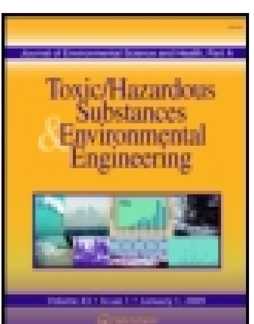

Journal of Environmental Science and Health, Part A

Toxic/Hazardous Substances and Environmental Engineering

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lesa20

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To cite this article: Carla Letícia Gediel Rivero-Wendt , Ana Luisa Miranda-Vilela , Inês Domingues , Rhaul Oliveira , Marta Sofia Monteiro , Monica A. M. Moura-Mello , Rosemary Matias , Amadeu Mortágua Velho Maia Soares & Cesar Koppe Grisolia (2020): Steroid androgen 17 alpha methyltestosterone used in fish farming induces biochemical alterations in zebrafish adults, Journal of Environmental Science and Health, Part A, DOI: 10.1080/10934529.2020.1790954

To link to this article: https://doi.org/10.1080/10934529.2020.1790954

Published online: 11 Jul 2020.

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JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART A
https://doi.org/10.1080/10934529.2020.1790954

Steroid androgen 17 alpha methyltestosterone used in fish farming induces biochemical alterations in zebrafish adults

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ABSTRACT

The 17 alpha methyltestosterone (MT) hormone is fed to *Oreochromis niloticus* larvae in fish farms with the purpose of inducing sex reversal. The aim of this study was to evaluate the toxicity and sub-lethality of MT (99.9% purity) and cMT (a commercial MT with 90% purity) in zebrafish (*Danio rerio*) adults, where the animals were exposed to concentrations of 0, 4, 23, 139, 833 and 5000 μg/L for 96 hours. Genotoxicity was evaluated by micronucleus test (MN), nuclear abnormalities (NA) and comet assay. A low genotoxic potential of MT was showed, inducing micronucleus, nuclear abnormalities and DNA damage in *Danio rerio*, depending on the use of MT or cMT, gender and tested concentrations. In the sub-lethality trials, there was a basal difference in the activity of the enzymatic biochemical markers for males and females, while the Glutatione S transferase (GST) activity decreased in all analyzed tissues, and for males the enzymatic activity decreased only in the intestine. Results suggest that MT has a toxic potential to fish because it alters enzymatic metabolic pathways and may pose a risk to the ecosystems.

ARTICLE HISTORY
Received 10 March 2020
Accepted 25 June 2020

KEYWORDS
Glutathione-S-transferase; micronucleus; comet assay; biochemical markers; methyltestosterone

Introduction

The use of 17 alpha methyltestosterone (MT), a synthetic androgen, is very common in tilapia fish farms with the purpose of inducing sex reversal in female juveniles through a diet containing MT. However, the excessive use of this substance and the inadequate treatment of wastewater may pose human and environmental health risks.[1,2]

In the literature, it was verified that concentrations of MT can be found in sediments, water columns and in the environment, raising a concern about the effects of MT on non-target species and the aquatic environment.[3–5] MT residues were detected in wastewater from the Beijing area (4.1–7.0 ng/L) and, in laboratory studies with 96-h exposure, MT was detected in test water, demonstrating that unmetabolized androgen was still present in water.[6,7] Several authors have registered changes caused by MT in non-target and target species, such as alteration in the development of gonads of *Astyanax bimaculatus* and *Oreochromis niloticus*, decreased fertility in *Astyanax bimaculatus*,[4] masculinization of exposed females of *Pimephales promelas*[8] and changes in the reproductive cycle of the *Oryzias latipes* species.[9,10] Decreased posture in *Coturnix cotumix* japonica females and decreased fertility in males when exposed to 50–110 mg/L of MT for 3 weeks have also been reported.[11]

Animal models such as fish are often used to perform toxicity tests and evaluate eventual environmental risks of contaminants. Fish allow for controlled evaluation of mortality, behavioral changes, defect and tissue changes and genetic material analysis, from which it is possible to predict the adverse effects of the evaluated substance.[12] Zebrafish (*Danio rerio*, Hamilton, 1822) is a freshwater Asian fish species of the Cyprinidae family, which is believed to be a good model for toxicology studies due to being easy to manipulate/maintain and having a high rate of procreation, as well as high genetic homology with mammals.[13,14]

Genotoxic effects or DNA damage caused by exposure to MT in intact animal organs are not evident.[15] Steroid hormones are considered non-genotoxic and non-carcinogenic;[4,7,15] however, the results are still controversial. Ho and Roy[16] reported that testosterone combined with estrogens induces an increase in single strand breaks of DNA. In fish, the combination of micronucleus (MN) test, nuclear abnormalities (NA) and comet assay (CA) can be used to generate consistent data on the genotoxicity of environmental pollutants in *in vivo* systems.[17–21]

The toxicity evaluation through genotoxicity and mutagenicity, together with biochemical marker studies, is commonly used, presenting high potential for the detection of risks posed by chemical substances generating information

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on the effects at the sub-lethal level.[22] This will also contribute to understanding the mechanisms of chemical contamination in the aquatic environment. In this study, we analyzed different biochemical markers involved in physiological processes, such as phospholipoproteins – vitellogenin (VTG), lactate dehydrogenase (LDH), cholinesterase (ChE) and glutathione S transferase (GST). In toxicological processes, alterations of subcellular biomarkers may occur before the response of higher levels of organization, such as conventional histological responses.[23] For example, the induction of changes in vitellogenin (VTG) protein in males has been widely used as an early biomarker to evaluate exposure to endocrine-active compounds.[23]

VTG, the major precursor of egg yolk protein synthesized in the female liver and secreted into the blood to be incorporated into the egg, is produced through activation of the estrogen receptor, whose production is controlled by the axial gonadal hypothalamic pituitary gland.[24,25] The production of VTG in juvenile male fish is accepted as an estrogen marker, and it is a tool to detect the effects on the body caused by the action of endocrine disrupters in the field and in laboratory studies.[23]

LDH is a key enzyme in the anaerobic pathway of energy production, responsible for converting pyruvate to lactate in the absence of oxygen; it is activated in conditions of stress (e.g. exposure to chemicals or oxygen stress) when high levels of energy are required.[26] The enzyme ChE has an important role in the neurotransmission mediated by the transmitter acetylcholine and thus is essential to the maintenance of normal nervous system function.[27,28] GSTs are a family of proteins implicated in the phase II of the detoxification process, responsible for the biotransformation of xenobiotic compounds and endogenous substances.[28,29]

The aim of our study was to understand the sub-lethal toxicity effects of MT as an active principle and commercial compound (cMT) in *Danio rerio* adults. In order to achieve this goal, genotoxicity tests (micronucleus and comet) and biochemical markers (LDH, ChE, GST, VTG) were assessed after 96-hour exposure to MT. Data on MT genotoxicity and its biochemical effects *in vivo* will provide information to assist in the implementation of appropriate measures for disposal and maintenance of the substance (MT), preventing aquatic environment contamination and human health risks. The absence of information on the use and disposal of MT makes it very difficult to estimate its adverse effects on the environment.

Materials and methods

Origin and maintenance of zebrafish

Zebrafish were kept in a ZebTEC (Tecniplast, Buguggiate, Italy) recirculating system. Culture water was obtained through reverse osmosis and activated carbon filtration of tap water, complemented with 0.34 mg/l of salt ("Instant Ocean Synthetic Sea Salt," Spectrum Brands, USA) and automatically adjusted for pH and conductivity. Water temperature was $26.0 \pm 1\,°C$, conductivity 750 ± 50 uS, pH 7.5 ± 0.5 and dissolved oxygen equal to or above 95% saturation. A 10:14 h photoperiod cycle was maintained.

Chemicals and test solutions

The compounds used in this experiment were 17 alpha methyltestosterone (99.9% of purity, Empirical Formula: $C_2H_3O_2$, CAS: 58-18-4), purchased from Sigma Aldrich (Co., St. Louis, MO), and the commercial formulation (cMT) (90% purity), purchased from Bioativa (Paraná, Brazil). Stock solutions were prepared by dissolving 13 mg of compound in 200 ml of dimethylsulfoxide (DMSO). Test solutions were then prepared by diluting the stock solution in culture water (see below). A solvent control (containing 25 µl/L of DMSO, which corresponds to the concentration of solvent used in the highest MT concentration tested) was included in all assays.

Experimental design and treatments

The assay using adult fish followed the OECD Test Guideline No. 203[30] in static test conditions complemented with endpoints of genotoxicity and biochemical markers. The initial population comprising female and male adult zebrafish of similar length and age (2 ± 1 cm, 6 months old) was selected for the test. The animals were acclimatized for 24 h in a glass aquarium. After the acclimatization, 9 fish were equally distributed in each treatment (0, 4, 23, 139, 833 and 5000 µg/L MT or cMT), in 3 aquariums per treatment (each containing 2 L of test solution) and the test solution was maintained for 96 h. The fish were not fed during the test period and their mortality was recorded daily. At the end of the test fish were sampled for micronucleus and comet assay and organs removed for biochemical determinations.

Micronucleus assay

The test was carried out as described by Hooftman and Raat[31] for fish erythrocytes. Peripheral blood was obtained by cardiac puncture with a heparinized syringe and immediately smeared for MN analysis. After fixation in methanol for 5 min, slides were left to air-dry and then were Giemsa-stained at a concentration of 5%. Three thousand erythrocyte cells with complete cytoplasm were scored per fish for MN analysis. The criteria for the identification of fish micronucleated erythrocytes were as follows: (a) MN should be smaller than one-third of the main nuclei, (b) MN must not touch the main nuclei, (c) MN must be a non-refractive, circular or ovoid chromatin body showing the same staining pattern as the main nucleus. One thousand cells were scored for NA analysis. Several types of NAs are defined according to Carrasco et al.[32]: cells with two nuclei are considered as binuclei; blebbed nuclei present a relatively small evagination of the nuclear membrane, which contains euchromatin; evaginations larger than the blebbed nuclei which can have several lobes are classified as lobed nuclei; nuclei with vacuoles and appreciable depth into a nucleus that does not

contain nuclear material are notched nuclei. In this work NA types were not discriminated.

Comet assay

The assay was conducted in whole blood under yellow light, to prevent UV-induced DNA damage, and performed as described by Nogueira et al.[33] An aliquot of 50 µL was added in a microtube containing 500 µL HAM-F10 serum for the comet assay. Visual scoring of cellular DNA on each slide was based on the categorization of 100 randomly selected cells. The comet-like formations were visually graded into five classes, depending on DNA damage level adapted from Garcia et al.[34]: undamaged—no tail visible (class 0); low damage – tails with low fluorescence and head still round and bright (class 1); medium damage – head and tail equally bright (class 2); high damage – small head, and a long and very bright tail (class 3); extreme damage – very long tail, while head is no longer round (class 4). Each cell was assigned a value (from 0 to 4) according to its comet class, and the overall score for 100 cells ranged from 0 (100% of comets being in class 0) to 400 (100% of comets in class 4). The number of comets in each class was counted, and average DNA damage (DD) was calculated as follows:

$$ID = 1n1 \pm 2n2 \pm 3n3 \pm 4n4$$
$$\Sigma = 100$$

where n1–n4 is the number of comets in classes 1–4 and Σ is the sum of all counted comets (n1 + n2 + n3 + n4). DD is expressed in arbitrary units.[35] Positive controls were always included.

Biomarker assays

At the end of the treatment, the animals were euthanized and dissected for organ removal. The head and muscle were used for ChE analysis; head, muscle, gills and gut to assess LDH and GST. The organs were immediately frozen in liquid nitrogen and the samples were kept at temperatures of −80 °C until the analyses were performed. Fish livers and gonads were used to evaluate the VTG alterations. Previously, the gonads and livers were removed and weighed to determine the gonadosomatic (GSI) and hepatosomatic (HSI) index, determined as [liver weight (g)/whole body weight (g)] × 100. [36]

Vitellogenin quantification

Liver and gonad samples were sonicated using homogenization buffer (125 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA and 1 mM dithiothreitol at pH 8; 1 mL to 200 g tissue ratio). This was centrifuged for 20 min at 12,000 × g at 4° C. Supernatants were used to quantify the amount of total proteins and VTG binding protein levels. The VTG was determined by the indirect induction method of unstable alkaline phosphate following the Gagné and Blaise [37] protocol with modifications.[38] Rapidly, 100 µL of the supernatants were mixed with 54 µL of acetone (35% final volume) for 5–10 min at room temperature, subsequently vortexed at least 3 times and then centrifuged at 10,000 × g for 5 min. The supernatant containing acetone was removed. Approximately 50–100 µL (depending on the pellet size) of 1 M NaOH were added to the samples and maintained for 90 min at 70 °C[38] to proceed with the phosphate barrier hydrolysis process.[37] The free phosphate levels were determined in the aqueous phase according to the phosphomolybdenum method.[39] The results were expressed as µg PO_4/mg protein. The gonads and livers were removed and weighed to determine the gonadosomatic (GSI) and hepatosomatic (HSI) index; determined as [weight of gonad (g)/ body weight (g)] × 100%.

GST, ChE and LDH

The head, muscle, intestine and gill samples were defrosted on ice, homogenized in phosphate buffer (0.1 M, pH 7.4) using a sonicator (KIKA Labortechnik U2005 Control TM) and centrifuged for 20 min at 11,500 × g in order to isolate the post-mitochondrial supernatant (PMS).

GST activity was determined at 340 nm by monitoring the increase in absorbance for 5 min, following the general methodology described by Habig and Jakoby,[40] as modified by Frasco and Guilhermino.[41] Activity determinations were made using 100 µL of PMS of the sample and 200 µL of reaction mixture [10 mM reduced glutathione (GSH) and 60 mM 1-chloro-2.4-dinitrobenzene in phosphate buffer 0.05 M, pH 6.5]. Enzymatic activity was determined in quadruplicate and expressed as nanomoles of substrate hydrolyzed per minute (U) per mg of protein.

ChE activity was determined using acetylthiocholine as substrate and measuring the conjugation product of thiocholine (a product of the degradation of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (absorbance increase) in phosphate buffer at 414 nm for 5 min, according to the method of Ellman et al.[42] Activity determinations were made using 40 µL of PMS of the sample, 250 µL of reaction mixture (acetylthiocholine 7.5 mM) and DTNB (10 mM) in phosphate buffer (0.1 M, pH 7.2).

LDH activity was measured in the PMS at 340 nm, by continuously monitoring (for 5 min) the decrease in absorbance due to the oxidation of NADH, following the methodology described by Vassault[43] with modifications introduced by Diamantino et al.[26] Activity determinations were made using 40 µL of PMS of the sample, 250 µL of NADH (0.24 mM) and 40 µL of pyruvate (10 mM) in Tris–NaCl buffer (0.1 M, pH 7.2).

Protein concentration in the samples was determined in quadruplicate by the Bradford [44] method at 595 nm, using α-globulin as standard. All biochemical determinations were made spectrophotometrically in 96-well microplates using a Thermo Scientific Multiskan® Spectrum.

Statistical analyses

Statistical analyses were carried out using IBM SPSS statistics program version 22.0. Data were expressed as

mean ± SEM (standard error of mean) and values of $p < 0.05$ were considered statistically significant. The continuous variables were tested for normal distribution with Shapiro-Wilk. Possible differences between the control and treated groups were investigated by ANOVA or the Kruskal-Wallis test (data not normally distributed), followed, respectively, by Tukey's test (multiple comparisons) or the Mann Whitney *U* test (2-to-2 comparisons). Lethal concentration values (LC50) and effective concentration values (EC50) were calculated for each parameter by fitting logistic dose-response curves.

Results

Micronucleus test (MN), nuclear abnormalities (NA) and comet assay

The toxicity evaluation of MT (17 alpha methyltestosterone 99.9% purity) and commercial MT (cMT 90% purity) tested in *Danio rerio* was based on LC 50% assay, showing a value of 6366.90 ± 1746.72 µg/L (2943.40 − 9790.41 µg/L) which was used as a reference for genotoxicity and sub-lethality studies. Mortality occurred in only one concentration of cMT (833 µg/L), evidencing a low mortality rate in relation to the administered concentration of MT.

The MN frequency presented a significant increase compared to the negative control in relation to the concentrations of 4 µg/L ($p = 0.020$) and 5,000 µg/L ($p = 0.001$) of MT, and also as regards the concentrations of 23 µg/L ($p = 0.002$) and 139 µg/L ($p = 0.028$) of cMT. The positive control, as expected, showed increased MN frequency. Significant differences between MT and cMT appeared in the results of MN test for the concentrations of 139 µg/L ($p = 0.028$) and 5000 µg/L ($p = 0.005$) (Table 1).

After the 96-hour exposure to MT, nuclear abnormalities (NA) significantly increased in the male groups treated with concentrations of 139 µg/L ($p = 0.019$) and 833 µg/L ($p = 0.003$) in comparison to the negative control; the same occurred in the female groups for the concentration of 833 µg/L ($p = 0.006$). For the cMT, similar results were observed in males treated with 23 µg/L ($p = 0.044$) and in females treated with 4 µg/L ($p = 0.038$), 833 µg/L ($p = 0.001$) and 5,000 µg/L ($p = 0.002$). Significant differences between MT and cMT in the results of NA appeared for males in the concentration of 833 µg/L ($p = 0.040$), and for females in the concentrations of 833 µg/L ($p = 0.014$) and 5,000 µg/L ($p = 0.004$) (Table 2).

The results of a genotoxic study with MT through comet assay showed a significant decrease in DNA damage for the concentration of 4 µg/L ($p = 0.045$) for males, while in treatments with cMT the decrease in this damage occurred for the concentrations of 4 µg/L ($p = 0.001$), 23 µg/L ($p = 0.001$) and 5,000 µg/L ($p = 0.001$). For females there was increased DNA damage only for cMT in the concentration of 833 µg/L ($p = 0.027$).

Interestingly, results of the comet assay also showed a significant reduction in the DNA damage index for males treated with DMSO (solvent control) ($p = 0.001$ for MT and $p = 0.000$ for cMT), but not for females (Table 2).

Table 1. Frequencies of micronucleus (MN) in erythrocytes of zebrafish (*Danio rerio*) exposed to different concentrations of methyltestosterone (MT) and its commercial formulation (cMT). Negative control was exposed to water; solvent control to DMSO (25 µL/L); and positive control to potassium dichromate (25 mg/L).

Treatment groups	MT	cMT
Negative control	0.00 ± 0.00	0.00 ± 0.00
Solvent control	0.11 ± 0.11	0.11 ± 0.11
Positive control	$3.33 \pm 0.88^{**a,b}$	$3.33 \pm 0.88^{**a,b}$
4 µg/L	$0.75 \pm 0.31^{*a,c}$	$0.56 \pm 0.29^{*c}$
23 µg/L	$0.33 \pm 0.17^{**c}$	$1.00 \pm 0.27^{**a,b,*c}$
139 µg/L	$0.00 \pm 0.00^{**c,*d}$	$0.44 \pm 0.18^{*a,**c,\neq}$
833 µg/L	$0.00 \pm 0.00^{**c,*d}$	$0.11 \pm 0.11^{**c}$
5000 µg/L	$1.33 \pm 0.41^{**a,e,f,*c,d}$	$0.11 \pm 0.11^{**c,e,\neq}$
P-value	**0.000**	**0.000**

The data correspond to the means ± standard error of mean (SEM). *P*-values were generated by the Kruskall–Wallis test. The superscript letters indicate significant differences in the pairwise comparisons, detected by the Mann-Whitney U test, with a = significant compared to negative control; b = significant compared to solvent control; c = significant compared to positive control; d = significant compared to MT 4 ⌈g/L; e = significant compared to MT 23 ⌈g/L; f = significant compared to MT 139 ⌈g/L. Asterisks indicate significant differences at $^{*}p < 0.05$ and $^{**}p < 0.01$. The symbol- ≠ indicates significant differences between MT and cMT in the same concentrations.

Vitellogenin

The levels of vitellogenin-like proteins (Figure 1) measured in fish gonads were generally higher for males. In this work, the MT did not cause effects associated with MT exposure. The brief MT exposure did not affect the VTG expression; Tables 3 and 4 show the gonadosomatic and hepatosomatic rates of *Danio rerio* adults exposed to MT for 96 h. The results demonstrate that there were no significant changes induced by MT or cMT; the baseline differences in the index expression in male and female zebrafish are represented.

Biochemical marker analyses

GST analysis (Figure 2) revealed different baseline activities among males and females in all three tested tissues: while males present higher activities in gills and intestines, females present higher activities in the head. Regarding the response to the testing compound, GST activity presented a general tendency to diminish with increasing MT concentrations; however, the pattern of response diverged from males to females: while for females decreased activities were observed for all tested tissues, for males the differences were only verified in the intestine. This dose response suggested that GST was a sensitive biomarker of the individual exposures to MT (and cMT – Figure A1 Appendix) in *D. rerio*. The other biochemical markers assessed in zebrafish were not altered by the exposure to either MT (Figures 3 and 4) or cMT (Figures A2, A3).

Discussion

As previously mentioned, steroid hormones are considered non-genotoxic and non-carcinogenic,[4,7,15] although reported results are still controversial. In this respect, our results showed low genotoxic potential of MT, inducing

Table 2. Results of nuclear abnormalities (NA) and comet assay (total DNA damage and the correspondent % total damage) in erythrocytes of male and female zebrafish (*Danio rerio*) exposed to different concentrations of methyltestosterone (MT) and its commercial formulation (cMT). Negative controls were exposed to water of system; solvent controls, to DMSO (50 µL/2 L); and positive controls, to potassium dichromate (125 mg/5 L).

	NA			Total DNA damage (au)		%Total damage		
Treatment groups	Males	Females	*P*-values[1]	Males	Females	Males	Females	*P*-values[1]
MT								
Negative control	0.56 ± 0.24	0.11 ± 0.11	**0.193**	127.04 ± 9.02	129.72 ± 14.45	31.76 ± 2.26	32.43 ± 3.61	**0.525**
Solvent control	1.00 ± 0.37	1.00 ± 0.37^{*a}	**1.000**	66.74 ± 5.55^{**a}	138.15 ± 9.86	16.69 ± 1.39^{**a}	34.54 ± 2.46	**0.001**
Positive control	3.56 ± 1.44	3.56 ± 1.44^{**a}	**0.843**	168.23 ± 4.59^{*a,**b}	160.94 ± 2.78^{**a,*b}	42.06 ± 1.15^{*a,**b}	40.24 ± 0.69^{**a,*b}	**0.243**
4 µg/L	1.63 ± 0.73	0.71 ± 0.29	**0.733**	90.74 ± 8.42^{*a,**c}	132.99 ± 13.29^{*c}	22.69 ± 2.11^{*a,**c}	33.25 ± 3.32^{*c}	**0.012**
23 µg/L	1.56 ± 0.44	0.38 ± 0.26^{*c}	**0.052**	124.64 ± 5.43^{**b,*c}	153.71 ± 4.80	31.16 ± 1.36^{**b,*c}	38.43 ± 1.20	**0.003**
139 µg/L	1.89 ± 0.42^{*a}	0.22 ± 0.15^{*c}	**0.003**	129.43 ± 9.72^{**b,d}	141.79 ± 11.62	32.36 ± 2.43^{**b,d}	35.45 ± 2.91	**0.297**
833 µg/L	2.14 ± 0.26^{**a,*b}	1.22 ± 0.40^{**a,*f}	**0.036**	144.49 ± 13.54^{**b,d}	137.55 ± 15.21	36.12 ± 3.39^{**b,d}	34.39 ± 3.80	**0.637**
5000 µg/L	1.22 ± 0.60	0.56 ± 0.34^{*c}	**0.481**	147.18 ± 2.99^{**b,d}	147.41 ± 4.21^{*c}	36.79 ± 0.75^{**b,d}	36.85 ± 1.05^{*c}	**0.825**
***P*-values[2]**	**0.132**	**0.013**		**0.000**	**0.155**	**0.000**	**0.155**	
cMT								
Negative control	0.56 ± 0.24	0.11 ± 0.11	**0.193**	127.04 ± 9.02	129.72 ± 12.52	31.76 ± 2.25	32.43 ± 3.13	**0.531**
Solvent control	1.00 ± 0.37	1.00 ± 0.37^{*a}	**1.000**	66.74 ± 5.55^{**a}	138.15 ± 9.86	16.69 ± 1.39^{**a}	34.54 ± 2.46	**0.001**
Positive control	3.56 ± 1.44	3.56 ± 1.44^{**a}	**1.000**	168.23 ± 4.59^{**a,b}	160.94 ± 2.78^{**a,*b}	42.06 ± 1.15^{**a,b}	40.24 ± 0.69^{**a,*b}	**0.243**
4 µg/L	2.56 ± 1.23	1.00 ± 0.38^{*a}	**0.580**	103.19 ± 7.54^{**b,c}	145.42 ± 2.22^{**c}	25.80 ± 1.89^{**b,c}	36.35 ± 0.55^{**c}	**0.000**
23 µg/L	2.13 ± 0.58^{*a}	1.67 ± 0.92	**0.642**	92.15 ± 6.74$^{*a,**b,c,\neq}$	145.89 ± 5.57^{*c}	23.04 ± 1.69$^{*a,**b,c,\neq}$	36.47 ± 1.39^{*c}	**0.001**
139 µg/L	0.89 ± 0.45	2.83 ± 1.33	**0.370**	116.20 ± 6.71^{**b,c}	137.96 ± 15.48	29.05 ± 1.68^{**b,c}	34.49 ± 3.87	**0.039**
833 µg/L	1.22 ± 0.28$^{\neq}$	4.00 ± 0.80$^{**a,*b,d,\neq}$	**0.011**	126.99 ± 6.15^{**b,c,*d}	154.16 ± 4.56^{*a,d}	31.75 ± 1.54^{**b,c,*d}	38.54 ± 1.14^{*a,d}	**0.015**
5000 µg/L	1.56 ± 0.53	5.00 ± 1.35$^{**a,b,d,\neq}$	**0.027**	86.40 ± 12.78$^{*a,**c,\neq}$	148.02 ± 4.19^{*c}	21.60 ± 3.19$^{*a,**c,\neq}$	37.01 ± 1.05^{*c}	**0.009**
P-values[2]	**0.341**	**0.005**		**0.000**	**0.034**	**0.000**	**0.034**	

[1]The Mann–Whitney U test was performed to evaluate differences between the genders.
[2]The Kruskal–Wallis test was performed to evaluate differences among the treatment groups, except for the comet assay on males, where ANOVA was used.
The data correspond to the means ± standard error of mean (SEM). au = arbitrary units; NA = nuclear abnormalities. The superscript letters indicate significant differences in the pairwise comparisons, detected by the Tukey test (comet assay on males) or the Mann-Whitney U test (other variables), with a = significant compared to negative control; b = significant compared to solvent control; c = significant compared to positive control; d = significant compared to MT 4 µg/L; e = significant compared to MT 23 µg/L; f = significant compared to MT 139 µg/L. Asterisks indicate significant differences at $^{*}p < 0.05$ and $^{**}p < 0.01$. The symbol ≠ indicates significant differences between MT and cMT in the same concentrations.

micronucleus, nuclear abnormalities and DNA damage in *Danio rerio*, depending on the use of MT or cMT, gender and tested concentrations. The use of cMT in fish farms (e.g. Brazilian farms) may give rise to reproductive anomalies in *Oreochromis niloticus* juveniles, such as a decrease in oocyte maturation in juveniles after exposure for 28 days at a concentration of 0.06 µg/L MT.[7] Khalil et al.[45] carried out a study with tilapia treated with methyltestosterone to induce sex reversion, and four months later detected increased DNA fragmentation through the DNA gel electrophoresis laddering assay as compared to the untreated control. The frequency of micronuclei is related to the genetic instability produced by a toxic agent.[46] Abo-Al-Ela et al.[47] found an increase in the MN frequency in Nile tilapia injected with 25 µg/g MT compared to controls, which demonstrates a genotoxic effect for this androgen. However, in another study, no genetic damage was found after exposure for 96 h at the concentration of 10 µg/L of MT analyzed by the MN test,[4] suggesting that *Danio rerio* could have greater sensitivity to the analyzed compounds when compared to *Oreochromis niloticus* and *Astyanax bimaculatus*.

The comet assay is a rapid, sensitive and economically favorable test for genetic damage detection in natural biota,[48] and thus this bioassay is suggested as an appropriate tool for the detection of effects caused by oxidative environmental stressors in fish.[4] Nevertheless, it has been demonstrated that the lowest genotoxic dose (defined as the lowest dose at which each mutagen causes a positive response in each genotoxicity assay) in the comet assay is higher than that in the MN test, suggesting that the power of the MN test to detect a low level of genotoxic potential is superior to that of the comet assay.[49] As the nuclear abnormalities test is considered a secondary and cytologic evaluation, not showing real genotoxicity, our results of the MN test and nuclear abnormalities suggest that further testing is needed to elucidate the possible genotoxic potential of these compounds.

Considering the above, results of the comet assay suggested that, at least for males, the use of DMSO as solvent, which has been used in medicine as an active anti-inflammatory pharmaceutical agent and as an accessory to carry compounds under the skin,[50] could be mainly responsible for the significant reduction of DNA damage observed. On the other hand, for females, which have the natural protection of estrogens as antioxidant in the reproductive age,[51] Ho and Roy[16] reported that testosterone, combined with estrogens, induces an increase in single strand breaks of DNA, corroborating our results. Although these were non-significant for any concentration of MT as well as for cMT at 5000 µg/L, the large standard deviation in the negative control groups and in the group treated with MT 833 µg/L could explain them. Thus, while not ruling out the hypothesis that the differences in the results of genotoxicity and cytotoxicity between the two MT products could also be related to the manufacture origin, since cMT contains 10% of unspecified impurities, the most relevant result in this study was the evidence of lesions induced in the DNA by methyltestosterone in females. However, it should also be investigated further, including an increase in sample size and a chemical evaluation of cMT impurities, mainly considering that: (1) oxandrolone is the major impurity often found in commercial MT, according to the National

Table 3. The gonadosomatic index (GSI) of adult zebrafish after 96 hours of exposure to 17 alpha methyltestosterone (MT and cMT).

	GSI MT[a]		GSI cMT[a]	
Treatment groups	Males	Females	Males	Females
Negative control	1.42 ± 0.53	19.42 ± 9.07	1.42 ± 0.53	19.42 ± 9.07
4 µg/L	1.11 ± 0.28	13.02 ± 4.84	1.28 ± 0.76	15.85 ± 5.28
23 µg/L	1.40 ± 0.68	16.05 ± 8.54	1.07 ± 0.37	14.89 ± 6.48
139 µg/L	1.27 ± 0.33	13.11 ± 7.11	1.34 ± 0.28	17.76 ± 9.49
833 µg/L	1.81 ± 1.08	16.00 ± 7.66	1.15 ± 0.32	15.99 ± 9.32
5000 µg/L	1.08 ± 0.43*	12.00 ± 6.86	1.41 ± 1.49	11.51 ± 4.42

[a]values were shown as the mean ± standard deviation (SD) of three replicates per treatment (54 fish per gender per treatment).
[b]GSI 1/4 gonad weight (mg) _ 100/body weight (mg).
[c]Asterisks denote significant differences between the control and treatments (*$p < 0.05$).

Table 4. The gonadosomatic index (HSI) of adult zebrafish after 96 hours of exposure to 17 alpha methyltestosterone (MT and cMT).

	HSI MT[a]		HSI cMT[a]	
Treatment groups	Males	Females	Males	Females
Negative control	1.39 ± 0.66	4.44 ± 0.89	1.39 ± 0.66	4.44 ± 0.89
4 µg/L	1.84 ± 1.00	4.87 ± 1.14	15.54 ± 11.49*a	4.05 ± 1.26
23 µg/L	1.74 ± 0.56	3.86 ± 1.12	2.64 ± 2.98	5.40 ± 1.78
139 µg/L	2.02 ± 0.66	5.03 ± 1.57	1.89 ± 0.64	4.95 ± 1.74
833 µg/L	2.49 ± 0.85	4.25 ± 1.22	1.74 ± 0.56	5.42 ± 3.62
5000 µg/L	2.56 ± 1.13*a	6.26 ± 1.43	1.62 ± 0.67	4.21 ± 0.84

[a]values were shown as the mean ± standard deviation (SD) of tree replicates per treatment (54 fish per gender per treatment).
[b]HSI 1/4 liver weight (mg) _ 100/body weight (mg).
[c]Asterisks denote significant differences between the control and treatments (*$p < 0.05$).

Institute of Health (NIH) of the U.S. Department of Health & Human Services; and (2) oxandrolone is an anabolic androgenic steroid (AAS), a 17α-derivative modification, which resists hepatic metabolism and is therefore more active when administered orally, being associated with elevated levels of hepatotoxicity.[52] We likewise cannot rule out the hypothesis that the increased DNA damage observed at 833 µg/L of cMT (but not of MT) could have possibly been caused by contaminants such as oxandrolone, since cMT is only 90% pure.

To our knowledge, the methyltestosterone effects on *D. rerio* over a short period of exposure have not been reported yet. However, exogenous hormone treatment is known to disrupt various systems in the body. In addition to the endocrine, toxicological and environmental damage related to non-target species, MT alters the immune system, increasing the susceptibility to infections and opportunistic diseases.[53] The main way in which steroid hormones interact with cells is by binding to proteins called steroid receptors. When steroids bind to these receptors, proteins enter the cell nucleus and alter gene expression[54] or activate processes that send signals to other parts of the cell[55] causing genetic toxicity. On the other hand, it has been shown that the positive regulation of genes related to the immune system does not have a functional effect on fish.[56] So, the concern regarding the effects of this androgen should continue to be investigated, as other effects may be detected through evaluations with different species and methodologies.

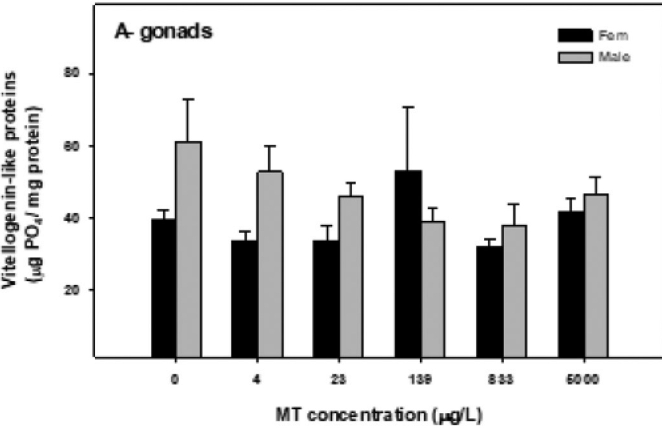

Figure 1. VTG level (mean ± standard error) measured in the gonads of male and female zebrafish exposed to MT. Asterisks denote differences from control (Holm Sidak test, $p < 0.05$).

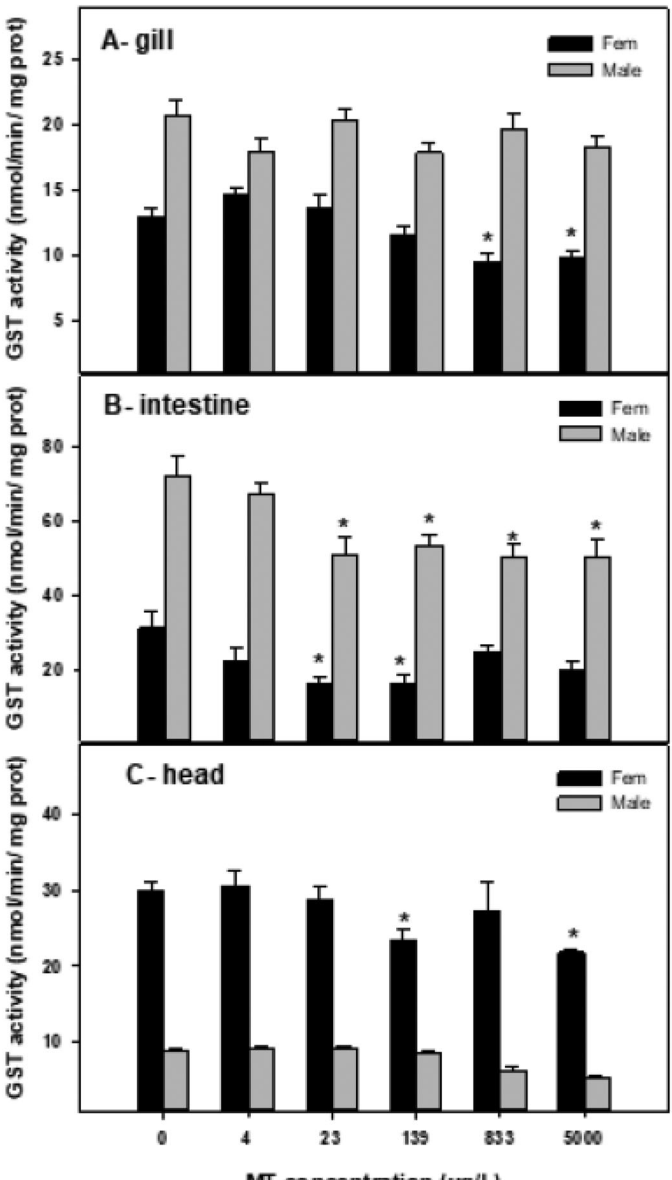

Figure 2. GST activity (mean ± standard error) measured in several tissues (A- gill, B- intestine and C- head) in male and female zebrafish exposed to MT. Asterisks denote differences from control (Holm Sidak test, $p < 0.05$).

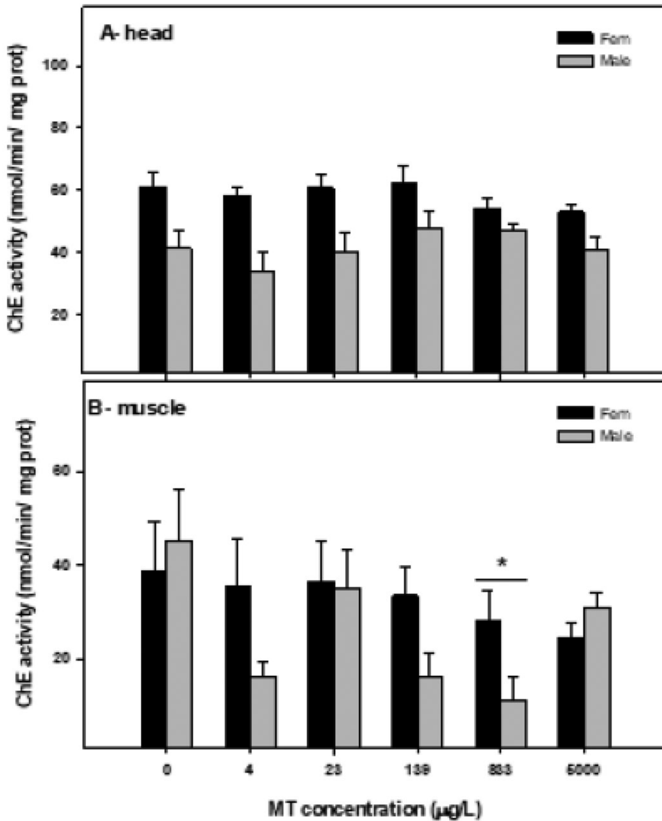

Figure 3. ChE activity (mean ± standard error) measured in several tissues (A- head and B- muscle) in male and female zebrafish exposed to MT. Asterisks denote differences from control (Holm Sidak test, $p < 0.05$).

The biochemical analyses of VTG-like protein levels revealed no reduction in fish exposed to the tested MT concentrations. Despite the present results with adult zebrafish, in the literature there are records of VTG level reduction by MT in the larval stage of the *D. rerio* species.[5] Although data on the environmental concentrations of MT are insufficient, the available information suggests that the effects of MT in zebrafish adults, albeit not inhibitory, should be taken into account due to the additional impact of MT on non-target species in fish farms and on population health.

GSI is broadly used as an unspecific biomarker to evaluate possibly adverse effects of environmental pollutants.[57] The index was developed as a parameter to describe the state of maturity of a specific exposure group, which is easy to quantify and record. Some studies have used GSI as an indirect parameter for evaluating reproductive capacity.[58] In this study, GSI values in female and male zebrafish did not change; thus MT did not affect the reproductive capacity in zebrafish adults. On the contrary, a tendency to decrease the GSI of male gonads was observed, suggesting that the androgen can cause atrophy of the male gonad.

MT did not affect HSI in adult zebrafish livers, which may be related to the short time of exposure to the hormone; in the literature, significant changes in HSI were detected after exposure to estrogenic substances, such as prochloraz and fenarimol for periods of 21 days or more.[24] These findings on VTG, GSI and HSI suggest that male zebrafish were more sensitive to the androgen in both tested products (MT and cMT).

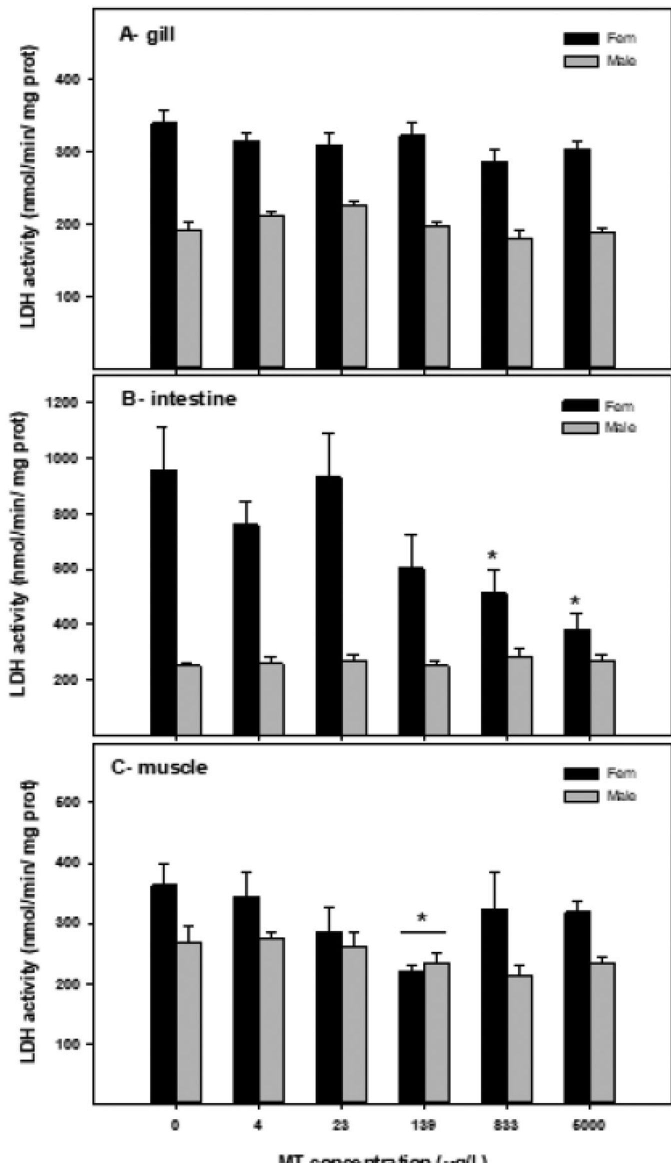

Figure 4. LDH activity (mean ± standard error) measured in several tissues (A- gill, B- intestine and C- muscle) in male and female zebrafish exposed to MT. Asterisks denote differences from control (Holm Sidak test, $p < 0.05$).

Enzymatic biomarker tests have been used as a routine tool to evaluate the potential effects of chemical substances, namely environmental contaminants, in biota.[59] It has already been described in the literature that the fish development stage is related to the greater or lesser sensitivity of hormones.[5,60–62] The effects of exposure to hormones during the developmental and adult stages have a complex pattern in nature, and in adulthood these effects may become irreversible due to previous exposure in earlier stages of fish development.

Thus, VTG has become an accurate endpoint to evaluate the reproductive risk of hormone exposure in adult models.[62] Nonetheless, the androgen and estrogen exposure effects during animal development do not persist until the adult stage.[5,63,64] MT and cMT do not cause toxicological effects on VTG expression; however, in previous studies it has been shown that MT influences VTG expression in zebrafish larvae after a 96-hour exposure.[5] VTG is indispensable for follicle development, oocyte maturation and gamete

biosynthesis, with prolonged exposure to estrogen being a cause of VTG activity inhibition in the early stages of development.[5,65]

GST, ChE and LDH are biomarkers much used in quality monitoring of aquatic ecosystems.[66–68] Effects of MT exposure on these markers are not sufficiently studied in aquatic organisms. In our work, these markers were chosen because they represent a variety of relevant biochemical pathways in fish (biotransformation, oxidative stress, energetic metabolism). GST is a phase II enzyme that facilitates detoxification.[29] MT is a lipophilic molecule that easily traverses the plasmatic membrane, and the GST pathway is expected to participate in the detoxification process in the organism. In our study, the enzymatic expression was different in relation to each evaluated organ. Thus, it is possible to understand the action of MT in different organs of this species. Overall, only the male zebrafish intestine presented a GST decrease. In females, the GST activity was inhibited in all organs, demonstrating a greater female susceptibility to the effects of MT on the GST pathway. Abo-Al-Ela et al.[69] reported that after exposure of *Oreochromis niloticus* to methyltestosterone for 21 days, there was decrease in transcription of the gstα1 gene, and even after a hormone administration interruption for 21 days, the gene expression remained low. Thus, it is evident that this substance influences the enzymatic alteration in fish,[70] in agreement with the results found in our work.

ChE activity analyses in muscle showed that basal activities in both sexes were similar, but in the head, ChE activity was higher in females. After MT exposure, ChE activity was only inhibited in the head of organisms exposed to the cMT (Figure A2). In the muscles, enzymatic inhibition occurred in males and females for both products, demonstrating that cMT presents greater interference in the metabolic pathway of this enzyme (Figure A2). ChE is an enzyme playing a significant role in neurotransmission and in neuronal and muscular development.[68,71] Our results suggest no neurotoxicity of MT on zebrafish adults. However, the ChE inhibition observed at 4, 23 and 139 μg/L concentrations in the head suggests a possible weak neurotoxic effect of MT on adult zebrafish and, thus, represents an unspecified secondary effect of MT. In the literature, a study regarding effects of testosterone in ChE of the fish (*O. latipes*) was found, which reported that muscular ChE of adults was unaffected after treatment with 100 g/L of testosterone for 6 days.[72]

LDH is involved in the anaerobic pathway of energy production, processing pyruvate to lactate in the glycolytic pathway and has been used as a general biomarker of stress in fish.[26] In our study, the basal activity of LDH was consistently higher in female fish than in male fish in all tested tissues (gill, intestine and muscle), and the highest tested concentrations (833 and 5000 μg/L) of MT caused an inhibition of LDH activity only in the intestine. Exposure to MT only produced a clear response in the intestine, where an activity decrease was recorded for females (Figures 5 and A3). Previous studies have shown LDH levels were inhibited by the application of testosterone in *Anabas testudineus* adult fish. However, in another study in ponds with and without MT contamination, no differences were detected in LDH activity.[73,74] This suggested that the androgen treatment may result in an influence on the aerobic metabolism, as seen by LDH decrease.

In general, biomarker analysis showed some alterations in GST and no major changes in ChE and LDH activities in adult zebrafish. Changes in these biochemical markers were more marked in the early stages of development of zebrafish.[5] This study has found that at the tested concentrations there was a weak but significant induction of ChE in the muscles, and significant inhibition of GST and LDH in the gut of fish.

Conclusion

The 17-alpha methyltestosterone hormone used in Brazilian fish farming (cMT) caused changes in biochemical markers in zebrafish adults, suggesting physiological disturbances. Furthermore, it showed more cytotoxicity than genotoxicity. The enzymatic alterations of GST, ChE and LDH in our findings suggest some interference in the detoxification processes that activate the zebrafish defense system. The biomarkers showed weak changes, with significant inhibitions depending on gender and evaluated tissue. Our data suggest that MT caused relevant effects in zebrafish adults, and attention should be given to these changes due to their impact on aquatic ecosystems and human health. Not only should environmental risk studies with chronic exposures be carried out, but more toxicological evaluations in non-target organisms ought also to be encouraged, considering the ever-increasing use of MT in aquaculture worldwide.

Funding

Research supported by Brazilian Research Council (CNPq), grant no. 305741/2015-2 FAPDF and University of Brasilia. Carla L G. Rivero-Wendt received a fellowship from CAPES (Brazilian Ministry of Education).

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Appendix

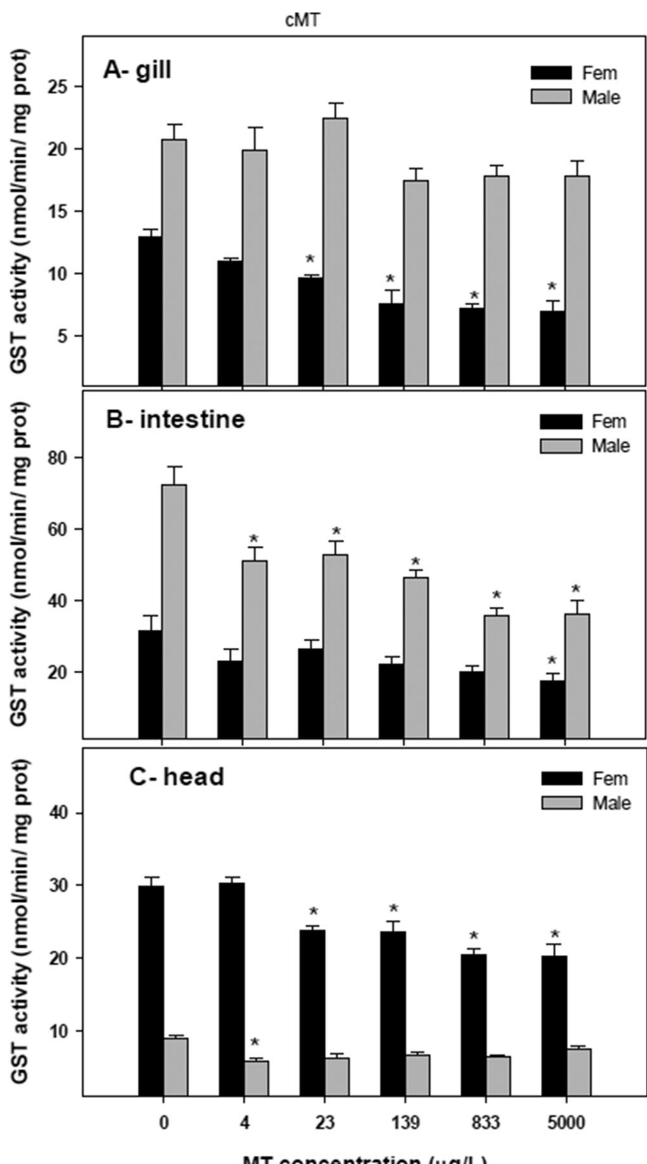

Figure A1. GST activity (mean ± standard error) measured in several tissues (A-gill, B-intestine and C- head) in male and female zebrafish exposed to cMT. Asterisks denote differences from control (Holm Sidak test, $p < 0.05$).

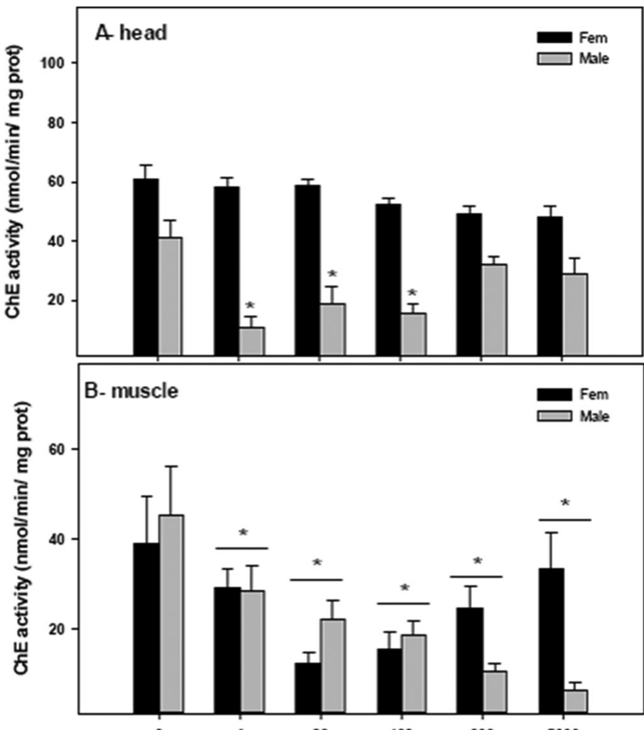

Figure A2. ChE activity (mean ± standard error) measured in several tissues (A-head and B-muscle) in male and female zebrafish exposed to cMT. Asterisks denote differences from control (Holm Sidak test, $p < 0.05$).

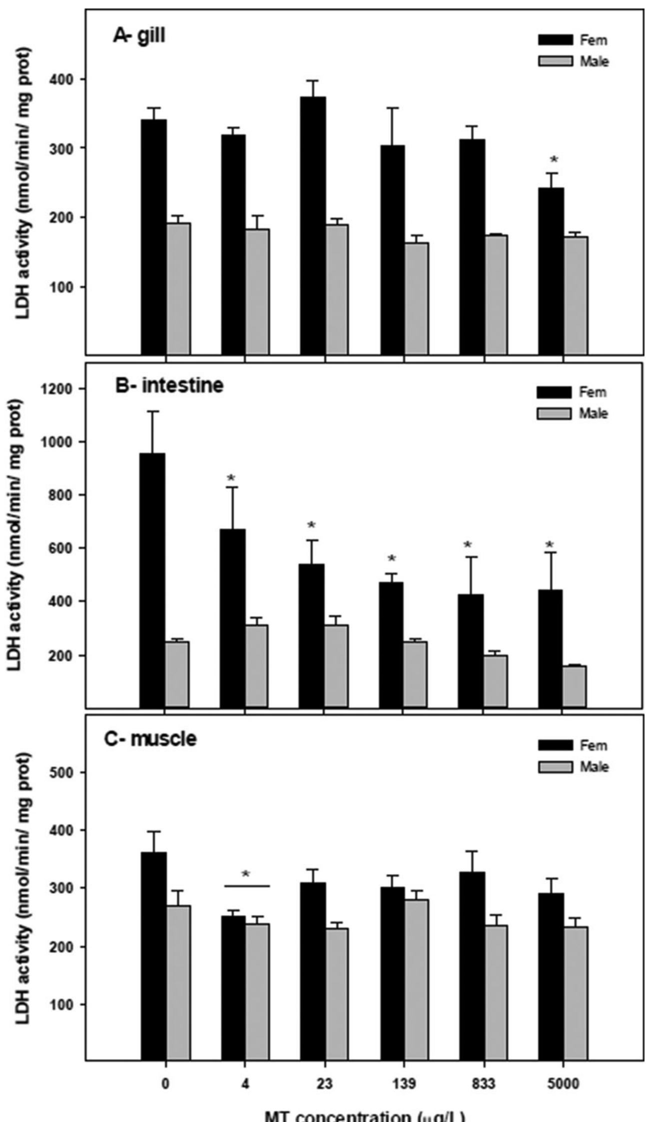

Figure A3. LDH activity (mean ± standard error) measured in several tissues (A-gill, B-intestine and C- muscle) in male and female zebrafish exposed to cMT. Asterisks denote differences from control (Holm Sidak test, $p < 0.05$).