Aquatic Toxicology 193 (2017) 9–17

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Research Paper

Combinatory effects of low concentrations of 17α-etinylestradiol and citalopram on non-reproductive behavior in adult zebrafish (*Danio rerio*)

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ARTICLE INFO

Keywords:
Combination effects
17α-Etinylestradiol
Citalopram
Zebrafish
SSRI
Endocrine disrupting chemicals
Stress behavior

ABSTRACT

Sewage effluents contain pharmaceuticals, personal care products and industrial chemicals, exposing aquatic organisms to complex mixtures. The consequences of exposure to combinations of different classes of drugs in fish are largely unknown. In this study, we exposed adult zebrafish (*Danio rerio*) males and females for two weeks to low, environmentally relevant concentrations of the endocrine disrupting chemical 17α-etinylestradiol (EE_2) and the selective serotonin re-uptake inhibitor (SSRI) citalopram, alone and in combination, and analyzed behaviors of importance for population fitness, scototaxis (light/dark preference), the novel tank test and shoal cohesion. Control water contained 0.4 ng/L EE_2 and the measured exposure concentrations were 0.9 ng/L EE_2 (nominal 0.1) and 1 ng/L EE_2 (nominal 0.5). The measured concentrations of citalopram were 0.1 (nominal 0.1) and 0.4 μg/L (nominal 0.5). Both EE_2 exposures increased anxiety in males in the scototaxis test, with significantly longer latency periods before entering and fewer visits to the white zone of the tank. The combined exposures (0.9 ng/L EE_2 + 0.1 μg/L citalopram and 1 ng/L EE_2 + 0.4 μg/L citalopram) resulted in abolishment of effects of EE_2, with shorter latency period and more transitions to white than for fish exposed to EE_2 alone. In the novel tank test, the results surprisingly indicated lower anxiety after both EE_2 and citalopram exposure. Significantly more transitions to the upper half of the tank observed in males exposed to 0.1 μg/L citalopram alone compared to control males. Males exposed to EE_2 (0.9 ng/L) had shorter latency period to the upper half. Combination exposure resulted in a longer latency and fewer transitions to the upper half compared to both control, EE_2- and citalopram-exposed males. Males exposed to the combination spent significantly less time in the upper half than males EE_2 or citalopram-exposed males. Females exposed to 1 ng/L EE_2 had fewer transitions to the upper half than the control group and females exposed to 0.4 μg/L citalopram. In the shoaling test, males exposed to 0.1 μg/L citalopram + 0.9 ng/L EE_2 showed more transitions away from peers than males exposed to 0.1 μg/L citalopram alone. In conclusion, low concentrations of EE_2, closely above the predicted no effect concentration (NOEC) of 0.1 ng/L, created anxiety-like behavior in zebrafish males. Citalopram showed marginal effects at these low concentrations but in the combination exposure the behavioral effects of EE_2 were abolished. This is an initial effort to understand the effects of cocktails of anthropogenic substances contaminating aquatic environments.

1. Introduction

Aquatic organisms are exposed to a wide range of chemicals from agriculture, industry and municipal sewage, often in complex mixtures. High concentrations of pharmaceuticals are present in effluents from sewage treatment plants (STPs) (Fick et al., 2011; Nikolaou et al., 2007; Weigel et al., 2004). The bioavailability and intended biological activity of pharmaceuticals give them high potential to cause sublethal effects on non-target organisms. Fish, as vertebrates, share many common physiological features with humans and are therefore very likely to be affected by waterborne pharmaceuticals designed to affect human physiology (Gunnarsson et al., 2008). Earlier studies on the effects of within-class mixtures of EDCs on fish exist (Brian et al., 2007; Kortenkamp, 2007; Lin and Janz, 2006; Santos et al., 2006; Thorpe et al., 2003). However we lack knowledge about the effects of mixtures of pharmaceuticals with different modes of action. In the present study,

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http://dx.doi.org/10.1016/j.aquatox.2017.10.001
Received 21 June 2017; Received in revised form 18 September 2017; Accepted 2 October 2017
Available online 04 October 2017
0166-445X/

we analyzed the effects of low levels of the endocrine disrupting chemical (EDC) EE_2, close to the NOEC of 0.1 ng/L, and the SSRI citalopram, as well as a combination of the two, on non-reproductive behavior in zebrafish (*Danio rerio*).

EE_2 from human oral contraceptives is present in effluents from STPs in concentrations from less than 1 ng/L up to 300 ng/L (Hannah et al., 2009; Kolpin et al., 2002; Laurenson et al., 2014; Sun et al., 2013). The predicted no effect concentration (NOEC) of EE_2 for water-living organisms is as low as 0.1 ng/L (Caldwell et al., 2012). EE_2 is environmentally persistent, bio-magnification has been observed (Aris et al., 2014) and is regarded as the EDC contributing the highest ecological risk in waste water (Laurenson et al., 2014; Sun et al., 2013). EDCs have the potential to interfere with the function of the hormone systems of all vertebrates (Guillette and Gunderson, 2001; Waring and Harris, 2005; Vos et al., 2000). Human fetal exposure to estrogenic compounds has been associated with depression (Wolstenholme et al., 2012) and distractibility, affected verbal skills, learning and memory, reduced masculine play (Xu et al., 2010). Behavioral variables shown to be affected by estrogenic compounds in rodents include aggression, anxiety, play behavior, attention, learning and memory and sexual behavior (Dugard et al., 2001; Ryan and Vandenbergh, 2006; Wolstenholme et al., 2012, 2011; Xu et al., 2010). Environmental-like levels of EE_2 caused alterations in female sexual behavior in adult rats exposed during development (Della Seta et al., 2008) and juvenile rats showed an anxiety-like response in a novelty preference test after developmental exposure (Zaccaroni et al., 2016). Developmental exposure of EE_2 has also showed to cause anxiety-related behavior, alter spatial memory, disturbed maternal behavior and a lack of discrimination between gonad-intact and castrated males in female mice (Ryan and Vandenbergh, 2006). Male mice developmentally exposed to low doses of EE_2 showed an increase in sexual behavior and modifications of neuronal networks. The effects were also transgenerationally transmitted to the F4 generation (Derouiche et al., 2015). In fish EE_2 has shown to cause reduced fertility and fecundity, feminization in male fish, skewed sex ratios and decreased egg and sperm production as well as behavioral changes (Aris et al., 2014). EE_2 exposure has caused alterations in risky behavior in the threespine stickleback (Bell, 2004) and guppies (Heintz et al., 2015) as well as boldness in Siamese fighting fish (Dzieweczynski et al., 2014). We have previously found that EE_2 increases anxious behavior in guppies (*Poecilia reticulata*) and zebrafish (*Danio rerio*) exposed as adults (Hallgren et al., 2011; Reyhanian et al., 2011) or during development (Volkova et al., 2015b; Volkova et al., 2012). Developmental exposure resulted in irreversible effects (Volkova et al., 2015b), which were shown to be transgenerationally transferred (Volkova et al., 2015a).

Citalopram has been detected in STP effluents in concentrations ranging from 9.2 ng/L (Vasskog et al., 2006) to 720 ng/L (Wahlberg et al., 2008) and in surface waters between 4 ng/L (Giebułtowicz and Nałęcz-Jawecki, 2014) and 76 µg/L (Fick et al., 2009); more typical concentrations in polluted recipients are around 10–150 ng/L (González Alonso et al., 2010; Grabicova et al., 2015; Metcalfe et al., 2010; Nödler et al., 2011). Several SSRIs are present in STP effluents, and the combined load of 7 major SSRIs and their metabolites was up to 3.2 µg/L downstream a Canadian STP (Brooks et al., 2005). The predicted water concentration needed to obtain human therapeutic levels in fish is 141 ng/L (Fick et al., 2010). Brain SSRI bioaccumulation has been observed in fish caught downstream an STP (Brooks et al., 2005), and citalopram has been found in the liver of perch (*Perca fluviatilis*) caught in the inner parts of the Stockholm archipelago (Woldegiorgis et al., 2006). SSRIs are psychoactive drugs prescribed for treating depression and other psychiatric disorders. SSRIs reduce the re-uptake of the neurotransmitter serotonin (5-hydroxy-tryptamine; 5-HT) into the pre-synaptic nerve terminal by inactivating 5-HT transporters (5-HTT), resulting in an increased concentration of extracellular 5-HT in the synapse. 5-HT is ubiquitous to all vertebrate groups and influences a wide range of behaviors and endocrine functions (Anon., 2010). In fish, SSRI have been shown to affect behaviors like feeding (Kellner et al., 2015), aggression (Winberg and Thörnqvist, 2016) and anxiety (Barbosa et al., 2012; Kellner et al., 2016; Olsén et al., 2014; Sackerman et al., 2010). Citalopram, an abundantly prescribed SSRI, has given an anxiolytic response in the novel tank test in zebrafish (Sackerman et al., 2010), guppies (Olsén et al., 2014) and three-spine sticklebacks (Kellner et al., 2016).

In this study, adult zebrafish were exposed to low, environmentally relevant concentrations of two pharmaceuticals commonly found in the environment, EE_2 and citalopram, and analyzed for impact on non-reproductive behavior. The aim of the study was to investigate if the behavioral effects previously found of the two substances could still be seen at very low concentrations. We also further wanted to investigate if the effects of the two compounds, the anxiolytic effects of citalopram and anxiogenic effects of EE_2 would counteract and affect the behavioral outcome in the combinatory exposure. We utilized two tests assessing anxiety, the scototaxis test (Maximino et al., 2010) and the novel tank (NT) test (Egan et al., 2009), and one test analyzing social behavior by mean of shoal cohesion (Moretz et al., 2007). We studied both effects on single-substance exposures as well as a combination of the two.

2. Materials and methods

2.1. Animals and treatments

Adult 6-month-old zebrafish (*Danio rerio*) of the wild type strain AB were obtained from the Karolinska Institute Zebrafish Core Facility, Stockholm, Sweden. Fish of different sex were kept separate under standardized conditions (tap water, 25–27 °C, pH 7.8, conductivity 20.7 mSi) with 12/12 h light/dark cycles, and fed three times daily with Sera Dry Flakes (Vipan, Germany) and newly hatched Artemia nauplii (Artemia International LCC, USA). The fish were allowed to acclimatize to the new environment for 7 days before the experiment was started. All treatment and handling of the animals was performed according to the Swedish Animal Care legislation and approved by the Southern Stockholm Animal Research Ethics Committee (DNR S28-15).

Solutions of EE_2 (Sigma-Aldrich, USA) and citalopram (a racemic mixture of the citalopram bromide R- and S-enantiomers, kindly donated by H. Lundbeck A/S, Copenhagen, Denmark) were made by stepwise dilutions from stock solutions of EE_2 in acetone and citalopram dissolved in distilled water. All stock solutions were kept refrigerated in dark bottles before the dilution with aquarium water. The final working solutions, obtained by a 1:1000 dilution from the refrigerator stocks with temperate aquarium water, had nominal concentrations of 0.1 and 0.5 ng/L EE_2 and 0.1 and 0.5 µg/L citalopram, respectively, and the two combinations of the two: 0.1 ng/L EE_2 + 0.1 µg/L citalopram and 0.5 ng/L EE_2 + 0.5 µg/L citalopram. Since EE_2 solutions contained acetone, all other solutions including the water control were adjusted to contain equal concentrations of acetone, 10 ppm.

Fish were exposed in 3L aquaria with a semi-static model of 1/2 volume exchange per day. We exposed seven fish per aquarium. For each sex, one aquarium started per exposure per day for three consecutive days, rendering 21 fish of each sex per treatment in total (3 replicate aquaria with 7 fish in each). When water was changed, the aquaria were also cleaned from feces and food residues. After the 14 days exposure period, the fish were subjected to three behavior tests, and locomotor activity was analyzed within one of the test sessions. Water samples for chemical analyses were collected at three occasions during the exposure period and stored at −20 °C until analysis.

2.2. Chemical analyses

All reference standards including citalopram, EE_2 and EE_2-d6 were purchased from Cerilliant Co (via Sigma-Aldrich Sweden AB, Sweden). Stock and working solutions were prepared in methanol and stored at

Table 1
Measured concentrations of EE_2 and citalopram shown as mean and SE in the water samples taken from the experiment aquaria at 3 occasions during the exposure.

	EE_2 ng/L	citalopram µg/L	EE_2 ng/L	EE_2 ng/L	citalopram µg/L	citalopram µg/L
Nominal concentration	0	0	0.1	0.5	0.1	0.5
Measured concentration	0.4	0.005	0.9	1.0	0.1	0.4
SE	0.1	0.003	0.1	0.1	0.004	0.01
	n = 13	n = 13	n = 11	n = 12	n = 12	n = 10

−20 °C. During sample preparation 100 mL water was spiked with 1 ng of internal standard and then applied to SPE cartridge (StrataX, 100 mg, 6cc from Phenomenex) for the extraction. Briefly, the column was conditioned with 2 × 2 mL methanol and 2 mL MilliQ water was added before 100 mL of the water sample was added under air pressure. The column was thereafter cleaned with 0.5 mL methanol and dried 30 min under vacuum pressure. The analytes were eluted with 5.5 mL acetonitrile. The volume was reduced to dryness and reconstituted with 70 µL of methanol. The final extract was transferred to an auto-sampler vial for the LC–MS/MS injection.

Instrumental analysis was performed on a TSQ Quantiva triple quadrupole mass spectrometer coupled to a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific). The instrument was operated in atmospheric pressure chemical ionization in positive mode with positive ion discharge current at 4.0 µA, ion transfer tube temperature 320 °C, vaporizer temperature 450 °C, sheath gas pressure 40 psi, ion sweep gas pressure 1.0 psi and aux gas pressure 2 psi. The achieved product ions were *m/z* 279 > 133, 154 for 17α-etinylestradiol and *m/z* 325 > 109, 262 for citalopram. The LC separation was performed on a hypersil gold C18 column (2.1 × 100 mm, 1.9 µm) (Thermofisher Scientific). The column temperature was set to 50 °C. The mobile phase flow rate was 450 µL/min operating in a gradient mode with total run time 4.3 min. Mobile phase A and B consisting of water and methanol, respectively, with both containing 0.05% formic acid. The method detection limit was 0.2 ng/L for EE_2 and 0.1 ng/L for citalopram.

2.3. Behavioral analyses

A scototaxis (dark/light preference) test was performed in a 20 × 20 × 40 cm test tank divided into a black and a white half with a water depth of 12 cm (Volkova et al., 2015b). The scototaxis test was observed from above. Two transparent sliding walls constituted a central compartment of 5 × 20 cm. The fish was placed in the central compartment and allowed to acclimatize. After a 5 min acclimatization period the walls were raised above water level allowing the fish to move freely in the aquaria (Fig. S1a). Behavior was registered for 5 min as latency to the first transition into the white half, total number of entries into the white half and total time spent in the white half.

Novel tank (NT) and shoaling behavior were studied in the same session as previously described in Volkova et al. (2015b). Briefly, the test tank (20 × 20 × 40 cm) filled with pre-heated tap water was divided by horizontal and vertical lines into top/bottom and right/left halves. The NT test and shoaling test were observed from the side. A transparent Plexiglas screen held 5 unexposed fish of the same sex as the subject fish in a separate compartment in the aquaria. A black sheet prevented visual contact with the test compartment during the NT test (Fig. S1b). A fish was introduced into the test compartment, and behavior was recorded for 5 min. Variables recorded were latency to crossing of the horizontal midline, number of transitions to the upper half and total time spent in the upper half of the aquarium. The black sheet was then removed for the shoaling test revealing the shoal for the subject fish (Fig. S1c). When the fish made contact with the shoaling fish, behavior was recorded for 5 min as latency to cross the vertical mid-line, number of crossings of the mid-line and total time spent in the opposite half of the aquarium. Fish that did not make contact with the shoal within 5 min were excluded from analyses. Locomotor activity was analyzed as the number of times the fish crossed the lines in a grid, both horizontal and vertical, during 1 min, starting 1 min into the NT session. All behavior tests were video recorded and analyzed manually on screen.

2.4. Statistical analyses

Behavioral data was analyzed with linear mixed-effects models using the statistical software R 3.01 (R Core Team, 2015) and package lme4 (Bates et al., 2015). The experimental effects on all response variables were analyzed with mixed models, using Treatment, Sex and Treatment × Sex interaction as fixed factors. Treatment starting day (n = 3) and aquaria were used as random factors to control for time effects and differences among aquaria. If the interaction Treatment × Sex was significant both sexes were also analyzed separately at each concentration combination for the two drugs. A Gaussian distribution was applied for time data, while a Poisson distribution was applied for counts (transitions across a line). If needed, data were log or square root transformed to improve normalization and heteroscedasticity. Post-hoc analysis was performed with Tukey contrasts using the package multcomp (Hothorn et al., 2008). Before plotting all means and confidence intervals were back-transformed using the package effects (Fox and Hong, 2009).

3. Results

3.1. Chemical analyses

The measured exposure concentrations of EE_2 and citalopram are shown in Table 1. The chemical analysis performed was sensitive, with a detection level of 0.2 ng/L for EE_2. Therefore, a background level of 0.4 ng/L in control water samples, obtained from Stockholm tap water, was observed (Table 1). Furthermore, while the measured concentration in the higher exposure group (1.0 ng/L) was close to the nominal value (0.5 ng/L) plus the concentration in control water, the nominal 0.1 ng/L exposure turned out to be almost as high (0.9 ng/L) and not the 20% of the high exposure. However, the two EE_2 groups were exposed and analyzed separately, and they are treated and presented as separate groups despite of the similarity of the measured concentrations. As the same water was used for the citalopram exposures, they also contained 0.4 ng/L EE_2 and no true control for the citalopram exposures were obtained. The levels of citalopram were very low, 0.0005 µg/L, in the control samples as well as in the samples without the additional citalopram. In the citalopram exposures the measured concentration was 0.1 µg/L in the nominal concentration 0.1 µg/L and 0.4 µg/L in the nominal 0.5 µg/L (Table 1).

3.2. Scototaxis behavior

The analysis of the complete data set with both exposure concentrations and both sexes revealed a Sex × Treatment interaction for the latency period before entering the white half, and for the number of transitions to the white half of the aquarium ($p = 0.004$, and $p < 0.001$, respectively, Table 2). Total time spent in the white compartment showed no statistically significant interaction between treatment and sex ($p = 0.85$) but there was a significant sex difference

Table 2
P-values from linear mixed-effects models for the effects of EE_2 and citalopram on the behavior of zebrafish in scototaxis, novel tank and shoaling tests. The treatment factor contains one control treatment, two concentrations of EE_2 (0.9 and 1.0 ng/L), two concentrations of citalopram (0.1 and 0.4 μg/L) and two combinations of both drugs, one with the low and one with the high concentrations. Results are also shown for separate models for each sex and the two levels of exposure. In these models the treatment factor contains one control treatment, one EE_2 and one citalopram concentration and the combination of these. P > 0.05 are shown as ns.

Scototaxis test		Latency to white half	Transitions to white half	Time in white half
Treatment		0.004	0.02	ns
Sex		< 0.001	< 0.001	< 0.001
Treatment x Sex		0.004	< 0.001	ns
Treatment effects, separate models				
	♂ EE_2 0.9 ng/L, cit 0.1 μg/L	0.002	0.007	ns
	♂ EE_2 1 ng/L, cit 0.4 μg/L	0.002	0.004	ns
	♀ EE_2 0.9 ng/L, cit 0.1 μg/L	ns	ns	ns
	♀ EE_2 1 ng/L, cit 0.4 μg/L	ns	ns	ns
Novel tank test		**Latency to upper half**	**Transitions to upper half**	**Time in upper half**
Treatment		< 0.001	0.008	ns
Sex		< 0.001	0.003	0.01
Treatment x Sex		< 0.001	< 0.001	< 0.001
Treatment effects, separate models				
	♂ EE_2 0.9 ng/L, cit 0.1 μg/L	< 0.001	< 0.001	0.003
	♂ EE_2 1 ng/L, cit 0.4 μg/L	ns	ns	ns
	♀ EE_2 0.9 ng/L, cit 0.1 μg/L	ns	ns	ns
	♀ EE_2 1 ng/L, cit 0.4 μg/L	ns	0.032	ns
Shoaling test		**Latency leaving peers**	**Transitions leaving peers**	**Time away from peers**
Treatment		0.03	0.054	ns
Sex		ns	ns	0.03
Treatment x Sex		ns	< 0.001	ns
Treatment effects, separate models				
	♂ EE_2 0.9 ng/L, cit 0.1 μg/L	ns	0.017	ns
	♂ EE_2 1 ng/L, cit 0.4 μg/L	ns	ns	ns
	♀ EE_2 0.9 ng/L, cit 0.1 μg/L	ns	ns	ns
	♀ EE_2 1 ng/L, cit 0.4 μg/L	ns	ns	ns

($p < 0.001$, Table 2). The significant interaction between sex and treatment for latency, was due to a higher latency for males in both exposure concentrations (Fig. 1a, Table 2) but not for females (Table 2). The same thing is seen for transitions to the white half, where male fish that were exposed to EE_2 had significantly fewer transitions (Fig. 1b, Table 2) while there were no treatment effects for females (Table 2). For the time spent in the white half there were no statistically significant treatment effects in neither males nor females. Since no treatment effects were observed for females in the mixed effects model, post-hoc analysis was performed on male behavior only.

For males, relevant groups were compared with each other using Tukey's multiple comparison test. The post-hoc analysis revealed that both 0.9 ng/L and 1 ng/L EE_2 significantly prolonged the initial period in the dark half of the tank compared to males in the control ($p = 0.003$ and $p = 0.032$ respectively, Fig. 1a). No significant effects of either 0.1 μg/L or 0.4 μg/L citalopram could be discerned (Fig. 1a). A combination effect was observed as the addition of 0.1 μg/L citalopram to 0.9 ng/L EE_2 abolished the dark-dwelling effect of the EE_2 exposure as shown by a significantly shorter latency period to first transition to white than in males exposed to EE_2 alone ($p < 0.001$, Fig. 1a). No significant effects on latency time could be identified in the comparison between 1 ng/L EE_2 with and without 0.4 μg/L citalopram ($p = 0.26$, Fig. 1a). Both citalopram exposures had significantly shorter latency period compared to EE_2 exposures (0.1 μg/L citalopram versus 0.9 ng/L EE_2: $p = 0.009$; 0.4 μg/L citalopram versus 1 ng/L EE_2: $p < 0.001$, Fig. 1a), but none of the citalopram exposures were significantly different from the control. Both EE_2 exposures also resulted in significantly fewer transitions to the white side when compared to the control ($p = 0.003$ and $p = 0.006$ for 0.9 ng/L and 1 ng/L, respectively, Fig. 1b). No significant differences in number of transitions caused by any of the citalopram exposures could be discerned (Fig. 1b). For the number of transitions citalopram abolished the anxiogenic effect of EE_2 in both combination exposures ($p < 0.001$ and $p = 0.003$, respectively, Fig. 1b). Also, the 0.4 μg/L citalopram exposure resulted in significantly more transitions than the corresponding concentration of EE_2 ($p < 0.001$, Fig. 1b). No significant effects in total time spent in the white compartment was noted (Fig. 1c).

3.3. Novel tank behavior

The mixed model of the complete NT dataset (Table 2) showed that there was a significant Treatment × Sex interaction for all NT parameters (all three $p < 0.001$). The interactions were due to significant effects for male fish in the exposure group 0.9 ng/L EE_2 and 0.1 μg/L citalopram for all response variables (Table 2) and in the 1 ng/L EE_2 and 0.4 μg/L citalopram exposure group in females for number of transitions to upper half of the aquarium (Table II) but not for the other response variables (Table 2).

Post-hoc analyses of the behavior in males exposed to the lower concentrations, 0.9 ng/L EE_2 and 0.1 μg/L citalopram, revealed that EE_2 significantly decreased the latency to first transition ($p = 0.035$, Fig. 2a), while no significant effects of citalopram could be discerned. The latency period for the combination treatment (0.9 ng/L EE2 + 0.1 μg/L citalopram, Fig. 2a) was significantly longer than for both the EE_2- and the citalopram-exposed males (both $p < 0.001$). The number of transitions to the upper half of the tank (Fig. 2b) was significantly increased by 0.1 μg/L citalopram ($p = 0.038$) exposure compared to the control while the EE_2 exposure did not significantly affect the number of transitions ($p = 0.084$). Males exposed to the combination of the two drugs (0.9 ng/L EE_2 + 0.1 μg/L citalopram, Fig. 2b) made significantly fewer transitions to the upper half than did both control males ($p < 0.001$), EE_2-exposed males ($p < 0.001$), and citalopram-exposed males ($p < 0.001$). Neither EE_2, citalopram, nor the combined exposure affected the time spent in the upper half

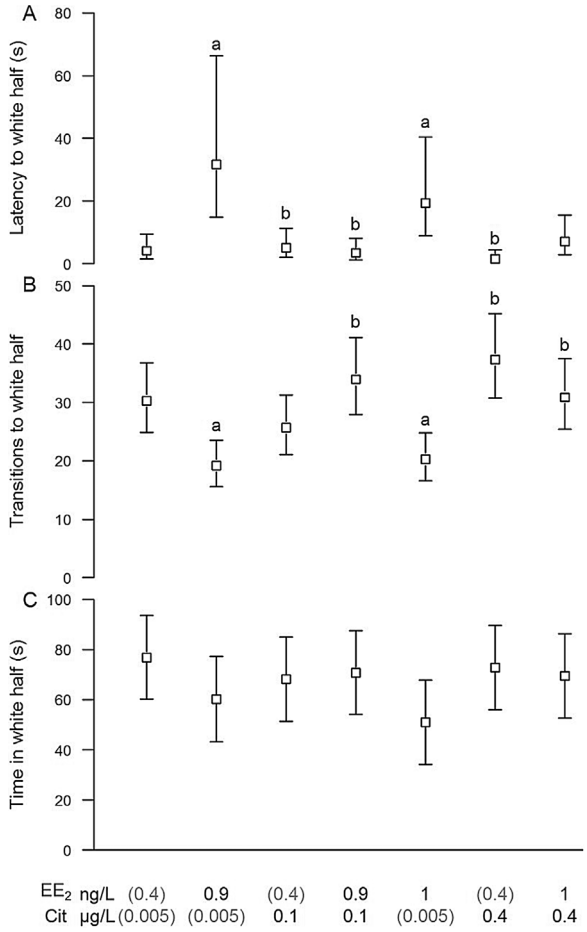

Fig. 1. Scototaxis behavior of zebrafish males exposed to EE_2 (0.9 ng/L and 1 ng/L), citalopram (0.1 µg/L and 0.4 µg/L) and combination exposures (background levels found in the water within brackets). A) Latency time (s) before entering the white half, B) Number of transitions to the white half and C) Total time (s) spent in the white half. Data represent mean ± 95% CI of 21 males/group. [a] Significantly different from control males, [b] significantly different from EE_2-exposed males.

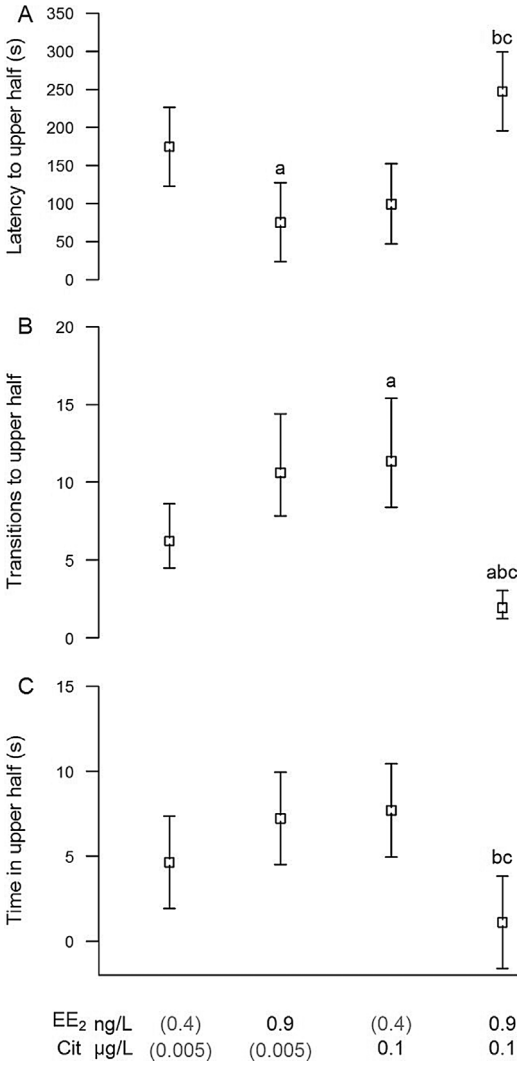

Fig. 2. Behavior of male zebrafish in the novel tank test after exposure to EE_2 (0.9 ng/L), citalopram (0.1 µg/L) and a combination of the two (background levels found in the water within brackets). A) Latency time (s) before crossing the midline to the upper half, B) Number of transitions to the upper half and C) Total time (s) spent in the upper half. Data represent mean ± 95% CI of 21 males/group. [a]Significantly different from control males, [b]significantly different from EE_2-exposed males. [c]significantly different from citalopram-exposed males.

compared to control males, but the males exposed to 0.9 ng/L EE_2 + 0.1 µg/L citalopram spent significantly less time in the upper half than both the EE_2- ($p < 0.001$, Fig. 2c) and citalopram-exposed males ($p < 0.001$, Fig. 2c).

For females exposed to 1 ng/L EE_2, post-hoc analysis showed that the number of transitions to the upper half was significantly lower in the EE_2-exposed fish compared to both the control ($p = 0.032$) and citalopram-exposed fish ($p = 0.011$, Fig. 3b).

3.4. Shoaling behavior

3 fish were excluded from the analyses due to failure to make contact with the shoal (1 fish in 0.9 ng/L EE_2, 1 fish in the lower combination treatment and 1 fish in the 0.4 µg/L citalopram). In the complete mixed model analyses of the shoaling behavior which include both exposure levels, and both sexes (Table 2), there was a significant treatment × sex interaction for the number or transitions over the vertical midline ($p < 0.001$). The interaction was due to a treatment effect on the number of transitions for males in the lower exposure group, 0.9 ng/L EE_2 and 0.1 µg/L citalopram ($p = 0.017$) but not for females or males in the high-exposure group.

Post-hoc analysis of the male fish showed that the number of transitions away from the peer group (Fig. 4b) were significantly lower in fish exposed to 0.1 µg/L citalopram compared to the control ($p = 0.004$) and compared to the combined exposure group 0.9 ng/L EE_2 + 0.1 µg/L citalopram ($p = 0.002$).

3.5. Locomotor activity

Locomotor activity, analyzed as the number of line crossings in a grid, both horizontal and lateral, were very similar between males and females. Both sexes made around 170 crosses within a one minute period of analysis. No significant differences in locomotor activity were observed in response to EE_2 or citalopram exposure, or to the combination of the two drugs (data not shown).

4. Discussion

In the present study, we observed anxiogenic effects, revealed as increased dark-dwelling in the scototaxis test after a two-week exposure to 0.9 and 1 ng/L EE_2 in adult zebrafish males. These results point out that the scototaxis test is very sensitive to EE_2, and suggests that it might contribute to the identification of exposure to environmental EDCs. We have previously found anxiogenic effects of developmental exposure to 1.2 and 1.6 ng/L EE_2 in zebrafish (Volkova et al., 2015b),

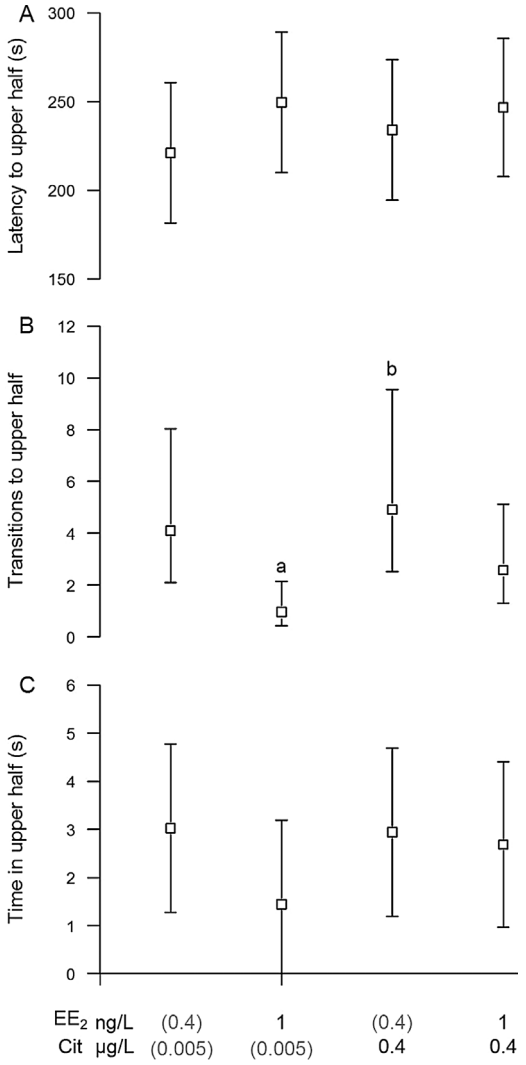

Fig. 3. Behavior of female zebrafish in the novel tank test after exposure to EE_2 (1 ng/L), citalopram (0.4 µg/L) and a combination of the two (background levels found in the water within brackets). A) Latency time (s) before crossing the midline to the upper half, B) Number of transitions to the upper half and C) Total time (s) spent in the upper half. Data represent mean ± 95% CI of 21 females/group. [a]Significantly different from control females, [b]significantly different from EE2-exposed females.

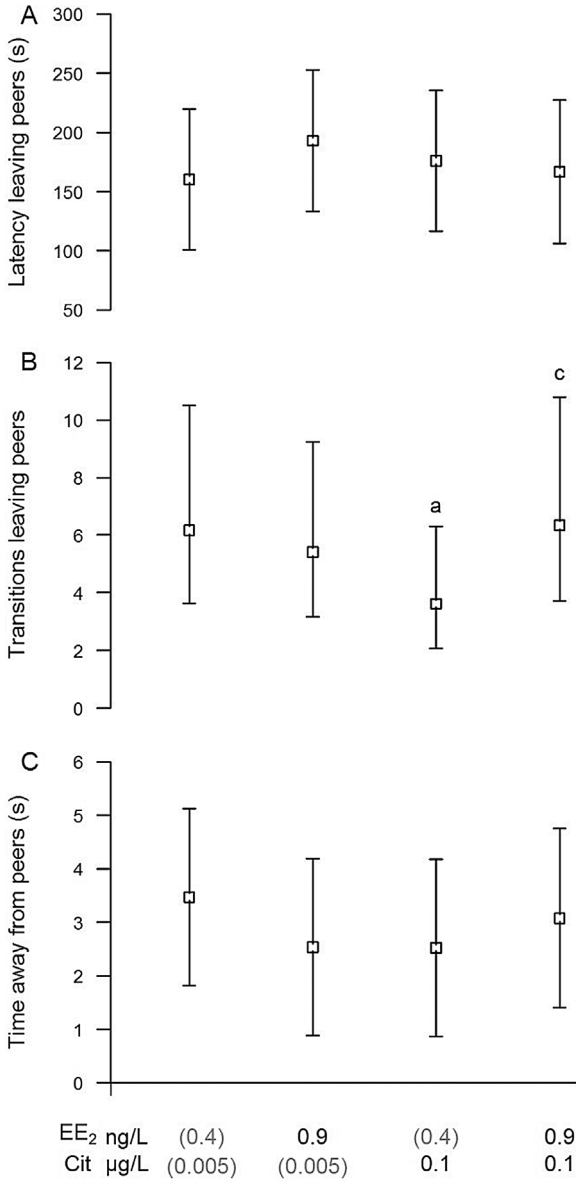

Fig. 4. Shoaling behavior of male zebra fish after exposure to EE_2 (0.9 ng/L), citalopram (0.1 µg/L) and a combination of the two (background levels found in the water within brackets). A) Latency time (s) before crossing the vertical midline away from the shoal, B) Number of transitions away from peers and C) Total time (s) spent away from peers in the opposite half. Data represent mean ± 95% CI of 21 males/group. [a]Significantly different from control males, [c]significantly different from citalopram-exposed males.

suggesting that this substance effectively affects scototaxis behavior in all life stages in fish. The more prominent effects in males than in females in this study suggest a higher sensitivity to EE_2-induced behavioral alterations in adult male zebrafish. This is, to the best of our knowledge, the first published study of anxiety behavior in female zebrafish, and conclusions await further studies. Developmental exposure does, however, affect females at least as much as males (Volkova et al., 2015a; Volkova et al., 2012), and developmental EDCs also affect fertility in both sexes (Hill and Janz, 2003; Xu et al., 2008).

In male fish, the response to EE_2 in the scototaxis test was clearly anxiogenic at both exposure levels, and in the NT test, 0.9 ng/L EE_2 increased anxious behavior in females. For males in the NT test, however, 1 ng/L EE_2 had no effect while 0.9 ng/L unexpectedly resulted in reduced anxiety. The lack of consistency between the anxiety tests is puzzling. These tests are not identical, however; while white is clearly an aversive stimuli in zebrafish, NT behavior is dependent on novelty and thus more sensitive to minor environmental fluctuations (Blaser and Rosemberg, 2012; Maximino et al., 2010). Non-monotonous concentration-response curves with different effects at very low concentrations is, not uncommon for substances with endocrine action (Vandenberg et al., 2012). We have previously observed that when adult zebrafish males were exposed to higher concentrations of EE_2, the behavior in the NT test shifted from anxiogenic at 5 ng/L to anxiolysis at 25 ng/L (Reyhanian et al., 2011).

Citalopram at the levels of 0.1 and 0.4 µg/L caused few effects on behavior in the present setting. Only two variables – number of transitions to the upper half in the NT test and number of transitions away from peers in the shoaling test – were significantly different for males exposed to 0.1 µg/L citalopram compared to control males. Our conclusion is that the current concentrations of citalopram were too low to efficiently affect the tested non-reproductive behaviors although it cannot be ruled out that the presence of EE_2 contamination in the water impaired the results. The citalopram concentrations used were lower than those previously demonstrated to be anxiolytic in zebrafish and other fish species (Kellner et al., 2016; Olsén et al., 2014; Sackerman et al., 2010).

We also found that combinatory exposure of two different classes of

drugs affected the outcomes compared to single-substance exposures. While no direct effects of citalopram were found in the scototaxis test, clear anxiogenic effects of EE_2 were observed in zebrafish males and these anxiogenic effects were counteracted by citalopram in both combination EE_2/citalopram exposures. In the NT test in males, both the exposure to 0.9 ng/L EE_2 and to 0.1 µg/L citalopram alone showed a result suggestive of anxiolysis, with more transitions to the upper half by 0.1 µg/L citalopram and shorter latency period before entering the upper half by 0.9 ng/L EE_2. When these exposures were given simultaneously, however, a combinatory effect was observed with significantly increased anxiety compared to fish exposed to each single substance. Furthermore, in the third variable in the NT test, where effects of neither drug alone were observed, fish given the combined exposure spent significantly less time in the upper half of the tank than fish exposed to citalopram or EE_2 alone. Thus, the combinatory effects observed were clear but not completely straight-forward. It should be expected, however, that combinations of drugs might have complex effects on living organisms, and combination effects will clearly further complicate the evaluation of environmental consequences of anthropogenic drug exposure.

The results from the combined exposures support that EE_2 and citalopram affected each other's effect on behavior, but the slightly conflicting data from the single-substance exposures prevent any speculations on the cause of the effects. An interaction between citalopram and EE_2 in the behavioral effects is a possible outcome, as both substances affect the hypothalamo-pituitary-interrenal (HPI) axis, the fish homologue to the mammalian hypothalamo-pituitary-adrenal (HPA) axis. Serotonin influences the development and function of the HPI-axis, resulting in an impact on metabolism and responses to acute and chronic stress (Winberg et al., 1997; Winberg and Nilsson, 1993). The HPI axis has shown sensitivity to SSRI by altered hypothalamic CRF expression in goldfish (*Carassius auratus*) (Mennigen et al., 2009), inhibition of anxious behavior and reduced whole-body cortisol levels in response to acute stress in zebrafish (Egan et al., 2009) and increased preference for open areas of the aquaria in Chinook salmon (*Oncorhynchus tshawytscha*) (Clements and Schreck, 2007). Estrogens also affect the stress axis. Estradiol-17β increases basal levels of adrenocorticotropic hormone and cortisol in blood plasma of juvenile rainbow trout (Pottinger et al., 1996), an effect which may be mediated by feedback on the monoaminergic systems, since serotonin receptor agonists increase cortisol release, and high brain serotonin turnover is normally associated with high stress levels in fish (Winberg et al., 1997; Winberg and Thörnqvist, 2016). Exposure to the test sessions in NT and scototaxis *per se* increase the level of both serotonin (5-HT) (Maximino et al., 2013), and cortisol (Kysil et al., 2017). Measurement of serotonin, ACTH and cortisol levels during exposure to single and combined substances would give more detailed mechanistic information. Furthermore, both SSRI and EE_2 also affect gonadotropin release (Lister et al., 2009; Mennigen et al., 2010), which would suggest that combination effects of citalopram and EE_2 on fertility could also occur.

In the shoaling behavior males exposed to 0.1 µg/L citalopram made fewer transitions away from peers than the control fish. They also made fewer transitions than the fish exposed to the combination of 0.9 ng/L EE_2 + 0.1 µg/L citalopram, suggesting a combination effect. Thus, it appears that 0.1 µg/L citalopram increased shoal cohesion in this setting while 0.9 ng/L EE_2 had no effect. We have previously detected strong effects on shoal cohesion of 5 and 25 ng/L EE_2 in adult male zebrafish, and of 1.2 and 1.6 ng/L EE_2 in zebrafish of both sexes exposed during the development (Reyhanian et al., 2011; Volkova et al., 2015b). Shoaling is, however, a complex behavior of group dynamics and social interaction and could thus provide information on potential effects on reproduction and survival. In summary, the results of the shoaling test are inconclusive and further studies are needed.

The detection limit of EE_2 of 0.2 ng/L resulted in a faithful demonstration of 0.4 ng/L in the control water. The presence of EE_2 in control water led to higher measured than nominal EE_2 concentrations. While the actual concentration in 0.5 ng/L correspond well to the nominal value + the level in the control, the measured concentration was substantially higher in the nominal 0.1 ng/L. Unexpectedly, this resulted in very similar concentrations for the two EE_2 exposure groups, stressing the need for sensitive chemical analyses in toxicological experiments. It also resulted in the presence of EE_2 in all citalopram exposures, somewhat hampering the value of this study, which might have contributed to the weak effects of citalopram on behavior. The EE_2 concentration in the control water, which is obtained from the drinking water of Stockholm, is at the same level as the 0.5 ng/L reported for German drinking water (Kuch and Ballschmiter, 2001). In light of the effects on anxiety of EE_2 in the range of 0.9–1.6 ng/L in developing (Volkova et al., 2015b) and adult zebrafish (this study), as well as fertility effects of 1 ng/L (Weber et al., 2003), it is doubtful whether such levels in drinking water is acceptable, especially during pregnancy in humans.

The behaviors studied are important for fish survival and reproduction in the wild. Increased anxiety makes the fish seek unnecessary shelter, which decreases foraging and reproduction opportunities. Anxiolysis due to drug exposure on the other hand increases the risk to be caught by predators. Formation of tighter shoals is an acute induced stress response in other species, such as sticklebacks (Wootton, 1984), but shoaling is also a complex social behavior involved in most aspects of fish life. The complex effects of exposure to cocktails of anthropogenic drugs are difficult to foresee, but would most likely affect the fitness of free-living aquatic vertebrates.

In conclusion, this study represents an initial attempt to address effects of low-concentration combinations of drugs from different classes on fish behavior. It showed that EE_2 slightly above the current NOEC level for water-living organisms increased anxiety in the scototaxis test, and combinatory effect of citalopram at low- or sub-active concentrations was observed, abolishing the anxiogenic effects of EE_2.

Acknowledgments

This study was supported by the Swedish Baltic Sea Foundation (Grant no: 1556/42/2011) and the Stockholm County Council (Grant no: 806/3.1.1/2014).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.10.001.

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