larvae per 5 grams of food, size of individuals and observation of abnormalities. In this study, eight chemicals at 200 ppm (mercury (II) chloride, phenol, toluene, metronidazole, bisphenol A- BPA, hydroquinone, hydrazine hydrochloride, and caffeine) were selected considering the toxicity reported for each of them and their mechanism of action (mutagenic and teratogenic).

Text-mining:

A text-mining step was carried out to detect citation-associated relationships between evaluated compounds and selected keywords, such as abnormalities, teratogenesis, malformations, genotoxic, developmental, reproductive and mutagenic effects. These keywords were recognized through literature reviews using a wide variety of academic databases and search engines available online, such as GoPubmed [19], pubGraph [20], helioblast [21] and Pubtator [22], and the analysis of associations among these terms was done in the PubMed/MEDLINE literature database was carried out using the Jaccard co-occurrence score, as a measure of the degree to which the two queries coincide among all publications.

Statistical analysis:

The data are presented as means ± standard deviation (SE), and the differences between means are considered to be significant at p-value ≤ 0.05. Comparisons between the control and exposed group at different time intervals were made with Bonferroni post-test [23]. GraphPad InStat 3.05 was used for data analysis. At this point, the data were entered into the statistical program to be analyzed and perform a posttest, which consists of a series of t-tests carried out on each pair of groups.

Results

During insect growth, it was observed a proportional increase in weight over time (Table 1) for both the control group and the group exposed to the different toxic substances. Mercury (II) chloride altered insect larvae weight at 28 days. However, this effect disappeared with time. The same effect was observed in the case of insects exposed to hydrazine hydrochloride. An effect on the weight of pupae was found in the group treated with phenol and hydroquinone group (Figure 1A). On the other hand, the weight of the adult insects was unaffected (Figure 1B). It is important to mention that, the slope of the regression line shows the relationship between the concentration of the toxic compound and the weight of the larvae, indicating how much the weight of the larvae changes in response to a unit change in the concentration of the toxic compound. Also, the ΔW is the change in larval weight after exposure to toxic compounds.

Table 1. Effects on the weight of *Tribolium castaneum* larvae.

Compounds (200 ppm)	**Day**				**Slope**
	20	28	36	44	
	Weight (mg), ΔW				
Control	3.90±0.95, --	14.73±2.25,--	22.23±2.48, --	26.03±1.18, --	--
Metronidazole	4.55±0.62, -0.65±0.18	10.68±0.92, 4.05±2.40	17.95±1.51, 4.28±2.66	21.80±1.28, 4.23±1.12	0.6291
Hydroquinone	9.65±1.25*, -5.75±0.53	14.65±0.45, 0.08±0.02	23.25±2.85, -1.02±0.11	24.75±1.55, 1.28±0.85	0.2499
Hydrazine hydrochloride	8.65±0.35, -4.75±0.11	17.55±4.95, -2.82±1.33	27.20±0.70, -4.97±0.11	25.50±0.90, 0.53±0.00	0.1711
Phenol	5.38±1.70, -1.48±0.10	12.88±1.25, 1.85±0.20	19.10±3.23, 3.13±1.33	25.73±0.94, 0.30±0.10	0.0828
Toluene	2.63±0.58, 1.27±0.46	9.08±1.34, 5.65±2.57	15.95±0.73, 6.28±2.20	22.83±1.70, 3.20±1.39	0.0803
Mercury (II) chloride	3.67±1.47, 0.23±0.10	3.20±0.14*, 11.53±0.99	17.10±3.66, 5.13±2.38	22.53±3.38, 3.50±1.74	0.1859
BPA	3.10±1.20, 0.80±0.08	8.98±0.91, 5.75±1.45	19.33±3.23, 2.90±1.48	24.63±1.26, 1.40±0.22	-0.0131
Caffeine	3.45±0.65, 0.45±0.11	12.90±0.30, 1.83±0.38	26.25±0.45, -4.02±0.11	24.85±0.55, 1.18±0.89	-0.0458

*Statistically significant compared to control. n=10.

The effect on the development of insects exposed to different toxic is shown in Figure 2. Of the eight compounds tested, only five affected the development of the beetle in various stages (larvae and pupae). In the case of adult insects, no abnormality was observed during the study.

Figure 3 shows a text-mining graph based on the PubMed search of the different keywords [24]. Where it stands out the association between the effects on reproduction and development and exposure to BPA with Jaccard co-occurrence of e(-4.6).

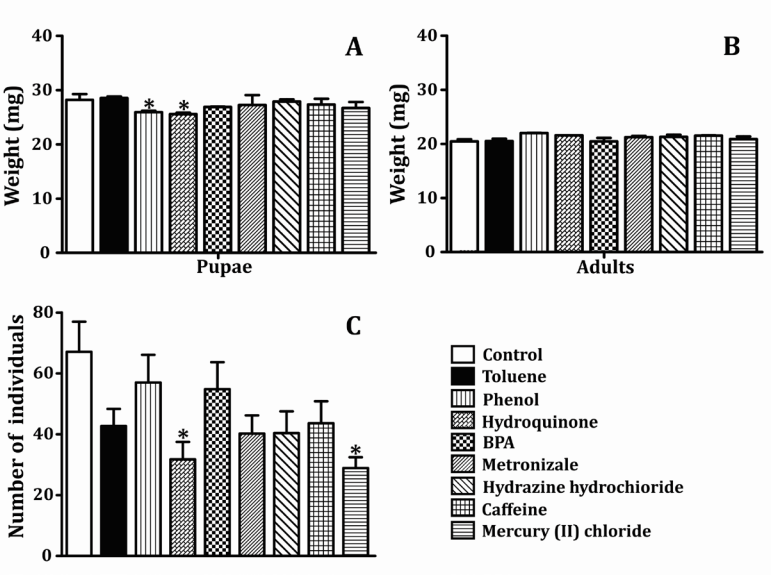

Figure 1. Morphometric changes insects F1 from adult *Tribolium castaneum* exposed to different toxic. The weight of F1 pupae (A) and adults (B). n=10 and the number of insects F1 found per 5 grams of food (C). *Statistically significant compared to control. The p-values for the Bonferroni test were $P < 0.05$.

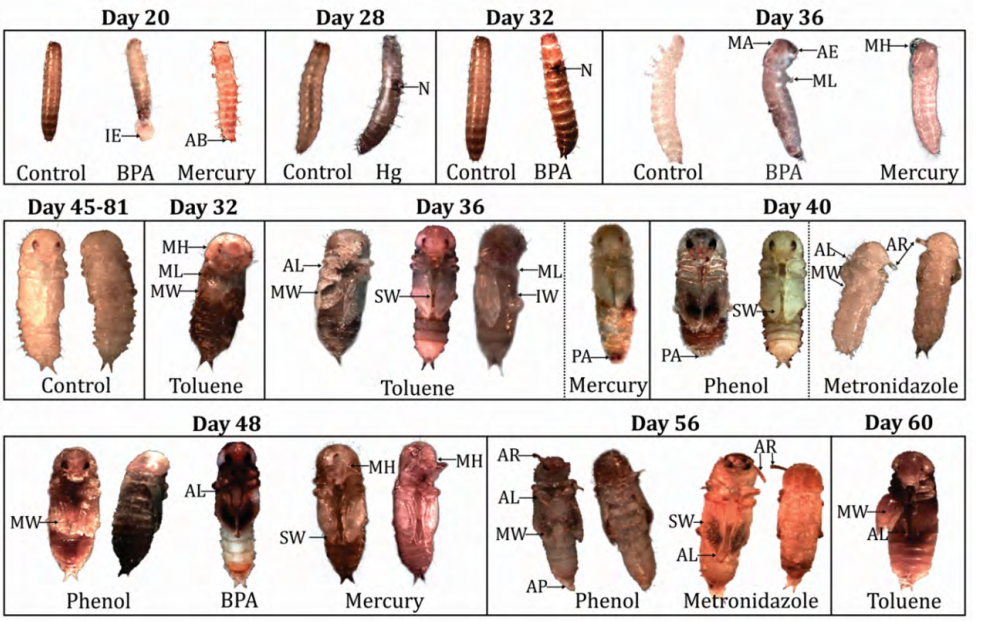

Figure 2. Abnormalities generated in one generation *Tribolium castaneum* after of exposure to various toxic. *Day. MH: Malformed head. ML: Missing legs. MW: Malformed wings. AL: Malformed legs. SW: Spread wings. IW: Missing wings. PA: Papillae for sex identification are absent. AR: Antennas present AP: Abnormal papillae. IE: Incomplete ecdysis. N: Necrosis. MA: Missing antennae. AE: Absent eyes. AB: appendages absent.

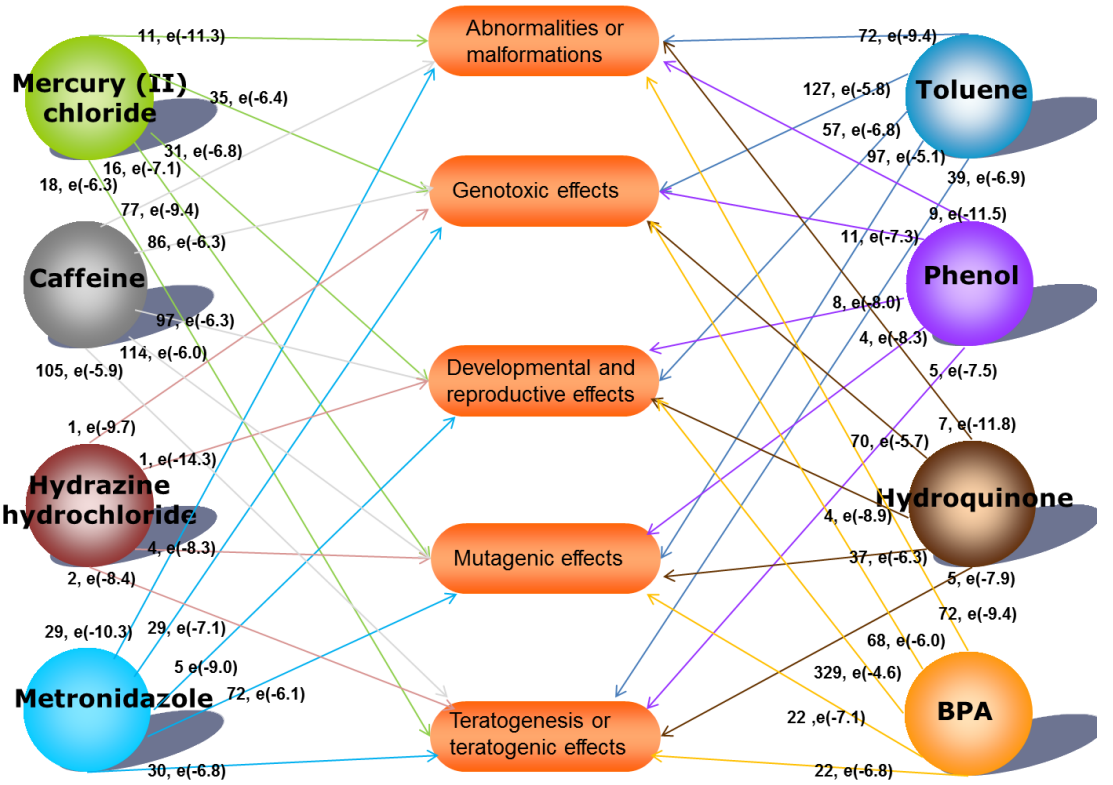

Figure 3. Text-mining graph based on the PubMed search of the different keywords, indicating the number of hits found in PubMed for this and each term found and Jaccard co-occurrence; updated September 2023.

Discussion

The substitution of laboratory animals by insects in toxicity testing is a reality in the framework of pre-screening [25]. Different studies have used these invertebrates to assess the toxicity of chemicals, such as *Chironomus riparius* [5,26], *Drosophila melanogaster* [27,28], and *Chironomus tentans* [29,30] have been used as a pre-screen system, especially for agrochemicals and environmental pollutants, genotoxins, and endotoxin detection [4]. In this study, we used *T. castaneum* to evaluate the toxicity eight chemical compounds, employing three endpoints: development, growth, and reproduction. There is evidence that the red flour beetle can be used to assess toxicity, as reported by Grünwald *et al*. [31], benzo[a]pyrene reduces the lifespan of *T. castaneum*. This is a relevant polycyclic aromatic hydrocarbon for the development of human cancers. was found to reduce the lifespan of *T. castaneum*. It has also been used as a model to evaluate toxicity and expression of oxidative stress genes induced by toluene, xylene, and thinner [32].

The compounds evaluated in this study have shown to produce defects in the formation of different species and they were used in this study as a model to assess toxicity in an invertebrate.

Bisphenol A: Is a synthetic chemical, most notably used as a component in the production of polycarbonate plastic, epoxy resins, and certain polymers. Laboratory studies show BPA causes developmental and reproductive effects in aquatic animals, including fish and shellfish [33]. In fact, exposure of zebrafish larvae to 80 µM BPA resulted in a teratogenic response, including craniofacial abnormalities and edema. BPA exposure might be on organ system development, particularly the central nervous system and prostate, in fetuses or young children [34]. Also, exposure to 0.1 µM BPA results in significant hyperactivity in 5-day-old larvae and learning impairment in adults exposed as embryos [35]. In *Xenopus laevis*, BPA affected embryonic development, larval metamorphosis, and induced malformation of the head region, scoliosis, and suppression of organogenesis [36]. Considering the data mining (Figure 3), there is an

association between the effects on reproduction and development and exposure to BPA (Jaccard co-occurrence e(-4.6)). In this study, also affected insect metamorphosis (incomplete ecdysis), and induced malformation eyes, antennae and limbs larvae, and wings pupae.

Phenol: Used as a disinfectant in household cleaning products and consumer products. Congenital disabilities have been observed in organisms born to females exposed to phenol during pregnancy. Those disabilities ocurred at exposure levels that were also toxic to the mothers [36]. Teratogenic effects more frequently produced by phenol on larvae of *Rhinella arenarum* were axial flexure, persistent yolk plug and different abnormalities which caused the death of blastulae. Other malformations included irregular form, acephalism, edema, axial shortening and underdevelopment of gills, among others. Phenol can produce lethal and teratogenic effects on some amphibian species [37]. In the model of *T. castaneum*, this chemical induced alteration in the pupae of the F1 generation at the level of the formation of the wings, legs, head, and papillae.

Metronidazole: Is a commonly used antimicrobial drug [38]. The administration of metronidazole in the mouse was associated with percentages of fetuses with skeletal defects, increased the incidence of axial skeletal variations, like full supernumerary ribs, short ribs, sternebral anomalies, cleft palate, dilated renal pelvis and distended bladder [39]. According to data mining (Figure 3), there are reports of the mutagenic activity (72 e(-6.1)) of metronidazole. This chemical induced malformation of wings and limb of pupae.

Toluene: Studies performed with toluene indicate that affects the reproduction of the fly by the reduction in the number of flies emerged and also a delay of 2 days in the emergence pattern when compared to control [40]. Inhalation studies with mice and rats have shown that toluene has the potential to cause growth retardation and skeletal anomalies in the offspring of exposed dams [41]. Toluene has reported being mutagenic, teratogenic, and genotoxic. In this study, toluene induced malformation head, wings, and ends of the pupae.

Mercury (II) chloride: Mercury is toxic to many species, in the case of the invertebrate; the larval stage is apparently the most sensitive of the life cycle. Levels of 1 - 10 µg/L usually causes acute toxicity for the most sensitive developmental stage of many different species of aquatic invertebrates [42]. These compounds also generated different abnormalities on the invertebrate *T. castaneum*, demonstrating its teratogenic effect.

In the case of hydrazine hydrochloride, hydroquinone and caffeine, these chemicals did not affect insect development in this study, though there are reports that indicate different toxicological effects in several species evaluated. Hydrazine is genotoxic in vitro but deemed not genotoxic in mice. International Programme on Chemical Safety concluded that hydrazine is not genotoxic in vivo in higher eukaryotes [43]. Hydroquinone is genotoxic in vitro and in vivo. IARC [44] concludes that hydroquinone is likely to be carcinogenic in humans in some in vitro systems and induces structural chromosome aberrations in mouse bone-marrow cells [43]. Studies conducted in *T. castaneum* exposed to caffeine showed decreased the duration of death-feigning. Caffeine increases activity in vertebrates, and it also increases activity in insects. These suggest that caffeine has a similar function in activity levels in invertebrates as vertebrates [45]. Embryos of *Xenopus laevis* exposed to caffeine for 96 hours had loose gut coils and abdominal and facial edemas in those that survived the exposure period [46]. However, other studies have reported that amphibian larvae did not appear to have any morphological abnormalities [47].

The reproduction of *T. castaneum* was assessed by estimating the number of insect offspring in the control group and exposed (Figure 1C), it can be observed that the progeny decreased as a result of the various toxic, but was only observed significant differences in the group of insects exposed to mercury (II) chloride and hydroquinone. Although, it has been reported that BPA reduced fecundity of the daphnids [48], in *T. castaneum* it was not observed this effect. The size of insects (length and width) at different stages (larvae, pupae, and adults) was not affected by the groups exposed to toxic (data not shown).

In summary, the eight chemical compounds evaluated only five had a visible effect on insect development, BPA and mercury II chloride induced abnormalities in the larval and pupal stages, while the phenol, toluene, and metronidazole, in the pupal stage only. Mercury (II) chloride was the most toxic due to the affected growth, development and reproduction of the insect. Hydroquinone

and hydrazine hydrochloride but did not affect the development, if it was observed an effect on the weight of the larva and pupa, respectively, hydroquinone also affected the reproduction of the insect.

Conclusions

Tribolium castaneum can be used as an alternative model for preliminary toxicity detection and can substantially reduce the use of vertebrate animals. Additionally, this insect was sensitive to five out of the eight chemicals analyzed at critical points evaluated (development, growth, and reproduction), which have been reported as toxic to other species of vertebrates and invertebrates.

Bisphenol A (BPA) and mercury (II) chloride induced anomalies in larval and pupal stages, while phenol, toluene, and metronidazole only affected the pupal stage. Significant anomalies were observed: necrosis in larval appendages, sclerotized or absent sex differentiation papillae in pupae, and anomalies in head formation, limbs, wings, and appendages. Mercury (II) chloride was the most toxic as it affected the growth, development, and reproduction of the insect.

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The authors declare no conflicts of interest.

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