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Evaluating the alterations of the estrogen-responsive genes in *Cyprinodon variegatus* larvae for biomonitoring the impacts of estrogenic endocrine disruptors (EEDs)

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ABSTRACT

Currently, endocrine disruptors (EDs) can be found in all the environmental compartments. To understand the effects of estrogenic EDs (EEDs), adults of *Cyprinodon variegatus* have been classically used as a marine model. However, it is during development that exposure to contaminants may generate permanent consequences. Thus, the aim of this study was to verify the effects produced by acute exposure to 17α-ethinylestradiol (EE2) in *C. variegatus* larvae. Quantitative PCR (qPCR) results revealed the induction of *vtg* and *zp* gene expression on exposure to 1000 ng/L EE2 and the induction of *vtgc*, *zp2*, *zp3* and *cyp19a2*, and inhibition of *vtgab*, *wap* and *cyp1a1* on exposure to 100 ng/L EE2. Lower concentrations inhibited the gene expression of *vtgab* and *wap* (50 ng/L), *cyp1a1* (25 ng/L) and *zp2* (12.5 ng/L). These alterations in gene expression allow us to affirm that larvae of *C. variegatus* are an efficient and sensitive model for biomonitoring EEDs.

1. Introduction

17α-Ethinylestradiol (EE2) is a synthetic estrogen used in contraceptive pills, and the most commonly found xenoestrogen (among other pharmaceuticals and industrial chemicals) in water samples from different sources (Laurenson et al., 2014). In the European Union, a meta-analysis of the available studies between 1995 and 2015 reported EE2 concentrations in urban wastewater treatment plants ranging from 0.2 to 50 ng/L (Tiedeken et al., 2017). Even so, studies on wastewater treatment plants in China have reported concentrations which reach levels of 4.1×10^3 ng/L (Zhou et al., 2012). Despite estrogens modulating a broad range of physiological processes (Huang et al., 2015; Segner et al., 2013), their main function in vertebrates is to regulate the female reproductive system. Hence, the presence of substances in the environment that activate the pathways controlled by estrogens may produce ecological alterations in the reproductive fitness of populations (Andersen et al., 2003; Kidd et al., 2007). Several studies have reported the endocrine disruptive effects of EE2 in freshwater fish such as trout (Hultman et al., 2015; Jobling et al., 1996), zebrafish (Andersen et al., 2003), carp (Huang et al., 2015) and the mangrove rivulus (Voisin et al., 2019). In marine species such as *Cyprinodon variegatus*, the effects of EE2 have been mainly studied in juveniles and adults (Bowman et al., 2000; Denslow et al., 1999, 2001; Folmar et al., 2000; Zillioux et al., 2001). Exposure to estrogen or estrogenic endocrine disruptors (EEDs) in adults can produce activational effects that can influence the reproductive parameters and consequently impair reproductive fitness (Bayley et al., 1999; Pradhan and Olsson, 2015). Due to the lack of sexual and thyroid development, larvae have not been broadly addressed as models for ED studies; however, exposure during development and maturation can generate organizational alterations which can remain permanently in the organism (Huang et al., 2015; Länge et al., 2001; Morthorst et al., 2016). Indeed, the Organisation for Economic Co-operation and Development (OECD) has developed test guidelines using transgenic fish embryos, such as OECD TG250, TG251 and REACTIV assays, that are under development for detecting estrogenic activity, androgenic activity and estrogenic activity, respectively in fresh water species (OECD, 2021).

Additional advantages related to the use of larval stages are directly

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linked to the reduction in exposure time, as well as in the production of residuals, consequently generating a higher throughput of the testing methods (Xiong et al., 2022).

The research done over the last decades on the physiological effects of EDs has allowed the identification of inherent problems when compared to other kinds of contaminants. One of those is that EDs may generate physiological responses at lower concentrations than those that generate acute toxicity (Zillioux et al., 2001); also, the majority of EDs have a non-monotonic dose-response curve (Pamplona-Silva et al., 2018). Another challenge is that adult males and females exposed to a given ED can display opposite responses in classical biomarkers as vitellogenins (*vtgs*), choriogenins (*chgs*) and estrogen receptor 1 (*esr1*) (Ye et al., 2018).

Among the biomarkers classically used to corroborate EDs at the molecular level, *vtg*, zona pellucida (*zp*) and aromatase (*cyp19*) genes stand out. Additionally, we estimate the gene expression of steroidogenic acute regulatory protein (*star*) and cytochrome P450 1 A (*cyp1a1*) as biomarkers of alterations in the steroidogenic (Prucha et al., 2020) and detoxification pathways (Hultman et al., 2015), respectively. Finally, the gene expression level of whey acidic protein-like (*wap*) has been assessed in order to verify the potential of this mRNA as a new biomarker for estrogenic substances (Schönemann et al., 2022b).

Vitellogenins (VTGs) are naturally synthesised proteins in the liver of mature female fish in response to estrogens, as a precursor to the egg yolk. In the liver of male fish, the presence of these proteins is not natural; despite this, the mechanisms of synthesis are present, and VTGs may be synthetised in male fish exposed to estrogenic substances (Denslow et al., 1999; Hara et al., 2016; Hutchinson et al., 2006). The rapid induction of VTGs has been broadly used as an indicator of the presence of xenoestrogens; however, the synthesis of VTGs can compromise other functions due to the energetic cost to the organism (Andersen et al., 2003). Because of this, the effects in the earlier stages of development may interfere not just in future sex determination but also in fundamental processes such as growth and development (Länge et al., 2001).

The zona pellucida (ZP) proteins, also known as zona radiata (Arukwe et al., 1997), are normally synthesised by females during oogenesis and are related to the production and maintenance of eggs. ZP production is also induced by the presence of estrogenic substances and used as a biomarker of endocrine disruption (Arukwe et al., 1997; Hutchinson et al., 2006; Qi et al., 2018).

CYP19A aromatase is the enzyme responsible for the autocrine production of estrogens from aromatisable androgens. Additionally, it mediates reproductive processes such as brain sex determination, ovarian growth, ovarian differentiation, vitellogenesis and sexual behavior (Senthilkumaran et al., 2015). Several fish species have two genes coding aromatases (*cyp19a1* expressed in the gonad and *cyp19a2* expressed in the brain) because of processes of genomic duplication. In fish, aromatase has a species-specific dimorphic expression (Okubo et al., 2011; Vizziano-Cantonnet et al., 2011), and its expression levels can be altered by ED substances (Hutchinson et al., 2006).

Cyprinodon variegatus is a suitable species for experimentation due to its small size, rapid development and great tolerance to salt and temperature alterations (which allows several options for experimental designs) (Schnitzler et al., 2017). Additionally, its marked sexual dimorphism and the availability of its whole sequenced genome (Lencer et al., 2017) provide widespread opportunities for research. Although it is a seasonal spawning species, its ovaries include ova in different stages (Kuntz, 1917), which allows it to reproduce several times during the summer. Under laboratory conditions, the incubation takes over 5–6 days. According to Kuntz (1917), the 2 days post-hatching (dph) larva is in a pre-metamorphosis stage, the yolk sac remains and it is characterized by the presence of 12 rays in the anal fin and the progression of melanophores on the edge of caudal fin rays.

Because of the issues mentioned above, there is a necessity to generate tools for environmental monitoring of EDs, oriented to overcome the difficulties identified until now. In this context, we designed a series of exposures, to verify if alterations of the classical biomarkers of EEDs (*vtgs*, *zps*, *cyp19a2*) previously reported in adults could be corroborated in larvae, with the aim of generating a cost-effective, faster and more accessible tool for environmental monitoring.

2. Materials and methods

C. variegatus larvae were provided by the ECIMAT marine station, an infrastructure of the Center for Marine Research (CIM-UVigo). Maintenance, handling and reproduction were done according to the plan approved by the Ethical Committee of the University of Vigo (REGA code: ES360570181401) and the method recommended by International Standard ISO 7346–1:1996 (Determination of the acute lethal toxicity of substances to a freshwater fish), considering the recommendations of the United States Environmental Protection Agency (methods 1004.0 and 1005; US EPA, 2015).

2.1. Reproduction

For reproduction, adult females and males (in a proportion of 2: 1, respectively) were transferred to aquariums previously prepared with a 500 μm mesh at the bottom for egg collection. In order to induce reproduction, 29 ppm salinity and a temperature of 26 °C were maintained. After 24 h, the produced eggs were collected, and the fertilized and viable eggs were selected. Subsequently, selected eggs were transferred to PVC cages with seawater in optimal conditions for growing (pH = 7.5, dissolved oxygen > 6.5, salinity 29 ppm, 25 °C and a 16: 8 light/dark photoperiod).

2.2. Exposure to EE2

Larvae were exposed to EE2 (Sigma-Aldrich, Switzerland) for 24 h in an isothermal room at 25 °C. Five replicates per treatment were done with pools of larvae (n = 10), to assess the expression levels of *vtgc*, *vtgab*, *zp2*, *zp3*, *star*, *cyp19a2*, *wap* and *cyp1a1*. Larvae were exposed to EE2 in 100 mL beakers at a density of 1 larva/10 mL (2 dph) in a volume of 100 mL. During the exposure, no aeration or food was supplied; the environmental parameters were the same as used for growing.

Separate exposures to EE2 were performed, the first for testing the responsiveness of the selected biomarkers to EE2 (non-exposed (Control) against 1000 ng/L), and the second for testing the effects of EE2 at environmental levels (Solvent Control (SC) DMSO (Scharlau) at < 0.005% v/v, 12.5, 25, 50 and 100 ng/L EE2).

In order to obtain the desired concentrations, a stock solution of 2 mg EE2 in 10 mL of DMSO was prepared and serially diluted. After 24 h, all larvae were anesthetised (1 g/L) with tricaine methane sulfonate (MS-222; Sigma- Aldrich, GmhB,) dissolved in seawater and subsequently euthanised (2 g/L).

2.3. Determination of EE2 stability in saltwater

The stability of EE2 in seawater was verified by measuring the concentration at 0 h and after 24 h. To do this, seawater samples were filtered through a 0.22 μm nylon filter. Due to the low concentration level of EE2 (10 and 1000 ng/L), solid phase extraction (SPE) with Oasis HLB cartridges was performed. First, cartridges were conditioned with 5 mL of methanol (MeOH) followed by 5 mL of Milli-Q water (same polarity as samples). Subsequently, 500 mL of sample was loaded at a flow rate of 1 mL/min using a vacuum pump. Then, cartridges were dried for 30 min and eluted with 8 mL of MeOH. Samples were then evaporated to almost-dryness in a TurboVap under a current of N_2 at 25 °C and transferred to a 2 mL chromatographic vial with 1 mL MeOH as washing solvent. Finally, samples were evaporated to dryness and reconstituted to 300 μL of MeOH.

The chromatographic analysis was performed using an ultra-high

performance liquid chromatograph (UHPLC) coupled to a triple quadrupole detector mass spectrometer (MS/MS; Xevo TQ-S micro, Waters, Milford¸ MA, USA). An Acquity UPLC BEH C18 column (100 mm × 2.1 mm ID, 1.7 μm particle size; Waters) was employed. The oven temperature was set at 30 °C. The mobile phase was a binary composition of solvent A (Milli-Q water acidified with 0.1% formic acid (FA)) and solvent B (acetonitrile, the organic mobile phase). Initially, the gradient started at 20% B and increased to 100% B in 4.5 min. After being held for 1.5 min at 100% B, initial conditions (20% B) were restored in 1.5 min, and held for 2.5 min. Total run time was 10 min. The flow rate was set at 0.250 mL/min and the injection volume was 10 μL.

Regarding the mobile phase (MS) conditions, the ionization source used was an atmospheric pressure chemical ionization source, in positive mode (APCI+). The acquisition was performed in multiple reaction monitoring (MRM) mode. The precursor ion ($[M+H]^+ = 297$), its fragmentations (297 < 159, 297 < 107) and, therefore, the optimum cone voltage (CV = 32 V) and collision energies (CE = 10 and 20 eV, respectively) were optimized by flow injection analysis (FIA). The system and data management were processed using the MassLynx v4.1 software package (Waters, Manchester, UK).

2.4. Gene expression analysis

2.4.1. Primer design

Specific primers were designed with the NCBI's primer designing tool, Primer-BLAST, based on the sequences reported for *C. variegatus* in the NCBI (Table 1). General characteristics of the primers were restricted to product size (90–150 bp), melting temperature (59.0–60.5 °C) and primer length (20–25 bp).

2.4.2. mRNA extraction and cDNA synthesis

Whole mRNA extraction was done following the RNeasy® Plus mini kit instructions (Qiagen, Hilden, NRW, Germany), with minor modifications detailed below. Every sample was transferred to a 1.5 mL tube with 200 μL of RLT lysis buffer and homogenized; the plastic pestle was previously cleaned with RNase Away reagent (Invitrogen, Carlsbad, CA, USA). After the whole homogenization, 150 μL of RLT buffer was added; the sample was mixed by vortex and centrifuged at 15,000 rpm for 15 min. After centrifugation, the precipitate was removed by micropipetting; the supernatant was transferred to a new tube and precipitated with 70% ethanol. The next steps were performed according to the kit guidelines. Immediately after extraction, total mRNA (1 μL) was quantified, and the purity of the samples was determined by NanoDrop2000 spectrophotometer (Thermo Scientific, USA).

The cDNA synthesis was done with RevertAid Reverse Transcriptase (200 U/μL; Thermo Scientific, Vilnius, Lithuania), RiboLock RNase Inhibitor (40 U/μL; Thermo Scientific, Vilnius, Lithuania) and Random Primers (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' specifications.

2.4.3. qPCR

The qPCR was performed with GoTaq® qPCR Master Mix enzyme (Promega, Madison WI, USA) according to the manufacturer's guidelines, with a thermocycler standard program (initial denaturation 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s) in an Applied Biosystems QuantStudio 6 PRO system. The stability of the housekeeping genes was verified for each experiment by comparison of the mean Ct values among all experimental groups.

2.5. Statistical analysis

Data are presented as mean values and standard deviations. For gene expression, the $2^{-\Delta Ct}$ analysis was developed according to Schmittgen and Livak (2008). Paired tests (between exposed and non-exposed groups or SC and exposed groups) were performed by a Mann–Whitney test, to assess statistical significance ($p < 0.05$). The fold change (FC) between treatments with statistical differences is given as the mean values' ratio of the treated/control for induction or −1/(treated/control) for inhibition (Schmittgen and Livak, 2008).

3. Results

3.1. EE2 concentration and stability

The chromatographic analysis (Table 2) confirmed that EE2 concentrations were close to nominal values. Additionally, both concentrations of EE2 remained stable (98–99%) in seawater after 24 h.

3.2. Expression levels of control larvae vs. larvae exposed to 1000 ng/L EE2

A preliminary test was performed in order to assess the gene expression of the selected biomarkers on exposure to high levels of EE2 (1000 ng/L); these results were compared against the gene expression in Control organisms (Fig. 1). *vtg* and *zp* were strongly induced by the EE2 exposure, having an FC of 4710 (*vtgc*), 22.28 (*vtgab*), 9.44 (*zp2*) and 15 (*zp3*). On the other hand, the gene expression of *star*, *cyp19a2*, *wap* and *cyp1a1* remained unaltered.

Table 2
EE2 concentration (ng/L) in seawater initially (0 h) and after 24 h.

Sample (ng/L)	Mean (n = 3) (ng/L)	SD (ng/L)	CV (%)
10 (0 h)	8.5	2.0	23
10 (24 h)	8.4	2.9	34
1000 (0 h)	957	44	5
1000 (24 h)	950	112	12

SD: standard deviation; CV: coefficient of variation.

Table 1
qPCR primers. List of housekeeping and biomarker genes: TATA-box binding protein (*tbp*), hypoxanthine-guanine phosphoribosyl transferase (*hprt*), vitellogenin C (*vtgc*), vitellogenin Ab (*vtgab*), zona pellucida 2 (*zp2*), zona pellucida 3 (*zp3*), steroidogenic acute regulatory protein (*star*), brain aromatase (*cyp19a2*), whey acidic protein-like (*wap*) and cytochrome P450 1A (*cyp1a1*). The efficiency percentages for *wap* could not be verified due to primer contamination (ND).

Gene	Accession number	Forward	Reverse	Efficiency %
tbp	XM_015386445.1	AGAACCAGTGTGCGTCTCAA	GACCAAACAGCGAATGTCGG	95.16
hprt	XM_015371023.1	CCTTTTGCATCGTGTCACTTCA	TGCAGAACAGCTCAACTCAAAG	96.37
vtgc	XM_015378677.1	TAGCCCTGACTCTGGCCTTT	GAAGGCCACCCAGGATTGAT	97.54
vtgab	XM_015378671.1	TTCCTCTTCACGCCGCAAAA	ACGTTGGCTAGAAGAGCTGC	97.24
zp2	AY598615.1	GATTGGGGGCTCCACAAAGA	TCAGTTGATTTTACGCACATCAAGA	91.78
zp3	AY598616.1	GGTCCGAGGGACTTTGTGAG	CGTTCTCGGCTCTGATGACA	106.6
star	XM_015387377.1	TTCCGCTCCAGCAGTTGAAT	CTTGGACGCTGAAAGGGGAT	103.61
cyp19a2	XM_015393753.1	GACCTTTCCTGTCCTACGGC	AGGACCTGCTGAAAATGATGGT	100
wap	XM_015379063.1	TGTGCTGAACTTTGCACCCA	TCGCAGCTTCCAGGTTTTACT	ND
cyp1a1	EF535032.1	TCTGAATGGCTACTTCATCCCC	CGAAGACGGGTCTTTCCACA	90.98

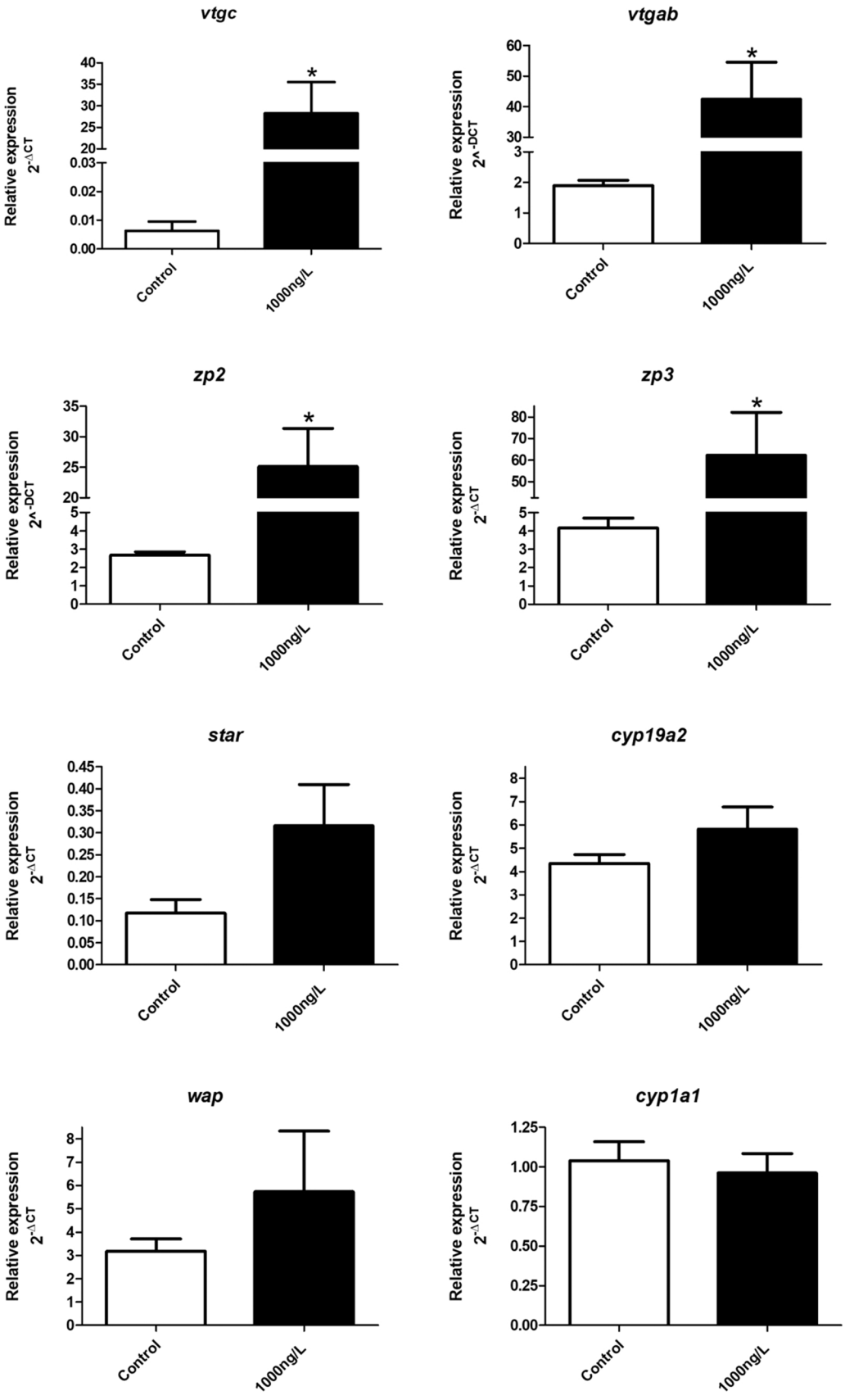

Fig. 1. Expression levels of non-exposed larvae (Control) against larvae exposed to 1000 ng/L EE2. Relative expression levels ($2^{-\Delta Ct}$) of vitellogenin C (*vtgc*), vitellogenin Ab (*vtgab*), zona pellucida 2 (*zp2*), zona pellucida 3 (*zp3*), steroidogenic acute regulatory protein (*star)*, brain aromatase (*cyp19a2)*, whey acidic protein-like (*wap*) and cytochrome P450 1A1 (*cyp1a1*). TATA-box binding protein (*tbp*) and hypoxanthine-guanine phosphoribosyl transferase (*hprt*) were used as housekeeping genes. Mann–Whitney paired tests were performed to assess statistical significance ($p < 0.05$). Significant differences are indicated by an asterisk ($n = 4–5$).

3.3. Differential gene expression at concentrations of environmental relevance

The patterns of response of the target genes at environmentally relevant concentrations of EE2 differed from those obtained at 1000 ng/L. Exposure to 100 ng/L EE2 (Fig. 2) produced a similar response to that observed for exposure to 1000 ng/L EE2, with a significant induction (FC) of the estrogen-responsive genes *vtgc* (34.8), *zp2* (1.87), *zp3* (3.43) and *cyp19a2* (1.24) with respect to the SC group. However, the same concentration of EE2 produced significant inhibition of *vtgab* (−11.36), *wap* (−3.92) and *cyp1a1* (−1.93). In the same way, 50 ng/L EE2 produced inhibition of *vtgab* (−4.76) and *wap* (−4.18). Lower concentrations (25 and 12.5 ng/L) produced weaker but significant inhibition of the gene expression of *cyp1a1* (−1.92) and *zp2* (−1.14).

4. Discussion

Small fishes with a short life cycle are excellent models for ecotoxicological studies because reproductive stocks are easily maintained in the laboratory. Fish share, with other vertebrates, the basic functions of the reproductive system and other metabolic pathways. In this sense, and despite the obvious differences, the neuroendocrine system and hormonal mechanisms are highly conserved across vertebrates (Gonçalves and Oliveira, 2010).

Marine fishes, and especially euryhaline species, bring additional advantages because of the broad exposure scenarios that can be tested. *C variegatus* is a valuable species with a sequenced genome (Lencer et al., 2017) that has been used for more than 20 years as a model to understand the effects of estrogenic substances (Denslow et al., 1999). The knowledge and technologies for molecular biomarkers have evolved but so has the necessity for quicker and more efficient tools for environmental monitoring.

The use of adult fish to assess the toxicological and endocrine disruptive potential of a substance is a well-established and relevant approach (Wagner et al., 2017); nevertheless, these assays are time-consuming and challenging due to infrastructure requirements. Because of this, if the same response can be evaluated in the larval stages of development, the costs related to time, maintenance and amount of residue produced will be reduced by several orders of magnitude.

Taking into account that generating environmental data about different contaminants requires an enormous effort at the analytical chemistry level, designing assays that evidence physiological responses at concentrations with environmental relevance reduce this chemical effort and also increase the certainty of the results (Wagner et al., 2017). Thus, it is of great relevance to verify the nominal concentrations as well as the stability of the contaminants in the medium, as evidenced by our results.

Comparing the effect of EE2 at high concentrations (1000 ng/L) against unexposed animals allowed us to corroborate the results previously reported by Bowman et al. (2000) and Folmar et al. (2000) for *vtg* gene expression in adults of *C. variegatus* at the same concentration but for longer exposure. In this sense, the sensitivity of *zps* in larvae under the same conditions was verified. These results are supported by the fact that *vtg* and *zp* expression in fish is under the tight control of estrogens, mediated via estrogen-responsive elements (Schönemann et al., 2022a; Wagner et al., 2017).

Nonetheless, it is well known that the effects of EDs are not proportionally linked to the concentration of the contaminants (Pamplona-Silva et al., 2018). So, it is of great relevance to assess the information of the biomarkers at low concentrations and also at levels of environmental relevance. In this sense, our results (12.5–100 ng/L EE2) showed that in larvae, *vtgs*, *zps*, *cyp19a2*, *wap* and *cyp1a1* have a rapid response to EE2 exposure (within 24 h), reinforcing their high potential as biomarkers for short-term high-throughput screening, as postulated for *vtgs* by Hutchinson et al. (2006).

The use of a single biomarker is a reductionist approach that limits the information and knowledge generated with respect to the great complexity of the biological responses (Hultman et al., 2015). Therefore, looking for alterations in a group of related genes brings mechanistic advantages, even more taking into account that the emerging contaminants, and the complex mixtures of chemicals, that arrive in aquatic environments can generate different, more complex effects than the ones classically evaluated (Awuchi, 2019). In this sense, our *vtg* results evidence that the two isoforms exhibit opposite patterns of expression at a concentration of 100 ng/L EE2 (induction for *vtgc* and inhibition for *vtgab*). This result is in accordance with Yamaguchi et al. (2005), who established that the two *vtg* mRNAs exhibit different responses to the same concentration of estrogenic contaminants.

The responses to alterations in the trophic levels of steroid hormones are quick and mainly regulated by the avaliability of cholesterol. The main enzyme that provides cholesterol for this process is StAR (Stocco and Clark, 1996). For this reason, StAR has been previously proposed as a biomarker for EDs (Prucha et al., 2020; Ye et al., 2014). Even so, no significant alterations in the mRNA of *star* could be verified by our experiments.

In the particular case of aromatase, its functionality confers enormous potential as a biomarker for EDs. Although it is well described at the biochemical level, its role in fish brain sexual differentiation, as well as the dimorphic expression, is species-specific and so generating data on this line is highly valuable. The present results evidenced a significant increase in the larval relative expression of *cyp19a2* at 100 ng/L EE2, in the same way that has been reported for guppy (*Poecilia reticulata*) (Hallgren and Olsén, 2010) and rare minnow (*Gobiocypris rarus*) (Wang et al., 2010). In this sense, Hallgren and Olsén (2010) highlighted the impact of EDs in steroidogenic pathways in the early life stages and the consequent implications for adulthood. Exposure to EE2 in the earliest stages of development can generate permanent alterations which can even lead to sex reversal and consequently generate effects at the population level (Andersen et al., 2003; Hutchinson et al., 2006). Because of this, exposure of fish to environmentally relevant concentrations of EEDs (at pertinent life stages) is essential to effectively evaluate exposure/response relationships and produce realistic risk assessments (Jobling and Tyler, 2003). As an example, the exposure of pregnant females of the viviparous eelpout (*Zoarces viviparus*) has allowed verification that early exposure to EE2 (17.8 ng/L) may not only induce alterations in VTG levels but also a significant reduction of growth, liver somatic index and gonado-somatic index, and an increase in the ovarian sac index and in the number of individuals with malformations (Morthorst et al., 2016). In fathead minnow (*Pimephales promelas*), exposure to EE2 at different concentrations did not influence the hatching success of the embryos; however, concentrations above 16 ng/L reduced the survival of the larvae and generated deformities (anal protrusion, distended abdomens, upward curvature of the spine, hemorrhaging and problems in gonad differentiation) (Länge et al., 2001).

In recent publications of our group (Schönemann et al., 2022a,b), the proteomic analysis of males of *C. variegatus* (exposed to EE2 and other estrogenic substances) revealed strong induction of the synthesis of WAP as the most conspicuous result; additionally, these studies related WAP functionality as a protease inhibitor. Although WAP is not a classical biomarker for estrogenic substances, the advent of omics tools brings new and invaluable information regarding unexplored metabolic pathways that can be affected by pollutants. Though WAP functionality has been deeply explored in mammals, due to its implication in milk production, in fish, it remains unknown. WAP can be found in other groups of vertebrates and also in invertebrates, arranged as polymers in a variable number, which confers it with a versatile functionality (Smith, 2011). Our results confirm that the modulation of *wap* gene expression is a sensitive biomarker for exposure to low concentrations (50–100 ng/L EE2) of estrogenic substances. Even so, more studies must be performed to unveil the interaction of WAP and estrogen and to identify their functionality in fish.

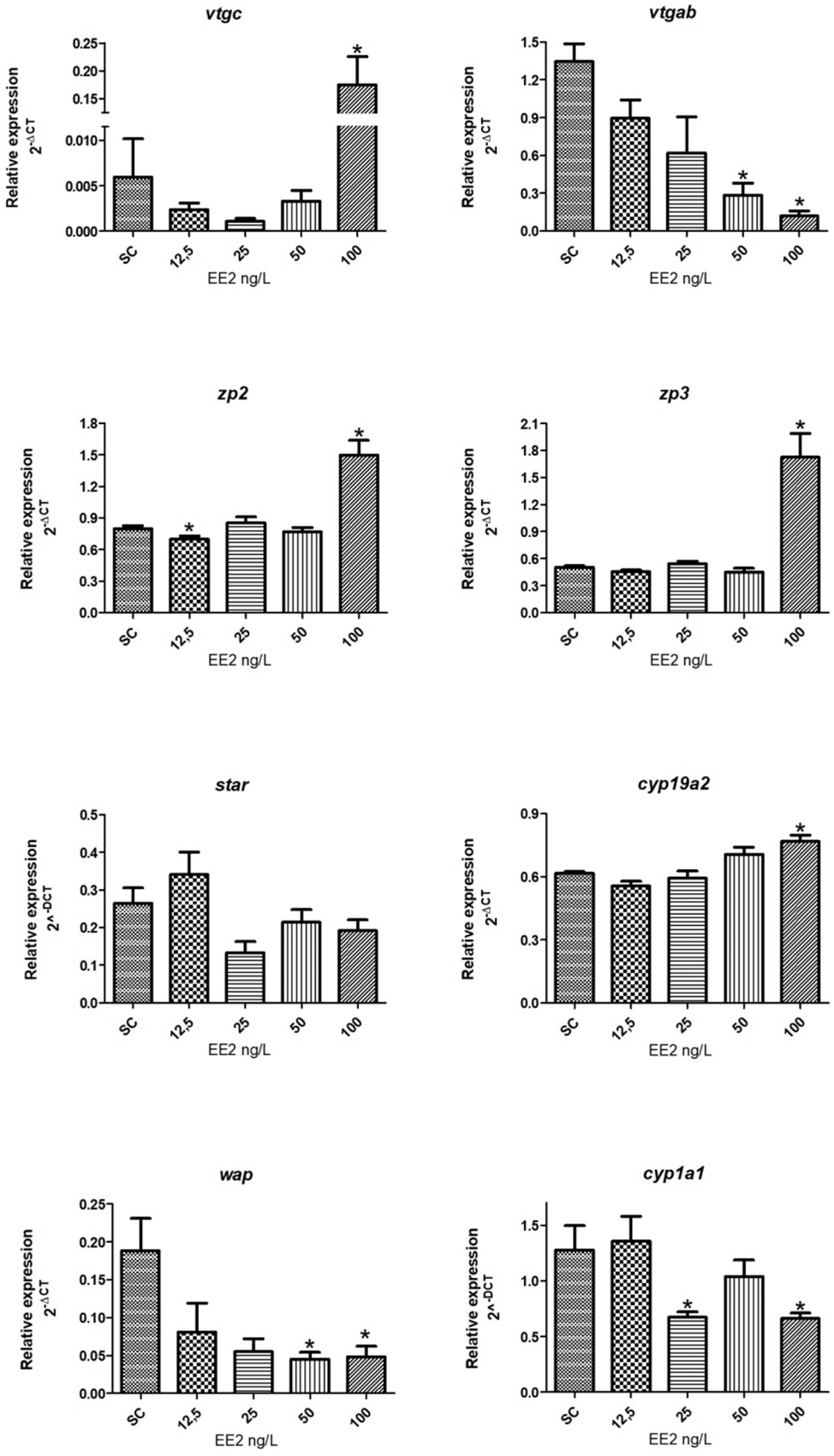

Fig. 2. Expression levels of larvae exposed to 12.5, 25, 50 and 100 ng/L EE2. Relative expression levels ($2^{-\Delta Ct}$) of vitellogenin C (*vtgc*), vitellogenin Ab (*vtgab*), zona pellucida 2 (*zp2*), zona pellucida 3 (*zp3*), steroidogenic acute regulatory protein (*star*), brain aromatase (*cyp19a2*), whey acidic protein-like (*wap*) and cytochrome P450 1 A (*cyp1a1*). TATA-box binding protein (*tbp*) and hypoxanthine-guanine phosphoribosyl transferase (hprt) were used as housekeeping genes. A Mann–Whitney paired test against the SC (DMSO > 0.005 v/v) was performed to assess statistical significance ($p < 0.05$). Significant differences are indicated by an asterisk ($n = 4–5$).

5. Conclusions

According to our results, we can affirm that 2 dph larvae of *C. variegatus* are a suitable in vivo model for rapid and cost-effective monitoring of EEDs in marine environments. Additionally, we can confirm that *wap* is a suitable biomarker for EEDs in *C. variegatus*, responding to environmentally relevant (50 ng/L) concentrations of EE2.

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CRediT authorship contribution statement

Sandra Isabel Moreno Abril: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Ana Olmos Pin:** Conceptualization, Methodology, Investigation. **Alexandre M. Schöneman:** Writing – review & editing, Visualization. **Marina Bellot:** Investigation, Methodology, Validation. **Cristian Gómez-Canela:** Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Project administration. **Ricardo Beiras:** Conceptualization, Methodology, Project administration, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: ECOTOX group reports financial support was provided by European Union through the European Regional Development Fund (ERDF), GLAUKOS Project. ECOTOX group reports article publishing charges was provided by CISUG University of Vigo. ECOTOX group reports equipment, drugs, or supplies was provided by Marine Research Centre (CIM) of the University of Vigo. ECOTOX group reports financial support was provided by European Union (Horizon 2020 research and innovation programme).

Data availability

No data was used for the research described in the article.

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