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Sublethal effects of carbendazim in *Jenynsia multidentata* detected by a battery of molecular, biochemical and genetic biomarkers

Jesica Y. Götte [a], Juan Cruz Carrizo [b], Ana M. Panzeri [c], M. Valeria Amé [b], Mirta L. Menone [c,*]

[a] Facultad de Ciencias Exactas y Naturales, Departamento de Ciencias Marinas, Mar Del Plata, Argentina
[b] Universidad Nacional de Córdoba - CONICET, Facultad de Ciencias Químicas, Dto. Bioquímica Clínica—CIBICI, Córdoba, Argentina
[c] Laboratorio de Ecotoxicología, Instituto de Investigaciones Marinas y Costeras (IIMYC), Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Mar Del Plata (CONICET- UNMdP), Dean Funes 3350, 7600, Mar Del Plata, Buenos Aires, Argentina

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ABSTRACT

The fungicide carbendazim (CBM) has been applied all around the world but its potential adverse effects other than its recognized activity as endocrine disruptor in non target organisms have been scarcely studied. The aims of this work were (1) to use a battery of biomarkers that can reflect potential negative effects such as oxidative stress, genotoxicity, neurotoxicity or altered immune response; and (2) to examine biomarkers of detoxification by analyzing the gene expression of cytochrome P4501A1 (CYP1A1) and the multi-xenobiotic resistance protein P-glycoprotein (P-gp) in the freshwater fish *Jenynsia multidentata* exposed to environmentally relevant concentrations of CBM during 24 h. Fish exposed to 5 μg/L showed inhibition of GST activity and an increase of TBARs contents in gills, the organ of direct contact with waterborne contaminants. Genotoxicity – measured in peripheral blood-was evidenced by the increases of micronuclei frequency when fish were exposed to 5, 10 and 100 μg/L CBM and of nuclear abnormalities (NA) frequency at 0.05, 0.5, 5, 10 and 100 μg/L CBM. The expression inhibition of interleukin (IL-1β) and tumor necrosis factor a (TNF-α) at 10, and 5 and 10 μg/L CBM, respectively, indicated an altered immune response. The expression of CYP1A1 was down regulated in liver at 10 μg/L and of P-gp at 5 μg/L CBM, indicating a possible slow on CBM metabolization. On the other hand, in gills CYP1A1 decreased at 5 and 10 μg/L while P-gp was induced at 5 and 100 μg/L CBM. Overall, most of these significant effects were detected below 10 μg/L CBM, in a range of realistic concentrations in aquatic ecosystems worldwide.

1. Introduction

Ecotoxicological studies of fungicides are not abundant in comparison to those made with insecticides and herbicides worldwide. The benzimidazole fungicide carbendazim [methyl-2-benzimidazole carbamate] (CBM) is a compound of particular interest due to its biological activity and the ongoing review program of active substances in biocidal products (Merel et al., 2018). The European Commission's Health and Food Safety Directorate-General (DG SANCO) and its Standing Committee on Pesticides have banned the fungicide CBM in October 2014 (EFSA, 2017), however, in other parts of the world this fungicide is still in use (CASAFE, 2017). In addition, benomyl and thiophanate-methyl also belong to the methyl benzimadazole carbamate class of fungicides used in agriculture and anthelmintic agents; and are pro-fungicides that generate CBM, the biologically active molecule of toxicological concern (EC, 2020). Environmental concentrations in the order of μg/L have been reported in areas where CBM was largely applied in agriculture, for example in Europe from 0.6 to 6 μg/L (Masiá et al., 2015) or in South America with maximum concentrations of 4.5 μg/L (Palma et al., 2014). Predicted no-effect concentrations (PNECs) values of 0.1 and 0.7 μg/L for long and short-term exposures in freshwater, respectively have been calculated for CBM using the EU Water Framework Directive protocols for developing environmental quality standards (Johnson et al., 2012). Risk assessment of this pesticide was calculated by using a risk quotient (RQ = PNEC/PEC) method in several parts of the world giving values RQ > 1, which indicates that adverse effects in non-target aquatic organisms could occur (Palma et al., 2004; Iturburu et al., 2019). Particularly in some areas of Argentina, CBM showed a contribution to RQ for current used pesticides as high as 95% (Iturburu et al., 2019). In addition, other sources than agriculture can add CBM to the environment. Recent data show that urban sources can also be very important, as it was documented at the mouth of the Rhine River, where the abundance

* Corresponding author.
E-mail address: mirta.menone@gmail.com (M.L. Menone).

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of CBM correlated poorly with that of other fungicides used as active ingredients in plant protection products but it correlated linearly with that of pharmaceuticals detected in the discharge of treated domestic wastewater (Merel et al., 2018). Similarly, domestic wastewater was the dominant input source for CBM in tributaries of the Dongjiang River Basin, South China (Liu et al., 2018).

This fungicide is able to reduce mice maternal levels of estradiol and progesterone and the number of live fetuses, as well as increase the number of dead and resorptions in addition to be classified as probable human carcinogen by the US-EPA (Farag et al., 2011). In CBM exposed embryos of the model fish *Danio rerio* the expression of genes that are involved in the hypothalamic-pituitary- gonadal/thyroid (HPG/HPT) axis were down-regulated and the expression levels of two cytochrome P450 aromatases, CYP19a and CYP19b, were increased significantly after 20 and 100 μg/L CBM, respectively (Jiang et al., 2014) denoting the endocrine disruption effect of this compound. However, other CBM potential adverse effects in non-target aquatic organisms have been scarcely studied. Most of the current use pesticides affect negatively cellular functions and processes, causing organ dysfunctions, energy depletion and even reduction of fitness. On one hand, oxidative stress and neurotoxicity are widespread effects among pesticides. Toxic reactive oxygen species (ROS) are known to be responsible for a variety of oxidative damages leading to adverse health effects and diseases (Valavanidis et al., 2006) while neurotoxicity of some compounds like organophosphate pesticides can conduce even to death. On the other hand, other relevant effects like genotoxicity and immunotoxicity are less studied. Overall, CBM occurrence, its associated risk for aquatic life and the scarce amount of studies about potential adverse effects to fishes motivated the present work based on the hypothesis that CBM could cause oxidative stress, neurotoxicity, genotoxicity and changes in immune response in fish. One of the aims of this work was to use a battery of biomarkers that can reflect these potential negative effects in female freshwater fish *Jenynsia multidentata* exposed to environmentally relevant concentrations of CBM. In addition, detoxification of CBM was examined by analyzing the response of biomarkers like the gene expression of CYP1A and the multi-xenobiotic resistance protein P-glycoprotein (P-gp).

2. Material and methods

2.1. Material and chemicals

Adult female fish were collected in freshwater bodies around "Tres Arroyos" city (Buenos Aires Province, Argentina, 37° 53' South, 57° 59' West) characterized by low levels of pesticides and metals (Peluso et al., 2014). Fish were acclimatized for 1 month to laboratory conditions in 140 L tanks. Male specimens were not considered for the study because of their small size, which is not enough to analyze each fish individually. In addition, using only one sex avoids sex-specific differential behavior of the biomarkers, as it was previously demonstrated in *J. multidentata* (Ballesteros et al., 2007). A total of 108 healthy specimens with approximately the same size (mean total length (±SD): 5.36 ± 0.55 cm; mean weight (±SD): 2.30 ± 0.63 g) were selected. For each treatment 18 organisms were exposed in 3 independent 20 L-tanks using tap water, each containing 6 fish. Then, two organisms were taken randomly from each tank resulting in 6 individuals per treatment (n = 6) for the analysis of each biomarker type (biochemical, molecular, genetic). The experimental room was illuminated with fluorescent lamps with 12:12 light: dark periods. The water temperature was 21 ± 1°C and the pH = 8.3 ± 0.1. A stock solution of CBM (CAS number 10605-21-7, Sigma-Aldrich, Saint Louis, USA) was prepared by diluting 0.04 g in 25 mL in dimethyl sulfoxide- DMSO (Mallinckrodt Saint Louis, USA). Afterwards, we prepared the appropriate exposure medium by diluting different amounts of CBM in tap water. A concentration-response curve using 0.05, 0.5, 5, 10 and 100 μg/L CBM was made, taking into account the reported values in freshwaters of Argentina and other parts of the world (Palma et al., 2014; Masiá et al., 2015; Corcoran et al., 2020) as well as the CBM quality guide level for the protection of aquatic biota of 0.1 μg/L (SRHN, 2014). A negative control (Controls) that consisted of tap water with DMSO at 0.004% (similar to the concentration of DMSO used for the CBM exposures) was also performed. In addition, one positive control (Co+) consisting of 50 mg/L of the mutagenic compound methyl methanesulfonate (MMS, CAS 66-27-3) was used using an extra 20 L-tank with six fishes. From each treatment a subsample of 1 L each was used to evaluate the chemical stability of CBM in the exposure media. Water samples were collected from the tanks at the beginning of the bioassay and after 24 h using 1 L pre-cleaned glass bottle with Teflon lined caps. Samples were stored at 4 °C until CBM concentrations analyses.

All fish were euthanized after an exposure period of 24 h by transecting the spinal cord, using a fresh razor blade. Liver, brain and gills were sampled, weighed, immediately frozen using liquid nitrogen, and stored at −80°C until analysis. The blood of each animal was obtained through heart puncture with heparinized tips; and peripheral blood smears, two per fish when possible, were immediately made by applying a drop of blood on clean slides, fixed in absolute methanol for 15 min, and air dried. For gene expression, organs were snap-frozen in liquid nitrogen and stored in RNA later (QIAGEN) at −80°C until analysis. All these procedures were previously approved by the Animal Ethical Committee at the National University of Mar del Plata (CICUAL/UNMDP, RD 387/17).

2.2. Analytical measurement of carbendazim concentrations in water

The concentration of CBM in aquarium water was measured by high performance liquid chromatography coupled to fluorescence detector (HPLC-F) according to Maldaner and Jardim (2012) at 0 and 24 h by duplicate. Carbendazim was extracted from filtered aquarium water samples (0.45 μm pore size; Millipore) by solid phase extraction (LiChrolut RP-18, 500 mg, Merck). C18 cartridges were conditioned with 3 mL of methanol and 3 mL of Milli-Q water (acidified with hydrochloric acid to pH = 4). Then, 500 mL of water samples (acidified with hydrochloric acid to pH = 4) were passed through the cartridges followed by vacuum drying for 30 min. The cartridges were eluted with 3x3mL of ethyl acetate and 1x3mL of methanol. The solvents were then evaporated and the extract was reconstituted with 500 μL of HPLC mobile phase. Chromatographic separation and detection was performed using a Supelco RP 18 column (5 μm particle size, length x I.D: 25 cm × 4.6 mm); mobile phase acetonitrile: water (acidified with hydrochloric acid to pH = 4), (40:60); flow: 1.2 mL/min; Fluorescence detection: excitation at 285 nm, emission at 317 nm. Recoveries of the complete analytical technique were obtained by laboratory fortified sample method reaching 95%. The obtained limits of the detection (LD) and quantification (LQ) in aquarium water were 0.013 and 0.040 μg/L, respectively.

2.3. Oxidative stress biomarkers

Activity of antioxidant enzymes. Preparation of cytosolic extracts in liver, gills and brain samples were carried out according to Wiegand et al. (2000) with modifications proposed by Cazenave et al. (2006). Approximately 250–500 mg of tissue was extracted with 2.5–5 mL sodium-phosphate buffer (0.1 M, pH 6.5), containing 14 mM DTE and 1 mM EDTA. Cell debris was removed by centrifuging at 10,000 g for 10 min. The activity of the soluble (cytosolic) glutathione S- transferases (GST) was determined using 1- chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Catalase (CAT) activity was assayed according with Claiborne (1985), measuring the decrease of H_2O_2 concentration. The total protein content for each sample was assessed spectrophotometrically by means of the method of Bradford (1976), using bovine serum albumin solution as standard. Enzymatic activities are reported in nano katals per milligram of protein (nkat/mg

prot), where 1 katal correspond to the conversion of 1 mol of substrate per second. Each enzymatic assay was carried out by triplicate.

Lipid peroxidation. Lipid peroxidation was determined in liver and gills by measuring the formation of thiobarbituric reactive substances (TBARs), according to the procedures of Oakes and Van der Kraak (2003). The supernatant containing TBARs was measured spectrophotometrically (λabs = 532 nm) and it was expressed as nanomoles per mg of fresh tissue using a molar extinction coefficient of 1.56 × 105 M^{-1} cm^{-1}. Brain tissue was not analyzed for TBARs because the weight of sample was not enough to prepare the extract.

2.4. Genotoxicity

Two slides per fish were prepared by placing one drop of blood and performing the smear technique. The smears were fixed in absolute methanol 15 min, and then allowed to dry at room temperature. They were stained using Giemsa solution (Merck, 15%) during 15 min. To analyze the micronuclei (MN) and other nuclear abnormalities (NA) frequencies, 2000 erythrocytes were counted by sample using an optic microscope (Olympus CX31) with a magnification of 1000×. The smears were evaluated by blind review of only one observer and randomly. Only cells with intact nuclear and cell membranes and with erythrocyte morphology were included. A MN was identified as a non-refractory cytoplasmic particle, with well-defined borders and the same stain pattern than the nucleus. The frequency of MN was quantified on 1000 erythrocytes, averaging the two smears by fish. The NA was grouped in two category called notched nucleus (with a notch in the membrane with absence of chromatin) and buds (nucleus with an evagination in its membrane with presence of chromatin) according to Bolognesi et al. (2006). The frequencies of each category of NA was calculated as the number of NA every 1000 erythrocytes, averaging both smears.

2.5. Neurotoxicity

The activity of acetylcholinesterase (AchE) was measured according to Ellman et al. (1961). Muscle and brain tissues were homogenized individually (20 mg tissue/mL buffer) in 0.1 M phosphate buffer (pH 8.0) using a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 10,000×*g* at 4°C during 15 min. The resulting supernatant was centrifuged at 100,000×*g* at 4°C during 60 min to separate the microsomes and the pellet was discarded. The supernatant was used to measure AchE activity. The reaction mix consisted on sodium phosphate buffer (100 mM, pH = 8), DTNB (10 mM), acetylthiocholine and the sample alicuot. The product of the reaction between DTNB and thiocholine was used to estimate the AchE activity which was calculated in terms of the protein content of the sample (Bradford, 1976), and was reported in in nano katals per milligram of protein (nkat/mg prot).

2.6. Immune response (IL-1β and TNF-α) and biomarkers of detoxification (CYP1A and P-gp)

For interleukin IL-1β and tumor necrosis factor TNF-α expression, liver samples were analyzed. For cytochrome P4501A1 (CYP1A) and the multi-xenobiotic resistance protein P-glycoprotein (P-gp) quantification, liver and gills were analyzed. Total RNA was extracted from the tissues by the guanidine thiocyanate–phenol chloroform extraction method in accordance with Chomczynski and Sacchi (1987). The absorbance of total RNA in water at 260 and 280 nm was measured with a Take3 Micro-Volume Plate in a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, USA) to validate the purity and concentration of RNA of each sample. A 1% non-denaturing gel electrophoresis was performed to check the RNA integrity. Nonspecific reverse transcription was performed with an Oligo (dT)15 primer (Bio-dynamics SRL), and M-MLV Reverse Transcriptase (Invitrogen) according to Amé et al. (2009). Quantitative polymerase chain reaction was performed with a Bio-Rad iQ cycler and was used to amplify and measure the transcript abundance of both enzyme systems in the samples. We have previously characterized the partial cDNA sequences for CYP1A1 and P-gp of *J. multidentata* (Amé et al., 2009; Bonansea et al., 2017); and have designed specific *J. multidentata* primers for real-time polymerase chain reaction as shown in Table 1 Supplementary Data. The primers were designed using Primer Express Software (Applied Biosystems), and obtained from Sigma-Aldrich. *J. multidentata* β-actin was evaluated and used as reference gene (Amé et al., 2009) and Cts are shown in Fig. 1 in the Supplementary Data. The relative expression levels (fold change) of the genes were based on mean quantification cycle differences between the sample and the control group. The amplification efficiency of each primer pair was considered for the calculation as developed by Pfaffl (2001). All samples were tested in triplicate.

2.7. Statistics

Normality and homoscedasticity of variances were verified by D'agostino & Pearson's and Bartlett's tests, respectively. To test differences among concentrations, one-way ANOVA was applied (followed by a post hoc Dunnett test) if assumptions were satisfied. If not, the non-parametric Kruskal-Wallis (followed by a post hoc Dunn test) test was applied (Zar, 1999).

3. Results

3.1. Carbendazim concentrations

Carbendazim levels were below the LD in the aquarium water of control treatment at 0 and 24 h (Table 2 Supplementary Data). Concentrations from 0.05 to 5 μg/L CBM showed a drop of 0.4–5.4% after 24 h showing high stability. On the other hand, at 10 and 100 μg/L, CBM levels showed an increase in the fungicide concentrations after 24 h (9.0 and 2.1% increase, respectively, Table 2 Supplementary Data). Nevertheless, none of the changes in fungicide concentration was statistically significant.

3.2. Oxidative stress biomarkers

The concentration-response relationship plots corresponding to GST in liver, gills and brain are shown in Fig. 1 (A,B, C). From the three tissues, only gills was sensitive showing an inhibition of GST activity at 5 μg/L CBM ($p < 0.05$) (Fig. 1B). Concomitantly, a peak in TBARs at 5 μg/L CBM was observed ($p < 0.05$) (Fig. 1 E). On the other hand, CAT activity did not show changes in comparison with controls in any tissue (Fig. 1F and G).

3.3. Genotoxicity

The MN frequency varied from 0 to 0.5‰ in the negative control samples. MN frequency increased when fish were exposed to 5, 10 and 100 μg/L CBM during 24 h ($p < 0.05$) (Table 1). Total NA frequency increased in the positive control and at 0.05, 0.5, 5, 10 and 100 μg/L CBM ($p < 0.05$) (Table 1). When NA were separated in different classes, both notched and buds showed also differences in comparison with the negative control. Thus, notched nucleus increased at 0,5; 10 and 100 μg/L while buds increased only at 5 μg/L ($p < 0.05$) (Table 1).

3.4. Neurotoxicity

The enzyme AchE was not significantly affected by the short-term sublethal exposure to CBM ($p > 0.05$) (Table 2).

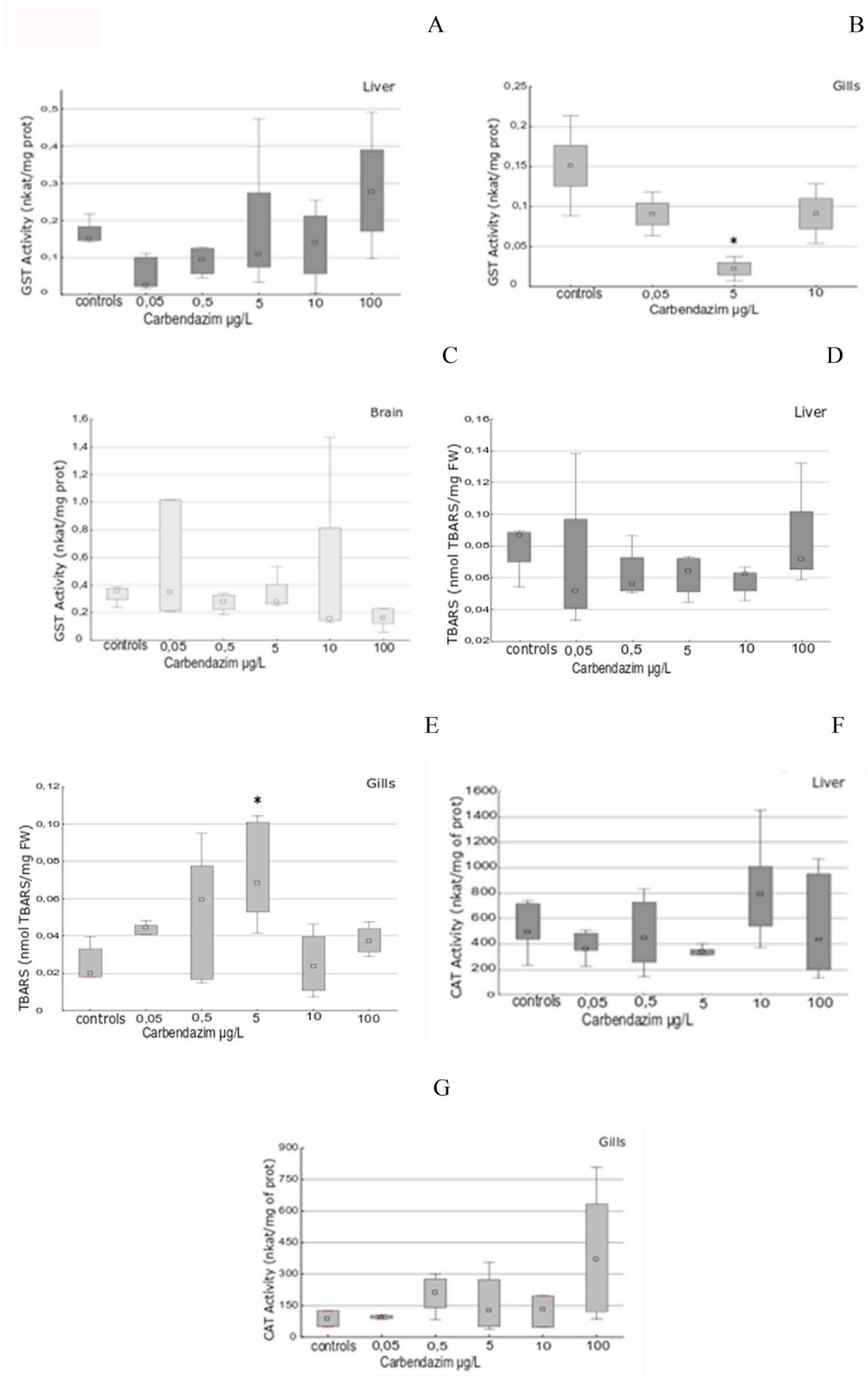

Fig. 1. Mean of Glutathione- S- Transferase (GST) activity in liver (A), gills (B) and brain (C), thiobarbituric reactive substances (TBARS) in liver (D) and gills (E) and catalase (CAT) activity in liver (F) and gills (G) of *Jenynsia multidentata* exposed to carbendazim (CBM). n = 6. Asterisk (*) indicates significant difference from negative control (p-value < 0.05), non parametric Kruskal- Wallis test, except ANOVA test for GST in gills.

Table 1
Genetic biomarkers in the fish *Jenynsia multidentata* exposed to carbendazim (CBM). Micronucleus and nuclear abnormalities frequencies expressed as mean ± standard deviation. n = 6. Asterisk (*) indicates significant difference from negative control (p-value < 0.05), non-parametric Kruskal-Wallis test.

Treatment	Micronuclei (‰)	Other Nuclear Abnormalities (‰)		
		Total	Notched	Buds
Negative Control	0.2 ± 0.27	9.83 ± 0.57	8.00 ± 3.26	3.98 ± 2.52
Positive Control	2.36 ± 0.25*	40.51 ± 5.39*	25.00 ± 2.07*	14.84 ± 3.37*
CBM 0.05 μg/L	0.59 ± 0.38	28.59 ± 18.65*	21.00 ± 14.49	6.97 ± 4.43
CBM 0.50 μg/L	0.81 ± 0.52	23.10 ± 7.06*	18.00 ± 5.58*	5.14 ± 1.99
CBM 5.00 μg/L	1.00 ± 0.35*	24.78 ± 12.13*	15.00 ± 8.75	9.40 ± 3.35*
CBM 10.00 μg/L	1.04 ± 0.42*	31.10 ± 13.21*	22.00 ± 8.03*	9.75 ± 5.32
CBM 100.00 μg/L	1.47 ± 0.68*	29.46 ± 12.94*	22.00 ± 9.10*	9.00 ± 4.73

Positive Control = 50 mg/L methyl methanesulfonate.

Table 2
Mean (±standard deviation) of acetylcholinesterase (AchE) activity (nkat/mg protein) in the fish *Jenynsia multidentata* exposed to carbendazim (CBM). n = 6, non-parametric Kruskal-Wallis test.

Treatment	AchE (nkat/mg prot)	
	Brain	Muscle
Negative Control	230 ± 180	0.9 ± 0.1
CBM 0.05 μg/L	280 ± 110	1.1 ± 0.1
CBM 0.5 μg/L	300 ± 180	1.1 ± 0.3
CBM 5.00 μg/L	660 ± 510	0.6 ± 0.2
CBM 10.00 μg/L	410 ± 210	0.1 ± 0.07
CBM 100.00 μg/L	400 ± 40	5.2 ± 4.8

3.5. Immune response

The expression of genes related to the innate immune system is shown in Fig. 2. The biomarkers studied resulted down-regulated by CBM. The expression inhibition, in the case of the interleukin (IL-1α) was observed at 10 μg/L CBM and of the tumor necrosis factor (TNF-β) at 5 and 10 μg/L CBM ($p < 0.05$).

3.6. Biomarkers of detoxification: CYP1A and P-gp expression

In liver, a general decrease of cytochrome P4501A1 (CYP1A1) expression from 0.5 to 100 μg/L CBM was observed, although it was significant only at 10 μg/L CBM ($p < 0.05$) (Fig. 3 A). A similar behaviour was observed in gills, being significant at 5 μg/L CBM ($p < 0.05$) (Fig. 3 B). Lower expression levels of P-gp in liver were also observed compared with the control, in this case at 5 μg/L CBM ($p < 0.05$). On the other hand, an induction of P-gp expression in gills was observed at 5 and 100 μg/L CBM ($p < 0.05$) (Fig. 3 D and E). Finally, no changes in brain CYP1A or P-gp expressions were observed (Fig. 3 C and F).

4. Discussion

Nowadays, a battery of biomarkers is recommended to evaluate potential effects of xenobiotics on celular functions and processes or cell damage. In the present work several potential negative effects were explored in a freshwater fish. Genotoxicity, changes in immune response and in biomarkers of detoxification in *J. multidentata* were conclusively demonstrated.

Changes in biochemical biomarkers observed in gills can be explained as oxidative damage caused by CBM since it has been described that GST activity could conjugate glutathione to inactivate highly reactive aldehydes produced from lipid peroxyl radicals (West and Marnett, 2006). Thus, the inhibition of GST could cause the peak of toxic TBARs observed. Similar results have been previously documented in liver of the milkfish *Chanos chanos* exposed to a range of 5.45–45.31 μg/L CBM (Palanikumar et al., 2014). Although CAT activity did not show any changes in *J. multidentata*, evidence from the literature supports the CBM ability to generate oxidative stress, such as the increased expression of CAT, GPX, and Mn-SOD enzymes during zebrafish *D. rerio* development (Jiang et al., 2015).

The lack of response in AChE activity indicated that CBM was not neurotoxic, at least measured through this biomarker at the concentrations tested. Results on AChE responses have been controversial in other fishes exposed to CBM; while *C. chanos* exposed at 2.85–45.31 μg/L CBM showed inhibition (Palanikumar et al., 2014), in embryos of *D. rerio* exposed at concentrations equal or above 4 μg/L CBM increased activity was observed (Andrade et al., 2016). Therefore, other biomarkers of neurotoxicity should be explored.

Concerning genotoxicity, we first analyzed the ability of the fish species selected to respond to mutagenic compounds. We found an

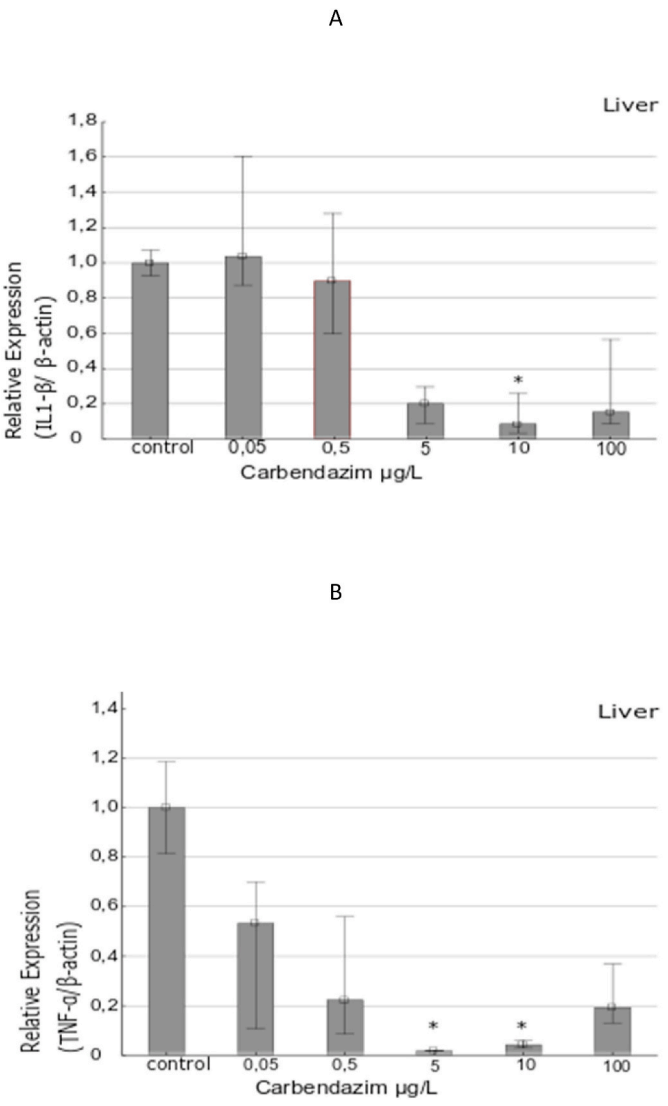

Fig. 2. Mean of expression of interleukine IL-1β (A) and tumor necrosis factor TNF-α (B) in liver in the fish *Jenynsia multidentata* exposed to carbendazim (CBM). n = 6. Asterisk (*) indicates significant difference from negative control (p-value < 0.05), non-parametric Kruskal-Wallis test.

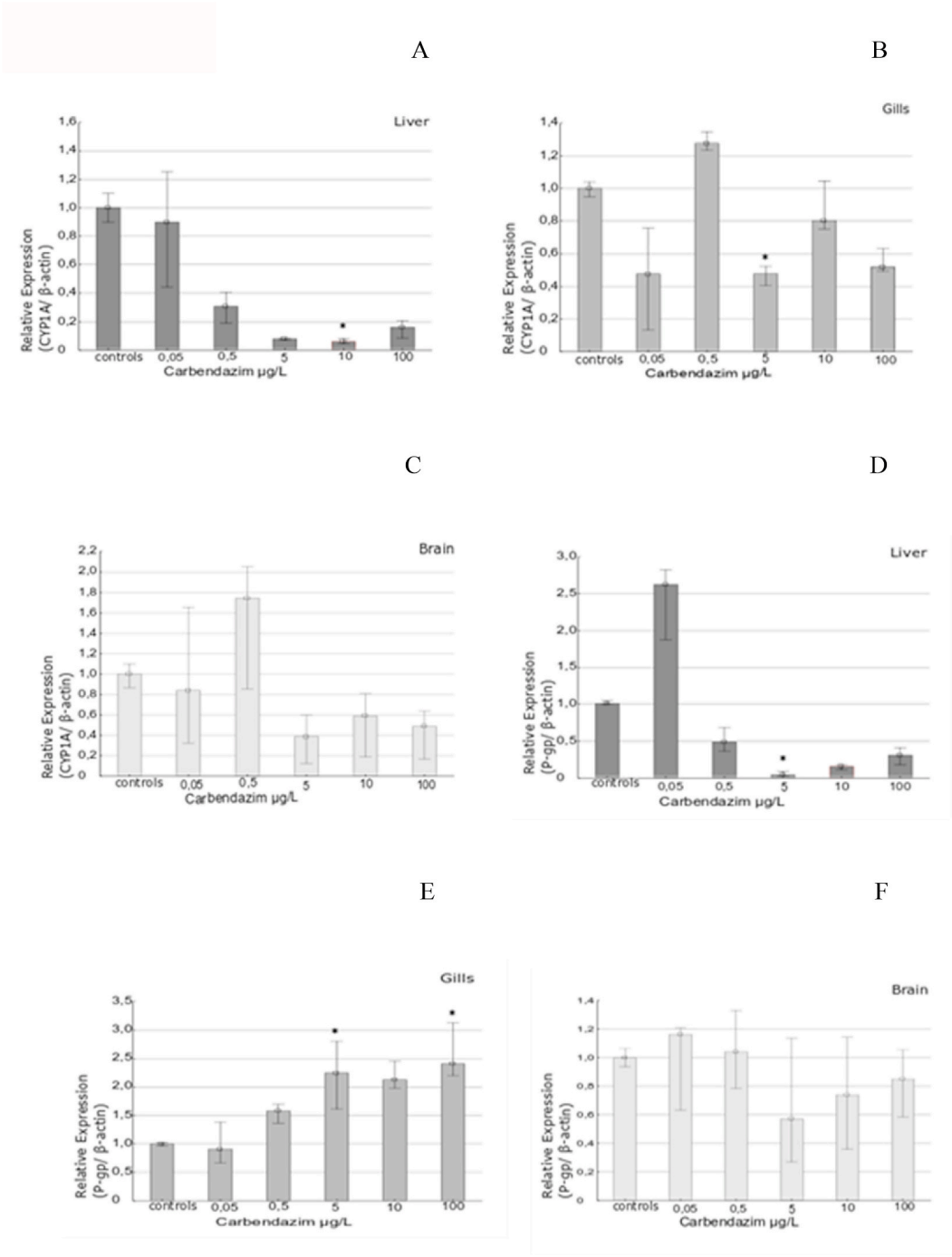

Fig. 3. Mean of expression of cytochrome P4501A1 (CYP1A1) and multi-xenobiotic resistance protein P-glycoprotein (P-gp) in liver, gills and brain in the fish *Jenynsia multidentata* exposed to carbendazim (CBM). n = 6. Asterisk (*) indicates significant difference from negative control (p-value < 0.05), non-parametric Kruskal-Wallis test.

induction of MN and other NA in peripheral erythrocytes by the positive control MMS. Noteworthy, *J. multidentata* data showed 3- fold higher total AN frequency compared to the cichlid *A. facetus* (Iturburu et al., 2017) denoting the sensitivity of the species selected for this study. For both fish species MMS produced significant notched nuclei, a category of NA that has been associated to aneuploidy (Carrasco et al., 1990) or cytotoxicity (Bolognesi et al., 2006). It is now well-established that MN mainly originate from acentric chromosome fragments, acentric chromatid fragments (clastogenicity) or whole chromosomes (aneunogenesis) that fail to be included in the daughter nuclei at the end of mitosis because they did not attach properly with the spindle during the anaphase (Fenech et al., 2011). The genotoxicity observed in *J. multidentata* from 5 to 100 µg/L CBM is in the same range previously observed for the fish species *C. chanos* (2.85–45.31 µg/L CBM) (Palanikumar et al., 2014). The fungicide CBM is known to induce chromosome aberrations and micronuclei in various cell types, primarily through an aneugenic mechanism (Sarrif et al., 1994) by the inhibition of the polymerization of tubulin, but whether CBM genotoxicity in *J. multidentata* was caused only by aneunogenesis (interference with mitotic spindle proteins) or also by clastogenesis (fragmentation of the DNA molecule) cannot be distinguish in the present study. Recent studies provide exciting evidence that MN act as key platform for

chromothripsis and a trigger of innate immune response, suggesting that MN could affect cellular functions contributing to chromosome instability, inflammation, senescence and cell death (Guo et al., 2019). Therefore, the consequences of the increased MN frequencies observed should be studied more deeply. Buds represent the process of elimination of amplified DNA, DNA repair complexes and possibly excess chromosomes from aneuploid cells. Whether buds are a mechanism to eliminate excess chromosomes in a hypothesised process known as aneuploidy rescue remains unclear as there is only limited evidence for this possibility (Fenech et al., 2011). Therefore, the mechanism behind the increased frequency of buds and the other AN observed under exposure to CBM in the present work is still difficult to interprete.

Innate immune mechanisms act as a first line of defense against infections. The IL-1β was the first interleukin characterised in bony and cartilaginous fish. IL-1β has diverse physiological functions and its roles in regulating the inflammatory process are conserved in fish, acting as a chemoattractant for leucocytes. Similarly, TNF-α is one of the early immune genes expressed at an early stage of infection in fish and has a key role in regulating inflammation (Zou and Secombes, 2016). Considering that both cytokines enhance the phagocytic activity of fish leucocytes, its down-regulated expression in *J. multidentata* can be considered detrimental for the liver that would not be able to get free of potential necrotic cells. On the other hand, particularly TNFs are key regulator of liver homeostasis in mammals and they seem to be essential for liver development in zebrafish (Qi et al., 2010). Taking into account that a knockdown of TNF-α in zebrafish caused reductions of liver size (Qi et al., 2010), future studies of fishes chronically exposed to CBM are suggested in order to explore potential consequences of changes in TNF-α expression in *J. multidentata*.

Although in a general basis in humans, rats and rabbit, data show that CBM increased mRNA and protein expressions and promotes activity of CYP1A1 (Rey-Grobellet et al., 1996; Backlund et al., 1999; Rudzok et al., 2009; Wei et al., 2016) other studies showed that human hepatic CYP2D6 activity (an enzyme implicated in the metabolism of several pharmaceutical compounds) was completely inhibited by CBM (Abass et al., 2009). In fishes, a lack of information about CBM detoxification through P450 enzymes is noteworthy, and at the best of our knowledge this is the first report on the evaluation of CYP1A1 expression in this taxa. The down regulated expression of CYP1A1 observed in liver and gills of *J. multidentata* at environmentally relevant concentraction of CBM could imply a delay on the elimination of CBM from the fish body, and consequenly, facilitation of its accumulation despite its hidrophylicity (log Kow = 1.49, Singh et al., 2016). In this sense, in the same species, previous work have shown that in the organs where the pesticides cypermethrin and chlorpyrifos accumulation occurred, biotransformation enzymes were inhibited (Bonansea et al., 2017). Similarly, the phase III P- gp transporter showed a decrease in liver at 5 μg/L CBM, may be contributing also to its bioacumulation. On the other hand, an increment of P –gp expression in gills at the same concentration was observed, indicating a possible excretion of CBM or of a CBM-metabolite from the cell.

Although GST could play an important role on the oxidative stress process, its central role is in the biotransformation of endogen compounds and xenobiotics. Previous works on fishes have suggested the detoxification of CBM by GST. Indeed, embryos of *D. rerio* showed increased GST activities after exposure to CBM at concentrations equal or above 4 μg/L in an acute exposure (Andrade et al., 2016). Moreover, 5 weeks-exposure to benomyl concentrations higher than 100 μg/L increased in a dose- and time-dependent manner the GST level in the Nile tilapia *Oreochromis niloticus* (Min and Kang, 2008). It is worthy to mention that GST activity in gills of *J. multidentata* significantly decreased at 5 μg/L CBM, similarly to the phase I enzyme analyzed, suggesting that 5 μg/L CBM would be a critical concentration at which the detoxification can fail in gills.

The results of this work demonstrated that acute exposure of *J. multidentata* to CBM in the range of realistic concentrations (<10 μg/L) lead to significant effects such as increased frequencies of DNA damage and the response of biomarkers of detoxification and of immunity. Some data encourage a more detailed study of processes that would be negatively impacted by CBM in fish as well as the measurement of CYP P450 activity.

Author contributions

Jesica Y. Gotte- Investigation, Formal analysis, Methodology, Visualization; Juan Cruz Carrizo- Investigation, Methodology; Ana M. Panzeri- Investigation, Methodology; María V. Amé- Investigation, Methodology, Writing - Original Draft (+ Review), Resources; Mirta L. Menone- Investigation, Writing - Original Draft (+ Review), Visualization, Project administration, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111157.

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