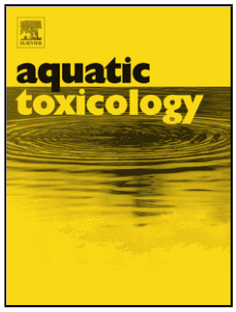
Endocrine disruptors affect larval zebrafish behavior: Testing potential mechanisms and comparisons of behavioral sensitivity to alternative biomarkers



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Endocrine disruptors affect larval zebrafish behavior: Testing potential mechanisms and comparisons of behavioral sensitivity to alternative biomarkers

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Running title: Endocrine disruption in zebrafish larvae

Highlights

- Locomotor behavior appears less sensitive as an indicator of endocrine disruption than molecular methods

- No evidence androgens or estrogens effected behavior via nuclear receptors
- Thyroid disruption had no effect on behavior
- The timing of experimentation between 96 and 118 hpf can affect behavioral results

Abstract

Larval zebrafish (*Danio rerio*) are a tool for assessing endocrine disruption during early development. Here, we investigated the extent to which a simple light/dark behavioral test at five days post fertilization could compliment current methods within the field. We exposed fertilized embryos to hormones (17β-estradiol, testosterone, dihydrotestosterone, 11-ketotestosterone, thyroxine, triiodothyronine, progesterone, and hydrocortisone) and other relevant compounds (17α ethinylestradiol, bisphenol A, bisphenol S, nonylphenol, flutamide, nilutamide, linuron, drospirenone, potassium perchlorate, mifepristone, and fadrozole) to screen for behavioral effects between 96 and 118 hours post fertilization (hpf). With the exception of progesterone, all the hormones tested resulted in altered behaviors. However, some inconsistencies were observed regarding the age of the larvae at testing. For example, the xenoestrogens 17α- ethinylestradiol and nonylphenol had behavioral effects at 96 hpf, but not at 118 hpf. Furthermore, although thyroxine exposure had pronounced effects on behavior, the thyroid disruptor potassium perchlorate did not. Finally, we were unable to demonstrate a role of nuclear receptors following testosterone and 17α- ethinylestradiol exposure, as neither the androgen receptor antagonist flutamide nor the general estrogen receptor inhibitor fulvestrant (ICI) could rescue the observed behavioral effects, respectively. Similarly, molecular markers for androgen and estrogen

disruption were upregulated at concentrations below which behavioral effects were observed. These results demonstrate hormones and endocrine disruptors can alter the behavior of larval zebrafish, but the mechanistic pathways remain unclear.

Key words: androgens, estrogens, hormone, locomotor behavior, neurotoxicology, thyroid

1. Introduction

Research into endocrine disruption has traditionally focused on reproductive processes, but more recent concerns relate to the effects endocrine disrupting chemicals (EDCs) may have on early neuronal development. For example, there is a growing consensus that early life stages are particularly sensitive to endocrine disruption, as endogenous steroid hormones play a critical role in developing and organizing sexual dimorphisms within the brain (Frye et al., 2012). Therefore, disturbances to early endogenous steroid hormones may have lasting effects on brain morphology and juvenile/adult behavior. Indeed, a number of epidemiological studies have reported sex dependent links between EDCs such as bisphenol A (BPA) and adverse behavioral outcomes in children (Braun et al., 2009; Perera et al., 2012). Similar sex-specific effects in relation to EDC exposure have been found in other mammals, as well as birds, amphibians, reptiles, and fish (reviewed in Orlando and Guillette 2007; Zala and Penn 2004).

The list of EDCs is extensive with over 800 compounds (Bergmann et al., 2013) covering plastic contaminants, herbicides, pesticides, biocides, and pharmaceuticals amongst others (Frye et al., 2012). These compounds can result in alterations in hormone synthesis or metabolism, or receptor target modulation, via mimicking, antagonizing, or altering endogenous hormone levels (reviewed in Frye et al., 2012). Laboratory experiments have not only highlighted the endocrine

disrupting potential of these compounds, but have shown that organs such as the brain and thyroid, which rely on steroid hormones during development, are particularly at risk (Colborn et al., 1993). This risk to the brain is further increased by the lipophilic nature of numerous EDCs, which can lead to their bioaccumulation in neural tissue with its high lipid content (Geens et al., 2012; Renz et al., 2013). However, identifying EDCs as being neurodevelopmental toxins is notoriously difficult, as their effects may remain hidden until late in life (Colborn et al., 1993). More worryingly, many EDCs remain relatively untested (Bergmann et al., 2013), and new compounds continuously emerge. Therefore, there is a need to establish methods that can quickly identify those of potential risk to humans and wildlife.

The zebrafish (*Danio rerio*) is an established vertebrate model in toxicology and is becoming increasingly popular as a tool to study endocrine disruption. This includes the use of zebrafish larvae, as nuclear receptors are expressed within the first five days of life (i.e. Kinch et al., 2016) and embryos/larvae are seen as an alternative to animal testing (Strähle et al., 2012). Recently, the EDC community has adopted a number of automated high-throughput behavioral assays in zebrafish larvae making screening studies more feasible (i.e. Noyes et al., 2015). However, there is still a need to identify whether endocrine hormones influence behavior so as to compare responses to suspected EDCs. Furthermore, there is a need to understand how sensitive larval behavior is as a tool to detect potential endocrine disruption. For example, how sensitive is behavior compared to molecular biomarkers, and is larval behavior sensitive to environmentally relevant concentrations of pollutants? Finally, although methodology is known to influence basal larval zebrafish behavior, how methodology impacts on EDC research is relatively unstudied. Here, we recently found that the age of testing, even during a 24 h period, could influence behavioral responses to recognized EDCs (Fraser et al., 2017).

Our objective was to investigate whether hormone exposure during early development could lead to behavioral responses in larval zebrafish, and whether they were similar to the responses seen following exposure to compounds with known endocrine disrupting ability. Following this, for estrogenic and androgenic compounds, we compared the sensitivity of the behavioral test to molecular biomarkers of endocrine disruption and attempted to rescue the behavioral effects using pharmacological agents. Similarly, we compared the sensitivity of the behavioral test to a measure of intrafollicular thyroxine content to assess thyroid disruptors.

2. Materials and methods

2.1. Chemicals

Stock solutions of 17β-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA), bisphenol S (BPS), nonylphenol (NP), testosterone (T), dihydrotestosterone (DHT), 11-ketotestosterone (11-KT), linuron (LIN), thyroxine (T_4), triiodothyronine [T_3], potassium perchlorate ($KClO_4$), progesterone (P4), drospirenone (DRO), hydrocortisone (cortisol), mifepristone (MIF), fulvestrant (ICI), flutamide (FLU), nilutamide (NIL), and fadrozole (FAD) were prepared in dimethyl sulfoxide (DMSO). Fresh stock solutions were made on the day of testing, except for 11-KT, LIN, DRO, MIF, and FAD where the stock solutions were stored at -20°C. The final concentration of DMSO in test solutions ranged from 0.01 to 1% (see **Table S1** for final DMSO concentrations and chemical origin and purity), which are below toxic levels (Maes et al., 2012).

2.2. Fish husbandry

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences (NMBU), Oslo, Norway. The unit is licensed by the Norwegian Animal Research Authority (NARA) (www.mattilsynet.no) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (www.aaalac.org). The study was carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway.

AB wild-type zebrafish were maintained at 28°C under a 14:10 light/dark photoperiod. Adult care and breeding was in accordance with the local protocols. To generate embryos, adults were placed in spawning tanks in the afternoon, the fish were spawned after the lights came on (08:00) the next day, and the embryos collected (09:00) and maintained in sterile embryo media (60 µg/ml Instant Ocean® sea salts) until the time of exposure.

2.3. Chemical exposure

Fertilized embryos were transferred into clear polystyrene 96-well plates (Nunc™ MicroWell™) and continuously exposed under static conditions from 6-7 hpf until the time of testing. For the majority of test substances, four nominal concentrations with a 1000-fold range, and a solvent control were equally distributed across one or two 96-well plates (n=12-16/concentration). For EE2, BPA, NP, and BPS, we initially included a 1 000 000-fold (pM - µM) range for each time point, but saw no effects in the low nM or pM range (see **Figures S1-4** for representative data). Therefore, consequent analysis focused on the higher concentrations only. For $KClO_4$, only one concentration (1 mM) was used. With the exception of BPS and

$KClO_4$, pilot studies were used to determine the highest concentrations that were non-teratogenic based on ×10 dilution series and the assessment of spinal aberrations, yolk sac or cardiac edema, aberrations in pigmentation, and loss of equilibrium. Generally, the highest concentration used was non-teratogenic, with the exception of T_3 and T_4 where 100 nM exposure led to spinal aberrations, craniofacial deformities, loss of pigmentation, and cardiovascular abnormalities, whereas 10 nM induced a loss of pigmentation (**Figure S5**). For mechanistic studies, embryos were exposed from 6 hpf simultaneously to test substances (i.e. EE2 or T) and the respective pharmacological substance (i.e. ICI, FLU, FAD). Each experiment was repeated 3-4 times. Prior to and following the exposure, embryos were reared in an incubator at 28°C. The light cycle within the incubator was 14:10 light/dark (lights on 07:30/lights off 21:30).

2.4. Larval behavior

Behavioral tests were conducted using a ViewPoint® Zebrabox and the accompanying video tracking software (ViewPoint Life Sciences, Lyon, France). Behavioral screening was undertaken at three time points, 96, 100, and 118 hpf, except for $KClO_4$ where data was only collected at 118 hpf and for mechanistic studies where data was collected at 96 hpf only. These corresponded to tests beginning 90 minutes (09:00), 330 minutes (13:00), and immediately after the cessation of light (07:30) in the incubator, for 96, 100, and 118 hpf, respectively. These time points were chosen as they cover "day 5", the most common age at which locomotor activity is tested. Larval behavior, including the cumulative distance travelled and the time spent active per minute, were simultaneously measured for all larvae on a plate during a light-dark-light cycle that lasted for a total of 40 minutes and consisted of 20 minutes of light (the first 10 minutes of which is considered as an acclimation period and not considered for analysis), 10 minutes of

darkness, and a final 10 minutes of light. Here, although light cycles are commonly used to assess zebrafish behavior, there is no "standard" protocol for the length or number of cycles. However, the one used in the current study is commonly used in our lab (Fraser et al., 2017; Khezri et al., 2017) and by others (Fetter et al., 2015a), but see Noyes et al. (2015) for an example of an alternate protocol. The average swimming speed was calculated by dividing the cumulated distance travelled by the total time spent active. The light level was set to 100 % on the ViewPoint software (7.45 Klux, TES 1337 light meter). During the dark period, infrared light is used to track larval activity. After the behavioral test, the larvae were inspected with a stereo microscope to identify dead or deformed larvae. Deformities included spinal aberrations, yolk sac or cardiac edema, aberrations in pigmentation, and loss of equilibrium. Different larvae were used for each time point (i.e. no individual larvae was tested more than once in the behavioral test) and the results of each compound are pooled from 3-4 independent experiments.

2.5. RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Larvae exposed to a solvent control, 10 µM 11-KT (6-96 hpf), or 1 µM EE2 (6-118 hpf) were sampled for RT-qPCR. Total RNA was isolated from 11-12 larvae per biological replicate using the QIAzol lysis reagent (Qiagen, Hilden, Germany) followed by on column purification by NucleoSpin RNA kit (Macherey-Nagel) including a DNase treatment after RNA isolation according to the manufacturer's instructions. Three independent batches of embryos were used for each treatment. For EE2, two samples of 11-12 larvae per batch were taken whereas for 11-KT only one sample of 11-12 larvae was taken.

RNA concentration was measured using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, USA). All samples had an OD A260/A280 ratio of ≥ 2.0. A total of 200 ng of RNA was used for cDNA synthesis reaction using a Tetro cDNA synthesis kit (Nordic BioSite, Norway), and 5 ng was used in the qPCR reaction in triplicate per sample using Express SYBR GreenER SuperMix with premixed ROX (Invitrogen) according to the manufacturer's recommendations. Transcript levels were analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and the standard cycling program: 50 °C for 2 minutes, 95 °C for 2 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute, and the melting curve analyses were applied. The transcript levels of *cytochrome P450, family 2, subfamily K, polypeptide 22* (*cyp2k22*), *sulfotransferase family 2, cytosolic sulfotransferase 3* (*sult2st3*), and *vitellogenin 1A/B* (*vtg*) were analyzed with *beta actin 1* (*ßactin*) and *elongation factor alpha* (*elfα*) included as housekeeping genes. Primer sequences are presented in **Table S2**.

Negative controls with no added template were included for all primer pairs (no template control), and no RT control reactions for each sample and each primer pair were run in qPCR in order to check for genomic DNA contamination (no RT control). Initial analysis of the RT-qPCR data was performed using RQ Manager 1.2 (Applied Biosystems). A standard deviation of ≤ 0.3 per triplicate was accepted. The fold change was calculated by ΔΔCt method i.e. transcript levels relative to the control and normalized to the two housekeeping genes.

2.6. Immunohistochemistry

To confirm that 1 mM $KClO_4$ abolished intrafollicular T_4 content we followed the protocol of Thienpoint et al. (2011). Briefly, 120 hpf larvae were fixed in 4% paraformaldehyde

overnight at 4°C before being transferred to methanol for long term storage at -20°C. After rehydration, larvae were depigmented in 3% H_2O_2 and 1% KOH in phosphate buffered saline (PBS) for 50 min, cracked in acetone at -20°C for 20 min, pre-treated with 0.1% collagenase in PBS at room temperature for 15 min, immersed in blocking buffer (4% goat serum, 1% BSA, 1% DMSO, 0.8% Triton X-100, and 0.1 % Tween in PBS) for 2 h, and incubated overnight at 4°C in a polyclonal anti-thyroxine antibody (anti T_4, 1:1000; MP Biochemicals, Illkirch, France). Larvae were then washed with blocking buffer for 15 min and incubated with fluorescent secondary antibody (1:300, Alexa Fluor 555 goat anti-rabbit IG) at room temperature for 2 h followed by ×3 washes in PBS. Fluorescence images of thyroid follicles were obtained from zebrafish larvae mounted in 5% methyl cellulose using a Zeiss LSM 710 Confocal Microscope (Carl Zeiss, Jena, Germany) and images acquired using a ×40 objective and LSM ZEN version 2009 software. All larvae exposed to 1 mM $KClO_4$ (n = 12) had either no or reduced fluorescence at the expected location of the thyroid follicles compared to controls (n = 12) where staining was strong.

2.7. Statistical analysis

Behavioral data were transferred to R version 3.2.3 (R Development Core Team, http://www.r-project.org). All dead and deformed larvae (with the exception of 10 and 100 nM T_4 and T_3) were discounted for behavioral analyses (≤5 % for any given exposure group). Deformed larvae were included in the T_4 and T_3 analyses as these deformities were seen over a relatively large range of concentrations and could be expected based on other vertebrate models. For all test compounds, only motility during the dark phase was analyzed as movement was minimal during the light periods. We used linear mixed effect (LME) models within the "nlme"

package of R to assess behavior. The dependent variable was either the cumulative time spent active (seconds), the cumulative distance travelled (mm), or average swimming speed (calculated as the cumulated distance travelled/cumulated time spent active), with concentration and larval age (96, 100, 118) as categorical independent variables, and replicate as a random effect. In the initial model, concentration and larval age were allowed to interact. We then used the "dredge" command within the "MuMIn" package to compare all possible model outcomes before selecting the model with the lowest Akaike information criterion (AIC) score, a method of describing data fit. The "Anova" command within the "car" library was used to extract the results for the main effects whereas the "lsmeans" command within the "lsmeans" library was used as a post-hoc test to compare groups against controls. Type II sum of squares were used for models without interactions, whereas main effects were calculated using type III sum of squares when interactions were present within the final model. The raw data (endo.csv and endo_mech.csv) and the R script (Endo.R) used can be found within the **supplementary material**. To determine whether ICI could rescue the behavioral effects of EE2, we used LME models with time active as the dependent variable, concentration as a continuous variable, ICI (Yes vs No) as a categorical independent variable, and replicate as a random effect. For all models, examination of the residual plots verified that no systematic patterns occurred in the errors (e.g. q-q plots). For q-PCR data, log transformed fold change values were used and groups were compared using *t*-tests. Significance was assigned at $P = <0.05$.

3. Results

Statistical output for the main effects or interactions within the final model, and plots of the mean data, can be found in **Figures S6-S23**. The results of post-hoc tests for concentration and larval age can be found in **Table S3**.

3.1. Estrogen and xenoestrogens

The highest concentration of E2 decreased the time spent active and the total distance moved, but had no effect on mean swimming speed. The highest concentration of BPA also decreased the time spent active and the total distance moved, but also increased the mean swimming speed. EE2, NP, and BPS showed inconsistent effects with larval age. For example, EE2 and NP both affected behavior at 96 hpf, but not 118 hpf. For BPS, the highest concentration resulted in a significant decrease in the mean swimming speed at 118 hpf, but not at any earlier time point.

For EE2, exposure to 1 µM resulted in a significant increase of *vtg* expression at 120 hpf compared to controls (**Figure 1A**), but there was no effect on behavior (**Figure 1B**). In addition, ICI co-exposure did not rescue the behavioral effects of EE2 at 96 hpf (**Figure 1C**).

3.2. Androgens and anti-androgens

Androgens had a concentration dependent effect on behavior. The highest concentration of T, DHT, and 11-KT reduced the time active and the distance moved irrespective of larval age. For DHT, although the highest concentration reduced both the time spent active and the distance moved at all time points, there was an interaction with age as the effect was more pronounced at 96 compared to 118 hpf.

The highest concentration of the anti-androgen FLU led to a significant reduction in the time spent active and an increase in mean swimming speed irrespective of larval age. A narrower

concentration response curve was produced for swimming speed, with the lowest observed effect concentration being 5 µM (**Figure 2A**). In contrast, a second anti-androgen NIL had no effect on behavior. The highest concentration of LIN significantly decreased the time spent active and total distance moved at 96 hpf, but there were no such effects at 118 hpf. Swimming speed was significantly increased at all time points for the highest concentration of LIN, but this effect was stronger at 96 compared to 118 hpf.

FLU was unable to rescue the effects of testosterone on larval activity (**Figure 2B**). Similarly, ICI and FAD were unable to rescue the behavioral effects of testosterone (**Figure 2C**). For 11-KT, molecular markers of androgen exposure (*cyp2k22* and *sult2st3*, Fetter et al., 2015b) were up-regulated at 96 hpf following exposure to 10 µM 11-KT (**Figure 2D**), a concentration at which no behavioral effects were observed (**Figure 2E**).

3.3. Thyroid hormones and thyroid disruptors

T_4 had a non-linear concentration effect, as well as a significant interaction effect with time. Here, 1 nM increased the total distance moved whereas 100 nm led to a decrease. For swimming speed, the three highest concentrations significantly increased swimming speed, but the effect of the two highest concentrations were significantly greater at 96 and 100 hpf than at 118 hpf. The highest concentration of T_3 significantly reduced the time spent active and the total distance moved irrespective of age, but there was no effect on swimming speed. The thyroid disruptor $KClO_4$ had no effect on behavior at 1 mM (**Figure 3A-C**), although fluorescent staining demonstrated reduced intrafollicular T_4 content (**Figure 3D-J**).

3.4. Progestins

The progestins P4 and DRO had no effect on behavior.

3.5. Cortisol and mifepristone

The highest concentration of cortisol led to a significant decrease in the time spent active, whereas the two highest concentrations significantly increased swimming speeds. The glucocorticoid and progesterone receptor antagonist MIF had no effect on behavior.

3.6. Fadrozole

The highest concentration of fadrozole led to a significant reduction in the time spent active, the total distance moved, and swimming speed irrespective of age.

4. Discussion

We investigated the effects of hormones and suspected EDCs on behavior using a common light/dark test in larval zebrafish. We demonstrate that exposure to hormones and environmental pollutants can lead to consistent behavioral responses. However, although some xenoestrogens had similar behavioral responses to their respective hormones (i.e. E2 vs EE2), others did not (i.e. E2 vs BPS). Furthermore, mechanistic studies suggested nuclear receptors or aromatase B could not explain behavioral responses to estrogens and androgens. Finally, for estrogenic and androgenic compounds, as well as thyroid disrupting chemicals, the behavioral test was less sensitive as a tool to identify endocrine disruption compared to RT-qPCR and immunohistochemical techniques, respectively. Therefore, further work is required to understand

the mechanistic pathways that lead to altered behavioral responses following EDC exposure in larval zebrafish.

We found no evidence that either estrogenic or androgenic compounds acted on larval behavior via estrogen or androgen nuclear receptor pathways, respectively. In addition, molecular biomarkers demonstrated endocrine disruption was evident at concentrations that elicited no behavioral effect. Previously, we also found low concentrations of EE2 (10 nM) could upregulate *vtg* and *aromatase B*, but did not affect zebrafish larval behavior (Fraser et al., 2017). We also investigated whether testosterone could influence larval behavior via estrogen receptors, as testosterone upregulates *aromatase B* expression via aromatization in the zebrafish brain (Mouriec et al., 2009), but found no evidence for such a pathway. Finally, it was notable that BPS and BPA had contrasting effects on behavior, even though they have similar estrogenic potential in larval zebrafish (Cano-Nicolau et al., 2016a). As hormones/EDCs may influence a number of biological processes that may lead to behavioral aberrations, other than neural control (e.g. oxidative stress, de Wit et al., 2010), further work is required into the mechanistic pathways in zebrafish larvae exposed to estrogenic and androgenic compounds.

We tested several environmentally relevant xenoestrogens, of which EE2 had the lowest observed effect concentration at 100 nM. This is above the median concentrations of EE2 observed in sewage effluents (approx. 1-3 ng/L or 3-10 nM, Heberer 2002). BPA (Fromme et al., 2002), BPS (Yamazaki et al., 2015), and NP (Careghini et al., 2015) have all been found in the low (10-100) nM range in natural water bodies. Although we included such concentrations in the current study, behavioral effects of these compounds where only found in the µM range. However, in adult zebrafish, 1.7 pM EE2 (Colman et al., 2009), 0.2 nM BPA (Li et al., 2017) and 450 nM NP (Xia et al., 2010) have been found to influence behavior. Therefore, the more

expansive repertoire of behaviors that can be assessed in adult fish appear to be more sensitive than the measures of locomotor activity reported in the current study.

Two out of three anti-androgens led to an increase in swimming speed. FLU is commonly used as a reference for the anti-androgenic potential of contaminated water, and we found a lowest observed effect concentration of 5 µM that is only marginally higher than the reference values found in some surface waters (anti-androgenic potential equivalent to 3 µM FLU, Pottinger et al., 2013). This may indicate that anti-androgen concentrations found in natural waters could be a concern for fish neural development. However, for LIN, the single anti-androgen tested which is of environmental concern, behavioral effects were only observed at 10 µM which is considerably higher than the low nM levels that have been detected in natural water bodies (i.e. 4 nM, EPA 1992). Previously, anti-androgens have been found to influence male reproductive behavior in sticklebacks (Rouse et al., 1977; Sebire et al., 2008), guppy (Baatrup and Junge 2001) and seabass (Dey et al., 2010), but again at levels much lower than those we found affected larval behavior. NIL and FLU, which are both anti-androgens, showed different behavioral results, which would suggest different mechanisms of action and/or uptake.

T_4 was the most potent compound tested in the current study. Previously, Fetter et al. (2015a) found T_4 had no effect on behavior at 118 hpf, but these authors did not assess swimming speed, which we found to be the most sensitive behavioral response to T_4, or any other concentration than 30 nM. Behavioral effects following exogenous T_4 exposure could be expected based on behavioral alterations in children from mothers with hypothyroidism (Andersen et al., 2014) and hyperthyroidism has been found to lead to altered neurological development in mice (Evans et al., 2002). In contrast, we found the thyroid disruptor $KClO_4$ had no effect on behavior at a concentration that impaired T_4 production in the thyroid follicles.

Similarly, Fetter et al. (2015a) found an EC_{50} of 5.2 mM for the thyroid disruptor methimazole (MMI) on swimming distance in zebrafish larvae, whereas intrafollicular T_4 production is known to be impaired at concentrations as low as 0.5 mM MMI (Raldúa and Babin 2009). This would suggest that either reduced intrafollicular T_4 content does not influence behavior during early development or our behavioral endpoints are not specific to thyroid disruption. Here, it is noted that zebrafish eggs are supplied by maternal T_4 (Chang et al., 2012), therefore the exogenous production of T_4 may not be essential for brain development until all the maternal stores are depleted.

Cortisol is a known neuromodulator and perturbations in this hormone during pregnancy can lead to neurological disease in children (Weinstock 2007). Studies in zebrafish have also shown that whole body cortisol content is associated with anxiety-like behavior in adults (Egan et al., 2009) and embryos injected with cortisol show reduced levels of thigmotaxis, which is an indicator of anxiety-like behavior (Best et al., 2016). We found cortisol exposure led to behavioral aberrations characterized by an increase in swimming speed, but the glucocorticoid receptor antagonist mifepristone had no effect on behavior. As several environmental contaminants have been found to interact with glucocorticoid receptors (GR) (i.e. Cruesot et al., 2014), further work is warranted as to whether early activation of GR receptors influences early brain development and behavior in aquatic systems.

Progestins are an emerging environmental concern in part due to their inclusion in the human contraceptive pill (Cano-Nicolau et al., 2016b), but we found no effect on larval behavior. This is despite progestins being neuro-modulating (Schumacher et al., 2014) and capable of altering the transcription profile (Zucchi et al., 2014), upregulating aromatase B in neuronal

progenitor cells (Cano-Nicolau et al., 2016b), and influencing the circadian rhythm network (Zhao et al., 2015) in the brains of zebrafish at concentrations within the range tested here.

Cytochrome P450 aromatase is an enzyme that catalyzes the conversion of C19 androgens to C18 estrogens and therefore plays a significant role in sexual dimorphism and reproduction in fish. A number of environmental pollutants are known to inhibit aromatase (e.g. tributyltin, Cooke 2002; Lyssimachou et al., 2006). Although the aromatase inhibitor fadrozole had significant effects on behavior, this was at concentrations approx. 1000-fold higher than those previously found to alter global transcriptional response in the brain (approx. 100-500 nM, Villeneuve et al., 2009) and impair reproductive development (approx. 50-200 nM, Andersen et al., 2004; Luzio et al., 2015) in zebrafish.

Similar to our previous work with tetrabromobisphenol A (Fraser et al., 2017), we found larval age within a 24 h period could affect the lowest observed effect concentration of a given compound (i.e. EE2, NP). Therefore, it is essential researchers accurately report the age of the larvae at testing. Here, larval age was found to have relatively consistent effects on behavior, with peaks in activity at 100 hpf, but a general reduction in swimming speed with age. As yet, it is unclear if this is due to the time after fertilization, or differences in activity during circadian cycles. In addition, we found some of our responses are in contrast to already published literature. For example, Saili et al. (2012) reported BPA to increase the time spent active in larval zebrafish whereas we observed a decrease. This is most likely due to methodology, as we have reared our larvae on a day/night cycle whereas Saili et al. (2012) reared their larvae in constant darkness, which influences basal activity and the responses to EDCs (see Fraser et al., 2017). Therefore, it appears different methodology can result in different behavioral responses to a given compound in larval zebrafish. As yet, it is relatively unknown as to how these

methodological artefacts impact on mechanistic studies or the translation of data from zebrafish to other vertebrates.

In conclusion, the simple light/dark test employed here can detect behavioral perturbations following exposure to a number of hormones and EDCs. Generally, hormones and EDCs did lead to behavior effects, some within the range of environmental concern, but the mechanistic pathway is unclear. Furthermore, the behavioral endpoints tested here appear less sensitive than molecular or physiological biomarkers for the detection of endocrine disruption, as well as the more expansive repertoire of behaviors studied in adult zebrafish. Combined, this work suggests further work is required to understand the mechanistic pathways that lead to behavioral responses following exposure to hormones or EDCs.

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Figures

Figure 1. Exposure to 1μM 17α ethinylestradiol (EE2) increased the expression of vitellogenin in 120 hpf larvae (**A**), but had no effect on larval behavior (**B**). At 96 hpf, EE2 had an effect on larval behavior, but the concentration response was not effected by co-exposure to the general estrogen receptor antagonist ICI (**C**). For (**A**), the results are from a *t*-test, each point is an individual data point, and the horizontal line represents the mean. For (**B**) and (**C**), the results are the main effects from linear mixed effect models and data are means ± 95% CI. In (**B**), *N* = 69-70/group. In (**C**), *N* = 51-54/group.

Figure 2. Exposure to flutamide led to a concentration dependent increase in swimming speed at 96 hours post fertilization (hpf) (**A**). The behavioral response at 96 hpf following 10 μM testosterone exposure could not be rescued by co-exposure to two concentrations of flutamide (**B**), 1 μM ICI, or 1 μM fadrozole (**C**). Exposure to 10 μM 11-ketotestosteorne had no effect on behavior (**D**), but upregulated androgen

response genes at 96 hpf. For (**A-D**), the results are the main effects from linear mixed effect models and data are means ± 95% CI and an asterisk represents a significant effect of the concentration compared to the mean (lsmeans). *N* = 36-48/group. For (**E**), the results are from *t*-tests, each point is a biological replicate, and the horizontal lines represent the mean.

Figure 3. Exposure to 1 mM potassium perchlorate ($KClO_4$) had no effect on behavior at 120 hours post fertilization (hpf) (**A-C**), but 1 mM $KClO_4$ was found to reduce intrafollicular thyroxine content (**D-J**). For (**A**), results are the main effects from linear mixed effect models and data are means ± 95% CI. *N* = 48/group. For (**D-J**), images are representative images (*n* = 12/group).