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Bisphenol A and 17 α -ethinylestradiol-induced transgenerational differences in expression of osmoregulatory genes in the gill of medaka fish (*Oryzias latipes*)

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Abstract

Embryonic bisphenol A (BPA) and 17 α -ethinylestradiol (EE2) exposure can have far reaching health effects in fish, including adult onset transgenerational reproductive abnormalities, anxiety, and cardiac disorders. It is unknown whether these two environmental estrogens can induce transgenerational abnormalities in the gill. The present study examined transgenerational effects of BPA or EE2 exposure on genes that are critical for osmoregulation in fish. Medaka (*Oryzias latipes*) embryos were exposed to either BPA (100 μ g/L) or EE2 (0.05 μ g/L) for the first 7 days of embryonic development and never thereafter for the remainder of that generation (F0) or in subsequent generations of this study (F1, F2, or F3). Expression of osmoregulatory genes (*NKAa1a*, *NKAa1b*, *NKAa1c*, *NKAa3a*, *NKAa3b*, *NKCC1a*, and *CFTR*) were examined in gills of the first-generation (F0) adults which were directly exposed as embryo and in the fourth-generation adults (F3), which were never exposed to either of these environmental estrogens. Significant alterations in expression of osmoregulatory genes were observed in both F0 and F3 generations. Within the F0 generation, a sex-specific expression pattern was observed with a downregulation of osmoregulatory genes in males and an upregulation of osmoregulatory genes in females. At the F3 generation, this pattern reversed with the majority of the osmoregulatory genes upregulated in males and downregulated in females, suggesting that exposure to BPA and EE2 during embryonic development induced transgenerational impairment in molecular events associated with osmoregulatory functions in subsequent generations. These adverse outcomes may have impacts on physiological functions related to osmoregulation of fish inhabiting contaminated aquatic environments.

1. Introduction

Bisphenol A (BPA) is a component of plastics, produced in high-volumes around the globe, and is routinely found in both surface and ground waters (Barnes et al. 2008; Loos et al. 2010). BPA is an endocrine disrupting chemical (EDC), causing adverse effects: in male reproduction among a wide range of vertebrates (Bhandari et al. 2015a; Flint et al. 2012;

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Vandenberg et al. 2009); prostate development in mammals (Timms et al. 2005); induction of obesity (vom Saal et al. 2012); and impairment of spermatogonial stem cell function in mammals (Vrooman et al. 2015). BPA exposure induces not only direct adverse effects in exposed organisms or populations, but also in future generations through transgenerational inheritance (Bhandari et al. 2015b; Drobná et al. 2017; Goldsby et al. 2017; Lombo et al. 2015; Wolstenholme et al. 2012; Wolstenholme et al. 2013). 17 α -ethinylestradiol (EE2) is a synthetic estrogen used as an oral contraceptive (i.e. birth control pills) of which approximately 16–68% is excreted in the urine or feces (Johnson and Williams 2004). As such, EE2 is frequently detected in the environment (Brooks et al. 2006; Kolpin et al. 2002). EE2 exposure leads to reproductive impairment, intersex development, impaired gill function, and in some instances, transgenerational reproductive and neurobehavioral effects in subsequent generations of fish (Bhandari et al. 2015b; Volkova et al. 2015). Both BPA and EE2 can reach concentrations in surface or ground waters that are sufficient enough to affect development and reproduction in aquatic organisms (Bhandari et al. 2015a; Canesi and Fabbri 2015; Flint et al. 2012).

Evaluation of BPA and EE2 effects on aquatic organisms has largely focused on adverse outcomes from direct exposures; however, developmental exposures to either of these EDCs can produce both latent and transgenerational effects in fish. In a previous study, developmental (0–7 days) exposure of medaka to 100 μ g/L BPA or 0.05 μ g/L EE2 produced transgenerational abnormalities of reduced fertilization rate in F2 and F3 generations and reduced embryo survival in F3 and F4 generations (Bhandari et al. 2015b). It was also demonstrated that these transgenerational phenotypes appeared in F2 generation adults and persisted in F3 and F4 generations. Transgenerational abnormalities are initiated in germ cells as a result of direct exposure and can be distributed to both germ cells and somatic cells in the subsequent generation because a single zygote gives rise to a complete individual after fertilization (Bhandari 2016; Skinner et al. 2011). Embryonic development is vulnerable to EDCs exposure during the two waves of epigenetic reprogramming that occur during this period (Labbé et al. 2017; Messerschmidt et al. 2014). The first epigenetic reprogramming event occurs in embryos from fertilization to the blastula stage, and the second wave occurs in primordial germ cells (PGCs) during sex determination and gonad differentiation. Perturbations that occur during the first reprogramming period cause a majority of somatic effects observed either immediately during development or later in life, while the epigenetic reprogramming that occurs during PGC development can be transmitted to offspring through the fertilized zygote (single cell) which ultimately gives rise to a complete individual (Bhandari 2016; Skinner et al. 2013). As a result, epigenetic reprogramming that occurs in the PGCs have outcomes not only in germ cells and their derivatives, but can also lead to transmissible outcomes in somatic cells (such as gills tissues) in subsequent generations.

The gills of fish are critical organs for ion transport which are continuously exposed to external conditions of the surrounding aquatic environment, including chemical contaminants (Evans et al. 2005). The osmoregulatory system of fish is regulated by endocrine system and maintains physiological homeostasis by movement of fluids and electrolytes (McCormick 2001). Osmoregulation occurring in gill ionocytes of fish allows rapid adaptation to salinity differences by exchanging sodium (Na⁺), potassium (K⁺), and chloride (Cl⁻) ions. Gill ionocytes (also referred to as mitochondrion-rich cells or chloride

cells) secrete Na^+ and Cl^- while fish are in seawater, and take up these ions in freshwater fish (Hwang et al. 2011). Osmoregulatory proteins, such as Na^+/K^+ -ATPase (NKA), $\text{Na}^+/\text{K}^+ - 2\text{Cl}^-$ cotransporter (NKCC) and apical cystic fibrosis transmembrane conductance regulator (CFTR) play important roles in osmoregulation in euryhaline fish. Gill Na^+/K^+ -ATPase (NKA) in teleost fishes is involved in ion regulation in both freshwater and seawater (McCormick et al. 2009). NKAa1a is involved in freshwater adaptation, whereas NKAa1b is found to be associated with seawater adaptation in salmonids (Bystriansky et al. 2006; McCormick et al. 2009; McCormick et al. 2013b; Tipsmark et al. 2011). Recent study suggested that non-salmonid fish have other isoforms of Na^+/K^+ -ATPase genes that play important roles in osmoregulation. In medaka, NKAa1b was upregulated in SW in anterior intestine (Wong et al. 2016). Active ion secretion from ionocytes in fish acclimated to seawater is mediated by NKA and $\text{Na}^+/\text{K}^+ - 2\text{Cl}^-$ cotransporter 1a (NKCC1a) in the basolateral membrane, and by the Cl^- channel, CFTR, found in the apical membrane (Hwang and Lin 2013).

Two types of NKCCs cotransporter proteins are found in vertebrates; NKCC1, a secretory-type present in basolateral membranes, and NKCC2, an absorptive-type cotransport protein present on apical surfaces gills. However, the expression of NKCC2 was detectable at very low levels in the tilapia gills, and were not detected in the gills of brackish water medaka (*Oryzias dancena*) (Kang et al. 2010; Li et al. 2014). In salmon undergoing parr-smolt transformation, a critical developmental stage in anadromous fishes required for adaptation from freshwater to saltwater, gene expression of NKCC1a increases manifold in gills, allowing excretion of salts as fish enter sea water (Mackie et al. 2007; Pelis et al. 2001). CFTR is the protein that regulates chloride channel function, and localized in apical membranes of Na^+ , K^+ -ATPase-immunoreactive ionocytes in gills of brackish medaka (*Oryzias dancena*) (Kang et al. 2012). Of the two isoforms, CFTR1 mRNA is elevated in gills during a preparatory phase of seawater tolerance and increases steadily after exposure to seawater. Whereas, CFTR2 mRNA increases only transiently after exposure to seawater, suggesting CFTR1 is the critical functional isoform in the gill (Singer et al. 2002). NKCC and CFTR are the major transport proteins involved in branchial salt secretion of fish gills. The basolateral $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter (NKCC1) carries one sodium, one potassium and two chloride ions into the cell, down an electrochemical gradient generated by the action of $\text{Na}^+ - \text{K}^+$ -ATPase (NKA). Accumulated intracellular Cl^- is then extruded through an apical Cl^- channel (CFTR) and Na^+ ions are secreted through junctions between ionocytes and accessory cells (Evans et al. 2005; Hwang 2009; Hwang and Lee 2007). Thus, alterations in expression of various isoforms of osmoregulatory genes, such as NKAa1a, NKAa1b, NKAa1c, NKAa3a, NKAa3b, NKCC1a, and CFTR have the potential to indicate overall osmoregulatory dysregulation in the gills of fishes.

Osmoregulatory disruptions have been reported in fish exposed to EDCs (McCormick et al. 2005). Indeed, 4-nonylphenol (4-NP) and 17 β -estradiol inhibit smoltification and the osmoregulatory adaptations associated with this transformation process in Atlantic salmon (*Salmo salar*) (Madsen et al. 1997). Moreover, Fairchild et al. (1999) and Brown and Fairchild (2003) provided several lines of evidence that 4-NP, as a component of an insecticide treatment for spruce bud worm, was causally-linked to population declines of Atlantic salmon based on inhibition of osmoregulatory transformations during smoltification

(Brown and Fairchild 2003; Fairchild et al. 1999). In abalone embryos, direct exposure to BPA resulted in disturbances of cellular ionic homeostasis and osmoregulation via alterations in Na^+/K^+ -ATPase levels and oxidative damage (Zhou et al. 2011). However, it is not clear if ancestral exposures of fish can induce transgenerational alterations in the expression of genes that drive osmoregulatory function in fish. The present study, therefore, examined alterations in expression patterns of osmoregulatory genes (mainly NKA isoforms, NKCC isoforms, and CFTR) in the gills of adult, fourth generation (F3) medaka (*Oryzias latipes*, Hd-rR strain). The F3 adults were lineages of medaka which were exposed to the EDCs, BPA or EE2, as embryos (F0) and were never exposed in subsequent generations (F1, F2, or F3). Alterations in gene expression in the fourth generation of fish (F3), when there is no exposure in the intermediate generations (F1, F2, and F3), indicate truly transgenerational effects, and an as yet, uncharacterized potential ecological risk to fish species that inhabit euryhaline environments.

2. Methods

2.1 Fish and Exposure

The procedure for handling of fish and chemical exposure were previously described (Bhandari et al. 2015b). Briefly, Fertilized eggs (F0) were collected from 6 to 10 breeding pairs of Hd-rR strain of medaka (*Oryzias latipes*) that were maintained at the U.S. Geological Survey Columbia Environmental Research Center (CERC). Exposure of the fertilized eggs started 8 hours after fertilization, coinciding with late blastula stage. Exposure concentrations were 100 $\mu\text{g/L}$ for BPA or 0.05 $\mu\text{g/L}$ for EE2. Embryos were continuously exposed to these test chemicals for 7 days, spanning the critical window of sex determination in medaka (6–8 days post-fertilization) (Matsuda et al. 2002). Uptake of BPA and EE2 by medaka embryos was 132 picograms and 3.2 picograms per egg (pg/egg), respectively (Bhandari et al. 2015b). Three replicate exposures were conducted for each treatment. Control water and test chemical solutions were replaced daily until hatch. After hatching, there were no further chemical exposures of the fish (F0) or any subsequent generations (F1, F2, or F3). Newly hatched fries were raised in floating meshed containers until 30 days post fertilization (dpf) and then all juveniles were transferred to aquaria and maintained to adulthood. Feed included brine shrimp and flake food. Feeding was done three times a day. Water temperature was maintained at $26.6 \pm 0.5^\circ\text{C}$ and 14:10 hours of light and dark cycles was maintained throughout the study.

2.2 Mating and offspring production

Mating and offspring production have been previously described elsewhere (Bhandari et al. 2015b). Briefly, a total of 6 adult pairs from the same treatment group were randomly chosen and mated to produce offspring for each subsequent generation. F0 fish were either unexposed (controls) or directly exposed to either test chemical, whereas F1 fish were exposed only as germ cells while in the F0 gonads. Therefore, truly transgenerational effects can only be expressed in the F2 generation and thereafter. In each generation (F1, F2, and F3), fertilized eggs were collected from six breeding pairs of adults and placed in 50 mL petri dishes in well water with a daily change of 80% water until the embryos were hatched.

Fish were raised to adulthood in 10L glass aquaria (35 fish per tank) with flow-through water (approximately 7 turnovers/day).

2.3 Sample collection and RNA extraction

In each generation (F0 and F3), gill tissues were collected from three fish from each biological replicate. Each treatment had three biological replicate tanks. Therefore, tissue samples from total of 9 fish were isolated for each treatment group in each generation. So, the data represents 9 males and 9 females per treatment in each generation. Gill samples collected from each fish were snap frozen in liquid nitrogen and stored in -80°C freezer until analysis. Total RNA was extracted from gill samples using ZR-Duet™ DNA/RNA MiniPrep (Zymo Research) according to the user's guide. RNA samples were treated with DNase I to remove genomic DNA. The extracted RNAs were tested for concentration using a NanoDrop 2000 and stored in -80°C freezer until analysis.

2.4 Real-time Quantitative PCR (qPCR)

For each sample, 1µg total RNA were used for reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligo(dT) and random primer mix was used under the conditions suggested by the user's guide. Realtime qPCR was conducted using PowerUp SYBR™ Green PCR Master Mix (Applied Biosystems) with an Applied Biosystems StepOnePlus real-time PCR system. Seven medaka osmoregulation genes (*NKAa1a*, *NKAa1b*, *NKAa1c*, *NKAa3a*, *NKAa3b*, *NKCC1a*, and *CFTR*) were examined. Primers used in qRT-PCR were designed using Primer3web (Untergasser et al. 2012), and the primer sequences are shown in Table 1. At least one of the paired primer or amplified region covered a junction of exon and intron to avoid amplifying genomic DNA. *β-actin* was used as an endogenous reference gene to normalize gene expression results.

2.5 Statistical Analysis

Target gene expressions were analyzed by 2^{-C_t} method (Livak and Schmittgen 2001). Cycle threshold (C_T) data exported from the qRT-PCR results were used to calculate $-C_T$. Gene expression was calculated as fold change against control within that generation (F0 or F3). Experimental values were compared to controls by Students' t-tests. Differences between treatments within the same generation was compared by one-way analysis of variance (ANOVA) followed by Tukey's Post-hoc test ($p < 0.05$). Data were represented as the mean plus minus standard error of the mean. Differences with $p < 0.05$ were considered significant.

3. Results

3.1 Changes in expression of *NKAa1* genes

In F0 generation, developmental BPA exposure decreased the expression of *NKAa1b* and *NKAa1c* in male ($p < 0.01$; Figure 1D and 1F), while EE2 exposure significantly increased the expression of *NKAa1b* in female ($p < 0.01$, Figure 1C), and decreased *NKAa1c* in male ($p < 0.05$, Figure 1F). In F3 generation fish, BPA exposure decreased the expression of *NKAa1a* in female ($p < 0.001$, Figure 1A), whereas it increased the expression of *NKAa1c* both in female and male ($p < 0.05$, Figure 1E and 1F). EE2 exposure at F3 generation

increased the expression of *NKAa1a* in male ($p < 0.05$, Figure 1B), *NKAa1b* in both male and female ($p < 0.01$, Figure 1C and 1D) and *NKAa1c* in both male and female ($p < 0.05$, Figure 1E and 1F).

3.2 Changes in expression of *NKAa3* genes

In F0 generation fish, BPA exposure significantly increased the expression of *NKAa3a* in female ($p < 0.05$, Figure 2A) and decreased in male ($p < 0.01$, Figure 2B). EE2 exposure decreased the expression of *NKAa3b* in male only ($p < 0.05$, Figure 2D). In F3 generation fish, BPA exposure lead to decrease in the expression of *NKAa3a* ($p < 0.05$) and *NKAa3b* ($p < 0.001$) in female (Figure 2A and 2C), whereas EE2 exposure lead to a decrease the expression of *NKAa3a* ($p < 0.001$) and *NKAa3b* ($p < 0.01$) in female (Figure 2A and 2C) and an increase the expression of *NKAa3a* in male ($p < 0.05$, Figure 2B).

3.3 Gene Expression changes of *NKCC1a* in medaka

In F0 generation fish, *NKCC1a* did not change in both BPA and EE2 lineage samples (Fig 3A and 3B). In F3 generation fish, BPA exposure lead to a decrease in expression of *NKCC1a* in female ($p < 0.001$, Figure 3A), whereas EE2 exposure lead to a decrease the expression of *NKCC1a* in female ($p < 0.001$, Figure 3A) and an increase in male ($p < 0.05$, Figure 3B).

3.4 Changes in expression of *CFTR* gene

In F0 generation fish, BPA exposure decreased the expression of *CFTR* in males ($p < 0.05$, Figure 4B), whereas EE2 exposure increased the expression in female ($p < 0.05$, Figure 4A). In the F3 generation fish, BPA exposure did not have effects on any of the genes examined, whereas EE2 exposure resulted in an increase in expression in male ($p < 0.05$, Figure 4B).

4. Discussion

Embryonic development is a critical period for environmental estrogens to induce organizational effects as the cells are being reprogrammed or in process of differentiation during this stage (Skinner 2014; vom Saal et al. 2012). Embryonic exposure leads to adverse health outcomes later in life of the exposed organisms or in offspring generations (Bhandari 2016; Skinner 2014). The present study demonstrated environmental estrogen-induced transgenerational alterations in expression of genes critical for osmoregulation in fish. The expression of genes encoding proteins that regulate osmoregulatory processes was taken as a marker to evaluate osmoregulatory health of fish. Gill ionocytes, which are mitochondrion-rich cells and also referred to as chloride cells, are important for secreting Na^+ and Cl^- in saltwater and uptake in freshwater (Hwang et al. 2011; Kaneko et al. 2008). An increase or a decrease in expression of these genes indicates alterations in osmoregulatory function in the gill.

Information on long-term organizational effects of environmental estrogens exposure on developing fish gills is limited. In Atlantic salmon, nonylphenol or estradiol-17 β (E2) exposure led to decreased gill Na^+/K^+ -ATPase activity and seawater tolerance one year after exposure; however, sex-specificity of exposure effects were not examined (Lerner et al.

2007). Similarly, Madsen *et al* observed E2 and 4-nonylphenol (4-NP) exposures caused delays in development of smolt characteristics and reductions in gill Na^+/K^+ -ATPase activities, as well as poorer hypoosmoregulatory performance of the treated Atlantic salmon (Madsen et al. 1997). In abalone (*Haliotis diversicolor supertexta*), embryonic BPA (2 and 10 mg/L) exposure led to increased Na^+/K^+ -ATPase activity in the whole larva (Zhou et al. 2011). *NKAa1a* abundance is found in fish acclimated to freshwater, while *NKAa1b*, *NKAa3b*, *NKCC1*, and *CFTR* abundance is observed during seawater adaptation (McCormick et al. 2013a; McCormick et al. 2009; Pelis et al. 2001). *NKAa1a* abundance seems to be opposite of other NKAs, *NKCC*, and *CFTR*. McCormick *et al* (McCormick et al. 2005) found alterations in thyroid hormones (T3, T4) and insulin-like growth factor 1 (IGF-1), but not cortisol in juvenile Atlantic salmon undergoing parr-smolt transformation directly exposed NP and E2, suggesting that parr-smolt transformation and salinity tolerance can be compromised by developmental exposure to estrogenic compounds. Although a sea water challenge test was not performed in the present study, the present gene expression results conform to literature and clearly demonstrate that direct exposure to BPA and EE2 can stimulate the molecular pathways associated with mechanism of saltwater adaptation in the freshwater fish. This alteration could have caused an osmoregulatory stress in gills, even if the fish was not experiencing saltwater. However, such studies are required to clarify if the transgenerational changes in expression of the *NKA*, *NKCC*, and *CFTR* genes would manifest themselves in functional alterations of osmoregulation. Altogether, EDCs-induced alterations in expression osmoregulatory genes indicate disruption in osmoregulatory function in fish caused by direct exposure.

The F3 generation which was never directly exposed to these two environmental estrogens showed an interesting pattern independent of F0 direct exposure responses. Gill samples were isolated from the fish that were previously demonstrated to have no effects of direct exposure on reproduction at F0 generation, but decreased fertilization success and increased embryo mortality in F2 and F3 generations (Bhandari et al. 2015b). Many other studies also have demonstrated transgenerational effects to be different from direct exposure effects and suggested such effects to be mediated by epigenetic mechanisms (Lombo et al. 2015; Manikkam et al. 2013; Volkova et al. 2015; Wolstenholme et al. 2012). The effects at F3 generation fish were only inherited from their F2 parents who were derived from F1 ancestors that were directly exposed as germ cells during the period of epigenetic reprogramming. Expression of *NKA* genes showed a robust change in F3 generation as compared to F0 generation. In males, all the changes in F3 generation were upregulation of genes, which were reversed compared to F0, indicating that direct effects and transgenerational effects were induced by different mechanisms. Interestingly, EE2 exposure lead to a significant increase in expression of *NKAa1a*, *NKAa1b*, *NKAa1c*, *NKAa3a*, *NKCC1*, and *CFTR* in males at F3 generation, whereas as F3 females showed a drastic decrease in *NKAa3a*, *NKAa3b* and *NKCC1a* expression. Function of *NKAa3a* is not well characterized, while *NKAa3b* is associated with release of Na^+ ions from the gills and intestine in hyperosmotic environment (Miyaniishi et al. 2016). Given all the genes play important role in seawater adaptation and excretion of salts from gill, present results suggest that the ionocytes that excrete salt from the body are transgenerationally activated in both males and females due to environmental estrogen exposure. To our knowledge, no studies

focused on exposure effects at multi- or transgenerational levels, so this study provides a baseline information on long-term organizational effects of past exposure to BPA and EE2 on current or future generation's osmoregulation via gills.

Direct effects may be different from transgenerational effects because a vertebrate embryo undergoes two rounds of developmental reprogramming- first immediately after fertilization and second during sex determination or gonad differentiation (Dean et al. 2003; Reik et al. 2001; Sasaki and Matsui 2008). Effects induced by environmental stressors may be corrected during reprogramming or may escape this process leading to multiple unprecedented health outcomes in offspring generation (Bhandari 2016; Skinner et al. 2011). Although to date, it is not yet clear what drives transgenerational inheritance, correlating epigenetic modifications have been found in the sperm suggesting involvement of epigenetic mechanisms in environmentally induced transgenerational inheritance of altered phenotypes (Carvan et al. 2017; Hao et al. 2016; Manikkam et al. 2012). Loss of function or gain of function studies with epigenome editing tools, such as CRISPR-dCas9, are required to confirm epigenetic regulation of environmentally induced transgenerational inheritance of phenotypes (Choudhury et al. 2016; Morita et al. 2018; Okada et al. 2017; Vojta et al. 2016; Xu et al. 2016).

Interestingly, sex-specific effects were observed in F0 generation gills of Hd-rR strain of medaka, which possesses euryhaline characteristics with intermediate adaptability to seawater (Miyaniishi et al. 2016). All significant changes induced by embryonic BPA or EE2 exposure were upregulation in female, while downregulation in male at F0 generation. Ionocytes that express *NKAa1a* are involved in adaptation to freshwater by facilitating uptake of Na⁺ ions from water (Kumai et al. 2012). Direct exposure to BPA did not change *NKAa1a* expression in F0 generation, whereas the gills of F3 BPA-lineage female fish maintained a suppressed state of *NKAa1a* expression and the male fish from F3 EE2-lineage expression was elevated. Thus, BPA and EE2 also induced sex-specific transgenerational effects on gill ionocytes both in direct exposure and transgenerational inheritance. Mechanisms underlying sex-specific alterations in these genes is currently a subject of future research. Studies suggest involvement of brain-pituitarygonad axis in regulation of osmoregulatory gene networks in the gills. In zebrafish and tilapia, pituitary hormone prolactin regulates osmoregulation (Breves et al. 2014; Breves et al. 2013), whereas in higher vertebrates, kisspeptin neurons directly regulate prolactin release by hypothalamic dopaminergic neurons (Szawka et al. 2010). In the same cohorts of fish, we observed transgenerational hyperactivation of kisspeptin expression in the brain, alterations in steroidogenic and sex hormone receptor genes in the gonad, and reproductive impairment in fish at F3 generation (Bhandari et al. 2015b), suggesting that hypothalamic changes and subsequent alterations in steroidogenesis may alter expression homeostasis of osmoregulatory genes in the gills (Thayil et al, unpublished data). Future studies will address sexual dimorphism of endocrine disruptor-induced transgenerational osmoregulatory dysfunction in medaka.

Our results demonstrate the adverse effects of BPA and EE2 exposure on osmoregulation of F0 and their subsequent generations. Potential adverse effects appeared in F3 offspring could not be predicted by the nature of adverse effects in their ancestors who were directly

exposed to environmental contaminants. The field of transgenerational epigenetic inheritance is still in infancy and molecular mechanisms underlying inheritance of unprecedented health effects by future generations need to be clarified (Heard and Martienssen 2014). The F0 effects differed from F3 generation effects and effects on males differed from that in females. This degree of variability in effects of EDCs exposure is not because of genetic diversity of medaka as Hd-rR strain of medaka are inbred. Studies have demonstrated that majority of epigenetic information on genome gets erased and is reestablished during embryogenesis. The remaining epigenetic information that is resistant in reprogramming can transmit parental effects to offspring (Hajkova et al. 2002; Jirtle and Skinner 2007; Sasaki and Matsui 2008; Seisenberger et al. 2012; Seisenberger et al. 2013; Soubry et al. 2014). How ancestral exposure effects can be inherited by offspring as altered health conditions can be based on the following hypotheses: 1) embryonic exposure may alter epigenetic programming of somatic cells after fertilization and these alterations remain stable throughout life causing somatic cells effects in juveniles and at adulthood; or 2) exposure during the period of primordial germ cells (F0, PGCs) reprogramming may result in perturbation of global erasure of epigenetic marks leaving behind the molecular scars in germ cells that escape erasure process during epigenetic reprogramming of embryo and PGCs at F1 generation. Not only do F1 germ cells harbor the reprogramming resistant alterations, but also all the somatic cells derived from that zygote. This will lead to both germline and somatic cell effects including shifts in gene expression patterns, which will be continuously inherited by subsequent generations. It is not known how long transgenerational effects can persist. Studies in *C. elegans* have demonstrated environmentally induced effects to last for up to 30 generations (Lev et al. 2017).

5. Conclusion

Taken together, the present study provides insights into global environmental problems, mainly effects of current exposure to BPA and EE2 on future generations or ancestral exposure effects on current generations. Similar to medaka, many other euryhaline organisms, tilapia and salmon share similar ionocyte structures in the gill and might be subjected to the same level of osmoregulatory stress caused by ubiquitous environmental contaminant BPA and EE2. This is the first study reporting that BPA and EE2 can produce osmoregulatory stress in the offspring past four generations and warrants studies in commercially important euryhaline migratory food fish, such as salmon, eels, and herrings. Transgenerational studies may be worth investigating in natural food fish population as many species migrate away from the ocean to spawning grounds, where their offspring are exposed to environmental contaminants while migrating downstream for development and feeding.

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Highlights

- Bisphenol A and 17-alpha ethinylestradiol induce transcriptional alterations in the medaka gill.
- Early life exposure led to sexually dimorphic alterations in expression patterns of genes involved in osmoregulation.
- Embryonic exposure led to transcriptional alterations in the gills four generations later.

Figure 1. Relative quantification (RQ) of transcripts for *NK4a1* genes in the gills of F0 and F3 generation medaka fish. Data are presented as mean $\pm$ standard error of the mean. Statistical significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 2.

Relative quantification (RQ) of transcripts for *NK4a3* genes in the gills of F0 and F3 generation medaka fish. Data are presented as mean $\pm$ standard error of the mean. Statistical significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3. Relative quantification (RQ) of transcripts for *NKCC* genes in the gills of F0 and F3 generation medaka fish. Data are presented as mean $\pm$ standard error of the mean. Statistical significance: *, $p < 0.05$; ***, $p < 0.001$.

Figure 4. Relative quantification (RQ) of transcripts for *CFTR* genes in the gills of F0 and F3 generation medaka fish. Data are presented as mean $\pm$ standard error of the mean. Statistical significance: *, $p < 0.05$.

Table 1:

Primers used in the present study

Gene	Reference	Forward primer	Reverse primer
<i>NKAa1a</i>	XM_004084864.3	GATTTGGATGATCACAAGTTGACAC	CCGGGCGAAGATCTCCTTTA
<i>NKAa1b</i>	XM_004066527.3	GCACCATGGCAAGAAAAGCA	GTGATCATCCAAATCGACCTCCTT
<i>NKAa1c</i>	XM_004066525.3	AAGGTGGACTCCTCTCTGACT	CAGCGGGTTTTTCGTTAGAGAA
<i>NKAa3a</i>	ENSORLT00000008844.1	ATGATGAGATGAGGGAGGCC	GAACAGGTGGCAGAATCCCA
<i>NKAa3b</i>	ENSORLG00000013191	AGAAGTGCCCATACAGAACA	TCAGTCCCTGCACGATGTC
<i>NKCC1a</i>	ENSORLT00000024013.1	ATCATCCTCATGGCTACCGT	TAATACGCTCCACCTCCTCG
<i>CFTR</i>	ENSORLG00000019555	AAAGCACTGGCTCGCTGTTT	AAGCTTCCCCGAGGTAGAGAA
<i>β-actin</i>	ENSORLG00000013676	CCCCACCCAAAGTTTAG	CAACGATGGAGGGAAAGACA