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Transgenerational effects of 17α-ethinyl estradiol on anxiety behavior in the guppy, *Poecilia reticulata*

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ABSTRACT

Environmental contaminants can cause alterations that can be transgenerationally transmitted to subsequent generations. Estrogens are among those contaminants shown to induce heritable changes that persist over generations in mammals. Results in other vertebrates are few. We have analyzed the effects on anxiety of 17α-ethinyl estradiol (EE_2) in the F1 and F2 generations in guppies, *Poecilia reticulata*, obtained from F0 fish maternally exposed to 0 or 20 ng/L EE_2 until birth. F0 males and females were bred with fish of the same treatment but different families producing F1 offspring. Behavior in the novel tank test at 6 months revealed that males with EE_2-exposed parents had significantly longer latency to the upper half of the tank than control males, while no EE_2 effects were observed in females. Also in F2, obtained from F1 as above, males in the EE_2 group had longer latency time compared to control males, with no differences due to EE_2-exposure of F0 observed in females. In the scototaxis (light/dark preference) test, latency to first transition to black compartment and total transitions to black were significantly altered in females due to EE_2 exposure of F0 while the total time in black was higher in males with EE_2-exposed F0 compared with controls. The increased anxiety in the F2 generation demonstrates a transgenerational anxiety phenotype and shows that non-reproductive behavior can be transgenerationally modified by estrogens in fish.

1. Introduction

Endocrine disrupting compounds (EDCs) are ubiquitously present in the aquatic environments, exposing fish downstream sewage treatment plants to a cocktail of substances of industrial and agricultural sources, as well as endogenous and synthetic hormones secreted in human urine. EDCs are present in sewage treatment plant effluents and reclaimed water all over the world (Sun et al., 2013). 17α-Ethinyl estradiol (EE_2) is, due to human oral contraceptive use, present in effluents from sewage treatment plants in concentrations from less than 1–300 ng/L (Sun et al., 2013; Kolpin et al., 2002; Hannah et al., 2009). EE_2 is believed to be associated with high ecological risks (Laurenson et al., 2014), due to its ubiquitous presence in waste water, and to the very low predicted No Adverse Effect level of EE_2, which has recently been lowered to 0.1 ng/L (Caldwell et al., 2012).

Fetal development is a vulnerable period, when environmental impact on the mother can affect the fate of her offspring. EDCs exposure during narrow windows of development leads to irreversible changes at the level of tissue organization, resulting in effects on, e.g., fertility and behavior (McLachlan, 2001; Li et al., 2003). These developmental changes do not result from genetic deviations in DNA sequence, but rather from alterations of the much more plastic epigenome, where hormones are key players (McCarthy and Nugent, 2013; Ho et al., 2012; Nugent et al., 2011). Modifications such as changes in DNA methylation, chromatin structure and micro-RNAs have been linked to the phenotypic alterations (Zhang and Ho, 2011).

It seems increasingly evident that developmental changes due to EDCs do not only persist during the lifetime of the individual organism, but also can be transmitted to their offspring for several generations. The demonstration of a transgenerational effect via

Abbreviations: EDCs, endocrine disrupting compounds; EE_2, 17α-ethinyl estradiol; NT, novel tank.

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the germ line requires effects persisting for at least two generations after the exposed generation, as the primordial germ cells giving rise to the first generation after the exposed animals have been exposed (McCarrey, 2014). The second generation after exposure have not been exposed in any other way than through their grandparents, and can thus demonstrate a transgenerational transmission of the affected trait. Transgenerational effects have been observed in an increasing list of organisms from plants to mammals (Skinner et al., 2010; Kleinmanns Julia et al., 2014; Guerrero-Bosagna and Skinner, 2012). The list of agents giving rise to effects in at least three generations is growing, including, e.g., diet and stress, high-fat diet, EDCs, drugs and chemotherapy (Guerrero-Bosagna and Skinner, 2012). Transgenerational effects of EDCs on fertility, reproductive as well as non-reproductive behavior such as anxiety have been demonstrated in mammals, in many cases with observed changes in the epigenome several generations after the exposure (Wolstenholme et al., 2012; Patisaul et al., 2012; Salian et al., 2009; Manikkam et al., 2013; Guerrero-Bosagna et al., 2012; Anway et al., 2005; Skinner et al., 2008; de Assis et al., 2012).

In fish, EDCs effects on gonadal development, reproduction and fertility have been observed both in wild fish and in laboratory experiments (Segner et al., 2003; Vos et al., 2000; Kidd et al., 2007; Nash et al., 2004; Saaristo et al., 2009; Ankley et al., 2009; Arukwe, 2001; Bayley et al., 2002; Fenske et al., 2005; Fenske and Segner, 2004). EDCs also affect non-reproductive behaviors such as anxiety, aggression, risky behavior and shoal cohesion (Espmark Wibe et al., 2002; Bell, 2004; Colman et al., 2009; Majewski et al., 2002; Ward et al., 2006; Xia et al., 2010; Hallgren et al., 2011; Reyhanian et al., 2011; Heintz et al., 2015; Vignet et al., 2014). Exposure during development to EDCs like estradiol, EE_2, nonylphenol and bisphenol A leads to persistent effects on fertility and behavior, discernible in adults after remediation since larval stages (Hill and Janz, 2003; Xu et al., 2008; Maack and Segner, 2004; Kinnberg et al., 2003; Van den Belt et al., 2002, 2003; Volkova et al., 2012, 2015). Transgenerational effects in fish have so far, to the best of our knowledge, been shown in four studies. Developmental dioxin exposure-induced malformations and reduced fertility persisted in two untreated generations of zebrafish, and mixtures of polycyclic aromatic hydrocarbons affected zebrafish larval photomotor activity in F1 and F2 progeny (Vignet et al., 2015; Baker et al., 2014). Benzo(a)pyrene was found to cause transgenerational effects on body and craniofacial deformities in zebrafish (Corrales et al., 2014). In medaka, both bisphenol A and EE_2 resulted in reduced fertility and embryo survival two and three generations after a brief developmental exposure of the first generation (Bhandari et al., 2015). So far, no studies examining transgenerational effects on adult behavior have appeared in fish.

In the current study, we extend the previously reported data on effects of developmental EE_2 exposure in guppies (Volkova et al., 2012) to a full three-generation study. The F0 fish, EE_2-exposed via their gravid mothers, who carry the fry until birth, showed increased anxiety as adults after being raised in clean water (Volkova et al., 2012). Here, we report the results from a second and third generation, obtained by within-group mating of these F0 fish, and receiving no further exposure. Anxiety was recorded in the novel tank test (NT) (Egan et al., 2009) and scototaxis test (F2 only) (Maximino et al., 2010a). The NT test, analyzing behavior in response to being introduced into an unfamiliar environment is well characterized, and shown to be sensitive to effects of anxiogenic and anxiolytic drugs in zebrafish (Egan et al., 2009; Maximino et al., 2010b; Cachat et al., 2011a,b; Stewart et al., 2011; Levin et al., 2007; Bencan et al., 2009; Sackerman et al., 2010). The scototaxis test, analyzing light–dark preference, is shown to detect anxiolytic effects of diazepam, buspirone and ethanol, and anxiogenic effects of stress, caffeine and EE_2 in zebrafish (Volkova et al., 2015; Maximino et al., 2010a,b; Champagne et al., 2010; Steenbergen et al., 2011). The aim of the study was to investigate if the significant increase in anxiety observed in the F0 fish (Volkova et al., 2012) could be transmitted to the F1 and F2 generations, thus demonstrating transgenerational inheritance in the F2 generation.

2. Methods

2.1. Animals

Wild type male and female guppies (*Poecilia reticulata*) were purchased from a fish importer in southern Sweden. The fish were originally caught from the wild and had been interbred but not inbred. Fish were maintained in aquaria enriched with bottom gravel and Java moss (*Vesicularia dubyana*) in 12:12 h light–dark cycle at 25–27 °C and fed twice daily with VIPA GRAN granules (Vipan, Germany) and once daily/once every second day with newly hatched live Artemia (*Artemia salina*). All experiments and handling of the animals were performed according to the Swedish Animal Care legislation, and approved by the Southern Stockholm Animal Research Ethics Committee (Stockholms Södra Djurförsöksetiska Nämnd, Dnr S130-09).

2.2. Experimental design

The three generation study was based on parts of the fish from a previously reported study of developmental EE_2 exposure of guppies (Volkova et al., 2012). The adult fish developmentally exposed to 0 or 20 ng/L EE_2 (measured concentration) from that study is referred to here as the F0 generation. The production of the F0 generation of EE_2 guppies is described in that article (Volkova et al., 2012). In brief, gravid guppy females were exposed to 0 (10 ppm acetone) or 20 ng/L EE_2 in 10 ppm acetone in a flow-through system. EE_2 (Sigma–Aldrich, Sweden) dissolved in acetone was injected to flowing aerated pre-heated (25 °C) tap water (pH 7.8, conductivity 20.7 mSi) with a peristaltic pump. The water levels were held constant to 16 L. Gravid females were exposed individually in breeding traps placed in one exposure or in one control tank, and exposed during their whole pregnancy (~28 days). The newly hatched fry were reared in sibling groups in clean water in 1 L aquaria enriched with gravel and java moss and fed as above until adulthood. Developing males were removed from the aquaria before sexual maturation and reared in aquaria together with their male siblings. Four families each of control and EE_2-treated groups was obtained. After behavioral testing at 6 months of age, males and females of the F0 generation, from the same experimental treatment but from different families, were put together in pairs to copulate. When born, the F1 fry were removed and reared in clean water, under the same conditions of daylight, temperature and feeding as the parental F0 generation. The production of the third generation (F2) followed the same procedure as the production of the second generation.

2.3. Behavior tests

All fish of the F1 and F2 generation were tested for anxiety in the NT test (Egan et al., 2009) at approximately 6 months of age, as described (Volkova et al., 2012). In brief, the test was performed in 20 × 20 × 40 cm glass aquaria, divided in equally sized bottom and upper half by a horizontal midline. The test was initiated by introducing the test fish into the aquarium by gentle netting, and the behavior was recorded for 5 min by video recording and manually analyzed. The latency to the first crossing of the line from below, the number of times the fish crosses the horizontal line

from below, and the time the fish spent in the upper, surface half, were recorded. Due to a technical failure, parts of the behavior data on F1 females were lost from analysis, and they had to be omitted from the study.

The F2 generation fish were also analyzed in another test for anxiety, the scototaxis test (Maximino et al., 2010a), recording light/dark preference, as described (Volkova et al., 2015). The test aquarium (20 × 20 × 40 cm) filled up to 10 cm with pre-heated tap water consisted of two compartments of equal size. White plastic sheets covering the bottom and the walls of one half, and black sheets the other half. Two transparent gliding doors formed a central compartment (5 × 20 cm). After introduction of the test fish into the central compartment for a 5 min acclimatization period, the sliding doors were raised, and latency to first entrance into the black area, number of visits and total time in the black half were recorded from video tapes filmed from above.

2.4. Statistical analysis

Differences in sex ratios between treatment and control groups for the different cohorts were analyzed with chi-square tests. All behaviors from both the NT and scototaxis tests were analyzed with mixed-effects models using the statistical software R version 3.0.1 (Team, 2013) and package lme4 (Bates et al., 2014). All response variables were analyzed with treatment, sex and the interaction treatment * sex as fixed factors. The variable family was used as random factor to control for dependency among siblings within treatment groups. The response variable latency to first movement was log transformed to improve normality and homoscedasticity. Models of number of transitions to upper half, or into black part, were analyzed with Poisson distributed residuals. Time spent in upper half or in black part was either square root transformed (F1) or log transformed (F2) to improve normality and homoscedasticity.

Further, we made pairwise contrasts to analyze treatment effects within sex, or sex differences within treatment groups, using the package phia (Rosario, 2013). All family-wise error rates were controlled with Holm's method. Fixed factor effects are displayed as back-transformed means and ±95% confidence limits using package effects (Fox, 2003), gplots (Warnes et al., 2013), and plotrix (Lemon, 2006).

3. Results

3.1. Sex ratios

At 6 months of age, the F1 generation consisted of 72 control fish (41 females, 31 males) and the EE_2 group of 58 fish (30 females, 28 males). The F2 contained 82 control (38 females, 44 males) and 76 fish in the EE_2 lineage (31 females, 45 males). No significant differences between gender distributions in treatment and control groups were observed (F1 Chisq = 0.24, $p = 0.62$ and F2 Chisq = 0.49, $p = 0.48$). A few animals were later lost before or during behavior analyses, mainly due to jumping out of the aquaria.

3.2. Behavior analyses, NT

The results obtained for the F0 generation have been described in Volkova et al. (2012) and will not be detailed here. However, some information is important for the understanding of this article and will be briefly recalled. In F0 fish, exposed during development and tested after remediation in clean water, there was an over-all anxiety effect of EE_2. The treatment significantly increased the latency to first transition to upper half and decreased the number of transitions to upper half compared with unexposed fish. A significant difference by sex was observed for all three response variables, and significant EE_2 effects were observed in both sexes.

In the F1 generation there was a significant treatment effect for latency to upper half (Table 1, Chisq = 4.82, $p < 0.05$). Even though there was no significant interaction (Chisq = 2.04, $p < 0.16$), a pairwise contrast across treatment within sex showed that the treatment effect was due to a significant increase in the latency period in EE_2-treated males (Chisq = 6.69, $p < 0.05$), but not in females (Chisq = 0.02, $p > 0.80$) (Fig. 1). For the F1 generation no significant treatment effects were observed in the number of transitions to upper half or in total time spent in upper half. No significant differences by sex or sex * treatment interactions were

Table 1
Novel tank behavior in the unexposed F1 and F2 generation of guppies from F0 fish exposed to EE_2 during development (20 ng/L) or control females (0 ng/L). All three behavior parameters were analyzed with linear mixed models using sex (females, males) and treatment (0 ng, 20 ng) as fixed factors and family as random factor to control for dependence among siblings. Log and square root transformed response variables were analyzed assuming Gaussian error distributions. The number of transitions to the upper half was analyzed assuming Poisson distributed errors. The degrees of freedom are equal to one for all Chi^2-values. Pair-wise contrasts for significant interactions are corrected with Holms method using the package phia for R. All confidence intervals are back transformed estimates from the linear mixed models.

	Treat. (ng/L)	F1						F2					
		Mean	CI low.	CI upp.	Esti.	Chi^2	p	Mean	CI low.	CI upp.	Esti.	Chi^2	p
Log latency to upper half													
Treatment	0	39.80[a]	28.22	56.14	0.06	4.82	0.028	51.56[a]	34.57	76.91	0.21	3.62	0.057
	20	64.93[a]	45.39	92.87				89.98[a]	59.80	135.40			
Sex					−0.00	1.57	0.210				−0.22	1.79	0.180
Treatment * sex interaction					0.64	2.04	0.153				0.60	3.84	<0.05
Transitions (Poisson reg.)													
Treatment	0	5.66[a]	4.29	7.47	−0.45	1.62	0.203	5.02[a]	3.37	7.48	−0.02	1.30	0.255
	20	4.24[a]	3.17	5.65				3.52[a]	2.30	5.37			
Sex					−0.04	0.20	0.651				0.25	0.21	0.648
Treatment * sex interaction					0.24	0.78	0.376				−0.59	11.39	<0.001
Time in upper half					(Sqrt)						(Log)		
Treatment	0	152.46[b]	121.78	183.14	−0.66	3.00	0.083	43.69[a]	20.84	91.61	0.60	0.23	0.632
	20	117.33[b]	85.20	149.46				33.24[a]	15.60	70.82			
Sex					−1.26	3.35	0.067				0.31	2.71	0.100
Treatment * sex interaction					−1.82	0.65	0.419				−1.50	7.02	0.008

[c] * = $p < 0.05$, ** = $p < 0.01$, ns = not significant.
[d] There exist significant higher order factors.
[a] Geometric mean.
[b] Arithmetic mean.

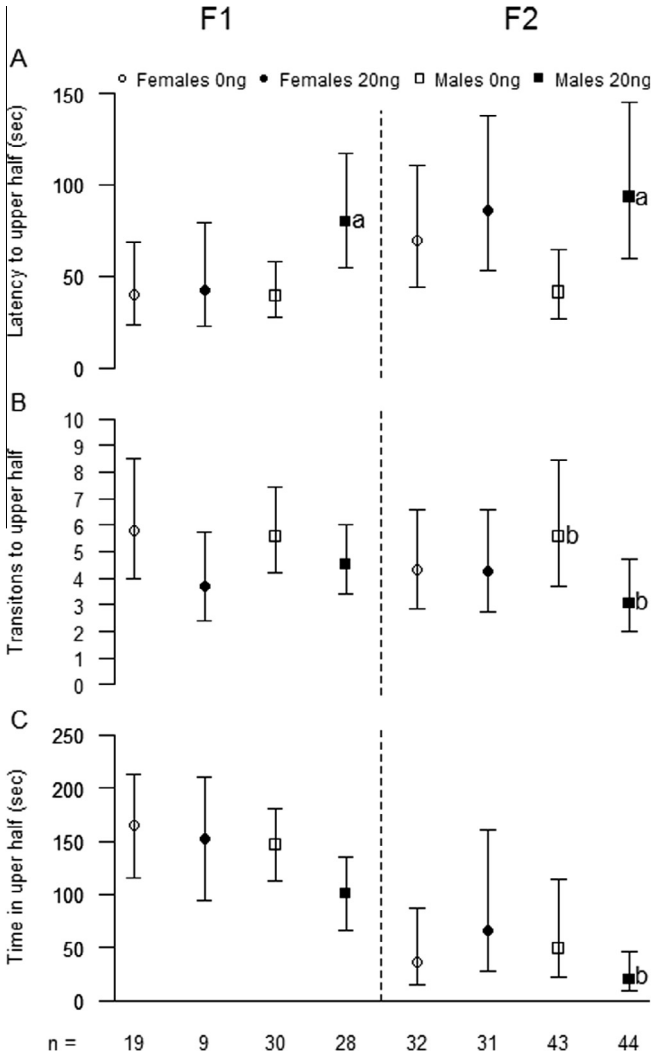

Fig. 1. Behavior in the novel tank of F1 and F2 descendants from F0 fish with or without developmental exposure to 20 ng/L EE_2. (A) Latency to first crossing of the horizontal midline, (B) total numbers of transitions of the horizontal midline from below and (C) total time spent in upper half. Data represent back-transformed mean ± 95% confidence interval using estimates from mixed effects models (see Section 2 for details). [a]Significantly different ($p < 0.05$) from control fish of the same sex, [b]significantly different from females in the same treatment group. Pairwise comparisons of treatment effects were corrected with Holms method using the package phia for R.

observed in the F1 generation (Table 1). As some filmed material from the female trials was lost, the resulting low *n* may explain the lack of statistical significance for the interaction between treatment and sex in the latency to first visit in the upper half, even though there was a significant treatment effect on latency in males but not in females in the pairwise contrasts.

In the F2 generation there were significant sex * treatment interactions for all three variables (latency to upper half: Chisq = 3.85, $p < 0.05$; number of transitions: Chisq = 11.39, $p < 0.001$; total time in upper half: Chisq = 7.02, $p < 0.01$, Table 1). These significant interactions have different explanations but they all showed a predominant male effect (Fig. 1). For latency to upper half the significant interaction effect was that males with EE_2-exposed F0 had significantly longer latency to upper half than control males with unexposed F0 (Chisq = 3.84, $p < 0.05$, Fig. 1A), while there was no significant treatment effect in latency for females (Chisq = 0.38, $p > 0.50$, Fig. 1A). For the number of transitions to upper half males were significantly more active than females in the control group (Chisq = 4.34, $p < 0.05$, Fig. 1B). However this behavior was reversed by the EE_2 treatment of F0 to significantly more anxious males compared to females (Chisq = 7.27, $p < 0.05$, Fig. 1B). For time spent in upper half there was no significant difference among males and females in control fish (Chisq = 0.58, $p > 0.40$) but significantly less time spent in upper half in EE_2 group males compared to EE_2 female fish (Chisq = 9.14, $p < 0.01$, Fig. 1C).

3.3. Behavior analysis, scototaxis

The scototaxis (light/dark preference) behavior in the F2 generation showed significant differences by sex for all three parameters studied (latency to black compartment Chisq = 8.96, $p < 0.01$, number of transitions to black Chisq = 8.07, $p < 0.01$, and total time in black compartment Chisq = 6.74, $p < 0.01$; Table 2). EE_2 exposure of the F0 resulted in significantly decreased latency to first transition in the F2 generation (Chisq = 7.47, $p < 0.01$, Table 2). The number of transitions to black (Chisq = 5.59, $p < 0.05$, Table 2) and total time spent in the black compartment (Chisq = 6.11, $p < 0.05$) increased significantly with EE_2 treatment of the F0. No treatment * sex interactions were observed in light/dark preference (Table 2). Post-hoc analyses of males and females separately revealed that females, but not males, in the EE_2 group had significantly shorter latency period (females: Chisq = 8.31, $p < 0.05$; males: Chisq = 1.25, $p = 0.26$, Fig. 2A) and more transitions to black side when compared with control females (females: Chisq = 5.02, $p < 0.05$; males: Chisq = 1.24, $p = 0.27$, Fig. 2B). Males, on the other hand, spent more time in the black compartment if the F0 generation was EE_2-treated compared with untreated, while no significant effect could be discerned in females (females: Chisq = 1.85, $p = 0.17$; males: Chisq = 5.65, $p < 0.05$, Fig. 2C). A significant difference between control males and females was observed in time in black compartment (Fig. 2C).

4. Discussion

In this study, we show a transgenerational effect of EDCs in a non-mammalian vertebrate, the guppy fish. An increased anxiety behavior, induced in the F0 generation by EE_2 exposure of their mothers during their development, was transmitted to two consecutive, unexposed generations. While the germ cells giving rise to the F1 was exposed to EE_2, the F2 represent a truly unexposed generation, demonstrating transgenerational effects on anxiety. This further strengthens the generality of the concept of transgenerational transmission of experiences during early development stages. The reports on transgenerational effects by environmental contaminants in fish are increasing over the past few years, and have been demonstrated in medaka and zebrafish on fertility, larval survival, malformations and photomotor response (Vignet et al., 2015; Baker et al., 2014; Corrales et al., 2014; Bhandari et al., 2015). The present study shows for the first time a transgenerational phenotype in behavior in fish, and extend previous findings to a third fish species, the guppy. It also confirms the capacity of EE_2 to induce transgenerational effects in fish, previously demonstrated on fertility in medaka (Bhandari et al., 2015). Transgenerational effects of environmental stressors in fish could be one of the essential components to be considered for ecological risk assessment.

The effects observed in the NT experiment are indicative of increased anxiety caused by EE_2 treatment. In the first generation (F0), anxiety in the NT increased significantly in both males and females in response to EE_2 exposure (Volkova et al., 2012). In F1 and F2 significant treatment effects were observed when the sexes were analyzed together. However, only male anxiety showed

Table 2
Scototaxis behavior in the F2 generation of guppies from F0 fish exposed to EE_2 during development (20 ng/L) or control females (0 ng/L). All three behavior parameters were analyzed with linear mixed models using sex (females, males) and treatment (0 ng, 20 ng) as fixed factors and family as random factor to control for dependence among siblings. Log and square root transformed response variables were analyzed assuming Gaussian error distributions. The number of transitions to the upper black half zone was analyzed assuming Poisson distributed errors. The degrees of freedom are equal to one for all Chi^2-values. Pair-wise contrasts for significant interactions are corrected with Holms method using the package phia for R.

	Treat. (ng/L)	Mean	CI low.	CI upp.	Esti.	Chi^2	*DF*	*p*
Log latency to black zone								
Treatment	0	8.66[a]	5.40	13.89	−1.48	7.47	1	0.006
	20	3.44[a]	2.14	5.51				
Sex					0.54	8.96	1	0.003
Treatment * sex interaction					0.98	2.09	1	0.148
Transitions to black (Poisson reg.)								
Treatment	0	3.28[a]	2.89	3.73	0.28	5.59	1	0.018
	20	4.00[a]	3.57	4.50				
Sex					−0.17	8.07	1	0.004
Treatment * sex interaction					−0.14	0.66	1	0.414
Time in black zone								
Treatment	0	154.59[b]	132.25	176.93	28.81	6.11	1	0.013
	20	193.99[b]	171.80	216.18				
Sex					−42.79	6.74	1	0.009
Treatment * sex interaction					16.85	0.40	1	0.525

[a] Geometric mean.
[b] Arithmetic mean.

significant increase when the sexes were analyzed separately. It thus seems like the transgenerational phenotype is discernible only in male progeny. Transmission only to male descendants have previously been observed in rats treated with Vinclozolin (Anway et al., 2005) and in the effects of dioxin on zebrafish on fertilization (Baker et al., 2014). NT is a well-established test, capable of detecting effects of both anxiogenic and anxiolytic drugs (Egan et al., 2009; Maximino et al., 2010b; Cachat et al., 2011a; Stewart et al., 2011; Levin et al., 2007; Bencan et al., 2009; Sackerman et al., 2010). Increased cortisol levels has been associated with increased bottom-dwelling (Egan et al., 2009; Ghisleni et al., 2012). Increased anxiety is a well-known effect of EDC in mammals (Skinner et al., 2008; Valkusz et al., 2011; Gonçalves et al., 2010; Gioiosa et al., 2013), although sex, age, and timing of exposure affect the outcome (Gioiosa et al., 2013). The studies of behavior in EDC-exposed fish are still few, but ecologically significant effects like decreased aggressive behavior, decreased shoal cohesion and risky behavior, and increased anxiety have been shown in fish of several species after adult EDC exposure (Espmark Wibe et al., 2002; Bell, 2004; Colman et al., 2009; Majewski et al., 2002; Ward et al., 2006; Xia et al., 2010; Hallgren et al., 2011; Reyhanian et al., 2011; Heintz et al., 2015). EE_2 exposure during development increase anxiety as adults in zebrafish and guppies (Volkova et al., 2012, 2015).

In the F0 generation, there was a significantly higher anxiety in control females in the NT test when compared with males. Such difference between the sexes in F1 and F2 was only observed in number of transitions in F2. Although reared similarly, there were differences between the generations. In F0, males were tested for reproductive behavior, which meant that they were raised together with virgin females to stimulate them to be reproductively active before the test. The endocrine effects of reproduction might have affected the NT behavior in these males. Also, the sibling groups were larger in F1 and F2, which might also reduce basal anxiety in control fish. Furthermore, in spite of the thorough characterization of the test with drugs with well-established modes of action, novelty in the NT is a conditional stimulus, that might be sensitive to minor fluctuations in external factors, as discussed in Maximino et al. (2010b), and Blaser and Rosemberg (2012).

In contrast to the NT results, analysis of the scototaxis behavior in F2 revealed that also females were affected by the EE_2 exposure of F0, indicating that anxiety behavior was affected for both genders in F2. A significant difference by sex was also observed in the total time spent in black compartment. The scototaxis test has like NT been well characterized by means of pharmacologic drugs, and anxiety been shown to decrease in zebrafish exposed to anxiolytic drugs and increases in caffeine-exposed or stressed fish (Champagne et al., 2010; Steenbergen et al., 2011). We have also observed increased dark-dwelling in unexposed progeny of EE_2-exposed zebrafish (Volkova et al., 2015). The mechanisms regulating dark dwelling in this system have been shown to involve serotonergic pathways as well as the nitrergic system (Maximino et al., 2014, 2015). The white compartment has been suggested to be aversive in zebrafish, thus a causal stimuli for anxiety, and no habituation of the behavior have been observed (Blaser and Rosemberg, 2012). Scototaxis and NT are, based on differences in stimuli, suggested to be complementary rather than identical tests for anxiety (Maximino et al., 2010b; Blaser and Rosemberg, 2012).

We observed different results in the two anxiety tests, supporting that they are not mechanistically identical. The transgenerational phenotype was observed only in males in NT, but in both sexes in the scototaxis test. Transgenerational effects have previously been observed to be passed to both males and females (de Assis et al., 2012), transmitted only to males (Anway et al., 2005), and both paternal and dual-gender transmission depending on the endpoint studied (Baker et al., 2014; Dunn et al., 2011). Clearly, sex differences in transmission and inheritance are important for transgenerational effects, as discussed in Dunn et al. (2011).

The guppies used in this study have been captured from the wild and kept by interbreeding for commercial trade. They are thus less inbred than the laboratory strains of different species that have previously been used for demonstrating transgenerational effects. This study represent the first findings of such effects in a more ecologically relevant setting, which might be significant as EE_2 can affect inbred and wild-caught zebrafish differently (Söffker et al., 2012). Increased anxiety due to EE_2 likely affect ecologically important traits like foraging, reproduction opportunities and anti-predator behavior in wild fish and could be expected to affect population fitness. The findings that EE_2 affect anxiety behavior as well as fertility (Bhandari et al., 2015) at different life stages and transfer these effects over generations represent a new

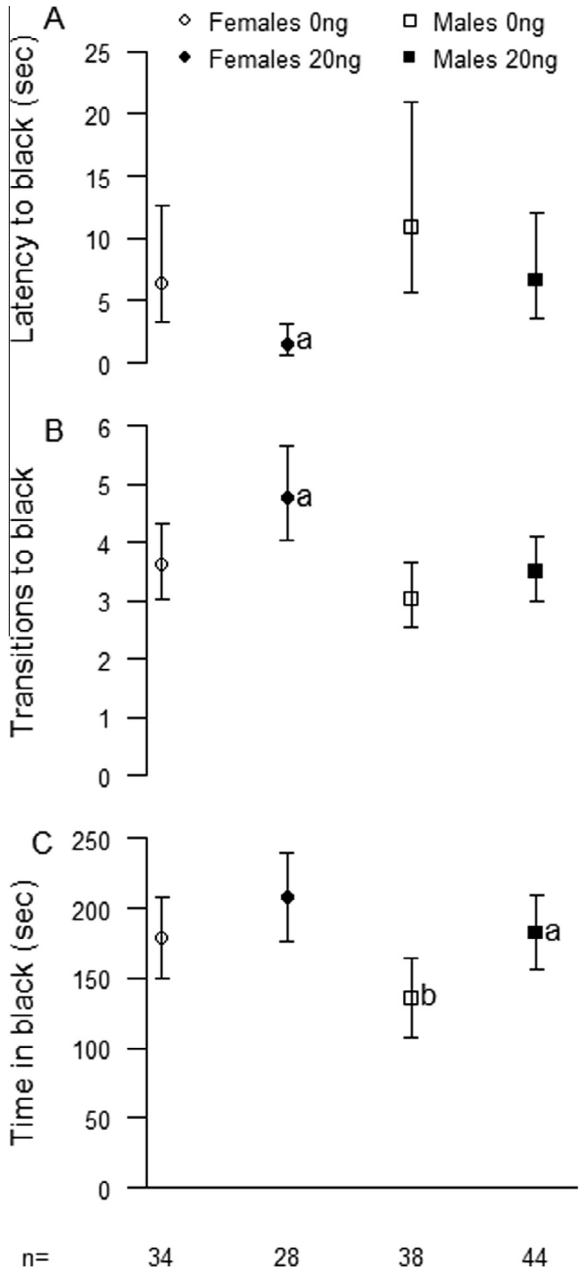

Fig. 2. Light/dark preference (scototaxis) in F2 descendants from F0 fish with or without developmental exposure to 20 ng/L EE_2. (A) Latency to enter black compartment, (B) number of transitions to black compartment and (C) total time spent in black compartment. Data represent back-transformed mean ± 95% confidence interval using estimates from mixed effects models (see Section 2 for details). [a]Significantly different ($p < 0.05$) from control fish of the same sex, [b]significantly different from control females. Pairwise comparisons of treatment effects were corrected with Holms method using the package phia for R.

component in ecotoxicology risk evaluation, as discussed in Vandegehuchte and Janssen (2014).

In conclusion, we found that increased anxiety, observed in F0 progeny exposed during development to EE_2, was transmitted to their F2 offspring. This provides evidence of a transgenerational behavioral phenotype induced by ancestral EE_2 exposure. This strengthens the concept that transgenerational inheritance can be induced by environmental contaminants, which can be of significance for the evaluation of toxicological risk in aquatic environments.

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