HHS Public Access

Author manuscript

Sci Total Environ. Author manuscript; available in PMC 2020 February 15.

Published in final edited form as:

Sci Total Environ. 2019 February 15; 651(Pt 2): 2424–2431. doi:10.1016/j.scitotenv.2018.10.079.

Evaluation of the estrogen receptor alpha as a possible target of bifenthrin effects in the estrogenic and dopaminergic signaling pathways in zebrafish embryos

Luísa Becker Bertotto[*], **Subham Dasgupta**, **Sara Vliet**, **Stacia Dudley**, **Jay Gan**, **David C. Volz**, and **Daniel Schlenk**

Department of Environmental Sciences, University of California, Riverside, CA 92521, USA

ABSTRACT

Bifenthrin (BF) is a pyrethroid insecticide widely used in urban and agricultural applications. Previous studies in embryos of zebrafish have shown that BF can affect estradiol biosynthesis and the dopaminergic system. To examine the role of the estrogen receptor (ER) in the endocrine effects of BF, embryos were exposed for 96 h to a mixture of 0.15 and 1.5 μg/L BF and an ER agonist (17α-ethynylestradiol – EE2) at 0.09 μg/L. Transcripts related to estrogenic (vitellogenin VTG) and dopaminergic (tyrosine hydroxylase (TH), dopamine receptor 1 (DR1), monoamine oxidase (MAO), and catechol-O-methyltransferase b (COMTb)) signaling pathways were investigated by qRT-PCR. Dopamine (DA) and its metabolites (homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC)) were also measured. There was a significant increase in VTG, DR1, MAO and COMTb mRNA levels and HVA-DA ratios within all zebrafish embryos exposed to EE2, including EE2 alone, 0.15 μg/L BF + EE2 and 1.5 μg/L BF + EE2. A significant decrease in homogenate concentrations of DA was observed within all zebrafish embryos exposed to EE2, which included EE2 alone, 0.15 μg/L BF + EE2 and 1.5 μg/L BF + EE2. Co-exposure of BF with EE2 failed to diminish estrogenic or dopaminergic signaling in embryos. Additionally, embryos with diminished ERα expression by morpholino injection were exposed to 0.15 μg/L BF, 1.5 μg/L BF and 0.09 μg/L EE2, with subsequent gene expression measurements. ERα knockdown did not prevent the effects of BF, indicating ERα may have a limited role in the estrogenic and dopaminergic effects caused by BF in zebrafish embryos.

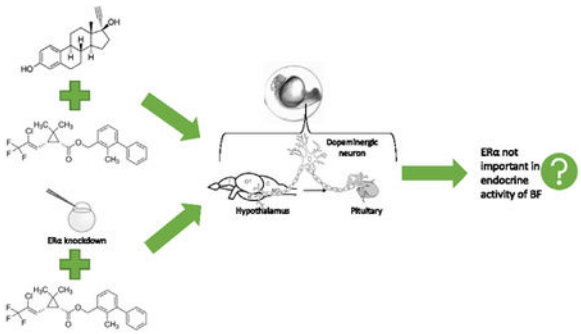
GRAPHICAL ABSTRACT

[*]Corresponding author at: 2258 Geology, 900 University Ave, Riverside, CA 92521, USA. lbeck006@ucr.edu (L.B. Bertotto).

The authors declare no conflict of interest

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.scitotenv.2018.10.079.



Keywords

Endocrine disruption; Pyrethroid pesticides; Aquatic toxicology; ERα knockdown; Developmental toxicology

1. Introduction

Pyrethroids are a family of synthetic insecticides used in urban and agricultural settings. Pyrethroids have low acute toxicity to mammals and relatively low persistence in the environment, when compared to other insecticides such as organochlorine derivatives. Consequently, pyrethroid use has increased in recent years (Weston et al., 2005; Spurlock & Lee, 2008). However, previous studies have detected pyrethroids in the water column after storm events in concentrations that cause estrogenic activity in aquatic organisms (Weston et al., 2005). One of the most frequently detected pyrethroids in surface waters is bifenthrin (BF), a type I synthetic pyrethroid, with concentrations varying from0.005 to 3.79 μg/L (Siepmann & Holm, 2000). A study that monitored BF in water from urban creeks in Sacramento, CA found concentrations ranging from 5 to 17 ng/L, with a maximum concentration of 73 ng/L (Weston et al., 2009).

The primary molecular target of BF is the voltage-gated sodium channel (VGSC) (Soderlund et al., 2002). Binding to VGSC leads to depolarization of the neuronal membrane, causing enhanced neurotransmitter release, and potentially to increased mortality from acute neurotoxicity (Van den Bercken & Vijverberg, 1980). BF also affects estrogen signaling pathways, demonstrating both estrogenic and anti-estrogenic activity (reviewed in Brander et al., 2016a). Brander et al. (2012) exposed cell-lines containing both estrogen receptor alpha (ERα) and beta (ERβ) to concentrations of BF ranging from 1 to 100 ng/L and observed anti-estrogenic activity. *In vivo*, BF decreased reproductive output, choriogenin, and ERα mRNA levels in adult inland silverside (*Menidia beryllina*) (Brander et al., 2016b). In contrast, BF caused estrogenic activity through induction of vitellogenin (VTG) expression in male Japanese medaka (*Oryzias latipes*) (Wang et al., 2007) and rainbow trout (*Oncorhynchus mykiss*) (Crago & Schlenk, 2015). Induction of the zona radiata-protein choriogenin was also observed in juvenile inland silverside exposed to concentrations as low as 1 ng/L of BF (*M. beryllina*) (Brander et al. 2012).

BF also affects the dopaminergic signaling pathway. Crago & Schlenk (2015) showed decreased levels of DR2A mRNA in rainbow trout (*O. mykiss*) after exposure to 0.15 and

1.5 μg/L of BF for 2 weeks. In adult male Wistar rats, BF treatment for 30 days at 3.5 and 7 mg kg^{1} doses significantly reduced levels of dopamine (DA) and 3,4-dihydroxyphenyl acetic acid (DOPAC), but significantly increased levels of homovanillic acid (HVA) in the frontal cortex, hippocampus and corpus striatum of the rats (Syed et al., 2018). Additionally, after administration of 5 to 20 mg/kg BF, mice showed decreased levels of tyrosine hydroxylase (TH) (Han et al., 2017).

Estrogenic and dopaminergic signaling pathways are intimately associated in vertebrates, through the hypothalamus-pituitary-gonadal (HPG) axis (Zohar et al., 2010). Dopaminergic neurons are activated by increases in E2 concentrations, further inhibiting release of gonadotropin releasing hormone (GnRH), and gonadotropin hormones (GtH), both synthesized by the hypothalamus and the pituitary, respectively (Zohar et al., 2010), reducing levels of E2. Fontaine et al. (2013) showed that, when sexually regressed female zebrafish were co-exposed with a DA receptor 2 (DR2) antagonist (domperidone) and a GnRH agonist, an increase in LHβ mRNA transcript levels, gonadosomatic index and ovarian vitellogenesis was observed. These results indicate that only removal of the inhibitory effect of dopamine allows GnRH to exert its stimulatory function, showing that DA can consequently modulate E2 levels in zebrafish. Dopamine is synthesized from tyrosine by TH which is the rate-limiting step enzyme in DA biosynthesis. Dopamine is catabolized to DOPAC by monoamino oxidase (MAO), and then to HVA by catechol-*O*-methyltransferase (COMT), which is targeted for excretion (Ashcroft, 1969). In the brain, DA activates at least two metabotropic receptor families, dopamine receptor 1-like (DR1) and dopamine receptor 2-like (DR2) (Ashcroft, 1969). Estrogenic signaling primarily occurs through the activation of nuclear ERs. In contrast to GnRH neurons, the dopaminergic neurons of the preoptic area express ERα in rainbow trout (*O. mykiss*) (Linard et al., 1995) and in other species (Zohar et al., 2010), demonstrating crosstalk between each signaling pathway.

Our previous study showed anti-estrogenic effects of BF in embryos of zebrafish with significant decreases in mRNA levels of TH and DR1, as well as significant decreases in HVA levels. VTG gene expression and E2 levels were also diminished although not significantly (Bertotto et al., 2018). The objective of the present study was to investigate the role of the estrogen receptor in the estrogenic and dopaminergic pathways impacted by BF in zebrafish embryos. To investigate if ER is the target of BF anti-estrogenic effects, we co-exposed BF with a known potent ER agonist, 17α-ethynylestradiol (EE2). We hypothesized that EE2 will reverse BF antiestrogenic and corresponding DA effects in embryos. To further evaluate the role of ER in the endocrine toxicity of BF, we also used a morpholino to knockdown ERα expression, which would also mimic anti-estrogenic responses observed with BF in zebrafish embryos and modulate DA metabolism. Overall, neither experimental system indicated a significant role of ERα in the endocrine effects of BF in zebrafish embryos and suggests other targets may be more important in the endocrine effects of BF.

2. Materials and methods

2.1. Chemicals

A racemic mix of BF isomers (98% purity) was purchased from Chem Service, Inc. Ethinylestradiol (EE2 ≥ 98% purity) was purchased from Sigma Aldrich. Ethanol (Decon Laboratories Inc.) was 200 proof. A stock solution of BF in 100% ethanol (EtOH) was prepared, and diluted solutions in embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$, in pH 7) were made at every exposure day. Methanol (MeOH) and acetonitrile (MeCN), both OPTIMA™ grade, were from Fisher Scientific. Deionized water of 18.2 Ω was prepared with an Milli-Q water purification system (Millipore). Formic acid (FA - OPTIMA™ grade) was obtained from Fisher Scientific. Dopamine hydrochloride (DA) (1 mg/mL in MeOH), and neat solid standards of 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were from Sigma Aldrich. Standard of isotopically labeled compound used as internal standard (IS), dopamine-1,1,2,2-d4 hydrochloride (DA-d4), was from Sigma Aldrich. All standards were >98% purity. Stock solutions of DOPAC, HVA, and DA-d4 were prepared at 1 mg/mL in methanol, and stored at −80 °C. The concentrations were not corrected for purity or salt content. Solutions of working standards and internal standard (IS) were prepared in MeOH. Calibration standards were prepared in 0.1% formic acid in DI water. All the solutions were kept in −80 °C.

2.2. Animal handling

Adult wildtype (strain 5D) zebrafish were maintained on a 14-h:10-h light:dark cycle within a 242-tank recirculating system (Aquaneering, Inc.) containing conditioned reverse osmosis water, and fed *ad libitum* twice per day with GEMMA Micro 300 diet (Skretting USA). Dissolved oxygen, pH, conductivity, salinity, alkalinity, and temperature within recirculating water were maintained at 4 to 6 mg/L, 7.0 to 7.5, 950 to 1000 μS, <1 ppt, 50 to100 mg/L, and 28 °C, respectively; in addition, levels of ammonia, nitrite, and nitrate within recirculating water were consistently below 0.1 mg/L, 0.05 mg/L, and 2 mg/L, respectively. Adult females and males were bred directly on-system using in-tank breeding traps suspended within 3 L tanks. For all experiments described in sections “Bifenthrin + 17α-ethynylestradiol co-exposure” and “ERα knockdown experiments”, newly fertilized eggs were staged according to previously described methods (Kimmel et al., 1995). All fish were handled and treated in accordance with approved Institutional Animal Care and Use Committee protocols at the University of California, Riverside (animal use protocols #20150035 and #20130005).

2.3. Bifenthrin + 17α-ethynylestradiol co-exposure

Twenty viable non-dechorionated embryos (3 h post-fertilization (hpf)) were placed in 60 × 15 mm Petri dishes containing 10 mL of the working solution. Treatments consisted of vehicle control (0.2% EtOH), nominal concentrations of 0.09 μg/L (0.32 nM) EE2, 0.15 μg/L(0.35 nM), 1.5 μg/L (3.5 nM) BF and co-exposures of 0.15 μg/L BF and0.09 μg/L EE2 and 1.5 μg/L BF and 0.09 μg/L EE2. The EE2 concentration was the maximum tolerated dose on a dose-response curve that did not significantly show mortality nor increased deformities (Supplemental Information - Fig. 1). Actual BF concentrations were not measured during exposure. A similar study in our lab by Bertotto et al. (2018) using the same 96 h treatment

regimen of BF at the same nominal concentrations (0.15 μg/L and 1.5 μg/L) found that initial measured concentration of BF were 0.34 μg/L for the 0.15 μg/L and 3.1 μg/L for the 1.5 μg/L dose. Differences in nominal concentrations are likely due to sample handling, or the process of extraction. For qPCR assays after BF co-exposure with EE2, each replicate pool consisted of 20 embryos with 4 to 7 replicate pools per treatment. For qPCR assays after ERα knockdown, each replicate pool consisted of 20 embryos with 4 to 7 replicate pools per treatment. For measurements of DA and its metabolites, 200 embryos (20 embryos per petri dish) were used for each replicate pool, with 3 replicates pool per treatment group. Following exposure of 96 h, fish were euthanized in ice and flash frozen in liquid nitrogen, and then kept in −80 °C until assays were performed.

2.4. ERα knockdown experiments

Morpholino (MO) antisense oligos were synthesized and obtained from Gene Tools, Inc. (Philomath, OR). A translational-blocking MO (zfesr1-MO: 5′-ACGAAGGTTCCTCCAGGGCTTCTCT-3′) based on the zebrafish ERα mRNA sequence (GenBank Accession ID: AF349412.1) was used for all experiments described below. Additionally, Gene Tools' standard negative control MO (nc-tMO:5′-CCTCTTACCTCAGTTA CAATTTATA-3′) was used to account for potential nontarget MO toxicity and to use as a base for the maximum tolerated concentration of the morpholino. Negative control-tMO were tagged with fluorescein at the 3′ end by Gene Tools to confirm MO delivery to embryos following injection, and a zebrafish-specific, fluorescein-tagged chordin MO (*chd*-MO: 5′-ATCCACAGCAGCCCCTCCATCATCC-3′) was used as a positive control during the injection and exposure experiments. Molecular-biology grade (MBG) water injections were used for the gene expression experiments. MO injection stocks (1 mM) were prepared by resuspending lyophilized MOs in MBG water, and stocks were stored at room temperature in the dark.

On the same day as injections, working solutions of nc-MOs, *chd*-MOs, and zfesr1-MOs were diluted to 62.5 μM, 125 μM and 62.5 μM in MBG water, respectively. Concentrations of nc-MOs and zfesr1-MOs chosen did not cause significant mortality nor deformities (data not shown). Newly fertilized (one-to-eight-cell stage) wild-type (5D) zebrafish embryos were microinjected with MOs or water (~3 nL per embryo) using a motorized Eppendorf Injectman NI2 and FemtoJet 4× similar to previously described protocols (McGee et al., 2013). At 3 hpf, embryos injected with water or zfesr1-MO were exposed to vehicle control (0.2% EtOH), a positive control of 0.09 μg/L EE2, 0.15 μg/L BF and 1.5 μg/L BF, as described above. Approximately 4 h after injection, embryos were evaluated using a Leica MZ10 F stereomicroscope equipped with a DMC2900 camera and a GFP filter cube to confirm nc-MO and *chd*-MO delivery. Embryos that did not have MO delivery confirmed were removed from exposure. Final pool (3–5 replicates) of zebrafish embryos was ~15 embryos. Following exposure of 96 h, fish were euthanized in ice and flash frozen in liquid nitrogen, and then kept at −80 °C until assays were performed.

2.5. RNA isolation, cDNA synthesis and RT qPCR

Expression of genes related to the dopaminergic and estrogenic pathways after exposure to BF and EE2, and after ERα knockdown was measured. Total mRNA was isolated from

embryos using the Lipid Tissue RNeasy kit (Qiagen) following the manufacturer's instructions. mRNA quantity and quality were determined using the ND-1000 (NanoDrop). mRNA (1 μg) was converted to cDNA using the Reverse Transcription System (Promega Corporation) according to the manufacturer's instructions. The primers used in this study (Supplemental Information – Table 1) were designed using IDT DNA PrimerQuest software. The exception was the vitellogenin set of primers, which was retrieved from Chen et al. (2010). Efficiency was calculated using PCR Miner (Zhao & Fernald, 2005). EF1α was used as the housekeeping gene, since its expression is stable throughout development (McCurley & Callard, 2008). qPCR was carried out with the iTaq Universal One-step RT-PCR kit with SYBR Green (Bio-Rad), on a CFX Connect thermos cycler (Bio-Rad). The samples were denatured, and the polymerase activated at 95 °C for 5 min, then 40 cycles of 10 s at 95 °C and 30 s of 55 °C. Samples were subjected to melting curve analysis from 54 to 95 °C in 0.5 °C increments with continuous fluorescence measurement. qPCR was analyzed according to Schmittgen and Livak (2008) and fold change was calculated against the vehicle control.

2.6. Ultra-performance liquid chromatography

To measure DA and its metabolites samples were stored in −80 °C prior to analysis, and on ice during handling and extraction. Sample preparation was based on Tareke et al. (2007) with slight modifications. Approximately 100 mg of homogenized zebrafish tissues (whole body) were placed into a 2 mL centrifuge tube, internal standard (IS) was added to yield 3 μg DA-d4 per mL of final extract, for recovery measurements. Ice cold 0.1% formic acid in water (270 μL) was added, samples were incubated in ice for 30 min, and the extraction was performed using a homogenizer (Fisherbrand) by vigorous blending. The tubes were centrifuged for 5 min at 12,000g at 4 °C. This process was repeat twice, however IS was not added to the samples on the second time. An aliquot (600 μL) of the extract was subjected to solid phase extraction (SPE) with Strata X polymeric reverse-phase cartridges (33 μm, 60 mg, 3 mL - Phenomenex), as described in Tareke et al. (2007). The SPE cartridges were conditioned with 1 mL of 0.1% formic acid in MeCN, followed by 1 mL of 0.1% formic acid in MeOH, and 1 mL of0.1% formic acid in water. After adding 600 μL of the extracts, analytes were eluted with 3 mL of 0.1% formic acid in MeCN/MeOH (1:1, v/v). The resulting extracts were evaporated to dryness with nitrogen gas (no heat) and reconstituted in 400 μL of 0.1% formic acid in water. Prior to analysis, samples were filtered using a PP 0.22 μm syringe filter (Tisch Scientific). After vortexing for 10 s, the extracts were transferred to autosampler vial inserts for UPLC analysis.

A Waters ACQUITY ultra-performance liquid chromatography (UPLC) combined with a Waters Micromass Triple Quadrupole mass spectrometer (qQq) equipped with an electrospray ionization (ESI) interface (Waters, Milford, MA) was used for analysis. Separation was achieved using an ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.7 μm, Waters.) at 40 °C. A binary gradient system was used to separate analytes comprising of mobile phase A, DI water (18 Ω) acidified using 0.1% FA, and mobile phase B composed of MeOH/MeCN (1:1, v/v) acidified using 0.1% FA. The solvent gradient program, in terms of mobile phase A, was as follows: initial condition began with 95% for 1 min when it was decreased linearly to 40% for 1 min, it was further decreased to 10% for 1.5

min then decreased linearly to 0% for 0.5 min, then increased linearly to 10% for 0.5 min, finally it was increased 95%. The mobile phase was then left to equilibrate for 0.5 min for a total run time of 5.50 min. The flow rate was 0.3 mL min^{-1} and the injection volume was set to 5 μL. Mass data was acquired using Intellistart® (Waters) in the multiple reactions monitoring (MRM) mode and product ion scan in the positive ESI mode. The specific instrument settings were as follows: capillary source voltage 3.00 kV, dwell time 0.028 s, source temperature 150 °C, desolvation temperature 600 °C, desolvation gas 1200 L h^{-1} and cone gas 150 L h^{-1} the collision gas was Argon 99.9% pure. Cone voltage and collision voltage were generated using IntelliStart software (Waters) and were as follows: 50 (V), and 20 (V). Quantification ions were also generated using IntelliStart software and are shown in Table 1. Individual compound peaks were detected and integrated using TargetLynx XS software.

2.7. Statistical analysis

Statistical analysis was performed using RStudio (version0.98.1091). Normality and homogeneity of variances were evaluated by Shapiro and Bartlett tests, respectively. All data were tested for extreme outliers using the Grubb's test. Two-way ANOVA were performed to evaluate statistical differences between control and treatment groups. Any non-normal data was log transformed. If interaction was significant after two-way ANOVA, Tukey HSD was performed. Survival and hatching was evaluated by generalized linear model. Pairwise comparison between zebrafish control and exposed to EE2 was performed to evaluate positive control effectiveness. Statistical significance was determined at $\alpha \leq 0.05$.

3. Results

3.1. Expression of genes within estrogenic and dopaminergic signaling pathways

There was a significant increase in VTG, DR1, MAO and COMTb mRNA levels within all zebrafish embryos exposed to EE2, which included exposures to EE2 alone, 0.15 μg/L BF + EE2 and 1.5 μg/L BF + EE2 (1.9-fold, *p*-value = 0.025, Fig. 1; 2.35-fold, *p*-value = 0.013, Fig. 2; 1.48-fold, *p*-value = 0.039, Fig. 3B; and 1.55-fold, *p*-value =0.029, Fig. 3C respectively, 4–7 replicates) when compared to embryos that were not exposed to EE2 (vehicle control, 0.15 μg/L BF alone and1.5 μg/L BF alone). There was no significant interaction between BF and EE2, after zebrafish embryos were co-exposed to BF and EE2 (Figs. 1–3, 4–7 replicates). There was a trend towards a dose dependent increase in mRNA levels of TH, COMTb and MAO after exposure to both concentrations of BF (Figs. 3, 4–7 replicates). Pairwise comparisons showed significant 4.2 and 3.6-fold increase in VTG and DR1 mRNA levels in embryos exposed to EE2 alone, compared to vehicle control embryos (*p*-value = 0.0008 and *p*-value = 0.009) (Figs. 1 and 2, 4–7 replicates).

3.2. Dopamine and metabolites concentrations in embryos

A modest but significant 1.73-fold decrease in homogenate concentrations of DA was observed within all zebrafish embryos exposed to EE2, which included exposures to EE2 alone, and in mixtures with BF (*p*-value = 0.005, Fig. 4A, 3 replicates) when compared to embryos that were exposed to BF, but not exposed to EE2. In addition, there was a significant 2.43-fold increase in HVA-DA ratios within all zebrafish embryos exposed to

EE2 alone and in mixtures with BF (p-value = 0.003, Fig. 4B, 3 replicates). There were no significant effects of BF, EE2 or a combination of both in the remaining metabolite levels and ratios (Supplemental Information - Figs. 2 and 3, 3 replicates). Recoveries of DA and metabolites ranged from 70 to 112%.

3.3. ERα knockdown followed by bifenthrin exposure

There was a significant 10.9-fold increase in MAO mRNA levels when water injected control embryos were exposed to EE2, compared to the vehicle control ERα knockdown embryos (p-value = 0.005). ERα knockdown embryos exposed to EE2 also had a significant 2.64-fold decrease in MAO mRNA levels when compared to the vehicle control injected embryos (p-value = 0.04) and a significant 11.7-fold decrease when compared to injected control embryos exposed to EE2 (p-value =0.0006, Fig. 7B, 3–5 replicates). Expression of COMTb mRNA was significantly higher with a 10.4-fold increase in injected control embryos exposed to EE2, when compared to vehicle control ERα knockdown embryos (p-value = 0.007). Expression of COMTb mRNA was also significantly higher with a 10.9-fold increase in injected control embryos exposed to EE2, when compared to ERα knockdown embryos exposed to EE2 (p-value = 0.002, Fig. 7C, 3–5 replicates). Injection (control or ERα morpholino) was a significant factor for VTG and DR1 mRNA levels (p-value = 0.002, Fig. 5 and p-value = 0.01, Fig. 6, respectively, 3–5 replicates). There were no significant differences nor a significant interaction between exposure and injection factors in relation to TH mRNA levels (Fig. 7A, 3–5 replicates).

4. Discussion

The mechanism of action for endocrine disruption of BF in zebrafish embryos at low (ng/L) concentrations is still uncertain (Crago & Schlenk, 2015; Bertotto et al., 2018). Bifenthrin has an estrogenic response in juveniles of zebrafish and silverside but is an antiestrogenic chemical in *in vitro* assays and embryos of zebrafish (Brander et al., 2012; Bertotto et al., 2018). In this study, the role of ER was evaluated to better understand this mechanism.

Since we demonstrated that BF had antiestrogenic effects at the embryonic stage (exposures starting at 3 hpf) of zebrafish (Bertotto et al., 2018), we sought to co-expose embryos to BF and an ER agonist, EE2, to determine if previously observed anti-estrogenic responses could be reversed. A dose of EE2 was used that provided a statistically significant estrogenic effect. However, BF and EE2 have very different binding affinities. Zhao et al. (2014) performed a molecular docking study with BF enantiomers with the human ERα, where *S*-BF and *R*-BF showed a binding score of −116.53 kJ/mol and −100.84 kJ/mol, respectively. Molecular docking was also performed with E2 and EE2 to human and rainbow trout ERα (Shyu et al., 2011). In this study, the authors reported binding affinities in pKd, which they calculated from the binding scores produced by the molecular docking simulations (in kJ/mol). Converting the scores giving by Zhao et al. (2014) using the same calculations, BF binding affinity to the human ERα is almost 300 times smaller than EE2, showing that BF is a weak ER ligand. The lower potential binding affinity may explain the inability of BF to reduce the ER activity of EE2. Whether this was due to the limited activation of ERα after ligand binding or other downstream effects requires additional studies.

Our results showed that EE2 exposure increased expression of VTG, after zebrafish embryos were exposed to EE2 alone or in mixtures of BF and EE2. EE2 is a model ER agonist (Ankley et al., 2010) used in other studies to antagonize antiestrogenic effects of various chemicals, like tamoxifen, fulvestrant (Hoffmann & Kloas, 2012), methyl-piperidinopyrazole (MPP) and pyrazolo 1,5-a pyrimidine (PHTPP) (Notch & Mayer, 2011), in concentrations varying from 0.001 to 0.03 μg/L. Since there was a significant difference in VTG mRNA between vehicle control and EE2-exposed zebrafish embryos without BF, EE2 appears to regulate E2-controlled genes in embryos and indicates that the ER signaling pathway is still conserved in this developmental stage and animal model.

As with VTG expression, only when animals were exposed to EE2 with or without BF was a significant change of DR1 mRNA observed. Previously, the endogenous ER ligand, E2, was shown to enhance DR1 activity in primary cultures of anterior pituitary cells from female rats (Maus et al., 1989). A two-fold increase in the DA-DR1 induced stimulation of adenylate cyclase was observed after 24 h exposure to 10^{-9} M E2 (Maus et al., 1989). Lévesque et al. (1989) showed that ovariectomy in female rats decreased striatal DR1 density by 17% compared to intact female rats, demonstrating that DR1 can be controlled by gonadal hormones, including E2, and that this relationship is conserved in zebrafish early developmental stages. Consequently, the lack of change when EE2 was combined with BF indicated ER may not contribute to the effects of BF.

Since a significant decrease in expression of TH and levels of HVA was observed in the previous study (Bertotto et al., 2018), we sought to analyze mRNA levels of MAO and COMTb, two important enzymes in the metabolism of DA. A trend towards dose dependent increase in mRNA levels of TH, MAO and COMTb, and increase in HVA levels after exposure to both concentrations of BF was observed indicating the non-significant increases of MAO and COMTb expression may be responsible for the trend towards increases in HVA levels. BF had significant effects in neurotransmitters and proteins involved in the dopaminergic pathway of rats treated with concentrations ranging from 5 to 40 mg/kg. HVA was significantly increased in the frontal cortex, hippo-campus and corpus striatum of the BF-treated rats (Syed et al., 2018). Additionally, mice showed decreased levels of the TH protein after administration of 4 doses of BF, ranging from 5 to 20 mg/kg (Han et al., 2017), in agreement with our previous results in zebrafish embryos (Bertotto et al., 2018). The lack of response observed in the current study may be due to its significantly weaker estrogenic potency at the ER relative to EE2 (Zhao et al., 2014).

MAO and COMTb mRNA levels, DA levels and the ratio of DA with HVA were altered in the same pattern when zebrafish were exposed to the ER agonist EE2 alone or in combination with BF. These data suggest ER activation had no impacts on the effects of BF on DA metabolism. Physiological (10^{-9}–10^{-7} M) concentrations of estradiol (E2) were shown to down-regulate steady-state 1.3-kb COMT mRNA levels in MCF-7 cells (Xie et al., 1999), as well as significantly reduce COMT protein and activity in MCF-7 cells (Jiang et al., 2003). The ER antagonist ICI 182780 blocked these estrogenic effects, and the study concluded that E2 decreased COMT activity through down-regulation of its mRNA and protein expression *via* ER interaction. Another study (Kumar et al., 2011) assessed changes in MAO activity in brains of female rats of 3, 12 and 24 months old, and whether these

possible changes are affected by administration of E2 (0.1 μg/g body weight for 1 month). E2 significantly decreased MAO activity in all age groups of rats, which contradict our results. Since EE2 exposures significantly increased MAO and COMTb gene expression, as well as decreased levels of DA and increased HVA-DA ratios, ER activation may have increased DA catabolism. However, since HVA and DOPAC levels were not significantly altered by EE2, DA may be converted to other metabolites not targeted for detection in this study. Possibilities include dopamine-3-*O*-sulfate, dopamine-4-*O*-glucuronide or norepinephrine and epinephrine (reviewed by Meiser et al., 2013).

Co-exposure of embryos to EE2 and BF failed to alter expression of VTG, DR1, MAO and COMTb, as well as levels of DA and ratios of DA/HVA. While this suggests ERα may not be a significant contributor to the endocrine effects of BF, co-exposure may have had significant effects on the pharmacokinetics of BF. Thus, a second manipulation of the ERα pathway was evaluated in our study. ERα knockdown experiments were employed and showed that morpholino injection of ERα was a significant factor in reducing the estrogenic response of VTG mRNA expression in embryos. Knockdown experiments utilizing the morpholino technique are widely used and well established as a mechanistic tool to evaluate the role of a protein in the toxicity of a chemical (McGee et al., 2013; Dasgupta et al. 2017). Water injected control embryos exposed to EE2 had a 5.7-fold increase in VTG expression, whereas ERα knockdown embryos exposed to EE2 had 2.12-fold decrease in VTG mRNA levels, when compared to vehicle control injected embryos, showing that our knockdown was effective at weakening the estrogenic effect of EE2 in zebrafish embryos. However, it should be noted that ERα knockdown may have had other effects on unknown targets that could also affect VTG expression. Nevertheless, given the lack of developmental abnormalities observed in control injections and plethora of studies (Hu et al., 2014; Griffin et al., 2013; McGee et al., 2013; Dasgupta et al. 2017) that have used this model in the same capacity, it is unlikely that non-target effects could explain the down regulation of VTG in morpholino treatments.

ERα morpholino treatment also affected DR1 gene expression. Control injected embryos exposed to EE2 had a 3.4-fold increase in DR1 expression, while ERα knockdown embryos exposed to EE2 had 2-fold decrease in DR1 mRNA levels, when compared to water injected controls. These results are in accordance with previously above-mentioned studies, where E2 increased DR1 activity (Maus et al., 1989) and ovariectomy (which decreases endogenous levels of E2) decreased striatal DR1 density (Lévesque et al., 1989). Again, these results are consistent with an effective ERα knockdown in our study.

Somewhat of a surprise was the effect of ERα knockdown on MAO gene expression. ERα knockdown embryos exposed to EE2 had a significant decrease in MAO gene expression when compared to the vehicle control injected embryos, and when compared to injected control embryos exposed to EE2. Gene expression of COMTb was significantly increased in vehicle control injected embryos exposed to EE2, compared to ERα knockdown embryo exposed to vehicle control and EE2. Although BF activities were not modified by ERα knockdown, MAO and COMTb expression were altered by EE2 through ERα activation. The relationship of ERα and VTG mRNA levels is well established in adult aquatic vertebrates (reviewed in Nagahama, 2002), but not for the expression of MAO and COMTb.

The human COMT gene was shown to have a putative ERE (Xie et al., 1999), and it can metabolize E2 as well (Dawling et al., 2001). MAO activity was also shown to be controlled by E2 in rats (Kumar et al., 2011). Therefore, regulation of MAO and COMTb gene expression by an ER agonist seems to be conserved in zebrafish early life stages, where response to EE2 exposure was significantly lost after ERα knockdown.

Contrary to our original hypothesis of ER involvement in the endocrine toxicity of BF, there was no difference in gene expression in ERα knockdown embryos exposed to BF. Several other studies have suggested that the effects of BF as an estrogenic/antiestrogenic compound might be due to its weak activation of the ER Although molecular docking studies indicated interaction between BF enantiomers and human ERα and ERβ, demonstrating that both enantiomers fit in the ligand binding domain for both receptor subtypes, the binding affinities were significantly less than E2 (Zhao et al., 2014). An alternative explanation may be related to metabolism. Previous research (DeGroot & Brander, 2014) has shown that the metabolite 4-hydroxy-bifenthrin is more estrogenic than its parent compound; however, this metabolite has yet to be identified in fish metabolism studies. It should also be noted that zebrafish express 3 ER subtypes: a single ERα and two ERβ, 1 and 2. In zebrafish embryos, ERα mRNA levels are higher than the other two ER subtypes, with an increase of 60-fold at 96 hpf in comparison to the <3 hpf stage. Similarly, ERβ1 mRNA expression increases 10-fold after 96 hpf in comparison to the <3 hpf stage, but ERβ2 expression is diminished during development with a small increase in expression at 96 and 120 hpf (Chandrasekar et al., 2010). Consistent with our results, knockdown experiments, with ERα and ERβ1 blocked E2 (0.1 μM) induction of VTG and ERα mRNAs (Griffin et al., 2013). However, knockdown of ERβ2 had no effect on the induction of VTG and ERα mRNAs by E2. Furthermore, Hu et al. (2014) performed loss-of-function analysis to identify a subtype of estrogen receptor that affects migration of primordial germ cells (PGC) in zebrafish after EE2 exposure. Only ERβ2 knockdown had an effect in EE2 disruption of PGC migration. Therefore, different ER subtypes could be involved in signaling pathways within this animal model. In reporter studies that expressed the 3 receptors in HeLa-cells, exposures to estrone, estriol and genistein elicited greater activity in the ERα construct compared to the other sub-types (Pinto et al., 2014). In contrast, Ferutinin elicited greater activity in the ERβ1 construct and Benzophenone 2 elicited greater activity in the ERβ2 cell-line (Pinto et al. 2014). In this study, Pinto et al. (2014) also demonstrated EE2 equal agonistic activity on all 3 zebrafish ER sub-types. Consequently, previous studies suggest that while ERα does not appear to play a substantial role in BF endocrine effects in zebrafish embryos, other ER subtypes may be involved, since exposure to EE2 seems to have different effects with different ER subtypes (Hu et al. 2014). Another possible target for BF effects could be the G protein-coupled estrogen receptor (GPER), a membrane-bound receptor, which expression was shown to be downregulated after juvenile Inland silverside (*M. beryllina*) was exposed to 0.5 ng/L BF (Brander et al., 2016b).

In conclusion, exposure to EE2 significantly altered gene expression in zebrafish embryos, but there was no significant interaction with BF. ERα knockdown did not alter the effects of BF in embryos of zebrafish, showing that ERα might not be a significant target of BF endocrine activity. ERα knockdown alter the effects of EE2 on DA metabolism. Further

analysis of other differentially expressed genes coupled with endocrine responses is needed to help assess other potential sublethal targets for BF endocrine activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

This work was supported by CAPES Foundation, Ministry of Education of Brazil, through the program Science Without Borders (grant n. BEX 99999.013554/2013–01); the AES/USDA Resource Allocation Program at the University of California, Riverside; the NRSA T32 Training Program [T32ES018827] to S.M.V.; and the USDA National Institute of Food and Agriculture Hatch Project [1009609] to D.C.V.

References

Ankley GT, Jensen KM, Kahl MD, Durhan EJ, Makynen EA, Cavallin JE, Martinovic D, Wehmas LC, Mueller ND, Villeneuve DL, 2010 Use of chemical mixtures to differentiate mechanisms of endocrine action in a small fish model. Aquat. Toxicol 99, 389–396. https://doi.org/10.1016/j.aquatox.2010.05.020. [PubMed: 20573408]

Ashcroft GW, 1969 Amine metabolism in brain. Proc. R. Soc. Med 62, 1099–1101. [PubMed: 5356862]

Bertotto LB, Richards J, Gan J, Volz DC, Schlenk D, 2018 Effects of bifenthrin exposure on the estrogenic and dopaminergic pathways in zebrafish embryos and juveniles. Environ. Toxicol. Chem 37, 236–246. https://doi.org/10.1002/etc.3951. [PubMed: 28815728]

Brander SM, He G, Smalling KL, Denison MS, Cherr GN, 2012 The in vivo estrogenic and in vitro anti-estrogenic activity of permethrin and bifenthrin. Environ. Toxicol. Chem 31, 2848–2855. https://doi.org/10.1002/etc.2019. [PubMed: 23007834]

Brander SM, Gabler MK, Fowler NL, Connon RE, Schlenk D, 2016a Pyrethroid pesticides as endocrine disruptors: molecular mechanisms in vertebrates with a focus on fishes. Environ. Sci. Technol 50, 8977–8992. https://doi.org/10.1021/acs.est.6b02253. [PubMed: 27464030]

Brander SM, Jeffries KM, Cole BJ, DeCourten BM, White JW, Hasenbein S, Nann FA, Connon RE, 2016b Transcriptomic changes underlie altered egg protein production and reduced fecundity in an estuarine model fish exposed to bifenthrin. Aquat. Toxicol 174, 247–260. https://doi.org/10.1016/j.aquatox.2016.02.014. [PubMed: 26975043]

Chandrasekar G, Archer A, Gustafsson JÅ, Lendahl MA, 2010 Levels of 17β-estradiol receptors expressed in embryonic and adult zebrafish following in vivo treatment of natural or synthetic ligands. PLoS One 5, e9678 https://doi.org/10.1371/journal.pone.0009678. [PubMed: 20300630]

Chen TH, Cheng YM, Cheng JO, Chou CT, Hsiao YC, Ko FC, 2010 Growth and transcriptional effect of dietary 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47) exposure in developing zebrafish (Danio rerio). Ecotoxicol. Environ. Saf 73, 377–383. [PubMed: 20074802]

Crago J, Schlenk D, 2015 The effect of bifenthrin on the dopaminergic pathway in juvenile rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol 162, 66–72. https://doi.org/10.1016/j.aquatox.2015.03.005. [PubMed: 25781393]

Dasgupta S, Vliet SM, Kupsco A, Leet JK, Altomare D, Volz DC, 2017 Tris (1,3-dichloro-2-propyl) phosphate disrupts dorsoventral patterning in zebrafish embryos. PeerJ 5, e4156 https://doi.org/10.7717/peerj.4156. [PubMed: 29259843]

Dawling S, Roodi N, Mernaugh RL, Wang X, Parl FF, 2001 Catechol-O-methyltransferase (COMT)-mediated metabolism of catechol estrogens: comparison of wild-type and variant COMT isoforms. Cancer Res. 61, 6716–6722. [PubMed: 11559542]

DeGroot BC, Brander SM, 2014 The role of P450 metabolism in the estrogenic activity of bifenthrin in fish. Aquat. Toxicol 156, 17–20. https://doi.org/10.1016/j.aquatox.2014.07.007. [PubMed: 25127356]

Fontaine R, Affaticati P, Yamamoto K, Jolly C, Bureau C, Baloche S, Gonnet F, Vernier P, Dufour S, Pasqualini C, 2013 Dopamine inhibits reproduction in female zebrafish (Danio rerio) via three pituitary D2 receptor subtypes. Endocrinology 154, 807–818. https://doi.org/10.1210/en.2012-1759. [PubMed: 23295741]

Griffin LB, January KE, Ho KW, Cotter KA, Callard GV, 2013 Morpholino-mediated knockdown of ERα, ERβa, and ERβb mRNAs in zebrafish (Danio rerio) embryos reveals differential regulation of estrogen-inducible genes. Endocrinology 154, 4158–4169. https://doi.org/10.1210/en.2013-1446. [PubMed: 23928376]

Han C, Liu S, Huang J, Liu L, Ma K, Guo X, Guo S, Wang L, Shen Y, Xia Y, Wan F, Xiong N, Wang T, 2017 Exposure to bifenthrin contributes to Parkinson's disease in mouse model [abstract]. Mov. Disord 32 (Suppl. 2). http://www.mdsabstracts.org/abstract/exposure-to-bifenthrin-contributes-to-parkinsons-disease-in-mouse-model/, Accessed date: 12 February 2018.

Hoffmann F, Kloas W, 2012 The antiestrogens tamoxifen and fulvestrant abolish estrogenic impacts of 17α-ethinylestradiol on male calling behavior of Xenopus laevis. PLoS One 7, e44715 https://doi.org/10.1371/journal.pone.0044715. [PubMed: 23028589]

Hu J, Sun S, Guo M, Song H, 2014 Use of antagonists and morpholinos in loss-of-function analyses: estrogen receptor ESR2a mediates the effects of 17alpha-ethinylestradiol on primordial germ cell distribution in zebrafish. Reprod. Biol. Endocrinol 12, 40 https://doi.org/10.1186/1477-7827-12-40. [PubMed: 24886565]

Jiang H, Xie T, Ramsden DB, Ho SL, 2003 Human catechol-O-methyltransferase down-regulation by estradiol. Neuropharmacology 45, 1011–1018. [PubMed: 14573393]

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF, 1995 Stages of embryonic development of the zebrafish. Dev. Dyn 203, 253–310. [PubMed: 8589427]

Kumar P, Taha A, Kale RK, Cowsik SM, Baquer NZ, 2011 Physiological and biochemical effects of 17β estradiol in aging female rat brain. Exp. Gerontol 46, 597–605. https://doi.org/10.1016/j.exger.2011.02.008. [PubMed: 21377519]

Lévesque D, Gagnon S, Di Paolo T, 1989 Striatal D1 dopamine receptor density fluctuates during the rat estrous cycle. Neurosci. Lett 98, 345–350. [PubMed: 2524681]

Linard B, Bennani S, Saligaut C, 1995 Involvement of estradiol in a catecholamine inhibitory tone of gonadotropin release in the rainbow trout (Oncorhynchus mykiss). Gen. Comp. Endocrinol 99, 192–196. [PubMed: 8536929]

Maus M, Bertrand P, Drouva S, Rasolonjanahary R, Kordon C, Glowinski J, Premont J, Enjalbert A, 1989 Differential modulation of D1 and D2 dopamine-sensitive adenylate cyclases by 17β-estradiol in cultured striatal neurons and anterior pituitary cells. J. Neurochem 52, 410–418. [PubMed: 2521359]

McCurley AT, Callard GV, 2008 Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. BMC Mol. Biol 9, 102 https://doi.org/10.1186/1471-2199-9-102. [PubMed: 19014500]

McGee SP, Konstantinov A, Stapleton HM, Volz DC, 2013 Aryl phosphate esters within a major PentaBDE replacement product induce cardiotoxicity in developing zebrafish embryos: potential role of the aryl hydrocarbon receptor. Toxicol. Sci 133, 144–156. https://doi.org/10.1093/toxsci/kft020. [PubMed: 23377616]

Meiser J, Weindl D, Hiller K, 2013 Complexity of dopamine metabolism. Cell Commun. Signal 11, 34 https://doi.org/10.1186/1478-811X-11-34. [PubMed: 23683503]

Nagahama Y, 2002 Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol 38,217–229.

Notch EG, Mayer GD, 2011 Efficacy of pharmacological estrogen receptor antagonists in blocking activation of zebrafish estrogen receptors. Gen. Comp. Endocrinol 173, 183–189. https://doi.org/10.1016/j.ygcen.2011.05.008. [PubMed: 21641908]

Pinto C, Grimaldi M, Boulahtouf A, Pakdel F, Brion F, Aït-Aïssa S, Cavaillès V, Bourguet W, Gustafsson JA, Bondesson M, Balaguer P, 2014 Selectivity of natural, synthetic and environmental estrogens for zebrafish estrogen receptors. Toxicol. Appl. Pharmacol 280 (1), 60–69. [PubMed: 25106122]

Schmittgen TD, Livak KJ, 2008 Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc 3, 1101–1108. [PubMed: 18546601]

Shyu C, Cavileer TD, Nagler JJ, Ytreberg FM, 2011 Computational estimation of rainbow trout estrogen receptor binding affinities for environmental estrogens. Toxicol. Appl. Pharmacol 250, 322–326. [PubMed: 21075131]

Siepmann S, Holm S, 2000 Hazard assessment of the synthetic pyrethroid insecticides bifenthrin, cypermethrin, esfenvalerate, and permethrin to aquatic organisms in the Sacramento-San Joaquin River system. Office of Spill Prevention and Response 00–6 Administrative Report. California Department of Fish and Game, Rancho Cordova, CA.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML, 2002 Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. Toxicology 171, 3–59. [PubMed: 11812616]

Spurlock F, Lee M, 2008 Synthetic pyrethroid use patterns, properties, and environmental effects In: Gan J, Spurlock F, Hendley P, Weston DP (Eds.), Synthetic Pyrethroids: Occurrence and Behavior in Aquatic Environments, 1st ed 991. American Chemical Society, Washington, DC, USA, pp. 3–25. https://doi.org/10.1021/bk-2008-0991.ch001.

Syed F, Awasthi KK, Chandravanshi LP, Verma R, Rajawat NK, Khanna VK, John PJ, Soni I, 2018 Bifenthrin-induced neurotoxicity in rats: involvement of oxidative stress. Toxicol. Res 7, 48–58. https://doi.org/10.1039/C7TX00205J.

Tareke E, Bowyer JF, Doerge DR, 2007 Quantification of rat brain neurotransmitters and metabolites using liquid chromatography/electrospray tandem mass spectrometry and comparison with liquid chromatography/electrochemical detection. Rapid Commun. Mass Spectrom 21, 3898–3904. [PubMed: 17979107]

Van den Bercken J, Vijverberg HPM, 1980 Voltage clamp studies on the effects of allethrin and DDT on the sodium channels in frog myelinated nerve membrane Proceedings, Society of Chemical Industry Symposium, London, UK, 9 3–7, 1979, pp. 79–85.

Wang L, Liu W, Yang C, Pan Z, Gan J, Xu C, Zhao M, Schlenk D, 2007 Enantioselectivity in estrogenic potential and uptake of bifenthrin. Environ. Sci. Technol 41, 6124–6128. [PubMed: 17937291]

Weston DP, Holmes RW, You J, Lydy MJ, 2005 Aquatic toxicity due to residential use of pyrethroid insecticides. Environ. Sci. Technol 39, 9778–9784. [PubMed: 16475366]

Weston DP, Holmes RW, Lydy MJ, 2009 Residential runoff as a source of pyrethroid pesticides to urban creeks. Environ. Pollut 157, 287–294. https://doi.org/10.1016/j.envpol.2008.06.037. [PubMed: 18676072]

Xie T, Ho SL, Ramsden D, 1999 Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. Mol. Pharmacol 56, 31–38. [PubMed: 10385681]

Zhao S, Fernald RD, 2005 Comprehensive algorithm for quantitative real-time polymerase chain reaction. J. Comput. Biol 12, 1047–1064. [PubMed: 16241897]

Zhao M, Zhang Y, Zhuang S, Zhang Q, Lu C, Liu W, 2014 Disruption of the hormonal network and the enantioselectivity of bifenthrin in trophoblast: maternal–fetal health risk of chiral pesticides. Environ. Sci. Technol 48, 8109–8116. [PubMed: 24938463]

Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O, 2010 Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol 165, 438–455. https://doi.org/10.1016/j.ygcen.2009.04.017. [PubMed: 19393655]

HIGHLIGHTS

- BF's antiestrogenic activity in zebrafish embryos might be mediated by ER.
- Zebrafish embryos were co-exposed to BF and EE2.
- Morpholinos were used to knockdown ERα.
- No interaction of EE2 and BF, and ERα knockdown did not alter effects of BF
- EE2 altered gene expression and DA levels, and ERα knockdown altered EE2 effects.

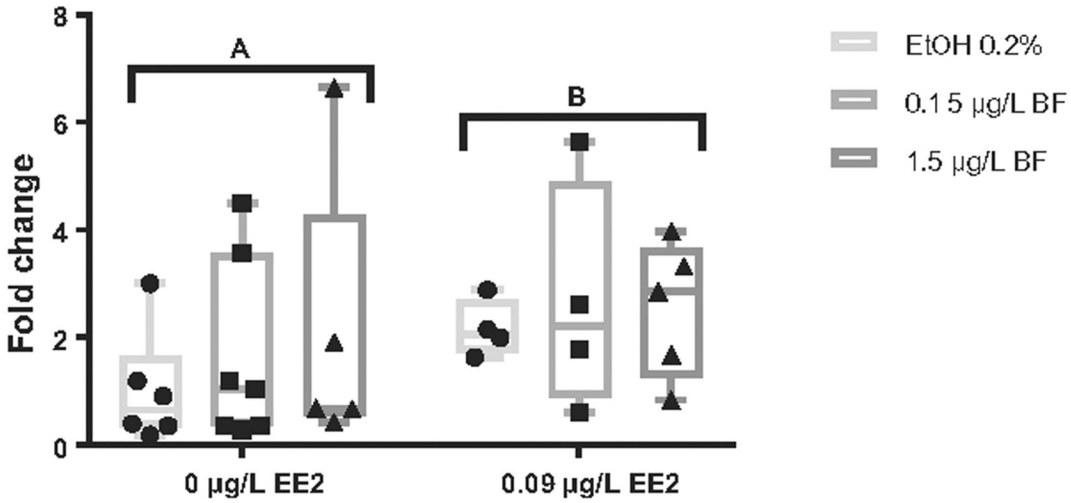


Fig. 1. -

Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on transcripts of vitellogenin in zebrafish embryos after 96 h of treatment. Box and whisker plot showing hinges from the 25th to the 75th percentiles, whiskers from the minimum to the maximum value, line in the box as median and points as each individual value. Sample size = 4–7. Treatment groups marked by letter have significantly different mRNA levels compared to the other group (two-way ANOVA).

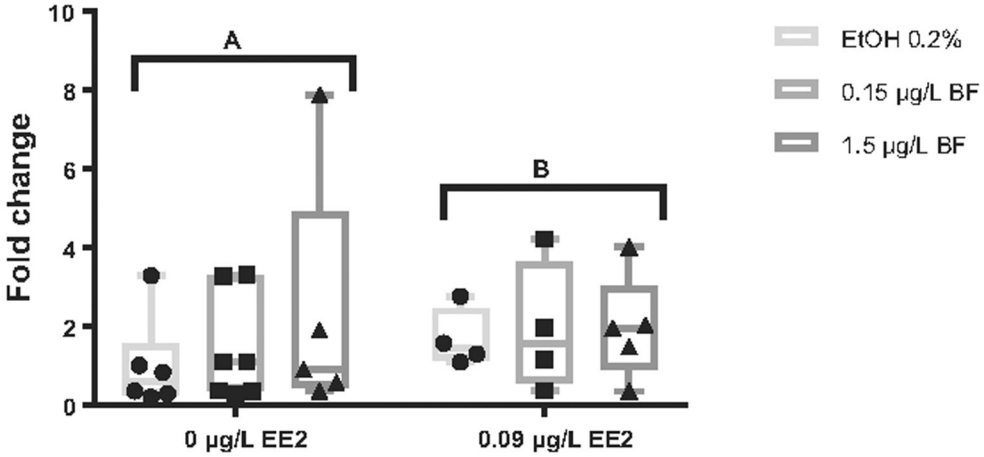


Fig. 2. -

Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on transcripts of dopamine receptor 1 in zebrafish embryos after 96 h of treatment. Box and whisker plot showing hinges from the 25th to the 75th percentiles, whiskers from the minimum to the maximum value, line in the box as median and points as each individual value. Sample size = 4–7. Treatment groups marked by letter have significantly different mRNA levels compared to the other group (two-way ANOVA).

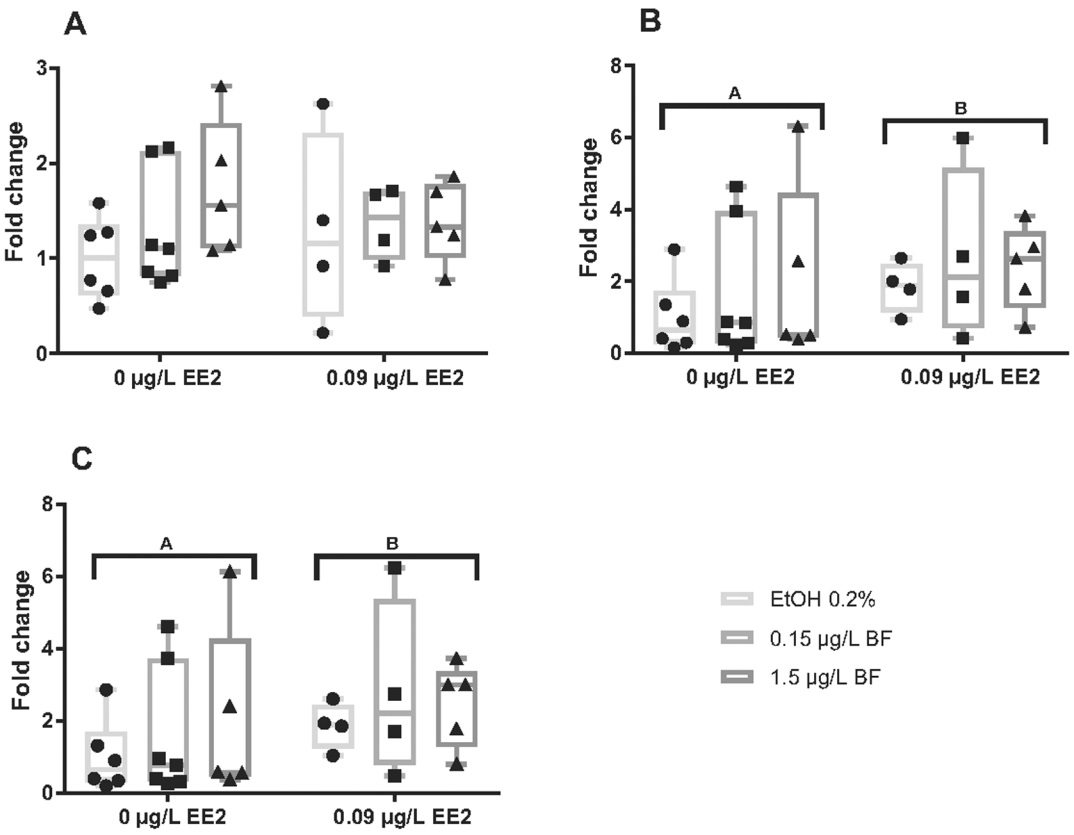


Fig. 3. -

Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on transcripts of A) tyrosine hydroxylase, B) monoamino oxidase, and C) catechol-*O*-amino transferase b in zebrafish embryos after 96 h of treatment. Each value represents the mean of replicates 4–7 ± SE. Treatment groups marked by letter have significantly different mRNA levels compared to other group (two-way ANOVA).

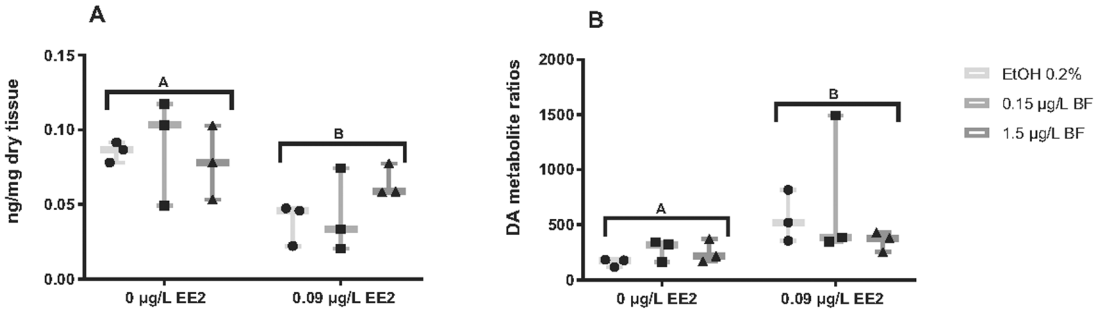


Fig. 4. -

Effects of bifenthrin, 17α-ethynylestradiol and a combination of both on A) dopamine levels and B) HVA-DA ratios in zebrafish embryos after 96 h of treatment. Box and whisker plot showing hinges from the 25th to the 75th percentiles, whiskers from the minimum to the maximum value, line in the box as median and points as each individual value. Sample size = 3. Treatment groups marked by letter have significantly different mRNA levels compared to the other group (two-way ANOVA).

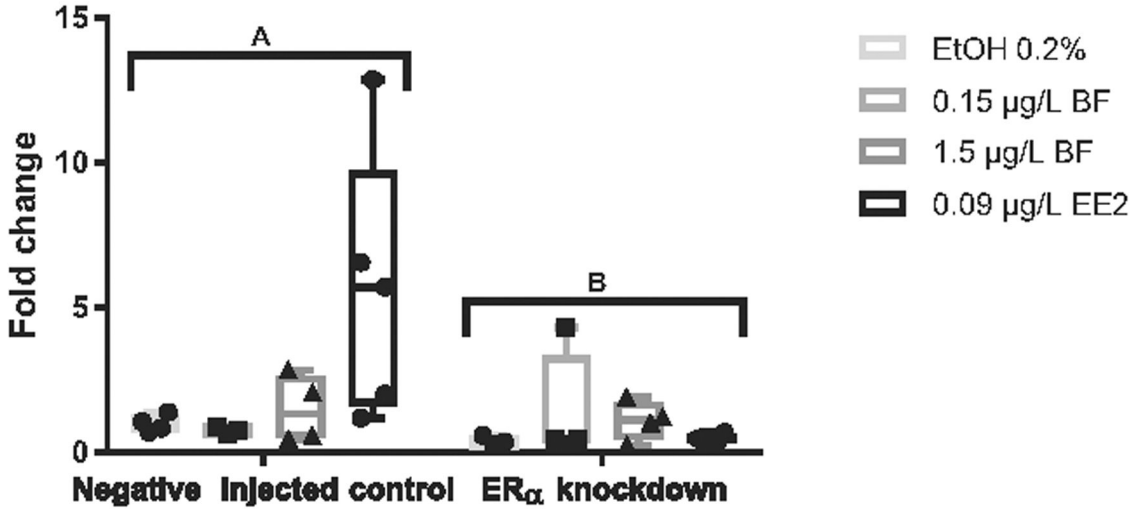


Fig. 5. -

Effects of bifenthrin on transcripts of vitellogenin in zebrafish embryos injected with water or an ERα morpholino after 96 h of treatment. Box and whisker plot showing hinges from the 25th to the 75th percentiles, whiskers from the minimum to the maximum value, line in the box as median and points as each individual value. Sample size = 3–5. Treatment groups marked by letter have significantly different mRNA levels compared to the other group (two-way ANOVA).

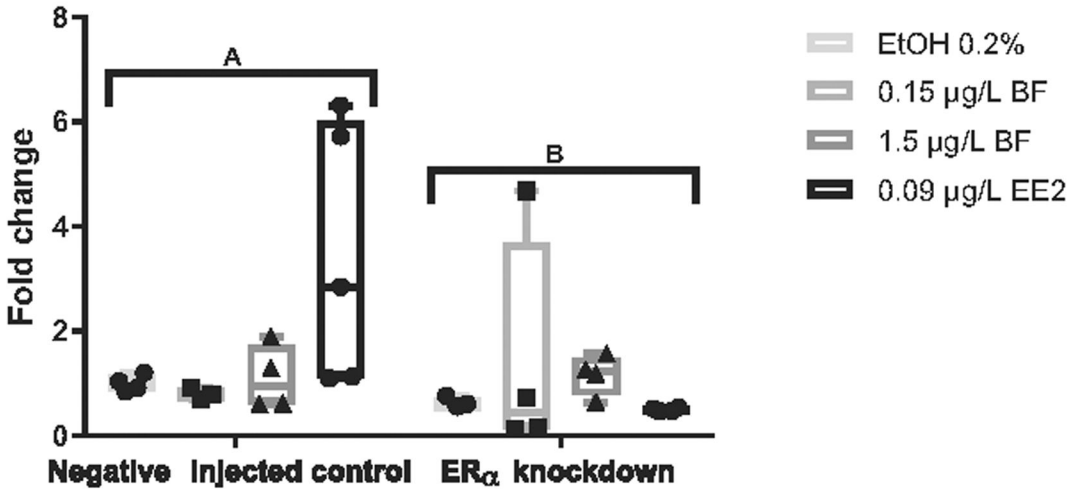


Fig. 6. -

Effects of bifenthrin on transcripts of dopamine receptor 1 in zebrafish embryos injected with water or an ERα morpholino after 96 h of treatment. Box and whisker plot showing hinges from the 25th to the 75th percentiles, whiskers from the minimum to the maximum value, line in the box as median and points as each individual value. Sample size = 3–5. Treatment groups marked by letter have significantly different mRNA levels compared to the other group (two-way ANOVA).

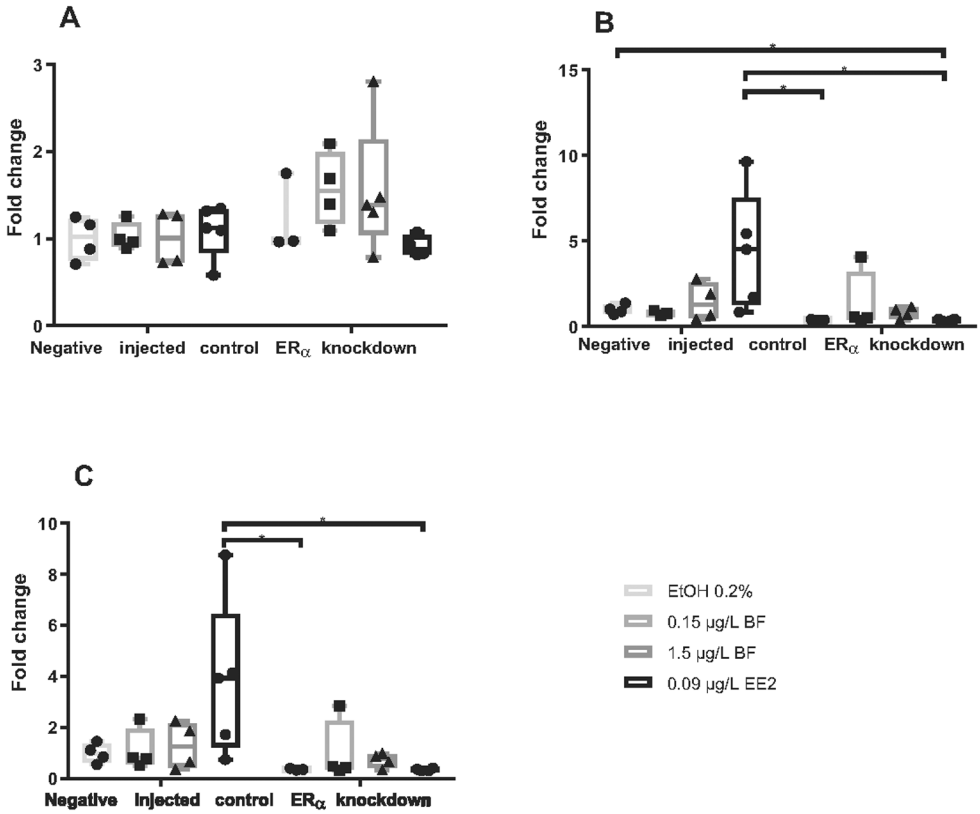


Fig. 7. -

Effects of bifenthrin on transcripts of A) tyrosine hydroxylase, B) monoamino oxidase, and C) catechol-O-amino transferase b in zebrafish embryos injected with water or an ERα morpholino after 96 h of treatment. Box and whisker plot showing hinges from the 25th to the 75th percentiles, whiskers from the minimum to the maximum value, line in the box as median and points as each individual value. Sample size = 3–5. Treatment groups marked by letter have significantly different mRNA levels compared to another group (two-way ANOVA). Treatments marked by an asterisk have significantly different mRNA levels compared to another treatment (two-way ANOVA, Tukey HSD post-hoc test).

Table 1

Quantification ions.

Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)
Dopamine	154.0381	90.9673
		118.9987
3,4-Dihydroxyphenylacetic acid (DOPAC)	169.0319	123.0351
Homovanillic acid (HVA)	183.0957	97.8735
		137.0271
Dopamine-I,l,2,2-d_4 hydrochloride (DA-d_4)	158.1043	94.9891