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Are fish populations at risk? Metformin disrupts zebrafish development and reproductive processes at chronic environmentally relevant concentrations

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Abstract

The antidiabetic drug Metformin (MET), one of the most prevalent pharmaceuticals in the environment, is currently detected in surface-waters in the range of ng/L to low μg/L. As current knowledge regarding the long-term effects of environmentally relevant concentrations of MET in non-target organisms is limited, the present study aimed at investigating the generational effects of MET, in concentrations ranging from 390 to 14 423 ng/L in the model organism *Danio rerio* (up to 9mpf), including the effects on its non-exposed offspring (until 60dpf). We integrate several apical endpoints, i.e., embryonic development, survival, growth and reproduction, with qRT-PCR and RNA-seq analyses to provide additional insights into the mode of action of MET.

Reproductive-related parameters in the first generation were particularly sensitive to MET. MET parental exposure impacted critical molecular processes involved in the metabolism of zebrafish males, which in turn affected steroid hormone biosynthesis and upregulated male *vtg1* expression by 99.78 to 155.47 folds at 390 and 14 432 MET treatment, respectively, pointing to an estrogenic effect. These findings can potentially explain the significant decrease in fertilization rate and the increase of un-activated eggs. Non-exposed offspring was also affected by parental MET exposure, impacting its survival and growth. Altogether, these results suggest that MET, at environmentally relevant concentrations, severely affects several biological processes in zebrafish, supporting the urgent need to revise the proposed Predicted No-Effect Concentration (PNEC) and the Environmental Quality Standard (EQS) for MET.

Synopsis: This research gathering multiple lines of evidence demonstrating that metformin, at environmentally relevant concentrations, severely affected important biological processes

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51 in zebrafish, supporting the need to revise the proposed PNEC/EQS, set several times
52 above the concentrations tested in the present study.

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54 **Keywords:** Contaminants of emerging concern; *Danio rerio,* Metformin full life-cycle
55 exposure; Endocrine disruption; Risk Assessment; Water framework directive; Watch List

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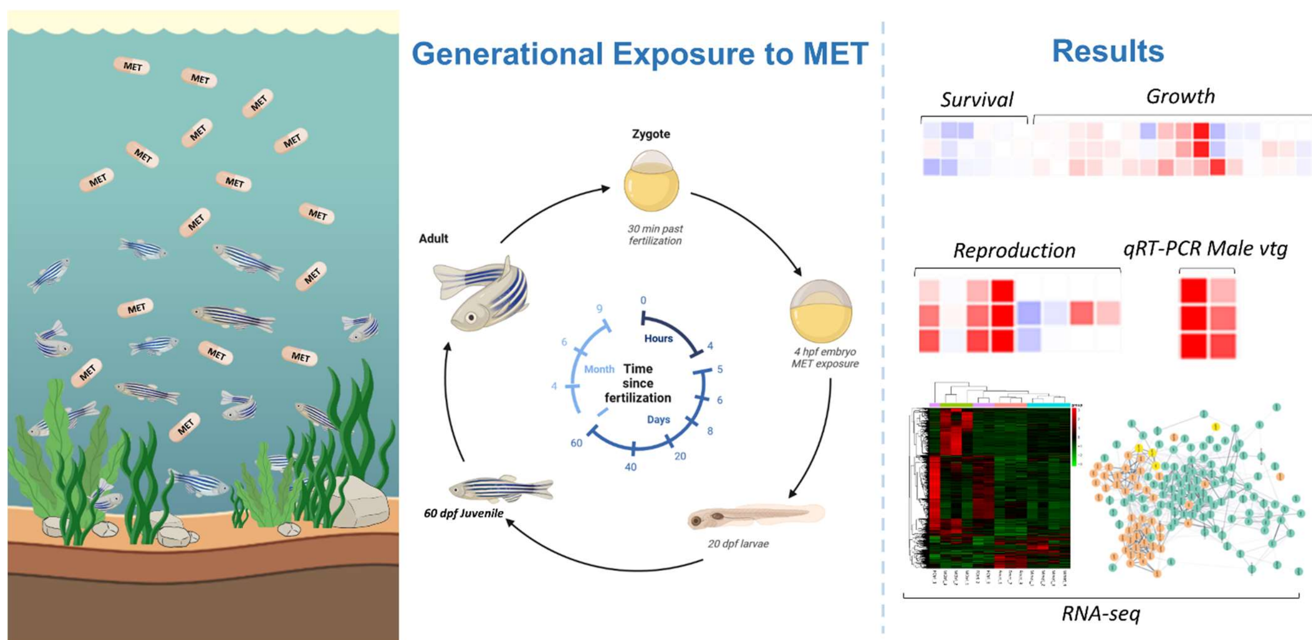
57 **Abstract Art**

58 

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60 1. Introduction

61 Metformin (MET), an antihyperglycemic pharmaceutical of the biguanides class, is used for
62 the treatment of type-II diabetes mellitus (T2DM) since 2009[1,2]. In T2DM patients, MET acts
63 by inhibiting the mitochondrial complex I of the electron transport chain, interfering
64 with several metabolic pathways, such as cholesterol and steroid hormone biosynthesis[3].

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6 65 Due to the increasing incidence of T2DM, MET is currently one of the most prescribed
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8 66 human pharmaceuticals worldwide[4–7]. As a result, high amounts of this drug are
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10 67 continuously discharged into aquatic environments. In the last decade, increasing levels of
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12 68 MET were detected in surface waters, with average concentrations of 145 ng/L in Canada[8],
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14 69 467 ng/L in Germany[9] and 613 ng/L in China[10]. However, a recent global-scale study, that
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16 70 monitored the presence of 61 pharmaceuticals along 258 rivers in 104 countries on all
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18 71 continents, reported that MET is one of the top three most frequently detected
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20 72 pharmaceuticals worldwide with an average concentration of 2 362.9 ng/L and extreme
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22 73 concentrations detected in Tunisia (56 600 ng/L), Pakistan (51 100 ng/L) and Bolivia (46
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24 74 800 ng/L)[11]. These studies supported the recent inclusion of MET in the 4th Watch List under
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26 75 the Water Framework Directive (WFD)[12], reinforcing the urgent need to get additional data
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28 76 on the risk of MET for aquatic environments. Therefore, the potential risk of MET in aquatic
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30 77 ecosystems should be addressed, as MET pollution can pose a serious global threat to
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32 78 environmental and human health. In fact, a growing body of studies have already
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34 79 investigated the effects of MET in different aquatic organisms, at different levels of biological
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36 80 organization, i.e., evaluation of survival, growth and reproduction parameters in *Brachionus*
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38 81 *calyciflorus, Daphnia similis, Pimephales promelas* and *Salmo trutta*[13–16]; endocrine
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40 82 disruption in *P. promelas* and *Oryzias latipes*[15,17,18] and biochemical and/or gene expression
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42 83 parameters in *Nothobranchius guentheri, P. promelas* and *O. latipes*[15,17,19]. In fish,
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44 84 contrasting findings have been reported regarding the nature and magnitude of MET effects
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46 85 associated with growth, reproduction and endocrine disruption. These divergences appear
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48 86 to be related with differences in the test species used, study duration, range of tested
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50 87 concentrations and exposure timing. Given the variability of effects observed following MET
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52 88 exposure and considering that only a few of the aforementioned studies tested
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54 89 environmentally relevant MET concentrations in long-term chronic tests, we performed a
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56 90 comprehensive long-term, low-level exposure investigation with the model species *Danio*
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91 *rerio* (zebrafish), to provide further insights on MET effects in aquatic ecosystems and to improve environmental hazard and risk assessment. This study assessed the long-term direct effects of environmentally relevant concentrations of MET on parental (F_0) zebrafish, from egg to 9 months old, and the effects that MET exposure exerts on the early-life stages of the non-exposed F_1 generation (intergenerational effects). Here we combined apical endpoints, i.e., embryonic development, survival, growth, and reproduction, with molecular analyses, i.e., qRT-PCR and RNA-seq to provide additional insights into MET mode of action (MoA).

2. Materials and Methods

2.1. Experimental design

All procedures with zebrafish during the bioassay were subjected to a previous ethical review process (Supporting Information - Section S1).

To investigate the generational effects of environmentally relevant concentrations of MET (CAS: 1115-70-4; 98% purity; Sigma Aldrich), zebrafish were continuously exposed to three MET nominal concentrations (in duplicate): 361, 2 166, and 13 000 ng/L, plus a control group (dechlorinated tap water, in triplicate), from 4 hours post fertilization (hpf) until 9-months post fertilization (mpf) (F_0). To evaluate the intergenerational effects of MET, the embryos resulting from F_0 exposure were raised in MET-free water (control water) until reaching 60 dpf – F_1 (Figure 1).

The experiment started by randomly allocating 400 newly fertilized wild-type zebrafish embryos (4 hpf, F_0), in 7 L aquaria. Each aquarium was maintained with a water temperature of 28 ± 1 °C, a 14:10 h (light:dark) photoperiod, pH 7.5 ± 0.2, and ammonia and nitrite concentrations <0.04 ± 0.02 and <0.03 ± 0.03 mg/L, respectively. MET exposure was performed under a flow-through system using two peristaltic pumps. MET working solutions were administered at a constant flow of 4.3 mL/h using a IP peristaltic pump (ISMATEC,

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6 117 Germany), while clean dechlorinated, heated and charcoal filtered water was supplied at a
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8 118 constant rate of 1.01 L/h using an ISM 444 peristaltic pump (ISMATEC, Germany). MET
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10 119 solutions and water were pumped into a mixing chamber before being released by gravity
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12 120 into aquaria[20]. Feeding started at 5 dpf, three times a day, with the commercial fish diet
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14 121 Tetramin (Tetra, Melle, Germany) and a supplement of decapsulated *Artemia* spp cysts. Up
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16 122 to 20 dpf, zebrafish larvae were fed *ad libitum* and from 20 dpf onwards, the amount and
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18 123 size of food supplied were adjusted to fish development, in equal proportion for all aquaria.
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20 124 At 20 dpf, animals were transferred to 30 L aquaria and fish density was reduced to 100 and
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22 125 35 larvae per aquarium at 20 and 60 dpf, respectively. Thereafter, F_0 fish continued to
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24 126 develop until 9 mpf (Figure 1).
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26 127 In order to evaluate MET effects on F_0 zebrafish reproduction, two reproductive trials were
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28 128 conducted. First, a tank breeding assay was performed at 4 mpf to evaluate MET effects in
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30 129 fecundity and fertilization rate, followed by a pairwise cross-breeding assay performed at
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32 130 4.25 mpf to assess if potential effects were caused by maternal or paternal exposure
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34 131 (detailed methodology in Supporting Information – Section S2).
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36 132 At 8 mpf, F_0 zebrafish exposed to 2 166 and 13 000 ng/L (nominal concentrations) were
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38 133 reproduced to start a second generation (F_1). F_1 embryos were raised in MET-free water
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40 134 and maintained until 60 dpf, following the methodology described for F_0 (Figure 1).
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42 135 Animals were sampled several times during the bioassay, i.e., 96 hpf (F_0 and F_1 – Section
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44 136 2.2), 20 dpf (F_0), 60 dpf (F_0 and F_1), and 9 mpf (F_0) (Figure 1). Survival was analyzed in all
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46 137 development stages, whereas total length, weight and the Fulton's condition factor
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48 138 [K=(weigth/length^3)×100] were determined at 60 dpf and 9 mpf (n= 70 for MET treatments
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50 139 and n=105 for control group; n=35 per replicate). Although at 60 dpf zebrafish are sexually
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52 140 differentiated, the distinction between males and females was very difficult without
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54 141 histological analysis. Therefore, to avoid errors in sex determination, the survival, length and
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56 142 weight parameters were only measured separately for males and females at 9mpf. At 9 mpf,
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sex ratio of each treatment was determined through direct inspection of the gonads using a stereomicroscope (Nikon) and gonads and livers from males and females of each treatment were excised. Gonads were weighted to calculate the gonadosomatic index [GSI=gonad mass/body mass×100]) (n ≃ 35 for males and ≃ 35 for MET treatments and n≃ 52 for males and ≃ 52 females in the control group). Livers were preserved in RNALater® at -80 °C for latter qRT-PCR and RNA-seq analyses.

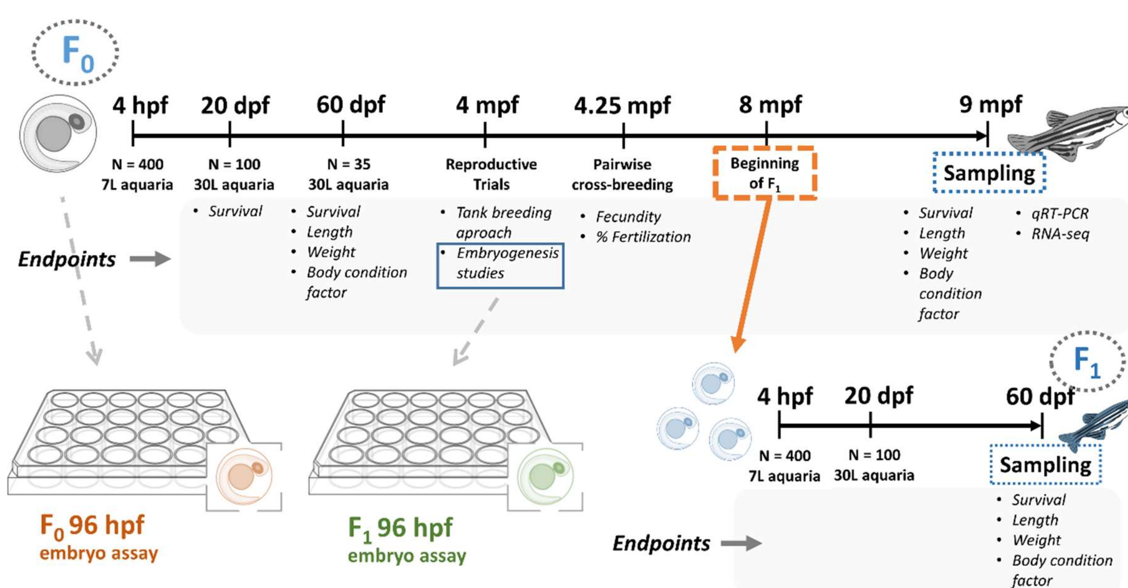

Figure 1. Schematic representation of the experimental setup: exposure period, temporal scale and endpoints analyzed in each sampling point.

2.2. Embryogenesis studies in F0 and F1 generations

Two different embryogenesis studies, with a duration of 96 h and replicated four independent times, were conducted using different conditions: F_0 96 hpf embryo assay - direct exposure of embryos to MET, where fertilized embryos obtained from unexposed parents of our zebrafish stock were exposed to the three MET concentrations tested in the present study, plus control (n=80, 20 per replicate); F_1 96 hpf embryo assay - indirect exposure of embryos, where fertilized F_1 embryos from the 4 mpf parental exposed treatments were reared in

MET-free clean water (n=80, 20 per replicate) (Figure 1). Both embryogenesis studies were carried out using slight modifications of the OECD Fish Embryo Acute Toxicity (FET) Test 236[21] (detailed description in Supporting Information - Section S3).

2.3. MET Analytical quantification

Actual MET concentrations were determined in water samples by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). As measured concentrations were slightly different from the nominal ones, from here onwards, a mean value of the actual MET concentrations measured, i.e., 390, 2 929 and 14 423 ng/L, will be used rather than the nominal ones (detailed description in Supporting Information – Section S4, Table S1).

2.4. F_0 Gene expression after 9 months of MET exposure

2.4.1. RNA isolation

At F_0 9mpf, RNA from livers was individually isolated from 12 males and 12 females of each treatment via Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare), according to the manufacturer's protocol. Quantification of isolated RNA was performed in a Take 3 Micro-Volume Plate Reader (Biotek Synergy HT) coupled with Gen5 (version 2.0). RNA quality was verified by 1.5% agarose gel electrophoresis and by λ260/λ280 nm absorbance ratio.

2.4.2. Fluorescence based quantitative real-time PCR (qRT-PCR) analysis

To evaluate the effects of MET at the molecular level, qRT-PCR was used in 8 males and 8 females liver samples from all MET treatments (390, 2 929 and 14 423 ng/L) and from the control group to quantify the transcription profiles of vitellogenin encoding genes, i.e. *vtg1* and *vtg*2, which are essential for egg production and a marker of estrogenicity. The ribosomal protein L 13 gene (*rpl13*) was selected as a housekeeping gene. Details of the

rational for genes selection and primers design is described in Supporting Information – Section S5, Table S2 and qRT-PCR methodology in Section S6.

2.4.3. Liver RNA-seq analysis

To gain further insights into the canonical pathways affected by MET beyond the findings of the exploratory RNA-seq analysis presented in our parallel study[3], we performed a more detailed transcriptomic analysis with biological replication focusing on zebrafish liver using 3-4 independent pools, composed by 3 individually extracted RNA livers, from the control and 3 livers from the 14 432 ng/L MET treatment for each sex (n=3-4). This RNA-seq approach, commercially obtained at Novogene (United Kingdom), was used to perform a mechanistic analysis on the genes and pathways involved in steroid biosynthesis and reproductive processes and to validate the genes/pathways related with energy/lipid metabolisms found to be altered in the exploratory RNA-seq analysis of our parallel study[3]. Samples were subjected to sequencing (Illumina Novaseq 6000 paired-end (2x150)) and subsequent bioinformatics analysis with parameters set to: false discovery rate (FDR) p-value (padj)<0.05 and a Log_2(FoldChange)>0. Raw dada was submitted to the NCBI SRA database under the BioProject PRJNA906165. Detailed methodology is described in Supporting Information - Section S7.

2.5. Statistical analysis

All statistical analyses were computed with Statistica 12.5 (Statsoft, USA). Obtained data was checked for normality and homogeneity of variances, using Kolmogorov-Smirnov and Levene's tests. Data were then analyzed by one-way ANOVA, and Post-hoc comparisons using Fisher's least significant difference (LSD) test. Significant differences were set as $p<0.05$.

3. Results and Discussion

Despite the ubiquitous presence of MET in aquatic ecosystems, uncertainties still remain about its long-term effects on non-target organisms. Therefore, in order to fill the knowledge gaps on the effects of environmentally relevant concentrations of MET in aquatic environments, the present study implemented a long-term multi-endpoint approach focusing on different zebrafish developmental stages to address direct effects of MET on parental exposed organisms (F_0) and the effects that parental exposure exerts on indirectly exposed generations [intergenerational effects (F_1)].

3.1. F_0 and F_1 Survival

First, we evaluated the effects of MET on survival of different developmental stages of zebrafish [i.e., larvae (96 hpf and 20 dpf), juveniles (60 dpf) and adults (9 mpf)] in the exposed F_0 generation; and in the indirectly exposed larvae (96 hpf) and juveniles (60 dpf) from the F_1 generation (Table 1). In the direct MET exposure (F_0), we found a significant decrease in survival on 96 hpf larvae at MET concentrations as low as 390 ng/L. At 20 dpf, a significant decrease in survival was also observed for larvae exposed to 2 929 and 14 423 ng/L of MET, when compared to control (45.2 and 24.4% survival, respectively) (Table 1). As expected, after 20 dpf mortality was very low until adulthood, since even under normal raising conditions, zebrafish are more sensitive up to 20 dpf, considering that during this period they develop from one cell stage into a swimming larva and change from vitellogenic reserves to external feeding[20,22]. Other recent studies with zebrafish are in agreement with the present findings, reporting that MET at concentrations from 1 to 10 µg/L significantly impacts zebrafish survival at the early phases of development[23,24]. However, these effects in mortality are not supported by other studies examining the effects of MET in other fish species, such as brown trout (*Salmo trutta*) and fathead minnows (*Pimephales promelas*), where MET exposure did not impact mortality at different development phases, including the

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6 239 larval stage[16,25,26]. Analyzing the few available studies, we hypothesize that zebrafish, at
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8 240 least in the early-life stages of development, are potentially more sensitive than the other
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10 241 few fish species tested so far that present longer periods of embryonic development.
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12 242 Together with the decreased survival in F_0 larvae, this parameter was also significantly
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14 243 impacted in F_1 96 hpf larvae (Table 1). Thus, the effects of MET exposure experienced by
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16 244 F_0 zebrafish during its lifetime led to decreased survival in the early stages of the non-directly
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18 245 exposed generation (F_1). Importantly, the decrease in larvae survival in both F_0 and F_1, at
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20 246 environmentally relevant MET concentrations, raises concerns on the potential effects in the
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22 247 recruitment and population fitness of the most sensitive fish species in aquatic ecosystems.
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26 249 **Table 1.** Effects of MET on survival (%) of *D. rerio* from F_0 and F_1 generations. Data expressed as mean ±
27 250 standard error. Significant differences are highlighted in bold and with asterisks (*) (p<0.05). Cardinals (#)
28 251 indicate differences with p<0.1.
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Treatment	Survival (%)					
	F0 96 hpf [a]	**F0 20 dpf [b]**	**F0 60 dpf [b]**	**F0 9 mpf [b]**	**F1 96 hpf [a]**	**F1 60 dpf [b]**
Control	95.8 ± 1.70	83.7 ± 3.10	91.0 ± 5.00	98.2 ± 1.79	83.3 ± 6.13	98.0 ± 1.00
390 ng/L	**75.6* ± 4.79**	52.4 ± 9.20#	71.0 ± 10.00	100.0 ± 0.00	76.0 ± 6.88	N.A.
2 929 ng/L	85.6# ± 3.41	**45.2 ± 12.20***	89.5 ± 3.50	95.8 ± 1.25	87.5 ± 3.80	99.0 ± 0.00
14 423 ng/L	**74.4* ± 3.93**	**24.4 ± 11.60***	87.0 ± 1.00	95.2 ± 1.07	**61.4* ± 2.78**	97.0 ± 2.00

30 31 32 33 34 35 36 37
38 252 a – Data obtained from embryogenesis studies
39 253 b – Data obtained from generational exposure study
40 254 N.A. – Data not available
41 255 Note – Data from generational exposure is presented for each stage of development:20 dpf represents the data
42 256 between 4 hpf and 20 dpf; 60 dpf represents the data between 20 dpf and 60 dpf, and 9 mpf represents the data
43 257 between 60 dpf and 9 mpf
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45 259 3.2. F_0 and F_1 growth effects
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47 260 Zebrafish growth parameters were also affected in F_0 and F_1. The F_0 zebrafish larvae (96
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49 261 hpf), exposed to 2 929 ng/L of MET, were significantly smaller than the control group (Table
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51 262 2). However, an opposite pattern in the juvenile phase (F_0 60 dpf) was detected, with an
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53 263 increase in length, weight and overall body condition factor (K) observed in the 390 ng/L
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55 264 MET treatment (Table 2). This trend continued until adulthood, where F_0 9 mpf males
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57 265 exposed to the highest MET concentration (14 423 ng/L), were significantly bigger and
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heavier than control males. Additionally, males and females exposed to 2 929 and 14 423 ng/L, respectively, displayed a significantly higher K. It should be noted that the significant increase in growth parameters observed for 60 dpf and 9 mpf zebrafish exposed to MET cannot be linked with higher food availability in exposed treatments due to the mortality observed in early phases. In fact, until 20 dpf, zebrafish larvae were fed *ad libitum* to ensure that all treatment larvae had equal food access to properly survive and grow; after 20 dpf (i.e., at 20 and 60 dpf), when the mortality was very low until adulthood, the number of fish and food were equally adjusted in all aquaria.

The growth findings here presented suggest that MET exposure impacted zebrafish differently, depending on the concentration tested, but also on the development stage of zebrafish, i.e., larvae, juvenile, and adult, with larvae exposed to MET being significantly smaller and juveniles and adults larger, heavier and with a higher K. Likewise, the studies available in literature addressing the potential impacts of MET show that the nature of the effects induced by MET on fish growth, reproduction and endocrine functions appear to differ depending on the species and developmental stage, study duration and tested concentrations. However, most of these studies are based in short partial life-cycle exposures, focus on one developmental stage (mainly early development phases) and present mixed results. For example, 28-day and 57-day MET exposures at concentrations of 1-100 µg/L and 1 µg/L have shown to induce decreased weight and length in early life stages of the Japanese medaka (*Oryzias latipes*)[18] and *S. trutta*[16], respectively. Yet, an increase in length or no effects on growth parameters were observed when embryos/larvae of fathead minnow (*P. promelas)* were exposed to similar MET concentrations for 5[27] or 21 days[26], respectively. A similar increase in length was reported for *P. promelas* larvae exposed to 31 µg/L of MET at day 30 and an increase of the condition factor after MET exposure of 322 µg/L at day 64[25].

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291 Ideally, chronic studies should test environmentally relevant concentrations of chemicals
292 and cover the entire life-cycle of the tested organism(s), in order to mimic the real case
293 scenarios of wild populations[3,22,28–30], which is what we aimed at the present study. However,
294 the studies available in the literature that assess the full life-cycle effects of environmentally
295 relevant concentrations of MET in different fish developmental stages are scarce. Beyond
296 the present research, Ussery et al. (2018)[18] and Parrott et al. (2021)[25] studies have reported
297 growth decrease in early life stages of *O. latipes* and *P. promelas* exposed to 3.2 and 31
298 µg/L of MET respectively, but no effects were visible at adulthood, after approximately 200
299 days. Additionally, Niemuth and Klapper (2015)[15] reported a decrease in weight and body
300 condition factor in adult males of *P. promelas* following a 365-day exposure to 40 µg/L of
301 MET. These findings contrast with the increased growth parameters reported in the present
302 study at the juvenile and adult stages. We cannot exclude the possibility that the Ussery et
303 al. (2018) study[18] failed to observe effects on growth of *O. latipes*, as only one concentration
304 was tested at adulthood (3.2 µg/L). On the other hand, the MET concentrations tested in the
305 studies of Parrott et al. (2021)[25] and Niemuth and klapper (2015)[15] were much higher than
306 those tested in the present study and above ecological relevance, which could justify the
307 contrasting results. We hypothesize that the stimulating effects on zebrafish growth
308 parameters (length, weight and K), observed at 60 dpf and 9 mpf, by environmentally
309 relevant concentrations of MET, could be associated with a phenomenon called hormesis.
310 Hormesis is an adaptive response well documented in aquatic organisms in response to
311 long-term exposure to low concentrations of a variety of environmental stressors[31,32], that
312 results from an overcompensation of the homeostatic regulatory mechanisms, leading to
313 stimulatory effects (e.g., improved growth and/or reproduction)[31,33,34]. This hormesis
314 phenomenon induced by MET was also suggested by Elizalde-Velazquez et al. (2021)[24] in
315 early developmental phases of zebrafish. Despite the potential hormetic effect here
316 observed in zebrafish growth and weight (F_0 60 dpf and 9 mpf males), other key ecological

endpoints revealed severe signs of toxicity, such as increased mortality at early stages of development and disruption of reproductive processes (discussed in sections 3.1 and 3.3). Growth was also affected in the F_1 generation. A significant length increase was observed in F_1 96 hpf larvae for all MET treatments, and in F_1 60 dpf juveniles for the 2 929 ng/L MET concentration (Table 2). This increase in length in the F_1 60 dpf juveniles, which was accompanied by a decrease of K in all MET treatments, shows that F_1 zebrafish were gaining less weight in proportion to their size. These results highlight the impacts of MET in F_1 early stages of development, even in the absence of direct exposure, which can potentially be linked with epigenetic modifications. In fact, our RNA-seq analysis (section 3.3.2) showed several differentially expressed genes involved in epigenetic modification in both males (e.g., *piwil2, tdrd9, prdm12b*, *znf541* and *mcm8*) and females (e.g., *dnmt1, uhrf1* and *suv39h1a*) (Supporting information – Table S8). This hypothesis is further supported by previous studies using mammalian and cancer cell lines that have shown that MET affects DNA methylation[35–37], histone modifications[35,38,39] and microRNAs[40–42]. The observations here reported are of particular concern from an environmental risk assessment perspective as MET adverse effects at environmental relevant concentrations are potentially passed to future generations. As such, future research is needed to uncover the potential molecular mechanisms underlying MET modulation of epigenetic markers and how these relate with the effects here reported.

Table 2. Effects of MET on sex ratio, weight, length, Fulton's condition factor (K) and gonadosomatic index (GSI) of *D. rerio* from F_0, F_1 generations (M, males; F, females; N.A., not-measured). Data expressed as mean ± standard error. Significant differences are highlighted in bold and with asterisks (*) (p<0.05). Cardinals (#) indicate differences with p<0.1

Generation	Sampling Time	Treatment	Sex ratio (M:F) %	Weight (mg)	Length (mm)	K	GSI
F_0	96 hpf	Control	N.A.	N.A.	3.91 ± 0.0206	N.A.	N.A.

		390 ng/L	N.A.	N.A.		3.86 ± 0.0192#		N.A.		N.A.	
		2 929 ng/L	N.A.	N.A.		**3.84 ± 0.0138***		N.A.		N.A.	
		14 423 ng/L	N.A.	N.A.		3.90 ± 0.0171		N.A.		N.A.	
	60 dpf	Control	N.A.	19.79 ± 1.963		1200.00 ± 31.000		0.80 ± 0.022		N.A.	
		390 ng/L	N.A.	**28.49 ± 4.253***		**1380.00 ± 60.000***		**0.92 ± 0.071***		N.A.	
		2 929 ng/L	N.A.	23.59 ± 1.786		1310.00 ± 35.000		0.85 ± 0.020		N.A.	
		14 423 ng/L	N.A.	22.67 ± 4.389		1300.00 ± 74.000		0.78 ± 0.023		N.A.	
				M	F	M	F	M	F	M	F
	9 mpf	Control	56.50 : 43.50 ± 5.010	510.30 ± 24.730	749.00 ± 29.510	3760.00 ± 70.000	4050.00 ± 49.000	0.94 ± 0.037	1.12 ± 0.034	0.84 ± 0.110	11.48 ± 0.867
		390 ng/L	56.00 : 44.00 ± 21.180	546.30 ± 13.230	832.50 ± 45.110	3860.00 ± 32.000	4120.00 ± 69.000	0.94 ± 0.016	1.16 ± 0.035	0.65 ± 0.085	14.17 ± 1.777
		2 929 ng/L	62.60 : 37.40 ± 1.710	551.50 ± 15.690	819.60 ± 40.250	3800.00 ± 61.000	4140.00 ± 48.000	**1.02 ± 0.033***	1.15 ± 0.032	0.83 ± 0.074	12.26 ± 1.273
		14 423 ng/L	55.90 : 44.10 ± 4.070	**609.80 ± 20.940 ***	848.90 ± 37.340	**3970.00 ± 40.000***	4070.00 ± 55.000	0.97 ± 0.035	**1.25 ± 0.037***	0.97 ± 0.103	15.59 ± 1.896
F_1	96 hpf	Control	N.A.	N.A.		3.90 ± 0.0142		N.A.		N.A.	
		390 ng/L	N.A.	N.A.		**3.99 ± 0.0160***		N.A.		N.A.	
		2 929 ng/L	N.A.	N.A.		**4.09 ± 0.0208***		N.A.		N.A.	
		14 423 ng/L	N.A.	N.A.		**4.07 ± 0.0200***		N.A.		N.A.	
	60 dpf	Control	N.A.	13.51 ± 0.812		1190.00 ± 26.000		0.73 ± 0.018		N.A.	
		2 929 ng/L	N.A.	15.25 ± 0.666		**1290.00 ±23.000***		**0.68 ± 0.013***		N.A.	
		14 423 ng/L	N.A.	13.97 ± 1.057		1230.00 ± 35.000		**0.70± 0.016***		N.A.	

3.3. F_0 Reproductive processes

In order to further disentangle the effects of MET in apical endpoints, we then focused on the potential effects of MET on zebrafish reproduction using breeding studies to assess the reproductive output and q-PCR/RNA-seq analyses to evaluate the expression of gene networks involved in endocrine processes.

3.3.1. Reproductive success

The results of the two F_0 reproductive studies are presented in Table 3. In the tank breeding approach, (tanks holding a number of males and females that replicate the treatment sex ratio), the reproductive success of F_0 4 mpf, indicates that no MET treatment affected fecundity or fertilization rates. However, this breeding approach can potentially increase data variability and may not allow sufficient statistical power to fully assess the fecundity rate, since this approach doesn't determine how many males and females contributed to eggs spawned at each reproductive event. Although both fecundity and fertilization rates weren't affected, a dose-dependent significant increase of un-activated eggs was recorded for all MET treatments compared to control. Furthermore, reproductive events with un-activated eggs were observed at all MET treatments, but not in the control group (Table 3, Supporting information - Figure S1). In zebrafish, eggs are activated once they come in contact with water[43]. The egg activation process is characterized by the expansion and hardening of the chorion, which is essential for egg fertilization and development, as it prevents polyspermy and creates a protective barrier for the developing embryo[43,44].

In order to clarify if the presence of un-activated eggs was due to reproductive disruption on males or females exposed to MET, a second reproductive study was performed in the F_0 4.25 mpf zebrafish, using a pairwise cross-breeding approach (one male X one female). This second reproductive study was conducted between fish from the control and from the highest MET concentration (14 423 ng/L), using three different combinations, i.e., males and females from control ($♂_{Ctrl}$ X $♀_{Ctrl}$) or males/females from the 14 423 ng/L MET concentration with control animals ($♂_{14\ 423\ MET}$ X $♀_{Ctrl}$ and $♂_{Ctrl}$ X $♀_{14\ 423\ MET}$) (Table 3). Interestingly, the results show a significant decrease in the fertilization rate ($p<0.05$), a tendency for lower fecundity ($p=0.08$) and the presence of un-activated eggs only in the breeding pairs with exposed males (Table 3). These findings indicate that the reproductive capability of males

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6 374 was particularly affected by MET, and that pairwise breading is a better approach than tank
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8 375 breeding to assess zebrafish reproductive output, as it yields better statistical power.
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10 376 Although short-term reproductive studies with adult *P. promelas* exposed in pairs failed to
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12 377 show MET disruption in fish reproduction[45,46], these studies may not represent the full effects
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14 378 of environmental exposure, since the critical period of sexual differentiation was not included
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16 379 in the exposure. Despite the short-term reproductive studies referenced above, the
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18 380 assumption that pairwise breading is a better alternative to the tank breeding approach to
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20 381 assess MET effects in fish reproductive success is largely in agreement with the two full life-
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22 382 cycle studies that addressed the reproductive output of *P. promelas* exposed to MET[15,25].
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24 383 Even though Parrot et al. (2021)[25] failed to find MET adverse effects in the reproductive
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26 384 success of *P. promelas* using a method similar to our tank breading approach, Niemuth and
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28 385 Klapper (2015)[15] observed decreased fecundity in MET exposed fish with a pairwise
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30 386 breeding approach. Thus, based on the available full life-cycle data (ours and Niemuth and
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32 387 Klapper (2015)[15]), MET appears to affect the reproductive output of model fish.
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35 388 Most available studies, in different *taxa*, largely associate reproductive output alterations
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37 389 induced by contaminants with maternal transmission[44]. However, in the case of MET, our
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39 390 findings show that male exposure history negatively impacts reproductive performance of
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41 391 zebrafish. Previous studies in zebrafish have described that male sex pheromones are
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43 392 essential for a successful reproduction due to its role in female attraction, triggering ovulation
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45 393 and oviposition[47–49]. Moreover, it is also known that male sex pheromones influence the
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47 394 quality and viability of embryos[48]. The absence of male pheromones at the time of
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49 395 reproduction results in egg spawning lacking chorion expansion, i.e., un-activated eggs[48]. A
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51 396 significant part of male pheromones are glucuronidated conjugates of steroid sex
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53 397 hormones[50], which have been shown to be modulated by MET, as part of its putative
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55 398 MoA[1,4,51,52] (this mechanism is further discussed in section 3.3.2 based on the qRT-PCR and
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57 399 RNA-seq data obtained in the present study).
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8 401 **Table 3.** Chronic effects of MET on F_0 zebrafish tank breeding and pairwise cross-breeding (Comparison
9 402 between control and MET 14 423 ng/L). Data on fecundity (number of embryos/female/day), % of fertilization (%
10 403 of fertilized eggs/female/day), % reproductive events with un-activated eggs, and % of un-activated eggs (n=10
11 404 reproductive events of *D. rerio* at 4 mpf). Data expressed as mean ± standard error. Significant differences are
12 405 highlighted in bold and asterisks (*) (p<0.05). Cardinals (#) indicate p<0.1.

		Fecundity	% Fertilization	% of reproductive events with un-activated eggs	% un-activated eggs/reproductive event
Tank breeding	**Control**	45.1 ± 3.80	93.3 ± 0.90	0.0 ± 0.00	0.0 ± 0.00
	MET = 390 ng/L	52.2 ± 4.40	92.0 ± 1.30	**29.0 ± 7.00***	**2.5 ± 1.24***
	MET = 2 929 ng/L	68.1 ± 3.30	96.7 ± 0.40	**56.0 ± 13.00***	**3.5 ± 0.97***
	MET = 14 423 ng/L	75.0 ± 10.80	89.8 ± 1.40	**73.0 ± 14.00***	**7.4 ± 3.62***
Pairwise cross-breeding	♂Ctrl X ♀Ctrl	286.4 ± 22,70	98.1 ± 0,70	0.0 ± 0.00	0.0 ± 0.00
	♂14 423 X ♀Ctrl	198.8 ± 62.90#	**88.5 ± 4.10***	**56.0 ± 5.77***	**22,6 ± 5,33***
	♂Ctrl X ♀14 423	226.9 ± 39.70	97.2 ± 0.80	0.0 ± 0.00	0.0 ± 0.00

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30 408 3.3.2. qRT-PCR and RNA-seq

409 Considering the findings obtained in the reproductive assays, that point to reproductive
410 changes linked to males, and previous studies indicating that MET can potentially act as an
411 endocrine-disrupting chemical in fish by increasing expression of estrogen associated
412 vitellogenin (VTG) encoding genes in males[15,17,53], we further evaluated the effects of MET
413 at the molecular level. First, a strong focus was given to a liver q-PCR screening of
414 expression of the *vtg1* and *vtg2* genes for all treatments. We found that, in males, *vtg1* was
415 particularly altered with a significant increase in all MET treatments (fold inductions from
416 99.78 for 390 ng/L MET to 155.47 for 14 423 ng/L MET) and *vtg2* mRNA levels were also
417 significantly upregulated by 8.28 and 8.80-fold, in 2 929 ng/L and 14 423 ng/L MET
418 treatments, respectively (Figure 2.B). In females, *vtg1* showed a significant upregulation with
419 a low fold induction of 1.8 for the lowest MET concentration tested (390 ng/L) and *vtg2* was
420 significantly downregulated by 2.09-fold for 14 423 ng/L MET treatment (Figure 2.A).

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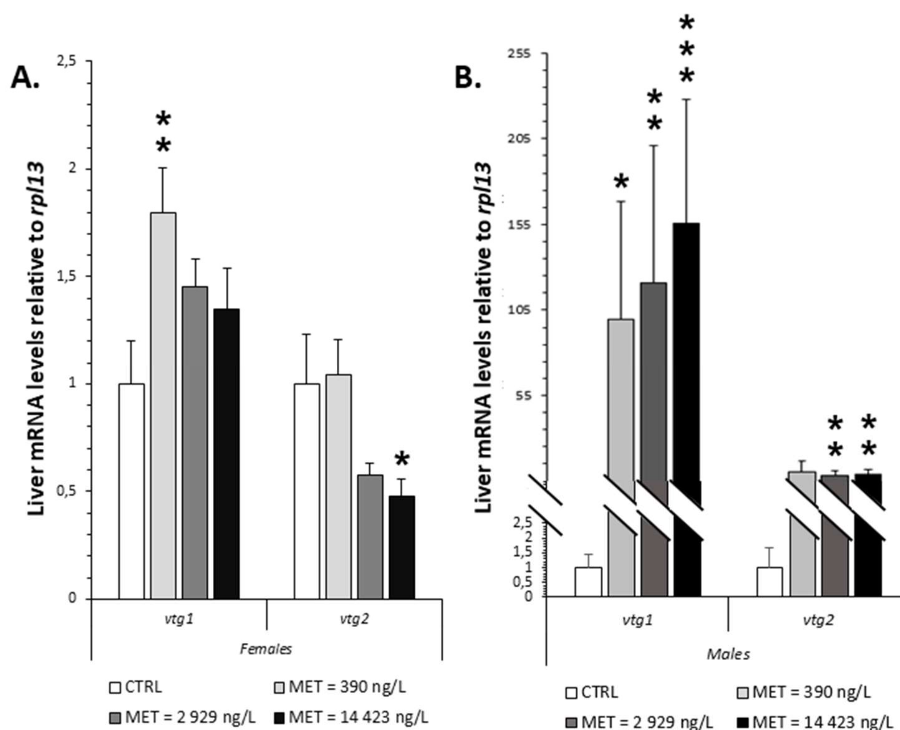

Figure 2. F_0 *D. rerio* liver mRNA levels of *vtg1* and *vtg2* relative to *rpl13*, after 9-months exposure to MET in both females (A) and males (B). Error bars indicate standard errors; asterisks indicate significant differences from de control group (1-fold) (*- p<0.05; **- p<0.01; ***- p<0.001) (n=6-8).

As VTG is typically synthesized as an egg yolk precursor[54,55], high levels of VTG or mRNA levels of its encoding genes (*vtg*) in males have been used over the years as a marker of endocrine disruption associated with estrogenic compounds [20,56,57]. Of concern is the 99-fold increase of *vtg1* here induced by 390 ng/L of MET in males' liver, more than double when compared to a 40-fold induction in male zebrafish exposed to 2 ng/L of ethinylestradiol, a well-known and potent estrogenic endocrine disrupting compound[20]. This 99-fold induction points to a severe feminizing effect of MET at concentrations as low as 390 ng/L. Previous studies have reported that MET exposure was able to modulate the expression of male *vtg* genes in other fish species, although at higher concentrations and much lower fold induction[17,46,58]. Since MET is commonly detected in surface waters worldwide in concentrations at the same range of those tested in this study or even higher[8,9,59–61], this

data suggests that freshwater ecosystems are facing massive chemical loads with the capability to trigger estrogenic responses, despite global efforts to decrease the estrogenicity of river waters[62–64]. Nonetheless, the MoA by which MET can upregulate *vtg1 and vtg2* gene expression is still unclear. Since MET does not have a hormone-like structure, a direct disruption of estrogen receptors (ERs) is therefore unlikely. Furthermore, the interaction of MET with transcription factors, such as retinoic acid receptor (*RAR*) or peroxisome proliferator-activated receptor gamma (*PPARg*), previously indicated to bind to retinoic acid responsive elements (RARE) in the promoter region of *vtg1* and *vtg2* for regulate their gene expression[55], are also unlikely. The RNA-seq analysis performed here and discussed below, showed no changes in the expression levels of *pparg* and *rar* genes nor in the downstream pathways regulated by these nuclear receptors (Supporting information – Table S3). Instead, it is hypothesized that MET may regulate VTG synthesis by modulating steroid biosynthesis[46,58,59,65], although future studies should demonstrate the detailed mechanism associated with VGTs induction.

To provide additional insight into the MoA of MET, we looked more closely for gene networks related with energy/lipid metabolism signaling, steroid hormone biosynthesis and reproductive processes, that can potentially be involved in the proposed estrogenic activity of MET in males. Thus, a comprehensive transcriptome analysis with biological replication was produced for liver samples of males and females from the control group and from the 14 423 ng/L MET treatment (Supporting information – Table S3 and S4) to strengthen the data of our previous exploratory RNA-seq analysis[3]. Importantly, the hierarchical clustering heatmap (Supporting Information – Figure S3) reported that exposed males presented a pattern of gene expression closer to the females (MET exposed and control) than to the control males. Furthermore, the RNA-seq findings showed multiple metabolic processes significant disrupted in males. A total of 121 genes were found to be differentially expressed, with 61 downregulated (e.g., *nme9*, *insl3, insl5b gpat2* – energetic metabolism and *fabp6,*

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lrp2b – lipid metabolism) and 60 upregulated (e.g., *cox5ab*, *pkmb* – energetic metabolism and *nr2f5, ebpl, ckmb, hadhaa* – lipid metabolism) (Supporting information – Table S5), which correlates well with the metabolic changes observed in our parallel study, both at molecular and biochemical levels[3]. Moreover, 3 steroidogenic genes, essential for the production of sex hormones, were found to be downregulated (*cyp11c1, cyp17a1* and *star*) and one upregulated (*ugt2a7)* (Supporting information – Table S5). The down regulation of *star*, a key rate-limiting enzyme of steroid hormones synthesis, together with *cyp11c1, cyp17a1*, could indicate a disruption of the homeostatic regulation of steroids[66]. These findings are also in agreement with the significant decrease in cholesterol levels in the livers of MET exposed males reported in our parallel study[3], as cholesterol is the sole precursor of steroid hormones[67,68], and is also associated with the upstream deregulation of genes coding energy/lipid metabolism processes. Finally, we noted 49 downregulated genes linked to reproductive processes, most of them involved in gametogenesis/sex differentiation (e.g., *amh, ddx4, dmrt1, piwil1* and *spata4*) and sperm motility (e.g., *dnaah1, dnai1.1, dnaaf2* and *spef1*) (Supporting information – Table S5). Although these reproductive genes are often associated with gonads, they also express in the liver of control animals, albeit with lower transcript levels[69–73]. Interestingly, although our reproductive trials did not find any effects associated with females, a large number of genes associated with energy/lipid metabolism signaling, steroid hormone biosynthesis and reproductive processes were also observed to have a differential gene expression between control and MET exposed females. This suggests that several females signaling pathways could also be disrupted by MET (Supporting information – Table S6).

Focusing more broadly on Protein-Protein Interaction (PPI) and in the Gene ontology (GO) enrichment analyses (Supplementary information - Section S8, Figure S3 and Table S7), several categories of gene networks were identified in males exposed to MET, showing

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further evidence that MET disrupts zebrafish reproduction by dysregulating its lipid/energy metabolism, in turn affecting steroid hormone biosynthesis pathways.

Taken together the findings of the present study with those of our parallel study[3], MET appears to deregulate critical biochemical and molecular processes in male zebrafish metabolism, which in turn could affects the steroid hormone biosynthesis and the expression of *vtg1* and *vtg2*. We hypothesize that this disruption can be linked with the significantly decreased in the fertilization rate and the non-activated eggs reported in the pairwise cross-breeding study.

3.4. Final Remarks and Implications for Hazard and Risk Assessment

According to the available data, the impact of MET in non-target organisms is not fully consistent among studies regarding the effects of MET on fish growth, reproduction and/or endocrine functions. These differences can reflect the limited number of studies performed so far and the use of distinct experimental designs, i.e., different MET concentrations evaluated, different test species and study duration. Apart from the present work, there is a paucity of data assessing long-term effects of environmentally relevant concentrations of MET in different time-points during fish life cycle. Most of the studies available tested MET concentrations above those reported in surface waters, and/or fish were exposed for short time-periods, covering only one stage of the life cycle[25,27,53,74].

In fact, there are still significant gaps in our understanding of the ecological impacts of MET in fish. However, our findings, together with previous studies, provide strong evidence that current MET concentrations in aquatic environments may be sufficient to disrupt survival, growth, reproduction and metabolic/endocrine functions of fish. Putting together the multiple lines of evidence addressed in the present study, MET, at environmentally relevant concentrations, impacted several biological processes in zebrafish. A concentration as low

as 390 ng/L of MET was able to affect early life stages of zebrafish in F_0 and F_1 generations, disrupt growth throughout its life cycle, impair reproductive output and massively induce *vtg1* gene expression in males. Furthermore, the transcriptomic responses observed in 9 mpf male zebrafish exposed to 14 423 ng/L of MET add to the growing body of evidence reported in literature that MET acts as an endocrine disrupting compound[15,17,18,46,53,75], by disrupting genes and pathways associated with metabolic processes, steroidogenesis and reproductive functions.

As indicated by the Fent (2006) and Wilkinson et al. (2022) studies[11,76], average MET concentrations in surface waters strongly increased from 2006 (100 ng/L) to 2022 (2 362.9 ng/L), with a tendency to further increases due to aging of the global population. Therefore, these findings suggest that MET should be considered a top priority chemical to be integrated in toxicity testing and hazard assessment strategies. In fact, MET was just recently integrated in the proposed 4th Watch List under the Water Framework Directive (WFD)[12] and in the Implementing decision (EU) 2022/1307 (http://EUR-Lex - 32022D1307 - EN - EUR-Lex (europa.eu)), which reflects the urgent need to further address the risk of this pharmaceutical to aquatic life. However, according to the proposed 4th Watch List the current Predicted No-Effect Concentration (PNEC) for MET is 1 030 µg/L, with a proposed environmental quality standard (EQS) of 160 µg/L[12] and the decision (EU) 2022/1307 settled a maximum acceptable quantification limit for MET at 156 µg/L. Thus, it is suggested that the current PNEC/EQS proposal for MET should be urgently reviewed, as the findings reported here, and in other previous studies, show that MET concentrations inducing adverse effects are several orders of magnitude lower than the proposed PNEC/EQS values in the EU 4th Watch List of WFD.

4. Associated content

- Additional experimental methods and results (PDF).

- List of differentially expressed genes (DEGs) and Gene Ontology (GO) terms involved in lipid/energy metabolism, steroid hormone biosynthesis and reproductive processes (XLSX).

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Supporting information

Are fish populations at risk? Metformin disrupts zebrafish development and reproductive processes at chronic environmentally relevant concentrations

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Material and methods

Section S1. Ethical review

The bioassay was carried out at *Biotério de Organismos Aquáticos* (BOGA) facilities, at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Matosinhos, Portugal) in compliance with the European Directive 2010/63/EU, on the protection of animals used for scientific purposes and with the Portuguese Decreto-Lei 113/2013. The research project has received approval for research ethics from Animal Welfare and Ethics body (ORBEA – CIIMAR).

Section S2. F_0 reproductive trials after 4 months of MET exposure

First, reproductive success was tested at 4 mpf using a tank breeding approach (tanks holding a number of males and females that replicate the treatment sex ratio). In the afternoon, before the beginning of the trials, aquaria from each treatment were divided in two separate sections in which a suspended cage with a net bottom covered with marbles was fixed; males and females were distributed through the cages (four reproductive units per treatment, n=4). A total of 12 reproductive events were performed for each treatment. Over the course of 6 days, 1-1.5 h after the beginning of the light period, breeding fish were removed and the eggs collected, cleaned, and conserved in 70% ethanol for subsequent counting. Reproductive success was assessed through the evaluation of two endpoints: fecundity (number of eggs per female per day) and percentage of fertilization (% of fertilized eggs per female per day). Unfertilized eggs were grouped with non-viable and un-activated eggs to determine the fertilization rate.

Furthermore, in order to determine whether the potential changes in reproductive success were due to paternal or maternal exposure to MET, a second reproductive study was conducted one week after the first reproductive trial, i.e. 4.25 mpf, using a pairwise cross breading approach

(one male X one female) with fish from the highest MET concentration (13 000 ng/L nominal concentration) and control. Ten different couples per combination were used for this reproductive study (n=10). Briefly, zebrafish couples were placed in breeding chambers with net bottom covered with marbles, inside aquaria filled with clean dechlorinated water. Three combinations of couples were made: (1) both male and female from the control group; and two crossed reproductions, (2) a MET exposed male with a control female; and (3) a MET exposed female with a control male. Once embryos were collected, fecundity and fertilization rate were analyzed as described above.

Section S3. Embryogenesis studies in F_0 and F_1 generations

After reproduction, newly fertilized eggs were collected and cleaned in order to initiate the F_0 and F_1 96 hpf embryo assays, that were replicated four independent times. Briefly, 20 embryos from each treatment, with approximately 1.5 hpf, were randomly distributed in 24-well plates (one egg per well), filled with 2 mL of exposure medium, i.e., 390 to 14 423 ng/L of MET or dechlorinated water (control) in F_0 96 hpf embryo assays; and MET-free dechlorinated water in F_1 96 hpf embryo assay. The 24-well plates were randomly placed in an incubator and maintained at 28 ± 0.5 °C with a 14:10 (light:dark) photoperiod for 96 h, to mimic the chronic assay development conditions. Every 24 h, embryo mortality was checked, and the exposure medium renewed. At the end of each assay (96h), embryos were checked for morphological abnormalities on the head, tail, eyes and yolk-sac, as well as screened for oedemas, abnormal cell growth, and developmental arrest (which was only compared to the control group, as the assay temperature did not match the one from the FET Test 236[1]).

Additionally, total larvae length of the F_0 and F_1 generations (n = 40, 10 larvae per replicate) was measured at 96 hpf under an inverted microscope (Nikon Eclipse TS100), coupled with a digital camera Nikon D5 – Fi2.

Section S4. Analytical quantification of MET

Actual MET concentrations were determined three times during the experiment, to assure that concentrations were within the expected range. Samples were collected (1) one week after the beginning of exposure, (2) in the middle of the study, i.e., 4 mpf, and (3) one week before the end. At each time-point, two bulk samples from each treatment replicates were collected and stored at -20 °C until quantification.

Metformin determination in water samples was performed by Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS) with an Acquity UPLC® liquid chromatography system from Waters (Milford, MA, USA). A sample volume of 45 µL was directly injected into an Acclaim Trinity P1 column (50 mm × 2.1 mm, 3 µm particle size) supplied by Thermo Fisher Scientific (Waltham, MA, USA) maintained at a constant temperature of 35 °C and using a 0.2 mL/min flow rate. The mobile phases consisted of (A) ultrapure water with 2% of acetonitrile and 2mM ammonium acetate at pH 5.5 and (B) acetonitrile with 20% of ultrapure water and 20 mM ammonium acetate at pH 5.5. The gradient was applied as follows: 0–2 min, 0% B; 2–10 min, linear gradient to 100% B; 10–15 min, 100% B and finally 15–20 min, 0% B [2]. The system was interfaced to a XEVO TQD® triple quadrupole mass spectrometer equipped with an electrospray interface (ESI). Nitrogen was used as a nebulizing and drying gas and Argon was used as collision gas. Metformin was determined in the electrospray positive polarity and multiple-reaction monitoring (MRM) mode of acquisition. Two MRM transitions were used: m/z 130 to m/z 60 as quantification transition and m/z 130 to m/z 71 as qualification transition. Under these chromatographic conditions, the retention time of metformin was 8.1 minutes. The method assured quantification limits (LOQ) of 10 ng/L. Quantification was performed by matrix matched calibration using standards prepared in dechlorinated system water in the 0.01-200 ng/mL range (which was checked to be linear, R^2=0.9992). Repeatability of the determination was checked in

terms of relative standard deviation (RSD) at 0.5, 5 and 50 ng/mL levels and the values were lower than 10% in all cases.

Table S1 summarizes the actual concentrations of MET measured throughout the assay in each treatment at the three sampling points. MET was never detected in the control group. In most cases, the detected MET concentrations were higher than nominal ones, except for T3 13 000 ng/L treatment where the MET level decreased. Due to technical constraints, the water samples collected at the third sampling point from the 361 ng/L MET were lost and the actual concentration of MET could not be determined. The concentration variations were slightly out of the expected range, presenting an average deviation of 28.8 % from the nominal concentrations. Since MET is not metabolized in vertebrates, being released in its native form, it is possible that the variation in concentrations here observed is a result of MET accumulation in the aquaria throughout the 9-month exposure assay.

Table S1. Nominal and measured concentrations of MET in water samples collected through the duration of the bioassay, from each treatment. Data is expressed as mean ± standard error (n = 2 replicates per sampling time). T_1 – first sampling time; T_2 – second sampling time; T_3 – third sampling time.

	Control	MET 361 ng/L[a]	MET 2 166 ng/L[a]	MET 13 000 ng/L[a]
T_1	N.D.[b]	437.5 ± 62.90	2 410.5 ± 18.10	18 796.7 ± 186.00
T_2	N.D.[b]	342.5 ± 19.00	2 767.0 ± 368.40	15 757.0 ± 1 182.40
T_3	N.D.[b]	N.A.[c]	3 610.6 ± 1 256.30	8 718.0 ± 1 747.60

[a] – nominal concentrations; [b] – not detected; [c] – not analyzed

Section S5. qRT-PCR primer design

Specific primers for qRT-PCR gene expression analysis of *vtg1, vtg2* and *rpl13* were designed using the Primer designing tool "Primer – BLAST" (NCBI) and Beacon Designer (Premier Biosoft International). To confirm the specificity of each primer, amplification products were run by 1% agarose gel electrophoresis and resulting bands were purified with GelPure (NzyTech), following

the manufacturers' protocol. Purified bands were then sequenced at GATC (Eurofins Genomics) and sequencing results were analyzed with the BLAST tool from NCBI in order to confirm the amplified sequence (Table S2).

Table S2. Primer sequences, forward (F) and reverse (R), and parameters used in the qRT-PCR for gene expression quantification in the liver (*vtg1* and *vtg2*) of 9-month *D. rerio* form control and MET treatments

Gene	Accession number	Sequence (5' – 3')	Expected band size (bp)	Combined annealing and extension temperature (°C)	Average efficiency (%)
vtg1	NM_001044897.3	F: CGCTGTTCCCATCAATCCAG R: TTGCAGTACAGCAGTGGTCTAA	183	60	97
vtg2	NM_001044913.1	F: TACTCCTCCGTTGTTGGTGC R: AGCTTGGATGAAGGCAGTGT	192	58	95
rpl13	NM_198143.1	F: GCTGAAGGAATACCGCACCA R: TCCAGTAAGCTGTGTTGCCAT	109	60	98

Section S6. Fluorescence based quantitative real-time PCR (qRT-PCR) analysis

For each treatment, cDNA was synthesized from 1 µg of extracted RNA using the iScript™cDNA Synthesis Kit (Bio-Rad). Each cDNA sample (n=8 per treatment and sex) was diluted by 1/5-fold and amplified with NZYSpeedy qPCR Green Master Mix (2x) (NZYTech) on a Mastercycler ep realplex system (Eppendorf), following the manufacturer instructions. A two-step qRT-PCR was performed as follows: initial denaturation at 95 °C (2 min), followed by 40 cycles of denaturation at 95 °C (15 s) and combined annealing and extension at 58 – 62 °C, depending on primer set (25 s) (Table S2). A melting curve was generated in each run to confirm the specificity of the reactions. Relative changes in the transcription abundance of target genes were normalized to the housekeeping gene (*rpl13*) expression and calculated using the $2^{-\Delta\Delta Ct}$ analysis method [3].

Section S7. Liver RNA-seq analysis

In the RNA-seq analysis commercially obtained at Novogene (United Kingdom), RNA samples were subjected to a primary quality control. Integrity of test samples was evaluated in an Agilent 2100 Bioanalizer system using the RNA Nano 6000 Assay Kit (Agilent Technologies, USA). Samples were shown to have an average RIN (RNA integrity number) of 9.8 and, as such, were used for library construction and sequencing.

Sequencing libraries were generated using the Novogene NGS RNA Library Prep Set Kit according to the manufacturer protocol. Briefly, poly-T oligo attached magnetic beads were used to isolate mRNA from total RNA, which was used as initial input material, and rRNA was removed using the Ribo-Zero Kit (Illumina, USA). mRNA was then fragmented with divalent cations under elevated temperatures in First Strand Synthesis Reaction Buffer (5x).. The first strand of cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-), followed by second-strand synthesis using RNase H and DNA polymerase I. Remaining overhangs were then converted into blunt ends using polymerase/exomerase activities. After adenylation of 3' ends of DNA fragments, adaptors with hairpin loop structure were ligated to prepare for hybridization. Second strands were enzymatically removed and resulting cDNA fragments were purified using the AMPure XP system (Beckman Coulter, USA) to select fragments of approximately 370~420 bp in length. Finally, a PCR was performed using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, USA), universal PCR primers and Index primers were used to attribute sequences to each sample. PCR products were then purified (AMPure XP system) and the cDNA library quality was checked with Qubit 2.0 fluorometer (Termo Fisher Scientific, USA) and real-time PCR for quantification and Agilent Bioanalyzer 2100 system for quality assessment.

Index coded samples were clustered with a cBot Cluster Generation System, using TruSeq PE Cluster Kit V3-cBot-HS (Illumina, USA) according to manufacturer instructions. After clustering,

resulting libraries were sequenced on an Illumina Novaseq 6000 platform, generating 150 bp paired-end reads (an average 35 million pair-end reads per sample). Raw reads were submitted to NCBI SRA database under the BioProject number PRJNA906165.

Raw reads obtained from sequencing were processed through in house pearl scripts (Novogene, UK). Through this process, clean reads were obtained by removing sequences containing adapters and/or poly-N above 10 % and low quality reads, i.e. reads with Qscore of over 50% bases below a score of 5, were also removed from raw data. GC content, Q20 and Q30 of clean data were also calculated. Further analyses were performed on clean and high quality data.

Index of *D. rerio* reference genome (GRCz11) was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using Hisat2. Quantification of reads mapped to each gene was performed using featureCounts v1.5.0-p3 and FPKM (Fragments per kilo base of transcript per million mapped fragments) of each gene was calculated based on gene length and the amount of reads mapped to that gene.

Differential expression analysis was carried out using DESeq2 R package v1.20.0 [4]. The Benjamini & Hotchberg method [5] was used to correct resulting p-values, in order to eliminate the False Discovery Rate (FDR). Differentially expressed genes (DEGs) were set at Log_2(FoldChange) > 0 and padj (FDR p-value) < 0.05 and can be observed in Tables S3 and S4. Gene Ontology (GO) enrichment analysis of DEGs was determined using ShinyGO v0.76.3 [6]. GO terms with padj < 0.1 were considered significantly enriched by DEGs (Tables S7 and S8).

Hepatic differently expressed genes involved in steroid hormone biosynthesis, reproductive processes and energy/lipid metabolism were selected from the overall data and a protein-protein interaction (PPI) analysis was performed with Cytoscape (v3.9.1) with the String plugin attached. As the analysis showed differential expression of genes typically associated with other tissues, their expression in liver tissues was confirmed through GeneCards (www.genecards.org) and ZFIN (https://zfin.org) platforms.

Results

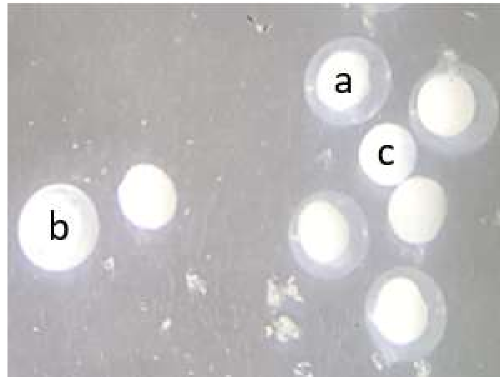

Figure S1. Zebrafish 2 hpf eggs preserved in 75 % ethanol - 4× magnification. A – fertilized egg; b – unfertilized egg; c – un-activated egg.

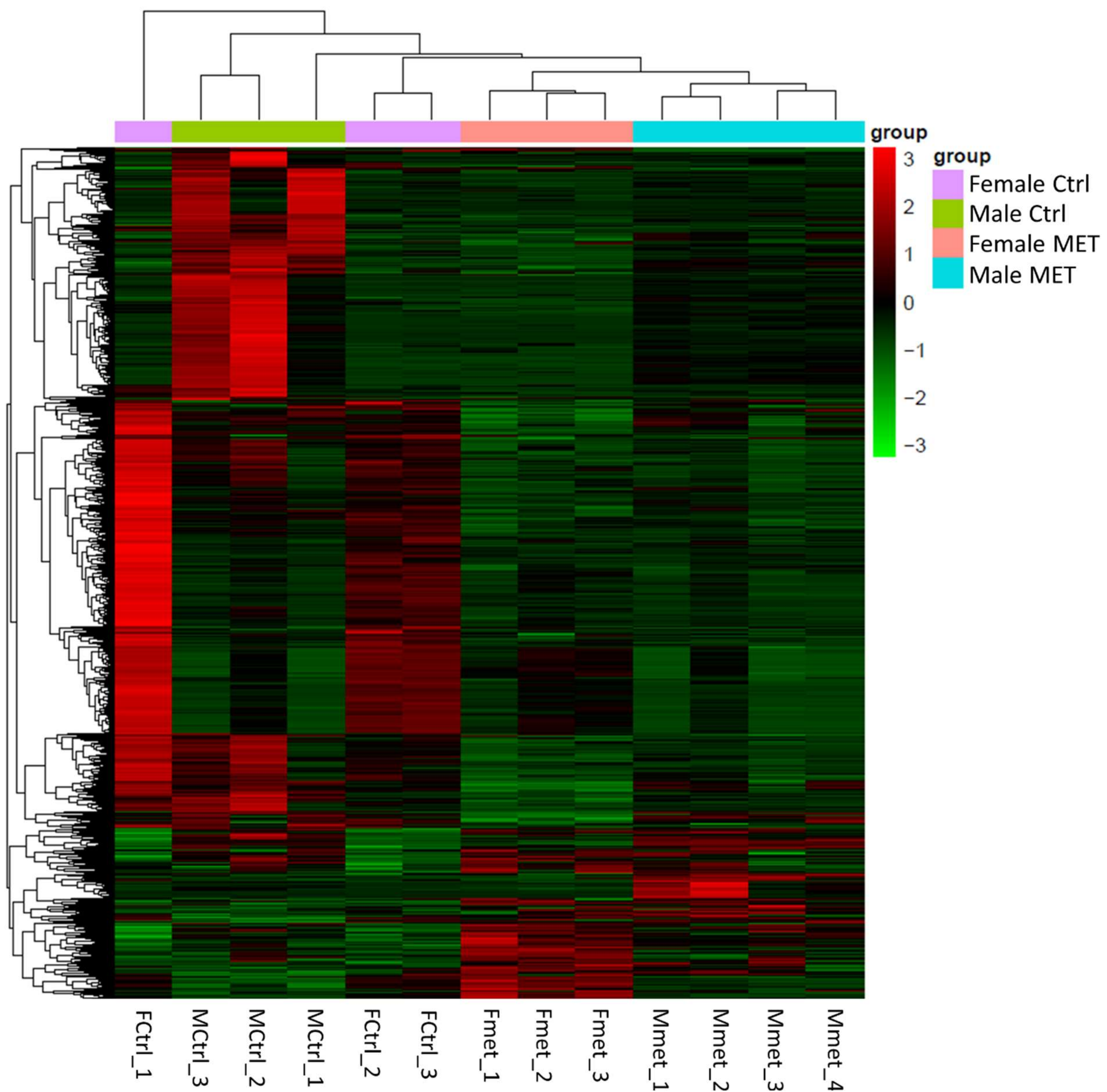

Figure S2. Hierarchical clustering heatmap depicting the patterns of gene transcription between pool samples of F_0 control and 14 423 ng/L MET treatment, in male and female *D. rerio* after a 9-month exposure to MET. FCtrl – Female control; Fmet – 14 423 ng/L MET exposed Female; MCtrl – Male control; Mmet - 14 423 ng/L MET exposed Male.

Section S8. Male Gene Ontology (GO) and Protein-Protein Interaction

The Gene Ontology (GO) enrichment analysis revealed a total of 14 biological processes (BP) significantly enriched (padj<0.1) and upregulated, all involved in energy/lipid metabolism (Table S7). Of those, the ones with bigger statistical differences were "Fatty acid b-oxidation" and "Carboxylic acid metabolic process", both expected as part of MET MoA. 15 BP were also found to be significantly enriched and downregulated. Of those, 2 are involved in steroid hormone biosynthesis, i.e. "Steroid hormone biosynthesis" and "C21-steroid hormone metabolic process", while the remaining 13 are involved in reproductive processes (Table S7).

The Protein-Protein Interaction (PPI) analysis performed for males, several interesting network of interaction between genes involved in energy/lipid metabolism, steroid hormone biosynthesis and reproduction were identified (Figure S3). The analysis revealed that energy metabolism and reproduction interact through several genes, such as *ak7a*, *ak7b dnah1* and *cfap43.* As expected, steroid hormone biosynthesis related genes (*cyp11c1, cyp17a1* and *star*) were shown to have strong interactions with *amh, star* and *dmrt1*, i.e., genes involved in reproduction, more specifically spermatogenesis.

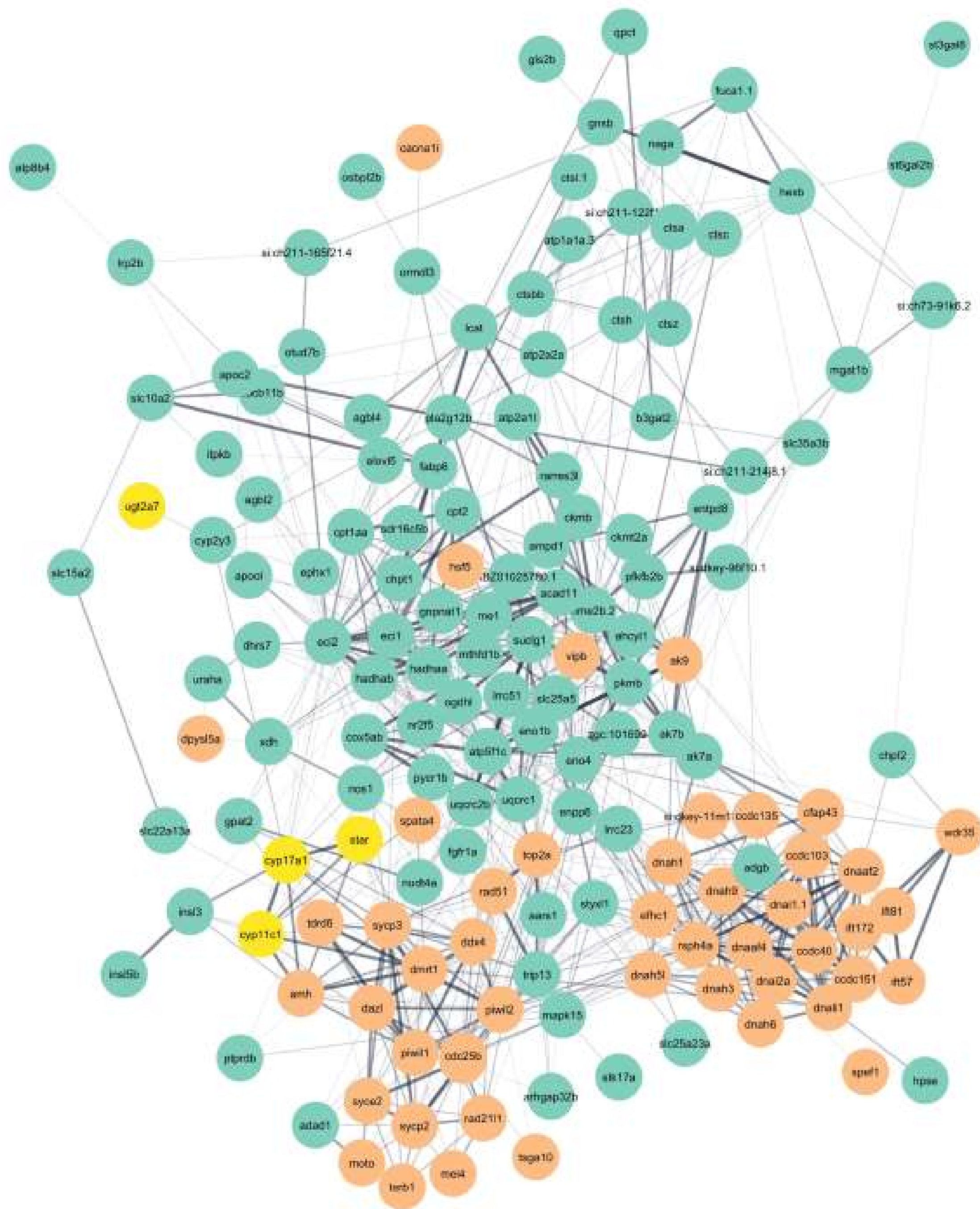

Figure S3. Protein-protein Interaction (PPI) of DEGs involved in steroid hormone biosynthesis (yellow), reproduction (orange) and lipid/energy metabolism (green) in the liver of F_0 *D. rerio* males exposed to 14 423 ng/L of MET. Thicker lines indicate stronger interactions (power from 0.7 to 1).

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