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Refinement of an OECD test guideline for evaluating the effects of endocrine disrupting chemicals on aromatase gene expression and reproduction using novel transgenic cyp19a1a-eGFP zebrafish

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Abstract :

Transgenic fish are powerful models that can provide mechanistic information regarding the endocrine activity of test chemicals. In this study, our objective was to use a newly developed transgenic zebrafish line expressing eGFP under the control of the *cyp19a1a* promoter in the OECD Fish Short Term Reproduction Assay (TG 229) to provide additional mechanistic information on tested substances. For this purpose, we exposed adult transgenic zebrafish to a reference substance of the TG 229, i.e. prochloraz (PCZ; 1.7, 17.2 and 172.6 µg/L). In addition to "classical" endpoints used in the TG 229 (reproductive outputs, vitellogenin), the fluorescence intensity of the ovaries was monitored at 4 different times of exposure using *in vivo* imaging. Our data revealed that 172.6 µg/L PCZ significantly decreased the number of eggs laid per female per day and the concentrations of vitellogenin in females, reflecting the decreasing E2 synthesis due to the inhibition of the ovarian aromatase activities. At 7 and 14 days, GFP intensities in ovaries were similar over the treatment groups but significantly increased after 21 days at 17.2 and 172.6 µg/L. A similar profile was observed for the endogenous *cyp19a1a* expression measured by qPCR thereby confirming the reliability of the GFP measurement for assessing aromatase gene expression. The overexpression of the *cyp19a1a* gene likely reflects a compensatory response to the inhibitory action of PCZ on aromatase enzymatic activities. Overall, this study illustrates the feasibility of using the *cyp19a1a-eGFP* transgenic line for assessing the effect of PCZ in an OECD test guideline while providing complementary information on the time- and concentration-dependent effects of the compound, without disturbing reproduction of fish. The acquisition of this additional mechanistic information on a key target gene through *in vivo* fluorescence imaging of the ovaries was realized without increasing the number of individuals.

Keywords: Transgenic zebrafish, aromatase, reproduction, prochloraz, endocrine disruptors, OECD TG 229

1. Introduction

Over the last two decades, the scientific community has been interested in the impact of chemicals called endocrine disruptors (EDs). They have been defined as "exogenous substances or mixture that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism or its progeny or (sub)population" (WHO, 2002). These EDs are of different origins and among them are natural and synthetic hormones, plant components, pesticides, compounds used in plastic industry and in consumer products, as well as industrial by-products (Lavado *et al.* 2004). Due to contaminated effluents, agricultural and urban soil runoff, the aquatic environment is considered to be the final sink for most of these EDs. Therefore, aquatic organisms appear particularly exposed to the potential adverse effects caused by EDs. Indeed, several studies have shown that exposure of wild aquatic organisms to EDs, especially fish, are associated with reproductive adverse effects at both individual and population levels in a variety of species (Jobling *et al.* 2002, Noaksson *et al.* 2003, Nash *et al.* 2004, Kidd *et al.* 2007, 2014, Sanchez *et al.* 2011,).

Most studies on EDs were dedicated to the (xeno-)estrogens, compounds able to interfere with the estrogenic pathway. Among these compounds, increasing attention is paid to those disturbing steroidogenesis, the process by which hormones, *i.e.* estrogens, androgens, and progestins, are produced. One of the key steroidogenic enzymes is cytochrome P450 aromatase, which catalyzes the irreversible conversion of androgens into estrogens (Kazeto *et al.* 2001, Lau *et al.* 2016, Tang *et al.* 2017). Aromatase is encoded by the *cyp19* gene. Apart from eel, most of the teleost fish studied so far possess two aromatase genes, *i.e. cyp19a1a* and *cyp19a1b*, due to a duplication event in the actinopterygian lineage (Tchoudakova and Callard 1998, Tong and Chung 2003, Blazquez and Piferrer 2004, Zhang *et al.* 2014, Chaube *et al.* 2015, Roy Moulik *et al.* 2016). The *cyp19a1a* gene, also known as "gonadal aromatase", is mainly expressed in the differentiating gonad and later in the ovaries and plays a key role in sex differentiation and reproduction in fish (Guiguen *et al.* 2010). The *cyp19a1b* gene, also called "brain aromatase", is mainly expressed in the radial glial cells of

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the brain. Locally produced estrogens may be involved in sexual behaviors and neurogenesis (Diotel *et al.* 2016, 2018, Pellegrini *et al.* 2016). Fish aromatases are known targets of EDs and both their enzymatic activities and their genes expressions can be disrupted (Monod *et al.* 1993, Fenske and Segner 2004, Kazeto *et al.* 2004, Hinfray *et al.* 2006, Wang *et al.* 2010, Le Page *et al.* 2011, Brion *et al.* 2012, Baudiffier *et al.* 2013, Shanthanagouda *et al.* 2013).

Considering the hazards and risks posed by EDs for the reproductive health of aquatic organisms; development, improvement and implementation of tests to identify endocrine active substances has become a major issue these years. To that end, the Organization for Economic Cooperation and Development (OECD) has developed a conceptual framework intended to provide a guidance on available tests for assessing the endocrine disrupting potency of chemicals (OECD, 2012, 2018). Among those tests, a Fish Short Term Reproduction Assay (FSTRA, OECD TG 229) and a 21-day Fish Assay (OECD TG 230) intend to identify estrogenic, androgenic and aromatase inhibiting substances (OECD, 2009, 2012b). Zebrafish, fathead minnow or Japanese medaka are the three recommended species for these tests. In these assays, androgenic substances are detected only in fathead minnow and Japanese medaka by studying secondary sexual characteristics. In the three species, estrogenic substances are detected at the end of a 21-days exposure, by measuring inductions of concentrations of vitellogenin (VTG), a protein synthesized by the liver under the control of estrogens. Aromatase inhibiting substances are detected indirectly by measuring decreases of VTG synthesis. However, without any direct measurement of gonadal aromatase expression and/or activity, such an inhibition of VTG might also reflect other endocrine mode of action such as androgenic or anti-estrogenic activity. Further, VTG inhibition as a consequence of liver toxicity has often been postulated but recent studies demonstrated that it is unlikely that hepatotoxic chemicals will interfere with the hepatic capacity for VTG synthesis (Ayobahan *et al.* 2020, Baumann *et al.* 2020). Consequently, developing models providing information on mechanisms of action of substances on gonadal aromatase appears relevant to refine the fish screening assay(s).

For that purpose, transgenic fish represent relevant tools that can provide additional mechanistic information on selected genes expression without increasing the number of animals and experimental costs (Carvan *et al.* 2000, Lee *et al.* 2014). Different fish species have been genetically modified during the last ten years and genetic constructs vary from one organism to another, coupling a reporter protein such as GFP or luciferase with the promoter of a gene of interest (e.g., *cyp19a1b*, *choriogenin H*) or with estrogen responsive elements (ERE) (Legler *et al.* 2000, Kurauchi *et al.* 2005, Bogers *et al.* 2006, Tong *et al.* 2009). These transgenic fish models have been efficiently used for the study of the estrogenic potency of chemicals, alone or in mixtures (Gorelick and Halpern 2011, Brion *et al.* 2012, 2019, Gorelick *et al.* 2014, Hinfray *et al.* 2016, 2018, Le Fol *et al.* 2017). In this study, we thus propose to use a transgenic fish, the *cyp19a1a-eGFP* zebrafish (Hinfray *et al.* 2018), in the frame of OECD TG 229 in order to obtain additional/complementary mechanistic information. To that end, prochloraz (PCZ), a fungicide known to inhibit steroidogenesis, including aromatase activities, was used as a reference compound for the TG 229.

2. Materials and methods

All experiments presented were performed in accordance with Directive 2010/63/EU. Study design and experimental procedures were submitted to the French Animal care and experiment committee for ethical review and were approved with the official license n° 11177.

2.1. Chemical analysis

Water samples (about 10 ml) were collected at the outlet of the needles at 24 hours of exposure and in 2 aquaria per condition at 24 h, 48 h and 7, 14 and 21 days. They were stocked in amber glass bottles at - 20°C until chemical analysis.

2.1.1. Solvents and standards

All solvents used in this work were at least of analytical grade: methanol (MeOH) (Baker HPLC Analysed) was provided by Atlantic Labo ICS (Bruges, France), ammonium acetate was purchased from Atlantic Labo (Bruges, France) and acetic acid from Sigma Aldrich (Saint-Quentin Fallavier, France). Water used for analysis was ultra-pure water (UPW) (Milli-Q Millipore Corporation, 18.2MΩ/cm).

Analytical-grade standards were of the highest available purity (>98%). Solid certified standards of prochloraz and irgarol d9 (internal standard) were obtained from Cluzeau Info Labo (Sainte Foy la Grande, France). Stock solutions of pesticides were prepared in MeOH and stored in the dark at -18°C. Working standards or experiment solutions were prepared by dilution from stock solutions and aqueous solution were freshly prepared before experiments.

2.1.2. Water analysis

Prochloraz concentrations were determined in water samples. For the most concentrated samples a dilution of 10 or 100 was applied to fit with the calibration curve, using mQ water. The analytical method described below was validated in terms of extraction recoveries (samples of fortified mineral water from 3 to 10000 ng/L) and of limits of quantifications (LOQ: signal to noise ratio of 10). During analysis, different controls were performed: blanks of

experiment (complete procedure with mineral water: Vittel water sold in glass bottles); control calibration standards (0.1 to 15 µg/L; injected every 15 samples) and analytical blanks (mQ water).

Prochloraz was analysed in water by LC/MS/MS (QQQ) (characterization and quantification). After filtration on GF/F glass fiber filters (0.7 µm – Whatman, Fisher Bioblock Scientific, Illkirch, France), 5 µL of water sample supplemented with the internal standard (Irgarol D9) were directly injected. Acquisition was performed in dynamic MRM in positive mode using electrospray with an HPLC Infinity 1290 and a triple quadrupole 6495 from Agilent Technologies (Santa Clara, CA, USA). Separation was performed using a reverse C18 phase Kinetex column (100 x 2.1 mm; 1.7 µm, Phenomenex) at 35°C with ultrapure water (phase A) (+ 5mM ammonium acetate and 0.1% acetic acid) and methanol as mobile phase (phase B) with constant flow of 0.5 mL/min. The gradient expressed as changes in mobile phase B was as follows: 0 to 7 min, a linear increase from 0% to 100% of MeOH held during 0.5 min; 7.5 to 8.5 min, a linear decrease from 100% to 0% of MeOH, held until the end of the run at 9.5 min. Two transitions were recorded for prochloraz compound, one transition for quantification (TQ) and one transition for confirmation (TC) in order to ensure the identification of the compound and quality control. The transitions for prochloraz were the following ones: TQ: m/z $376 \rightarrow 307.9$ (CE 4), TC: m/z $376 \rightarrow 70$ (CE 24); and for irgarol D9: TQ: m/z $263.2 \rightarrow 199$ (CE 20). The MS/MS parameters were the following ones: sheath gas temperature, 400°C; sheath gas flow rate 11 mL/min; drying gaz temperature, 200°C; drying gas flow rate 11 mL/min; nebulizer pressure, 40 psi; nozzle voltage, 300 V; Vcap 3000 V.

The quantification recovery for spiked mineral water was 90+/-10 % (n = 10). The limit of quantification for water sample was 7 ng/L (without dilution). Residues in blank samples were below quantification limits.

2.2. Transgenic zebrafish exposure

For this study, the transgenic zebrafish model used express an enhanced Green Fluorescent Protein (eGFP) under the control of the zebrafish ovarian aromatase promoter (*cyp19a1a*) (Hinfray *et al.* 2018).

Adult transgenic *cyp19a1a-eGFP* zebrafish (8-10 months old) were obtained from breeding stock in our laboratory at INERIS (Institut National de l'Environnement Industriel et des Risques, Verneuil-en-Halatte, France). 12 glass tanks each containing 7 liters of water and an aeration system, were placed in a continuous flow-through system. The water was renewed 10 times a day and flowed in aquariums by gravity. Water quality was monitored every day: temperature (27.03 ± 0.12), pH (8.11 ± 0.09), dissolved oxygen (101% ± 1.42%), nitrate (not detectable), nitrite (< 0.025) and ammonia (not detectable). Throughout the duration of the experiment, the photoperiod cycle has been maintained at 14:10 light/dark. Experimental design for this study was based on TG 229 of OECD. Fish were first acclimated for 4 days in the flow-through system. Then a 14-days pre-exposure period started, to determine the basal reproduction level of each aquarium. After the pre-exposure period, fish were exposed for 21 days to three nominal concentrations of PCZ (3, 30, 300 µg/L) (Sigma-Aldrich, Lyon, France, CAS 67747-09-5, purity 98%) or to the solvent alone (DMSO, 0.01%; Sigma-Aldrich, Lyon, France, CAS 67-68-5, purity 99.5%). Each stock solution was stored in an amber bottle, protected from light and renewed weekly. Three replicates per condition were used, each containing 5 males and 5 females. During this experiment, fish were fed twice a day, the morning with flake food (SDS400, Scientific Fish Food) and the afternoon with newly hatched brine shrimp (Sep-Art, Ocean Nutrition Europe).

2.3. Monitoring of reproduction and offspring

Eggs were collected daily at the same time during the experiment (morning), using rectangular glass nest box, covered with green mesh. Once the eggs were collected, they were disinfected for 5 minutes in water containing 0.2% bleach (2.6% commercial bleach). Then, the eggs were

sorted into three classes using a SteREO Discovery.V8 stereomicroscope (Carl Zeiss, Germany): unfertilized, nonviable (fertilized eggs that had not reached the 16-cells stage) and viable eggs. Then, the percentage of fertilization (number of fertilized eggs / the total number of eggs *100) and viability (number of viable eggs / the total number of eggs *100) were determined. Finally, 50 viable eggs were placed in crystallizers containing 35 ml of clean water, and observed at 24, and 96h, to determine the survival rate of the offspring.

2.4. Measure of *in vivo* GFP fluorescence

During the exposure, female fish from each condition were anesthetized with 200 mg/L MS222 (Sigma-Aldrich, Lyon, France, CAS 886-86-2) and then photographed using an IVIS Lumina II fluorescence imaging system (Perkin Elmer, Courtaboeuf, France). All the pictures taken were made under identical conditions (fish orientation, zoom, binning, excitation filter (465 nm) and emission filters (520, 540, 560, 580 nm)). The Living Image software (Caliper Life Sciences, Hopkinton, USA) was used to apply spectral unmixing on the images captured. This enabled the discrimination between spectral signatures and the extraction of the GFP signal from the tissue autofluorescence. GFP fluorescence quantification was then expressed as the Total Radiant Efficiency (number of photons per seconde normalized by the excitation light intensity, $[p/s]/[\mu W/cm^2]$) divided by the gonad area (cm^2).

Once the image was captured, the fish was placed in a vat with clean water for about 10 minutes to recover, and then, in its original aquarium. This GFP fluorescence was quantified at 4 different times: the first day of the contamination launch to obtain a basal expression of GFP, and then at 7, 14 and 21 days of exposure.

2.5. Fish sampling

After 21 days of exposure, fish were euthanized in ice-cold water (Wilson et al. 2009), weighted and measured. A picture of the whole fish was taken, to measure GFP fluorescence as describe previously. 5 µL of blood was collected and transferred to tubes filled with 45 µL of phosphate buffered saline (PBS) containing heparin (2000 U/ml), glycerol (20%) and PMSF (0.2 mM). Then, the tubes containing blood were stored at -80° C for subsequent circulating vitellogenin and estradiol concentration analysis. The gonads were removed and weighed to calculate the gonadosomatic index (GSI = gonad weight [mg] / body weight [mg] x 100). They were stored in RNA*later*® (Sigma-Aldrich, Lyon, France) at 4°C overnight and then at -80°C for subsequent gene expression analysis.

2.6. RNA isolation, complementary DNA (cDNA) synthesis and qPCR

Gonads were transferred into lysis buffer (20 µl / 1mg of tissue) and then mechanically ground with 1 mm glass beads (2 x 10 sec, speed: 7.5m/sec) with a FastPrep-24™ 5G (MP BIOMEDICALS©, France). To evaluate the gonadal aromatase gene expression (*cyp19a1a*), the total RNAs were extracted from the ovaries (n = 15 individuals per condition) using the MagMAX™ mirVana™ Total RNA Isolation Kit (ThermoFisher Scientific, USA), in accordance with the manufacturer's instructions. The quantity and quality (260/280 ratio) of the extracted RNA were evaluated using a Nanodrop™ 8000 (ThermoFisher Scientific, USA), before being stored at -80°C for further analysis. All samples presented a 260/280 ratio between 1.8 and 2.0. Then, 200 ng of total RNA were used to synthesize cDNA using Maxima H Minus Reverse Transcriptase (50 U) (ThermoFisher Scientific, USA) in accordance with the manufacturer's instructions. Briefly, the mix reaction was incubated for 30 min at 50°C and then heated at 85°C for 5 min to end the reaction. Gene expressions were measured by quantitative polymerase chain reaction using Taqman technique performed on LighCycler® 96 (Roche Diagnostic France, France).

The *cyp19a1a* primers (forward primer 5'-atcagacaccatcgaccaca-3', reverse primer 5'-acaacaggacccagaccaaa-3') were designed using the Assay Design Center (Roche LifeScience). The probe #85 (Roche LifeScience, cat n° 04689097001) from the Universal ProbeLibrary (Roche LifeScience, USA) was used. A qPCR was then carried out with TaqMan™ Fast Advanced Master Mix (ThermoFisher Scientific, USA) according to the following protocol: a first step of UNG activation to prevent genomic DNA contamination was carried out for 2 min at 50°C followed by an activation step of enzyme for 2 min at 95°C. A total of 40 amplification cycles were performed. During this period, a denaturation (10 s at 95 ° C), then a hybridization/elongation (30 s at 64 ° C) have been executed. To obtain the cDNA concentration of the target gene in each sample, the technique of absolute quantification was employed (use of a standard curve of 15 points).

2.7. Vitellogenin ELISA

The circulating vitellogenin concentrations were measured by indirect competitive ELISA according to the method described by Brion et al. (2002), adapted in 384-wells microplates with a liquid handling robot Freedom EVO® (Tecan, Switzerland) for whole blood samples.

384-wells microplates were coated with 75 µL per well of purified zebrafish VTG solution (100 ng/ml in 0.05 M carbonate/bicarbonate buffer, pH9.6). Nonspecific binding was determined by coating two wells with the same volume of coating buffer without zebrafish VTG. Plates were then incubated overnight at 4°C.

The zebrafish VTG standard was serially diluted by a factor 2 in phosphate saline buffer (PBS) supplemented with 1% bovine serum albumin (BSA), from 1000 to 0 ng/ml. Similarly, whole blood samples from females were tested in 12 serial dilutions with a step of 3 (dilutions ranging from 1:250 to 1:44,286,750). Standard / samples were pre-incubated with the primary antibody (1:1 with DR-264 zebrafish anti-VTG antibody, Biosense Laboratories, Norway) overnight at 4°C.

The ELISA plates were then washed four times with 110 µL of PBS containing 0.05% of Tween 20 (PBS-T) and blocked with 115 µL of PBS-BSA 2% 2 hours at 37°C to avoid nonspecific binding. Plates were washed four times with PBS-T and the standard / samples-antibody solutions were transferred to the coated and blocked plate (75 µL per well in duplicate). The plates were then incubated for 2 hours at room temperature.

After four washes with PBS-T, 75 µL of secondary antibody (horseradish peroxidase goat anti-rabbit IgG, 1:2000 in PBS-BSA 1%) were added to each well and the plate was incubated for 2 hours at 37°C.

Finally, after five washes with PBS-T, the peroxidase activity was revealed by adding 75 µL of tetramethyl benzidine enzyme substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The reaction was stopped with the addition of 37.5 µL of 1M phosphoric acid after 30 min. The plates were then read at 450 nm on a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Biotek™, USA). The raw data were analyzed on the BioTek™ GEN5 software. For the whole blood samples, this ELISA exerted limits of detection and quantification of 75 ng/ml and 155 ng/ml respectively.

2.8. Circulating estradiol concentrations analysis

Steroid extraction was performed on blood samples. Briefly, 150 µl of ultrapure water was added to 40 µl of each blood sample (already diluted in PBS buffer, cf. 2.5 section). Tubes were vortexed and centrifuged at 3000g for 10 minutes. The supernatant was removed, and the samples were then run on an OASIS® HLB plate (µElution Plate 30 µm, Water SAS, France) with a vacuum SPE system (Extraction plate manifold for Oasis 96-well plates, Waters, USA) to extract total blood steroids in accordance with the manufacturer's instructions. At the end of the extraction, steroids were eluted with 75 µl of methanol. Then, samples were evaporated using an EZ-2 Plus Genevac™ (SP SCIENTIFIC, USA). Finally, blood sample

steroids of 5 females were pooled for each condition and resuspended in 100 μl of PBS. Estradiol (E2) concentrations were measured using an enzyme immunoassay kit (Cayman Chemical Company, USA) according to manufacturer's protocol.

2.10. Statistical analysis

All the data are represented as mean ± standard error of the mean (SEM) as indicated in each figure and table legend. The Shapiro-Wilk and Levene tests were used to verify the normality of the data and the homogeneity of variances. When data did not follow a normal distribution, non-parametric tests (Kruskall-Wallis and Wilcoxon-Mann-Whitney) were performed. Alternatively, the analysis of variance (ANOVA) was applied on parametric data, followed by a Tukey post-hoc test to identify the differences between the groups. For all tests, significant differences are indicated by an asterisk (* $p < 0.05$).

3. Results

3.1. PCZ concentrations in water

To determine the actual concentrations to which zebrafish were exposed, PCZ concentrations were measured by LC/MS/MS in water of aquaria and in the water delivered to the aquaria by the flow-through system of exposure. The measured data are reported in Table 1. No PCZ was quantified in the control conditions.

Table 1: Nominal and measured concentrations of PCZ in water at different sampling times of the exposure. Data are mean ± SEM. n=2 for each condition. LOQ: Limit of quantification (9 ng/L). "Needle" indicates the water sampled at the outlet of the needle supplying the aquarium with water.

Nominal concentration (µg/l)	Measured concentration (µg/l)						Average concentrations over 21 days
	Needle (24h)	24h	48h	7d	14d	21d	
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3	1.7	1.6	1.6	1.8	1.9 ± 0.1	1.7 ± 0.1	1.7
30	18.3	17.4 ± 0.7	17 ± 0.2	18.6 ± 0.8	17.3 ± 1.1	15.7 ± 0.4	17.2 ± 0.4
300	174.5	156.5 ± 20.1	175.6 ± 23.3	222.3 ± 26	158.9	142.8 ± 12.2	172.6 ± 12.4

3.2. Fish survival and growth

No mortality was observed during the pre-exposure or the exposure period. Exposure to PCZ induced no significant change in body length, wet body weight, gonad weight and gonado-somatic index (GSI) in both males and females (Table 2). Repeated anesthesia did not generate any mortality of the fish nor disturbed fish reproduction all along the exposure period.

Table 2: Physiological parameters of control and PCZ-exposed fish. GSI: gonadosomatic index. Data are mean ± SEM (n = 15). PCZ: prochloraz.

	Conditions	Body length (cm)	Wet body weight (mg)	Weight gonads (mg)	GSI (%)
Female	DMSO	3.7 ± 0.1	476 ± 30	46 ± 4	9.9 ± 0.8
	PCZ 1.7 µg/L	3.7 ± 0.1	481 ± 25	50 ± 5.	10.2 ± 0.8
	PCZ 17.2 µg/L	3.6 ± 0.1	443 ± 20	36 ± 3	8.1 ± 0.4
	PCZ 172.6 µg/L	3.8 ± 0.1	521 ± 35	64 ± 8	11.7 ± 0.9
Male	DMSO	3.8 ± 0.1	464 ± 23	6 ± 1	1.3 ± 0.1
	PCZ 1.7 µg/L	3.9 ± 0.1	504 ± 20	7 ± 1	1.3 ± 0.1
	PCZ 17.2 µg/L	3.7 ± 0.1	477 ± 21	7 ± 1	1.4 ± 0.1
	PCZ 172.6 µg/L	3.7 ± 0.1	462 ± 19	5 ± 1	1.0 ± 0.1

3.3. In vivo monitoring and gene expression of *cyp19a1a* in female zebrafish

The gonadal fluorescence of GFP can be quantified in anesthetized *cyp19a1a-eGFP* females (Figure 1A), while no specific fluorescence is detected in males (data not shown). Ovarian fluorescence of GFP was thus monitored *in vivo* over the 21 days of exposure to PCZ (Figure 1B). No statistically significant difference was observed between all T0 conditions. Between T0 and T21, fluorescence levels of ovarian GFP remained stable over time for DMSO and low PCZ concentration while a significant increase was observed for the medium and high PCZ concentrations (17.2 and 172.6 µg/L). Indeed, after 21 days of exposure, GFP fluorescence was 1.59 and 1.78 times higher than at T0 in medium and high PCZ conditions respectively.

After 21 days of exposure, expression of *cyp19a1a* was also measured in ovary by qPCR (Figure 1C). *cyp19a1a* gene expression tended to increase in a concentration-dependent manner, with a statistically significant 2.61-fold induction at the highest concentration of PCZ (172.6 µg/L).
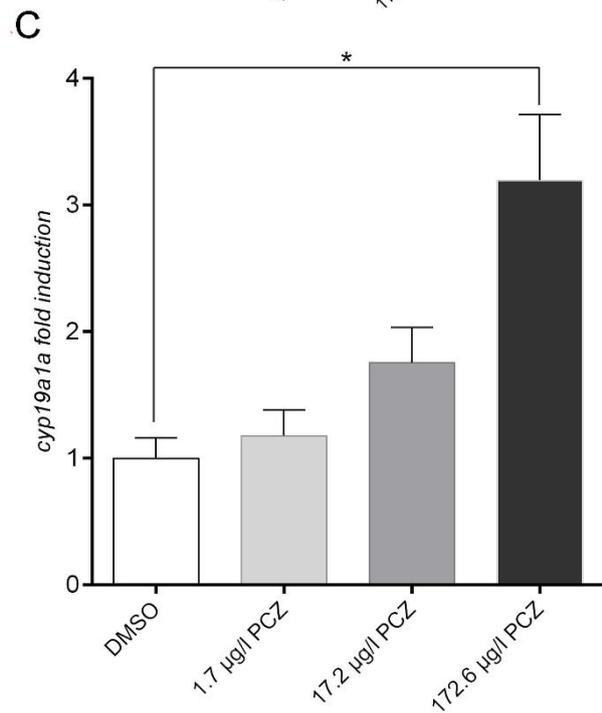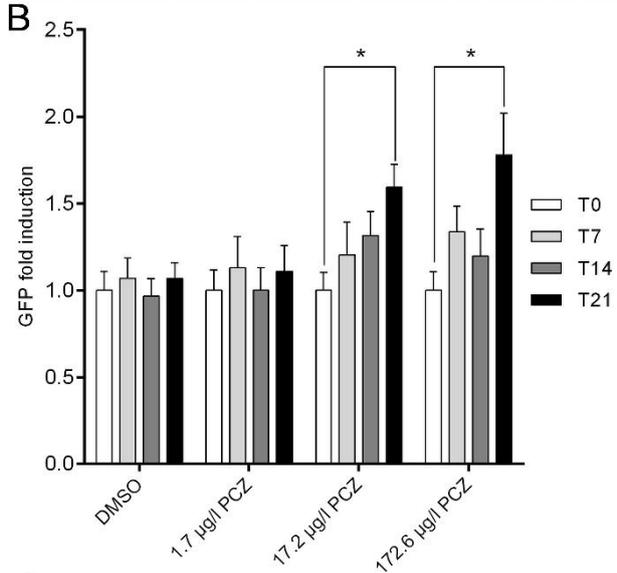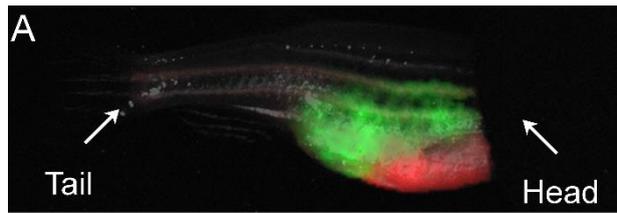
Figure 1: Fluorescence of gonadal GFP in *cyp19a1a-eGFP* transgenic zebrafish exposed to PCZ. (A) Picture of *in vivo* fluorescence of gonadal GFP in a female from the control group. Autofluorescence is in red and GFP fluorescence in green. Head of the fish is on the right and tail on the left. (B) Fluorescence of gonadal GFP in females over 21 days of exposure. Data are mean fold-inductions ± SEM (n = 15). * represent a significant difference from the T0 of each concentration (p < 0.05). (C) Expression of the cyp19a1a gene in ovaries of fish exposed to prochloraz (PCZ). Measurements were performed by qPCR. n = 14-15 for each condition. * represent a significant difference from the solvent control (DMSO) (*p < 0.05).

3.4. Circulating estradiol concentrations

After 21 days of exposure, circulating estradiol concentrations were measured in pools of 5 females zebrafish *cyp19a1a-eGFP* (Figure 2). Due to the few number of samples, no significant difference could be demonstrated between the different conditions. Nevertheless, a 48% inhibition was quantified between the control groups and the fish exposed to the highest concentration (172.6 µg / L).
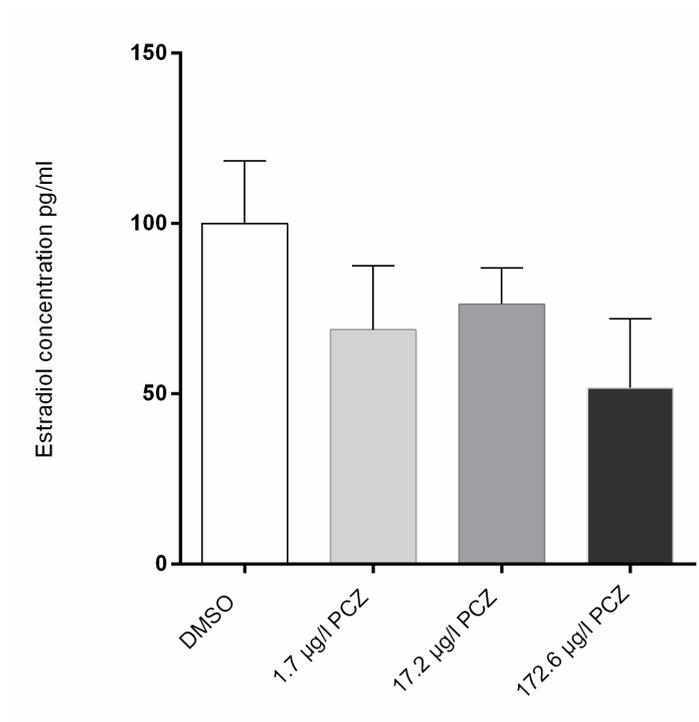

Figure 2: Circulating estradiol in *cyp19a1a-eGFP* female zebrafish exposed to PCZ for 21 days. DMSO: control fish only exposed to the carrier solvent. Data are mean ± SEM (n = 3).

3.5. Circulating vitellogenin concentrations

At the end of the 21-days exposure to PCZ, circulating concentrations of VTG were measured in female zebrafish (Figure 3). A significant decrease was measured in fish exposed at the highest concentration of PCZ, with a fall from 18.72 ± 0.23 mg/ml (DMSO) to 6.10 ± 0.74 mg/ml (PCZ 172.6 µg/l).
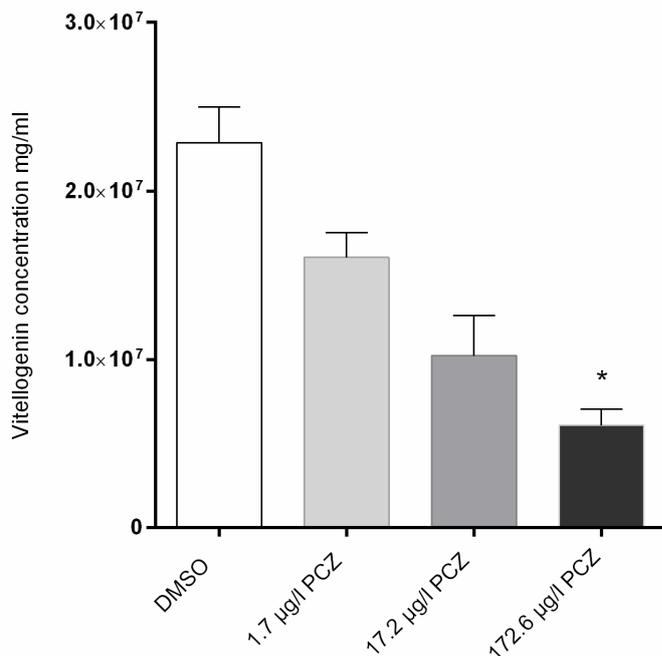
Figure 3: Circulating vitellogenin concentration in *cyp19a1a-eGFP* female zebrafish exposed to PCZ for 21 days. DMSO: control fish only exposed to the carrier solvent. Significant difference from the solvent control are represented by asterisk (*p < 0.05). n = 3.

3.6. Reproduction

During the pre-exposure period, no difference in egg production was observable between the different groups (Figure 4A,B). During the exposure period, a downward trend in cumulative egg production per female was apparent for fish exposed to the highest concentration of PCZ (Figure 4A; 220.6 ± 20.2 eggs for HC groups *vs* 339 ± 43 eggs for DMSO control groups after 21 days), but this change was not statistically significant. However, the comparison between the number of eggs laid per female and per day during the pre-exposure and exposure periods, showed a statistically significant decrease in eggs production for the highest concentration of PCZ (Figure 4B).

Embryonic development was monitored during the pre-exposure and exposure periods. We observed no modification of fertilization and viability rates (Table 3). Embryo survival was also assessed at 24 and 96 hours (Table 3). No significant difference could be found between the DMSO and the PCZ-exposed groups, either during the pre-exposure period nor during the exposure. Comparing the pre-exposure and exposure periods, survival of embryos from fish

exposed to the PCZ decreased significantly whatever the exposure concentration and the duration of parental exposure.
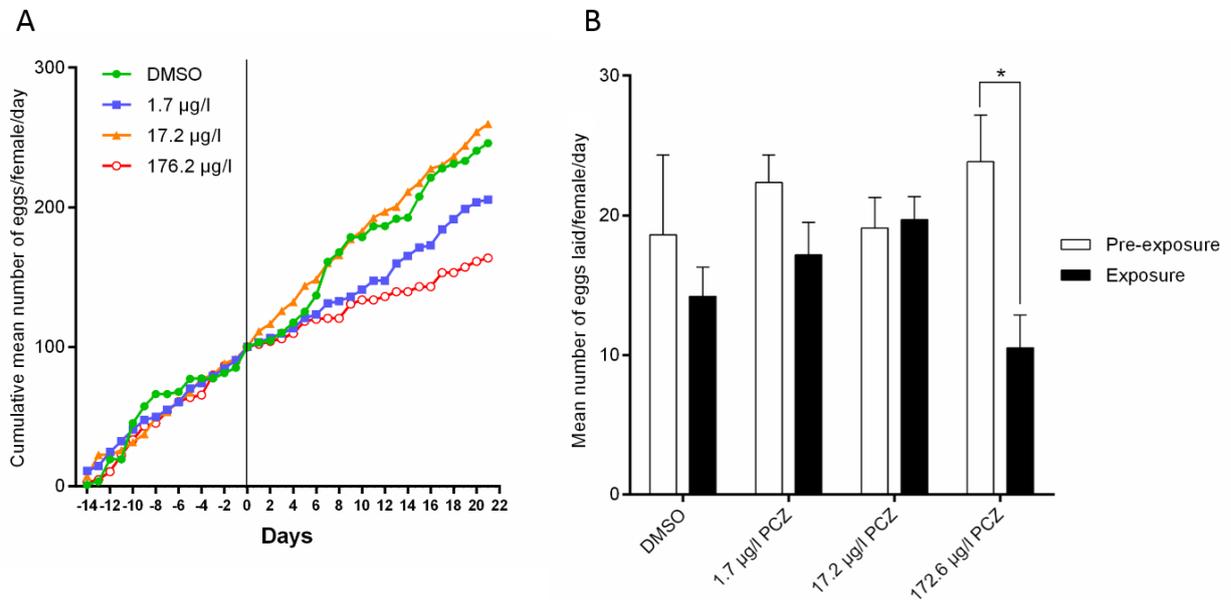

Figure 4: Reproductive effects in *cyp19a1a-eGFP* transgenic zebrafish after exposure to PCZ. (A) Cumulative mean number of eggs per female during the pre-exposure and exposure periods. (B) Mean number of eggs laid per female per day during the pre-exposure and the exposure periods. Data are mean ± SEM. Significant difference between pre-exposure and exposure is indicated by an asterisk (*p < 0.05).

Table 3: Percentage of fertilization, viability and survival of the offspring of eggs from *cyp19a1a-eGFP* zebrafish exposed to prochloraz (PCZ). Data are mean ± SEM (n = 12 - 21). Significant difference from the pre-exposure condition are represented by an asterisk and bold numbers (*p < 0.05).

Fertilization							
	DMSO		**1.7 µg/l**		**17.6 µg/l**		**176.2 µg/l**
Pre-exposure	99.9 ± 0.1		98.8 ± 0.4		99.6 ± 0.2		99.4 ± 0.3
Exposure	99.8 ± 0.1		99.0 ± 0.3		99.6 ± 0.1		96.0 ± 3.8
Viability							
	DMSO		**1.7 µg/l**		**17.6 µg/l**		**176.2 µg/l**
Pre-exposure	74.8 ± 6.6		83.6 ± 1.7		90.5 ± 1.4		90.9 ± 1.6
Exposure	69.1 ± 4.7		82.4 ± 2.4		88.8 ± 1.9		89.9 ± 3.7
Survival							
	DMSO		**1.7 µg/l**		**17.6 µg/l**		**176.2 µg/l**
	24h	**96h**	**24h**	**96h**	**24h**	**96h**	**24h**	**96h**
Pre-exposure	92.5 ± 4.1	74.0 ± 7.6	99.2 ± 0.2	96.3 ± 1.2	98.4 ± 0.5	93.1 ± 2.0	99.4 ± 0.2	97.0 ± 1.2
Exposure	89.7 ± 5.0	79.6 ± 6.2	**85.6* ± 3.9**	**72.5* ± 5.6**	**85.9* ± 3.2**	**73.3* ± 5.2**	**89.3* ± 5.4**	**63.6* ± 7.6**

4. Discussion

Effect of PCZ on classical endpoints of the OECD TG 229 in *cyp19a1a-eGFP* zebrafish

In the present study, PCZ was chosen as a model aromatase inhibiting substance because it has been extensively studied in fish screening assay (Monod *et al.* 1993, Ankley *et al.* 2005, Hinfray *et al.* 2006, OECD 2006). Classical endpoints of the OECD TG 229 (VTG, reproduction) were measured after exposure to PCZ to ensure that responses in the *cyp19a1a-eGFP* transgenic zebrafish were consistent with those of wild type zebrafish. In *cyp19a1a-eGFP* zebrafish, a decrease in circulating E2 concentrations accompanied by an inhibition of circulating VTG concentrations were measured in the females exposed to the highest concentration of PCZ. These effects are consistent with our expectations based on PCZ mechanisms of action. By inhibiting steroidogenic enzymes activities (c17α-hydroxylase,17,20-lyase (Cyp17) and aromatase) (Villeneuve *et al.* 2007, Nielsen *et al.* 2012), PCZ caused an inhibition of estrogen synthesis that led to a decrease in stimulation of the vitellogenin synthesis. In wild-type zebrafish, exposure to PCZ (200-300 µg/L) resulted in similar inhibitory effects on E2 and/or VTG synthesis (Kinnberg *et al.* 2007, Dang *et al.* 2015, 2016, 2018). To a larger extent, PCZ and other azole compounds such as ketoconazole, propiconazole or letrozole, have also been reported to decrease E2 and VTG synthesis in different fish species (Ankley *et al.* 2005, 2007, 2009, Skolness *et al.* 2011, 2013).

Accompanying the effects on E2 and VTG, PCZ exposure had marked effects on *cyp19a1a-eGFP* fish fecundity. Indeed, the average number of eggs laid per female and per day during the exposure at the highest concentration of PCZ decreased significantly compared to the pre-exposure period. These results agree with what has already been reported for different fish species exposed to similar concentrations of PCZ, such as fathead minnow, medaka or wild type zebrafish (Ankley et al. 2005, Zhang et al. 2008, Dang et al. 2018). Other azole compounds such as fadrozole, ketoconazole or propiconazole are also known to inhibit

fish reproduction (Ankley *et al.* 2002, Zhang *et al.* 2008, Zhang *et al.* 2008b, Skolness *et al.* 2013).

At the functional level, PCZ had no effect on the fertilization rate nor on the viability rate. However, 24 and 96 hpf survival of embryos from parents exposed to PCZ decreased compared to the pre-exposure period. In our study, even if most part of the effects of PCZ on embryos might be due to parental exposure, a direct effect of PCZ exposure could not be totally excluded since the eggs could be exposed during few hours before their daily collection. Some studies describe the ability of different azole compounds to decrease hatching and/or survival rates of embryos after a parental exposure (Teng *et al.* 2018) or when fish are exposed at embryo-larval stage (Thorpe *et al.* 2011, Domingues *et al.* 2013, Mu *et al.* 2013, Cao *et al.* 2016, Muth-Kohne *et al.* 2016). A recent study suggests that these effects may be due to impaired transcription of genes involved in growth, heart development, bone formation and/or lipid homeostasis (Mu *et al.* 2016). Regardless, further studies are needed to elucidate the mechanism of action of PCZ leading to survival alteration of embryos.

Exposure of *cyp19a1a-eGFP* transgenic zebrafish to PCZ caused a suite of responses consistent with the inhibition of circulating estradiol concentrations as already observed in wild-type zebrafish and other fish species. Collectively, the responses of this transgenic model suitably fit into the Adverse Outcome Pathways (AOP) which details the linkage between gonadal aromatase inhibition and reproductive dysfunction in female fathead minnow (Villeneuve 2016). In this AOP, the molecular initiating event "aromatase inhibition" leads to a decrease of circulating estradiol, an inhibition of VTG protein production and eventually in the adverse effect, *i.e.* impaired ovulation and spawning that could lead to a population sustainability alteration (Ankley *et al.* 2010, Perkins *et al.* 2015). From a quantitative point of view, the data we obtained in the *cyp19a1a-eGFP* zebrafish (48% inhibition of estradiol in the high concentration of PCZ compared to control females, leading to a 65% decrease of circulating VTG and finally to 60% less eggs produced) are in perfect adequation with the quantitative relationships existing between all key events of the AOP recently described for

wild-type zebrafish (Doering *et al.* 2019). Moreover, in wild type zebrafish, exposure to 300 µg/l (nominal) PCZ led to a 38 % inhibition of estradiol concentration, a 65% inhibition of circulating VTG and a 64% decrease of fecundity (Dang et al, 2018). In the end, the *cyp19a1a-eGFP* transgenic zebrafish proved to behave like a wild-type zebrafish and to respond with equal sensitivity in the suite of responses produced by aromatase inhibition.

Benefits of the *cyp19a1a-eGFP* transgenic zebrafish for the study of EDs

During the time course of the experiment, the effects of PCZ on gonadal aromatase expression was followed in individuals by measuring *in vivo* fluorescence of gonadal GFP at 4 different times of exposure (0, 7, 14 and 21 days). *In vivo*, GFP fluorescence have been detected only in females and not in males which is consistent with the known dimorphism of *cyp19a1a* expression between sexes in wild type zebrafish (Trant *et al.* 2001, Sawyer *et al.* 2006) and transgenic *cyp19a1a-eGFP* zebrafish (Hinfray *et al.* 2018). In females, a significant increase in ovarian GFP fluorescence appeared after 21 days of exposure for the medium and high concentrations (17.2 and 172.6 µg/L), indicating an increase in the gonadal aromatase gene expression. This induction was confirmed by the induction of the *cyp19a1a* gene expression measured in the ovaries by qPCR at the end of the exposure period. PCZ and other azole compounds (fadrozole, propiconazole) have already been reported to induce ovarian aromatase gene expression in wild type zebrafish and other fish species (Zhang *et al.* 2008b, Ankley *et al.* 2009, Skolness *et al.* 2011, 2013, Dang *et al.* 2015, 2016). These inductions of *cyp19a1a* gene expression are indicative of a compensation process resulting, at least in part, from inhibitions of aromatase enzymatic activities in fish exposed to azole compounds (Ankley *et al.* 2009, Villeneuve *et al.* 2009, Liu *et al.* 2011). The disruption of transcript levels of genes coding for steroidogenic enzymes (not only aromatase), in response to azole exposures, reflect a perturbation of the HPG axis functioning, seemingly occurring to restore circulating steroids concentrations (Ankley and Villeneuve 2015).

Our *cyp19a1a-eGFP* transgenic zebrafish line allowed us to follow the kinetic of appearance of PCZ effects at the gonadal level by measuring ovarian aromatase gene expression through non-invasively *in vivo* measurements of the fluorescence of GFP after repeated anesthesia of the fish. The use of the *cyp19a1a-eGFP* model provided additional mechanistic insight to the classical endpoints of the OECD TG 229 without utilizing the gonadal tissue that remains available for other analysis such as histology, and without increasing the number of animals needed. Indeed, in the present study, the *cyp19a1a-eGFP* model enables to confirm that PCZ effects on E2 and VTG circulating concentrations, and on reproduction, are mediated at least in part by a perturbation of the gonadal aromatase, even if due to PCZ known mechanism of action, inhibition of Cyp17 activities might accentuate the effects on E2 concentrations. Besides, the *cyp19a1a-eGFP* also possess interesting features such as being a stable transgenic line with only one transgene insertion and the possibility of inheritance of the transgene from both males and females rendering this line easy to maintain in laboratory (Hinfray *et al.* 2018). Altogether, these characteristics demonstrate this transgenic line is a valuable model to study the endocrine disrupting potential of chemicals and to refine actual mechanistic-based assays.

5. Conclusions

The present study details the effects of PCZ, on a recently developed *cyp19a1a-eGFP* transgenic zebrafish line. These experiments showed that the physiological responses observed in control and PCZ-exposed *cyp19a1a-eGFP* transgenic zebrafish (E2, VTG, reproduction) are similar to those of wild-type zebrafish, both qualitatively and quantitatively. Besides, the *cyp19a1a-eGFP* model reliably informs on the time- and concentration-dependent effects of PCZ on ovarian aromatase gene expression through *in vivo* GFP fluorescence of the ovaries, thereby providing novel mechanistic information to the OECD TG 229. Overall, the *cyp19a1a-eGFP* transgenic zebrafish line proved to be a relevant tool to study the effects of

EDs in fish, that might also be wisely used to refine mechanism-based assays such as OECD TG 229.

6. Declaration of interests

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

7. Acknowledgements

8. Author contributions

NH and HB acquired the funding; JDO, NH, FB, XC, HB conceived and designed the experiments; JDO, BP reproduced and maintained the transgenic zebrafish line in INERIS facility; JDO, NH, EC, EMM, CT performed *in vivo* experiments; OP performed VTG ELISA; PP and HB performed chemical analysis; JDO, NH wrote the manuscript; FB, XC, HB revised the manuscript. All authors have approved the final article.

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