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Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups

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Abstract

Galaxolide (HHCB) and tonalide (AHTN) are polycyclic musk compounds (PMCs) used in household and personal care products that have been included on the list as emerging contaminants of environmental concern due to their ubiquity in aquatic and terrestrial environments. There still exists a dearth of information on the neurotoxicity and endocrine disrupting effects of these contaminants, especially for marine and estuarine species. Here, we assessed the neuroendocrine effects of HHCB and AHTN using adult clams, *Ruditapes philippinarum*, and yolk-sac larvae of sheepshead minnow, *Cyprinodon variegatus*. The clams were treated with concentrations (0.005 - 50 µg/L) of each compound for 21 days. Meanwhile, sheepshead minnow larvae were exposed to 0.5, 5 and 50 µg/L of HHCB and AHTN for 3 days. Enzyme activities related to neurotoxicity (acetylcholinesterase - AChE), neuroendocrine function (cyclooxygenase - COX), and energy reserves (total lipids - TL) were assessed in *R. philippinarum*. Gene expression levels of *cyp19* and *vtg1* were measured in *C. variegatus* using qPCR. Our results indicated induction of AChE and COX in the clams exposed to HHCB while AHTN exposure significantly inhibited AChE and COX.

Gene expression of *cyp19* and *vtg1* in yolk-sac *C. variegatus* larvae exposed to 50 µg/L AHTN was significantly downregulated versus the control. The results of this study demonstrate that HHCB and AHTN might pose neurotoxic and endocrine disrupting effects in coastal ecosystems.

Keywords: Neurotoxicity, endocrine disruption, galaxolide, tonalide, transcriptional effects, biomarkers

1. Introduction

Galaxolide (HHCB) and tonalide (AHTN) are polycyclic musk compounds (PMCs) used in household and personal care products and are included on the list of emerging contaminants of environmental concern due to their ubiquity in aquatic and terrestrial environments (Schreurs *et al*., 2004; Zhang *et al*., 2013). HHCB and AHTN consist of aromatic structures consisting of acetylated and extremely methylated pyran and tetralin bases (Sumner *et al*., 2010). They possess high *n*-octanol – water partition coefficients (K*ow*) akin to most persistent organic pollutants and may persist in the marine environment. The presence and toxicity of PMCs in transitional and coastal ecosystems is yet to be fully understood. Most research regarding these compounds has been focused on freshwater ecosystems (Balk and Ford, 1999a, 1999b; Parolini *et al*., 2015; Yamauchi *et al*., 2008) with only a few reports available on the toxicity of PMCs in the marine environment (Breitholtz et al., 2003; Luckenbach et al., 2004; Wollenberger et al., 2003). Recently, Ehiguese *et al*. (2019) studied the avoidance behavior of the shrimp *Palaemon varians* and found that HHCB and AHTN potentially elicited avoidance behavior in this shrimp. Chronic exposure to environmentally relevant concentrations of these substances suggests that they may alter antioxidant enzyme activity and potentially trigger oxidative stress in Manila clams (Ehiguese *et al*., 2020). To the best of our knowledge, the neurotoxicity and endocrine disrupting effects of these contaminants in the marine environment are yet to be addressed.

About 30% of commercially available chemicals are estimated to possess neurotoxic and endocrine disrupting properties (Tilson *et al*., 1995). These chemicals can target neurotransmitter pathways and their

components such as neurotransmitters, receptors, biosynthetic enzymes, catabolic enzymes, and transporters (Basu, 2015). Neuroendocrine compounds promote diverse physiological and behavioral effects that alter the capacity of organisms to reach their biotic potential, cope with stress and other environmental challenges, and survive (Waye and Trudeau, 2011). Signals from environmental contaminants can interfere with neurotransmission and disrupt endocrine functions in marine organisms because of their potential to mimic the natural hormone estrogen, and can bind to estrogen receptors and influence estrogen biosynthesis (Waye and Trudeau, 2011). Many persistent organic pollutants have been implicated as neuroendocrine disruptors in the marine environment causing adverse effects related to changes in thyroid morphometry and functions, suppression of ovarian follicle development, altered sex differentiation, and mortality (Berg et al., 2016; Porte et al., 2006; Schnitzler et al., 2008). Furthermore, environmental concentrations of some pharmaceutical products have been shown to inhibit monoamine oxidase activity, increase plasma cortisol levels, and reduce feeding in aquatic organisms *(Maranho et al*., 2015; Melnyk-Lamont *et al*., 2014). Importantly, HHCB and AHTN have been demonstrated to disrupt neuroendocrine activity in several *in vitro* studies(Li *et al*., 2013; Mori *et al*., 2007; Schreurs et al., 2005, 2004, 2002) and significantly alter gene expression levels in male medaka fish (Yamauchi *et al*., 2008).

The aim of this study was to investigate the neurotoxic and endocrine disrupting effects of HHCB and AHTN in the marine environment. We assessed biochemical activities in Manila clams (*Ruditapes philippinarum*) using biomarkers of neuroendocrine toxicity (AChE, COX) and energy reserves (total lipids; TL) in a 21 day exposure. We also assessed and gene expression levels of *cyp19* and *vtg1* in yolk-sac larvae of sheepshead minnow (*Cyprinodon variegatus*) after 3 days of exposure to HHCB or AHTN. The suitability of *R. philippinarum* for ecotoxicological studies has been previously stated by Ehiguese *et al*. (2020). Sheepshead minnow are a suitable marine model used in ecotoxicological studies because they are easy to breed under laboratory conditions and spawn continuously with relatively large demersal eggs (Cripe *et al*., 2009). They have been used in the assessment of endocrine disrupting chemicals in transitional and coastal waters (Bowman *et al*., 2000; Folmar *et al*., 2000; Hemmer *et al*., 2001) as well as to

characterize alteration of immune pathways (Jones *et al*., 2017; Rodgers *et al*., 2020) and oxidative stress (Rodgers *et al*., 2018) after chemical exposures.

2. Materials and methods

2.1. Test chemicals

For the clam experiments, analytical grades of HHCB (85.0%) and AHTN (97.0%) were obtained from Sigma Aldrich Spain. The details of dissolution and preparations can be found in Ehiguese *et al*. (2020). In brief, stock solutions were prepared using DMSO (0.001% v/v) as the organic solvent to dissolve the test chemicals which were further diluted with distilled water to reach the concentrations needed (0.005, 0.05, 0.5, 5 and 50 µg/L). These concentrations were selected based on reported environmental concentrations measured in marine environments (Díaz-Garduño et al., 2017; Pintado-Herrera et al., 2013). For the fish experiments, 100 g of analytical grade AHTN (97%) was purchased from Sigma Aldrich, USA. 10 g of the product was dissolved in 0.001% v/v DMSO to form the stock solution. 25 g of HHCB dissolved in 50% diethyl phthalate containing 49% pure HHCB was purchased from TCI America, USA and the concentrations required were calculated based on the percentage of the active ingredient of HHCB in the solution. The stock solution was diluted using distilled water to create 0.5, 5.0 and 50.0 µg/L solutions for each compound.

2.2. Test organisms

The clams, *R. philippinarum* (550 specimens), were obtained from an aquaculture farm in the south-west of Spain and were transported to the laboratory of Marine Culture, Faculty of Marine and Environmental Science (University of Cadiz, Spain). The adult clams (average size of 43.2±1.6 mm) were acclimated in a 250 L aquarium and were fed with *Isochrysis galbana* once per day. During acclimation, aeration was

provided to improve the oxygen content (dissolved oxygen >5 mg/L) of the medium and other physiochemical parameters in the aquarium were monitored and controlled. The temperature, salinity, pH and photoperiod during the acclimation were 15±1 ^{0}C, 34.7±0.4‰, 7.8 – 8.2 and 12 h light/12 h dark, respectively.

Adult sheepshead minnows (*C. variegatus*) previously purchased and kept in artificial seawater (15‰), between the temperature range of 25-27 ^{o}C and photoperiod (12:12 light/dark) in 300 L static recirculating raceways at the Toxicology Building, Gulf Coast Research Laboratory, University of Southern Mississippi (Ocean Springs MS, USA) were used as brood stocks. Before breeding, the brood stocks were fed daily with *Artemia* nauplii and commercial flake food. Four Spawntex® Mats (15-20 cm; Pentair Aquatic Eco-Systems) were placed in the two holding raceways overnight for spawning, two per raceway, containing gravid females with a female:male at a ratio of 2:1. The fertilized eggs were collected by gently tapping the Spawntex® Mats into a clean laboratory dish. The embryos were gently rinsed and transferred into a hatching jar supplied with aeration to aid suspension of the fertilized eggs in the water column and they were incubated in an ISOTEMP 115 (Fisher Scientific) at 30 ^{o}C until hatching (Dangre *et al*., 2010; Griffitt *et al*., 2012). These yolk-sac larvae were then carefully collected in 100 ml beakers filled with artificial seawater (15‰) prior to exposure.

2.3. Biochemical effects: Experimental approach

R. philippinarum were exposed aqueously to either HHCB or AHTN in 10 L rectangular glass aquariums. The bioassay experiment was carried out in duplicate for all treatments including the controls (seawater and DMSO). Natural, filtered seawater was obtained from the Marine Culture laboratory of the University of Cadiz, and 8 L of the seawater was mixed with each test chemical. 16 clams were added to each aquarium, totaling 32 per treatment except for the seawater and solvent controls with 14 specimens each. The treatments were renewed every three days, during which the water was siphoned out of the holding tanks

and carefully cleaned and refilled with seawater spiked with freshly prepared contaminant. Any dead clams were immediately removed and recorded. The physical and chemical properties were adjusted to the same conditions as reported above during the acclimation period. Three clams were randomly collected from each replicate on day 3, 7, 14 and 21, and tissues (digestive gland and gonads) were immediately harvested on ice and stored at -80 ºC in the laboratory prior to homogenization.

Buffer was prepared for sample homogenization using 0.1 mM EDTA, 100 mM NaCl, 25 mM HEPES salt, and 0.1 mM DTT. The samples stored in the freezer were thawed on ice and the digestive glands and gonads of three clams from each aquarium were pooled together for homogenization. The pooled samples were homogenized, and a fraction of each homogenate (HF) was centrifuged to obtain supernatant portions at a speed of 15.000 x g for 20 min at 4 ºC (S_{15}) and 3.000 x g for 20 min at 4 ºC (S_3). The Bradford (1976) methodology was adapted to determine the corresponding total protein (TP) concentration with values expressed as mg/mL for different extracts (HF, S_3 and S_{15}). The biochemical analyses of the biomarkers were quantified using a kinetic microplate reader, Infinite® M200.

2.4 Collection and analysis of exposure water

Exposure water for each concentration was sampled using clean amber bottles on day 0 and 3 for analysis of initial and final concentrations and the samples were kept in -20 ºC prior to the chemical analysis. Details of the methodology for the chemical analysis and the results of the detection and quantification can be found in Ehiguese et al. (2020).

2.5. Biochemical analyses

2.5.1. Acetyl Cholinesterase (AChE) Activity

AChE activity was measured in the post-mitochondria fraction of the digestive glands according to the methodology described in Guilhermino *et al*. (1996). 20 µL of the centrifuged S_{15} fraction was added to 20 µl of 50 mM potassium phosphate buffer at a pH of 7.5 in 96 transparent, flat bottom wells. 130 µL of 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) solution was further added with 50 µL of acetylthiocholine iodide solution. The activity of AChE was determined by absorbance measured at 405 nm at every 40 – 52 sec for 5 – 7 min. The data were expressed as the formation of thiols in pmol DTNB/min/mg TP.

2.5.2. Cyclooxygenase (COX) Activity

COX activity was measured according to Gagné *et al*. (2015), following the oxidation of 2,7-dichlorofluoresceine in the presence of arachidonate (Fujimoto *et al*., 2002). Briefly, 96 dark, flat bottom well microplates were coated with 50 µL of the S15 sample. 200 µL of the assay buffer containing 50 µM arachidonic acid and 2 µM dichlorofluoresceine was added with 0.1 µg/mL horseradish peroxidase containing 50 mM Tris-HCl, pH 8.0 and 0.05% Tween 20. The reaction was incubated at 30 ºC for 0, 5, 10, 15, 20, 25 and 30 min and the fluorescence was measured at 485 nm (excitation) and 520 nm (emission). The data were expressed as µmol fluorescein/min/mg TP.

2.5.3. Total lipids (TL)

TL were measured in gonad and digestive gland tissues following the phosphovanilin method by Frings *et al*., (1972). Dark microplates with 96 flat-bottom wells were coated with 10 µL of samples diluted with 10 µL of MilliQ. 30 µL of concentrated sulphuric acid and 150 µL of phosphovanilin prepared with vanillin and phosphoric acid in water were added and incubated for 10 min at 80 ºC and cooled at 4 ºC for 2 min. The absorbance was determined at 540 nm. A standard solution of Triton X-100 was used for calibration and the results were expressed as µg TL/mg TP.

2.6. Molecular effects: Experimental approach

All tests were performed in static renewal bioassays in triplicate; 200 mL of seawater spiked with each treatment (0.5, 5.0 and 50 μg/L) was transferred into laboratory dishes. Then, 10 yolk-sac larvae of *C. variegatus* were randomly selected and transferred into each dish including the controls (seawater and DMSO). They were incubated in a Precision Scientific Incubator (Thermo, MA, USA) at 30 ºC and the exposure water in each dish was renewed every 24 h during the 3 days exposure. Upon termination of the experiment, the larvae were inserted into 1.5 mL tubes containing 500 µL RNALater® solution and stored at -80 ºC prior to RNA extraction.

2.7. Molecular analysis: Quantitative PCR (qPCR)

For RNA extraction, six larvae per replicate were pooled from each treatment and both controls (seawater and DMSO) for homogenization. Total RNA was extracted using RNeasy kits (Qiagen, Hilden, Germany) as described in the manufacturer's protocol, and the total RNA quantity and quality was assessed spectrophotometrically using a NanoDrop™ 2000 (Thermo Scientific, Wilmington, DE, USA). Reverse transcription of total RNA to single stranded cDNA was performed using RevertAid First Strand cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific, Wilmington, DE, USA).

Real-time qPCR was performed using the primers listed in Table 1. 18s was used as an endogenous control, while *cyp19* and *vtg1* were used as biomarkers of endocrine disruption. All qPCR reactions were performed in triplicates using an Applied Biosystems 7500 Fast Cycler with Fast SYBR Green Master Mix (Life Technologies, Carlsbad, CA). Relative quantification values compared to the control samples were determined by applying the ΔΔCT method. Fold changes in *cyp19* and *vtg1* genes were log transformed to normalize the data.

2.8. Statistical Analysis

All data were analyzed using SPSS (16.0) statistical package. Data normality and homogeneity were assessed before statistical tests were performed. One-way analysis of variance (ANOVA) coupled with Dunnett's multiple comparison tests were performed and significant differences between controls and clams treated with HHCB and AHTN were determined at $p < 0.05$. The relationships between effects and concentrations were checked using Spearman's rank order of correlation, and significant points were set at $p < 0.05$ and $p < 0.01$. For gene expression levels, significant differences were determined using a least-square difference test and significant difference was set at $p < 0.05$.

3. Results

3.1. Biochemical effects

No mortality was recorded in control clams, meanwhile 3% mortality was recorded in clams treated with HHCB and ~ 4% mortality in the clams treated with AHTN during the experiment.

The effects of HHCB and AHTN on AChE activity are presented in Figure 1. There was significant inhibition ($p < 0.05$) of AChE activity in the clams treated with HHCB at 5.0 µg/L and 50 µg/L after 3 days (Figure 1a). As the exposure continued, significant differences in AChE activity were further seen in the clams treated with 0.005 and 5 µg/L HHCB on days 14 and 21, respectively (Figure 1a). Prolonged significant inhibition of AChE ($p < 0.05$) was triggered by all concentrations of AHTN tested until day 7, but only 0.005, 0.05 and 5.0 µg/L concentrations produced significant AChE inhibition by day 14 (Figure 1b). Interestingly, at the end of the experiment (day 21), the 50 µg/L AHTN exposure showed a significant increase in AChE activity. (Figure 1b).

The impact of HHCB and AHTN on COX activity measured in *R. philippinarum* after 21 days exposure in a semi-static bioassay is presented in Figure 2. COX activity was induced in the clams treated with HHCB and this induction was significant in the 0.005 and 50 µg/L exposures on day 3. Continuous exposure to HHCB up to day 14 produced significant effects on COX activity at the highest concentration (50 µg/L) tested, but at day 21 only the 0.05 µg/L concentration significantly increased ($p < 0.05$) COX activity (Figure 2a). On the other hand, AHTN inhibited COX activity in *R. philippinarum* and the inhibition was significantly different from the control group ($p < 0.05$) on days 7 and 14 in all the concentrations tested except for 0.005 µg/L on day 7(Figure 2b).

The energy reserves, measured as total lipids, were determined in the digestive gland (DTL) and gonad (GTL) tissues of clams exposed to HHCB and AHTN. There was no significant difference in DTL in the clams exposed to HHCB (Figure 3a). For the clams exposed to AHTN, DTL increased significantly ($p < 0.05$) throughout the exposure period with the exception of the 0.05 µg/L treatment group on days 3, 7, and 14, and the 0.5 µg/L treatment on days 14 and 21 (Figure 3b). There was no clear pattern in GTL of the clams exposed to HHCB, as we observed both significant increases and decreases in GTL depending on the treatment and time point (Figure 3c). However, GTL decreased significantly ($p < 0.05$) after exposure to AHTN in all treatment groups except at the lowest concentration (0.005 ug/L) measured on day 7 (Figure 3d).

The activities of neuroendocrine biomarkers (AChE and COX) measured in the clams correlated significantly with the concentrations of HHCB ($p < 0.01$) over time (Table S1 – Supplementary Materials). For AHTN, a significant time and concentration-dependent correlation of AChE and COX was observed. In addition, the inhibition of AChE and COX activities correlated significantly ($p < 0.01$) (Table S2 – Supplementary Materials). Finally, gonad energy reserves (GTL) were significantly depleted over time (Table S2 – Supplementary Materials).

236 *3.2. Molecular effects*

237 There was no mortality in the yolk sac larvae of *C. variegatus* exposed to HHCB; for AHTN exposures,
238 3% mortality was recorded in the fish exposed to the 50 μg/L treatment.

239 The expression of *cyp19* in yolk sac larvae of *C. variegatus* exposed to HHCB was slightly upregulated,
240 though not significantly in any of the treatments (Figure 4a). However, concentration-dependent
241 downregulation of *cyp19* was observed in the larvae exposed to AHTN and was significantly different ($p <$
242 0.05) at the highest concentration (50 μg/L) with more than a 3-fold change in expression compared to the
243 controls (Figure 4b).

244 Expression of *vtg1* measured in the yolk-sac larvae of *C. variegatus* exposed to both substances had a
245 similar pattern to *cyp19* expression (Figure 5). HHCB slightly induced the expression of *vtg1* and the
246 induction was highest at 5.0 μg/L, though not significant (Figure 5a). For AHTN, a concentration-dependent
247 downregulation of *vtg1* was observed and decreased significantly ($p < 0.05$) by 3.40-fold versus the controls
248 at the 50 μg/L exposure concentration (Figure 5b).

249

250 **4. Discussion**

251 The current study assessed the neuroendocrine effects of environmental concentrations of HHCB and
252 AHTN in the marine environment by assessing enzyme activities and gene expression levels in marine
253 organisms from two taxonomic groups. Biomarkers of endocrine disruption (*cyp19* and *vtg1*) were
254 measured in *C. variegatus* and neurotoxicity (AChE) was measured in *R. philippinarum* together with
255 assessments of neuroendocrine and inflammation responses (COX) and energy reserves (TL).

256 *4.1. Biochemical effects*

Significant concentration-dependent AChE inhibition was observed in the clams treated with environmentally comparable concentrations of HHCB and AHTN, at the first time point (3 days of exposure - Figure 1). AChE is a well-established biomarker in toxicological studies of neurotoxicity and is the enzyme responsible for the deactivation of acetylcholine at the cholinergic synapses, preventing a build-up of acetylcholine, which is necessary for the normal functioning of sensory and neuromuscular systems (van der Oost *et al*., 2003; Sturm *et al*., 2000). AChE is also a target of many organic pollutants, toxic metals, human pharmaceuticals and personal care products, which have all been reported to inhibit AChE activity (Aguirre-Martínez *et al*., 2016; Maranho *et al*., 2015; Matozzo *et al*., 2005; Stefano *et al*., 2008). Our observations showed that these substances might possess the ability to bind with cholinesterase, preventing the breakdown of acetylcholine. It appears that HHCB and AHTN can inhibit AChE after a short exposure to environmental concentrations, but this does vary with the concentration and duration of the exposure (Figure 1). For HHCB, limited AChE inhibition was observed at days 14 to day 21 with no significant inhibition on day 7 (Figure 1a). This may be because the clams were able to metabolize HHCB to less toxic metabolites during the exposure (Balk and Ford, 1999a). On the other hand, all the AHTN exposure concentrations inhibited AChE activity until day 14 when all but the highest concentration of AHTN significantly ($p < 0.05$) inhibited AChE activity (Figure 1b). Consequently, chronic inhibition of AChE in clams could lead to high levels of acetylcholine, over-stimulation of cholinergic receptors, alteration of postsynaptic cell function, and signs of cholinergic toxicity such as morphological and behavioral changes may start to manifest (Nallapaneni *et al*., 2008; Pope *et al*., 2005; Song *et al*., 2004; Waseem *et al*., 2010). Furthermore, fatality may occur if AChE activity is depressed during exposure to cholinesterase-inhibiting chemicals due to the overstimulation of the target cells (Sancho *et al*., 2000). The nitro musk compound ambrette was previously reported to possess neurotoxic potential as well as elicit carcinogenesis in organisms, leading to its prohibition (Nair *et al*., 1986; Spencer *et al*., 1984). Our results are comparable with other studies reporting inhibition of AChE in bivalves after chronic exposure. Shan *et al.* (2020) reported significant inhibition of AChE in the digestive gland of Asian clams (*Corbicula fluminea*) exposed to 20 - 2000 μg/L imidacloprid for 30 days. Similarly, 0.1 – 1 μg/L carbamazepine, 5 and 50 μg/L caffeine,

and 50 µg/L ibuprofen significantly decreased AChE activity assessed in the digestive gland of *C. fluminea* after 21 days exposure (Aguirre-Martínez *et al.*, 2018). In contrast, environmental concentrations (15 µg/L) of ibuprofen and carbamazepine reportedly increased AChE activity assessed in the gills of *R. philippinarum* after 7 days exposure (Trombini *et al*., 2019). Although both HHCB and AHTN inhibited AChE activity at various points, AHTN appears to be more robust at inhibiting AChE because it exerted prolonged inhibition of AChE activity until day 14, although the clams seem to have recovered by day 21 (Figure 1b). These results demonstrate the potential of polycyclic musk compounds as neuroinhibitors and provide a baseline upon which neurotoxicity of HHCB and AHTN could be further investigated.

HHCB increased COX activity in clams, though this inhibition was also concentration and time-dependent (Figure 2a). COX catalyzes arachidonic acid to form prostaglandins which are responsible for several physiological and reproductive functions in aquatic organisms (Di Costanzo *et al*., 2019). It is an important indicator of inflammation in aquatic organisms exposed to environmental stressors (Gagné *et al*., 2015). Clams exposed to wastewater effluents in the Bay of Cadiz (Spain) exhibited significantly inhibited COX activity and triggered inflammatory responses in the gonad tissues, which correlated significantly with general stress, measured as lysosomal membrane stability (Díaz-Garduño *et al*., 2018). Recent studies have demonstrated that both HHCB and AHTN induce oxidative stress in clams (Ehiguese *et al*., 2020). In addition, COX synthesis of prostaglandins is involved in the control of oogenesis and spermatogenesis in aquatic invertebrates (Di Costanzo *et al*., 2019) and controls the effect of serotonin in the spawning process of bivalves (Matsutani and Nomura, 1987). Prameswari *et al*. (2017) demonstrated that arachidonic acid induced a significantly ($p < 0.001$) increased ovarian index, oocyte diameter and ovarian vitellogenin in the freshwater crab (*Oziothelphusa senex senex*). The authors reported that COX inhibitors, including indomethacin and aspirin, significantly ($p < 0.001$) reduced ovarian index, oocyte diameter and ovarian vitellogenin levels, corroborating the involvement of COX in the regulation of female reproduction in crabs. Given the concentration-dependent alteration of COX activity in clams exposed to HHCB and AHTN, reproductive success could be at risk, but additional research is needed to validate this hypothesis.

Furthermore, the functions of COX activity in marine bivalves is not fully understood but the significant correlation of COX and AChE activities in clams exposed to AHTN (Table S2 – Supplementary Materials) suggests that COX activity may be involved in neuroendocrine functions in marine bivalves. Consequently, AHTN might be a neuroendocrine disruptor in the marine environment.

To understand toxicity-driven energy deficit in clams, TL in the digestive gland and gonadal tissues were measured. It was hypothesized that chemical stress may trigger significant energy demand due to relatively high enzyme activities. For *R. philippinarum* exposed to HHCB, total lipids measured in DTL were unaffected and we did not observe any clear tendency in energy level measured in the gonads (Figure 3 a, b). The DTL measured in the clams exposed to AHTN was significantly ($p < 0.05$) higher than the control for most treatments and time points (Figure 3b), but GTL was significantly reduced in most treatments and time points (Figure 3d). Total lipids assessed in the gonad of *R. philippinarum* exposed to wastewater effluents was significantly reduced in other studies (Díaz-Garduño *et al*., 2018; Maranho *et al*., 2016). The variation in total lipids assessed in this study, especially for HHCB, did not reveal any clear trend.

4.2. Molecular effects

Exposure of fish to endocrine disrupting chemicals in the marine environment has serious consequences concerning survival and reproduction. In yolk-sac *C. variegatus* larvae exposed to HHCB, the expression of *cyp19* showed no concentration-dependent transcriptional effect (Figure 4a). Meanwhile, we observed concentration-dependent downregulation of *cyp19* expression levels after 3 days of exposure to AHTN (Figure 4b). Similarly, significant downregulation of *cyp19b* expression levels were measured in juvenile salmon exposed to 0.04 – 1 mg/L of the organophosphate flame retardant, tris(2-cloroethyl) phosphate for 7 days (Arukwe *et al*., 2016), and significant dose-dependent downregulation of *cyp19a* and *cyp19b* were recorded in the ovaries of adult marine medaka exposed to 2 – 5 ng/L 17β-trenbolone for 21 days (Zhang *et al.*, 2020). In contrast, *cyp19a* and *cyp19b* expression levels were upregulated in adult male and female *Danio rerio* exposed to 1 mg/L perfluorodecanoic acid (Jo *et al.*, 2014) and perfluorononanoate (Zhang *et*

al., 2016). *cyp19* is an important biomarker of endocrine disruption in teleosts because aromatase, the enzyme involved in the conversion of androgen to estrogen, plays an essential role in sexual differentiation, maturation, and reproduction (Cheshenko *et al*., 2008). *cyp19* is regarded as a major target for endocrine disrupting chemicals because modulation of its expression and function may potentially disrupt estrogen production (Cheshenko *et al*., 2008; Kazeto *et al*., 2004). Our results suggest that AHTN may be a more potent modulator of *cyp19* expression than HHCB (Figure 4). Previous reports in an *in vitro* study using the H295R cell line exposed to 25 μM HHCB demonstrated upregulation of *cyp19*, while AHTN downregulated *cyp19* by 43% of the basal control (Li *et al*., 2013). The modulation of *cyp19* by HHCB and AHTN in *C. variegatus* larvae may affect estrogen biosynthesis and, as a result, alter the survival, sexual behavior, and sex differentiation in fish. Although most studies of estrogen as an endocrine disruption biomarker in fish are related to reproductive functions or tissues, estrogen alteration may also affect tissue mineralization and mineral homeostasis (Suzuki *et al*., 2009; Yoshikubo *et al*., 2005), as well as delay development in early life stage fish (Rawson *et al*., 2006).

The induction and inhibition of *vtg1* in *R. philippinarum* exposed to HHCB and AHTN, respectively, was similar to *cyp19* (Figure 5). The basis for this similarity is not well understood but it appears that because vitellogenin is induced by estrogen (which is biosynthesized by the enzyme complex aromatase that converts androgen into estrogen), effects on *cyp19* may trickle down to have an impact on vitellogenin (Andersen *et al*., 2003). Previous studies have demonstrated correlations between aromatase and vitellogenin in fish exposed to endocrine disrupting chemicals (Andersen *et al*., 2003; Bizarro *et al*., 2014). However, further investigation is needed to help understand the relationships between the effects of contaminants on both biomarkers. Similar to our observation for *vtg1* measured in *C. variegatus* yolk-sac larvae exposed to HHCB, there was no statistically different increase in plasma vitellogenin levels in rainbow trout intraperitoneally injected with 1.41 X 10^{-5} mol/Kg of HHCB for 5 days (Simmons et al., 2010). Previous research on the effects of both contaminants have showed that a three day exposure of male medaka to 5, 50 and 500 μg/L of HHCB and AHTN led to a significant induction in the expression of *vtg1*

at 500 µg/L (Yamauchi *et al*., 2008). Meanwhile, the concentration of AHTN that elicited significant inhibition of *vtg1* in our study was much lower (50 µg/L), which may be attributed to age differences as fish larvae tend to be more sensitive to environmental contaminants than adults (Hutchinson *et al*., 1998). Reports of other chemicals inducing differential *vtg1* expression levels in fish abound. For example, three generations of *Oryzias melastigma* exposed to 20 and 200 µg/L benzo[a]pyrene demonstrated significant downregulation of *vtg1* expression (Sun *et al.*, 2020). In addition, female *Oryzias melastigma* exposed to lower concentrations (2 and 10 ng/L) of 17β-trenbolone (Zhang *et al*., 2020) and F1 generation male *Oryzias latipes* exposed to metformin (Lee *et al.*, 2019) all significantly downregulated *vtg1* expression levels. Contrarily, exposure of male *Oryzias melastigma* to 10 and 50 ng/L of 17α-ethynylestradiol significantly upregulated *vtg1* expression level (Zhang *et al*., 2020).

Generally, the concentrations of PMCs causing effects should be interpreted with caution due to their high volatility and potential to adsorb to aquaria walls. From our studies, the concentrations of HHCB and AHTN measured on day 0 were 74.26±18.38% and 88.50±19.09%, respectively and degraded to 22.00±1.41% and 26.37±4.24%, respectively on day 3 (Ehiguese *et al*., 2020). Tumová *et al.* (2019) suggested that the toxicity of PMCs for aquatic organisms under semi-static conditions could be underestimated due to the potential volatility in the aquarium, significantly lowering the concentration over time.

5. Conclusion

This study assessed the neuroendocrine effects of HHCB and AHTN using the marine bivalve *R. philippinarum* and yolk-sac larvae of an estuarine species, the sheepshead minnow, *C. variegatus*. Changes in AChE and COX activities as biomarkers of neuroendocrine effects were observed in the clams, though the exact effects varied with concentration and duration of exposure. *cyp19* and *vtg1* gene expression in yolk-sac *C. variegatus* larvae after 3 days of exposure to HHCB revealed no effect for the expression of either gene, but AHTN significantly downregulated the expression of both genes at 50 µg/L. The

biomarkers studied provided useful insights to understand the potential neuroendocrine toxicity of both substances in the marine environment. Although significant changes were detected in some of the biomarkers after exposure to each contaminant, AHTN seems to be a more potent inhibitor of neuroendocrine functions in marine organisms than HHCB.

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Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups

Friday Ojie Ehiguese[a,b], Maria L. Rodgers[b], Cristiano V.M. Araújo[c], Robert J. Griffitt[b], M. Laura Martin-Diaz[a]

Table 1. Details of forward and reverse primers

Gene	Primers	Amp. Length	Ref
18s	F: GCTGAACGCCACTTGTCC R: ATTCCGATAACGAACGAGACTC	100	Simning et al., 2019
cyp19	F: CTGTCCCCTGCAATCCCAAT R: AAAGGGGACCCAAACCCAAG	72	This study
vtg1	F: ATGTCACTGTGAAGGTCAACGAA R: ACCTGTTGGGTGGCGGTAA	68	Knoebl et al., 2004

Figure
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Figures

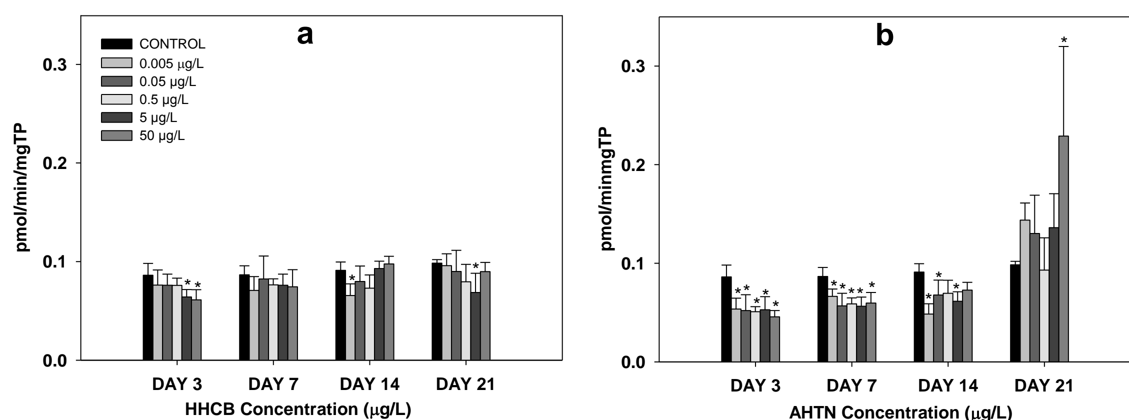


Figure 1: Acetyl Cholinesterase (AChE) activity measured in the digestive gland tissues of *R. philippinarum* exposed for 21 days to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN). Asterisks (*) show significant differences from control (p<0.05)

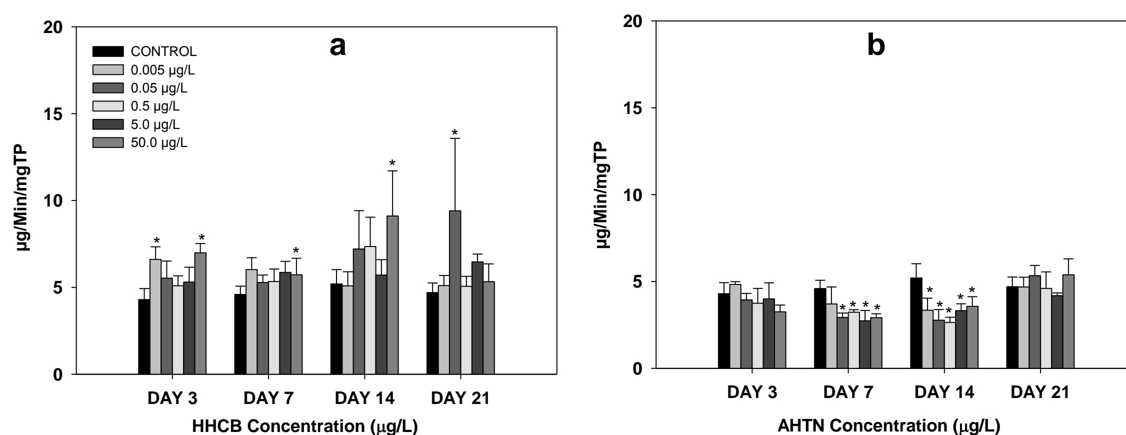


Figure 2: Cyclooxygenase (COX) activity measured in the digestive gland tissues of *R. philippinarum* exposed for 21 days to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN). Asterisks (*) show significant differences from control (p<0.05)

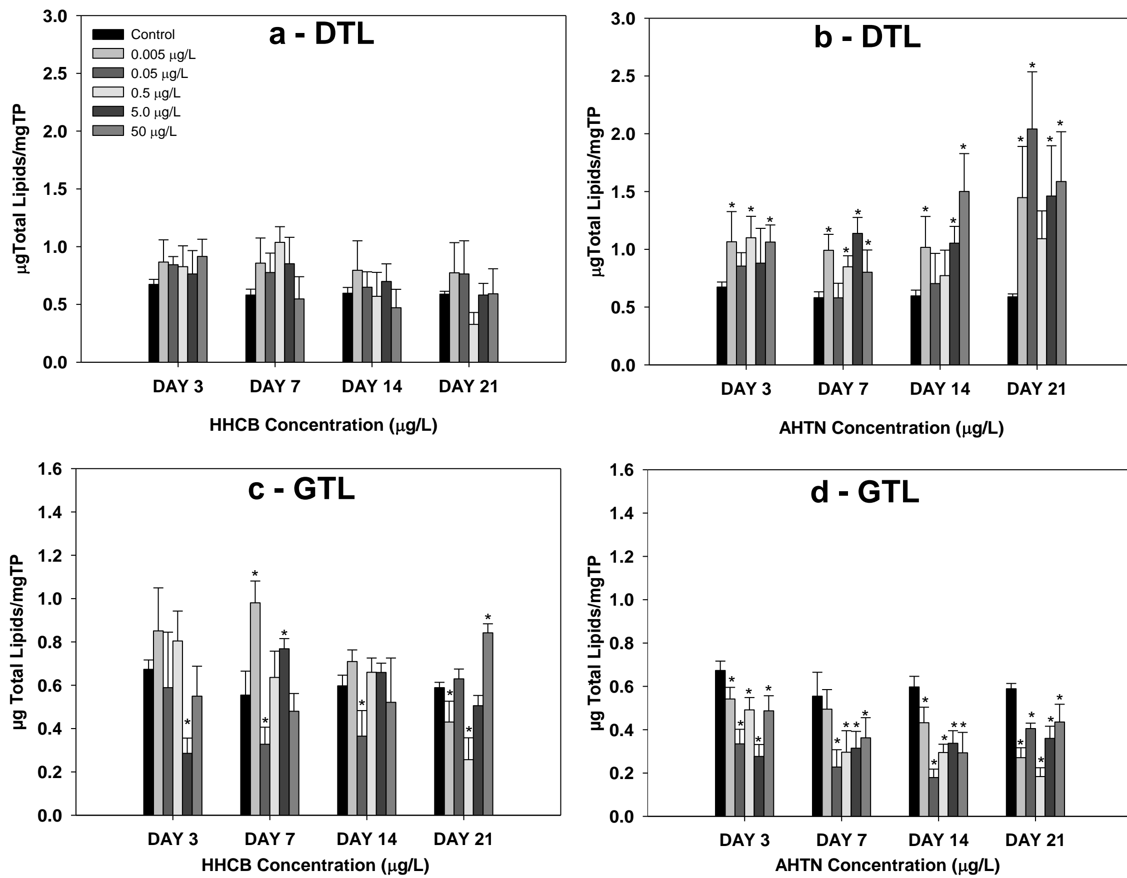


Figure 3: Energy reserves measured as total lipids in digestive gland (**a** & **b - DTL**) and gonad (**c** & **d - GTL**) tissues of *R. philippinarum* exposed for 21 days to galaxolide (HHCB) and tonalide (AHTN). Asterisks (*) show significant differences from control (p<0.05)

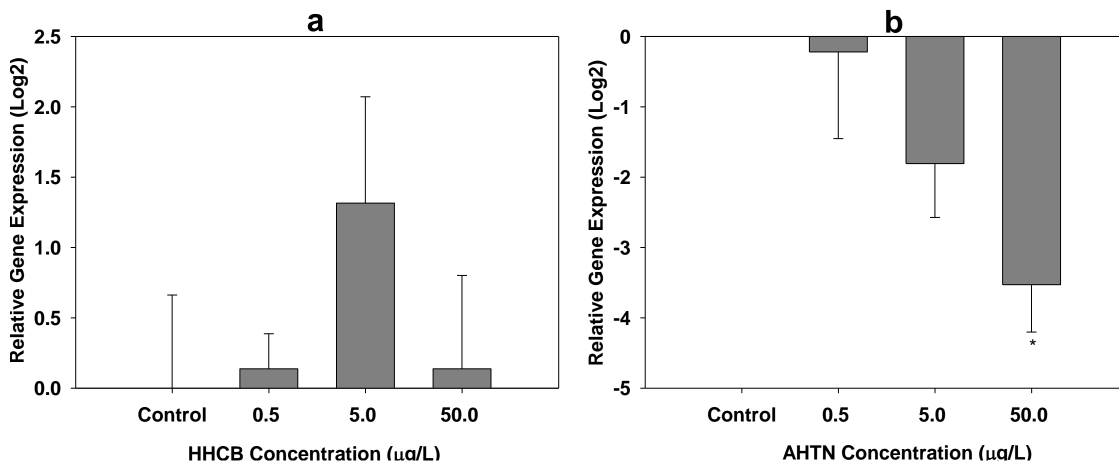


Figure 4: Relative gene expression s for *cyp19* in *C. variegatus* yolk sac larvae exposed to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN) for 96 h. Asterisks (*) show significant differences from control (p<0.05)

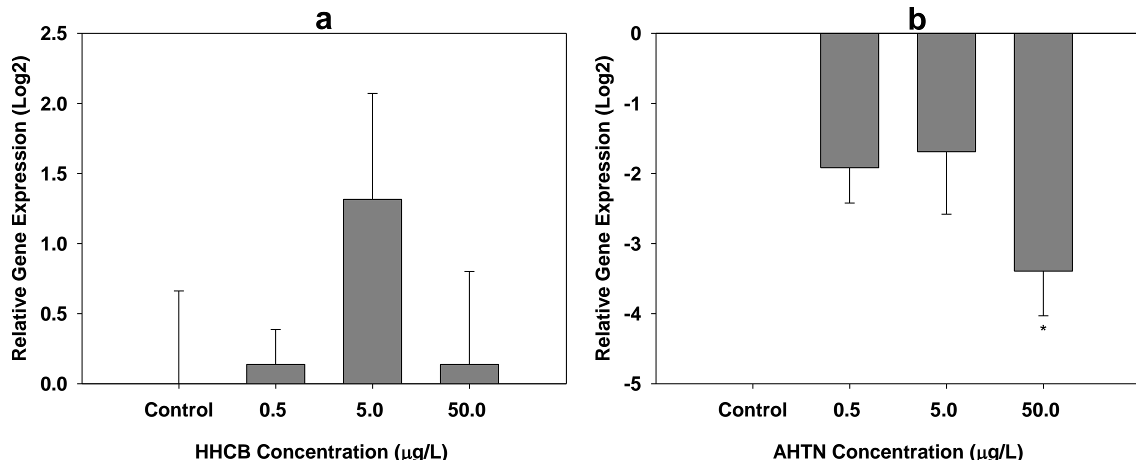
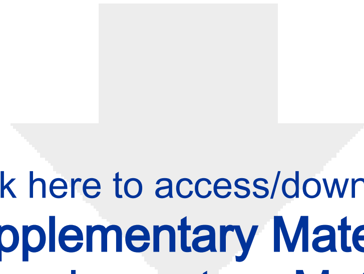


Figure 5: Relative gene expression for *vtg1* in *C. variegatus* yolk sac larvae exposed to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN) for 96 h. Asterisks (*) show significant differences from control ($p<0.05$)



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