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Estrogen receptors in medaka (*Oryzias latipes*) and estrogenic environmental contaminants: An *in vitro–in vivo* correlation☆

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ABSTRACT

In many vertebrates, estrogens are necessary to promote the growth and differentiation of the female reproductive system during development, and have important reproductive roles in both males and females. Medaka (*Oryzias latipes*) has three estrogen receptor (ER) subtypes, *ERα*, *ERβ1* and *ERβ2*. To evaluate the three medaka ER (mER)-ligand interactions, we applied the ERE-luciferase reporter assay system to characterize each ER subtype. In this transient transfection assay system using mammalian cells, the mER proteins displayed estrogen-dependent activation. 17β-Estradiol (E_2) and *op*'-DDT showed high activation irrespective of ERs. Endosulfan also exhibited activation; with less/no transactivity measured using other pesticides, i.e., heptachlor, carbendazim, deltamethrin, acephate, dimethoate and amitraz. It was generally observed that *ERβ2* had higher activation potential than *ERα* and *ERβ1*. To understand the molecular mechanism of estrogen action via ER, we also conducted E_2 treatment where we observed a trigger in *ERβ2* expression upon E_2 exposure. The present data suggest that *ERβ2* is essential for female gonad maintenance. The data were supported by induction of vitellogenin (*VTG*) mRNA in the liver and reduced *VTG* receptor mRNA expression in the gonad of both sexes. The present work will provide a basic tool allowing future studies to examine the receptor–ligand interactions and endocrine disrupting mechanisms, and also expands our knowledge of estrogen action on reproductive development in medaka.

1. Introduction

Endocrine disrupting chemicals (EDCs[1]) and their effect on the health of wildlife and humans have recently become a major concern. In recent years, substantial numbers of studies have confirmed that several chemical compounds, such as agriculture runoffs, prevalent in the aquatic environment exhibit endocrine disrupting activities in fish, potentially through bioaccumulation [1,2]. Therefore, the endocrine disrupting effects and their intensities must be evaluated in order to confirm the safety of both preexisting and novel chemicals. Many of such chemicals show higher estrogenic potential and display potential binding to human *ERα* in the 3-D QSAR[2] (three-dimensional quantitative structure-activity relationship) model, with 82% accuracy [2].

Estrogen is well known for its involvement in several essential phenomena, i.e. development, differentiation, reproduction and homeostasis in vertebrates. Thus, estrogenic chemicals are of particular concern with regard to undesirable effects on fish [3]. Fish show abnormalities along male or female developmental pathways exposed to sex steroid hormones and their chemical mimics, and other environmental factors (e.g. temperature), during early life, may result in functional sex changes against the genetic sex in fish [4]. Estrogen treatment, for example, leads to feminized gonads in roach (*Rutilus rutilus*) [5]. Studies on wild populations of

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[1] Endocrine Disrupting Chemicals.

[2] Three-dimensional Quantitative Structure-Activity Relationship.

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roach inhabiting UK rivers have shown that exposure to estrogenic effluents emanating from wastewater treatment works (WwTWs[3]) caused altered sexual development that can resulted in reduced fertility [6,7].

Biological effects of estrogens are principally mediated through estrogen receptors (ERs), which function as ligand-activated transcription factors. ERs exhibit broad tissue expression, consistent with the diverse roles of estrogens. In fish, tissue (mainly gonad and liver) specific expressions of *ERα* and *ERβ* subtypes have been shown to differ between species (e.g. Ref. [8,9]). ER expression is consistent with the pivotal role of estrogens in gonadal sex differentiation and development [4] and in the hepatic production of the egg yolk precursor protein, vitellogenin (VTG), and vitelline envelope proteins, required for oocyte development in teleosts [10].

EDCs can act at multiple sites through multiple mechanism of action. Importantly, using receptor binding assay and receptor based functional assay, researchers have shown that many of these chemicals interact with ERs [11,12]. These interactions mainly mimic or block the endogenous estrogen action [13], although additional modes of action cannot be discounted. Unfortunately, specific role of each ER subtype in exerting endocrine disruption are not well understood. Moreover, teleost fish has three ERs, which further complicates the situation. Full elucidation of the mechanistic pathways by which environmental estrogens are able to modulate endogenous estrogen signaling in fish requires a better understanding of the distinct roles of each ER subtype. Apart from this, medaka has been recommended as one of the model fish for ecotoxicological tests by the Organization for Economical Cooperation and Development (OECD[4]) and the potency of ERs in Japanese medaka (*Oryzias latipes*) have been demonstrated (e.g. Ref. [14]).

Keeping the recent investigations in mind, we concentrated on characterizing different ER subtypes for their specific transactivation potential using different estrogenic pesticides. We also focused on validating the *in vitro* data with *in vivo* experiment using medaka fish and their possible correlation with vitellogenin gene expression.

2. Materials and methods

2.1. Animals

QurtE strain of medaka was used for this study. All fish were maintained at 26 ± 2 °C under 14 h light and 10 h dark cycle. Eggs were collected within 30 min of fertilization and incubated in distilled water (Milli-Q) containing antifungal solution at 26 ± 2 °C. All fishes were fed with freshly hatched Artemia (brine shrimp).

2.2. Medaka genes

All medaka estrogen receptor (*mER*), vitellogenin (*mVTG*), vitellogenin receptor (*mVTGR*) and a housekeeping riposomal protein 18 (*mRPS18*) gene sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) or DFCI (http://www.compbio.dfci.harvard.edu) with accession numbers AY917147.1 (m*ERα*), AB 070901.1 (m*ERβ1*), AB428449 (m*ERβ2*), AB064320 (*mVTG1*), EF122597.1 (*mVTGR*) and TC115326 (*mRPS18*).

2.3. Chemicals

17β-Estradiol (E_2) was purchased from Sigma–Aldrich Co. (St Louis, MO, USA) and *o,p′*-DDT, heptachlor and endosulfan from Chem Service (West Chester, PA, USA). Carbendazim, deltamethrin, acephate, dimethoate and amitraz were obtained from Dr. Ehrenstorfer (Augsburg, Germany). All chemicals were dissolved in dimethylsulfoxide (DMSO) for *in vitro* assay. But for *in vivo* experiment, ethanol was used to dissolve E_2. These pesticides were selected based on data on current use of pesticides in India (www.indiastat.com/agriculture/2/consumptionofpesticides/206872). The concentration of DMSO in the culture medium did not exceed 0.1%.

2.4. Estrogen receptor transactivation assay

HEK-293 cells were seeded in 24-well plates at 5×10^5 cells/well in Dulbecco's modified Eagle's Medium (Sigma) supplemented with 10% charcoal/dextran treated fetal bovine serum (Hyclone, South Logan, UT, USA). Cells incubated for 24 h at 37 °C with 5% CO_2 were transfected with 400 ng of pGL3-basic-4X ERE-tk-luc, 100 ng of pRL-tk-luc (internal control to normalize the variation of transfection efficiency contains the *Renila reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter, Promega, Madison, WI, USA) and 200 ng of pCMV 3.1-medaka *ERα*/*ERβ1*/*ERβ2* using Fugene-6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instruction.

After 4 h, doses ranging from 10^{-17} to 10^{-6} M of E_2 and 10^{-11} to 10^{-6} M of *o,p′*-DDT, heptachlor, dimethoate, amitraz, carbendazim, deltamethrin, endosulfan and acephate were added to the culture media of transfected cells. Cells were collected 44 h after ligand addition, then, the luciferase activity was measured by a chemiluminescence assay with dual luciferase reporter assay system (Promega). Estrogenic activities of each chemical were calculated as firefly (*Photinus pyralis*) luciferase activity/sea pansy (*Renilla reniformis*) luciferase activity [15]. All transfections were performed at least three times, employing triplicate sample points in each experiment. The values were shown as mean ± SEM from three separate experiments, and dose-response data and EC_{50} were analyzed using GraphPad Prism (Graph Pad Software, Inc., San Diego, CA, USA).

2.5. In vivo estrogen treatment

2.5.1. Experimental design and sampling

Adult medaka (140 days post hatching, dph) were exposed to nominal concentration of 1 and 10 ng/ml E_2 for 7 days, while control fish were treated with vehicle ethanol. 100% water exchange was carried out for every 24 h interval. Three trials were performed. Gonad, brain, liver and kidney were dissected, separately, for RNA isolation and further analysis. Institutional Animal Care and Use Committee at the National Institute for Basic Biology approved all procedures and protocols related to treatment and maintenance.

2.5.2. Histology and in situ hybridization

Samples fixed in 4% paraformaldehyde were embedded in paraffin and sectioned at 5 μm followed by standard hematoxylin and eosin staining and *in situ* hybridization using each ER subtype genes. Probes of sense and anti-sense digoxigenin-labeled RNA strands were transcribed *in vitro* with RNA labeling kits (Roche Diagnostics GmbH, Mannheim, Germany) from plasmid DNA containing partial ORF (Primer details in Table 1) of medaka ERs. *In situ* hybridization was carried out as follows: sections were deparaffinized, hydrated and treated with proteinase K (10 mg/ml, Roche) and then hybridized with the sense or anti-sense DIG-labeled RNA probe at 58 °C for 22 h. The hybridization signals were then detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche) and NBT (Nitro blue tetrazolium chloride) as described previously [16].

[3] Wastewater Treatment Works.
[4] Organization for Economical Cooperation and Development.

Table 1
List of primers used in this study.

Primer name	Sequences	Purpose
ERα Realtime F	ATGGCCAAAGACACGCGTT	Real time PCR of respective gene
ERα Realtime R	TCATTGTGACCCTGGATGCTC	
ERβ1 Realtime F	ACCAACTTGGCCGATAAGGA	
ERβ1 Realtime R	AAACCCTGGAATCTTCTTGGC	
ERβ2 Realtime F	TCCGCCACGTGAGTAACAAA	
ERβ2 Realtime R	CCACGTTCTTCCTCTTCATGG	
mVTG1-Realtime F	CTCCAGCTTTGAGGCCATTTAC	
mVTG1-Realtime R	ACAGCACGGACAGTGACAACA	
mVTGR-Realtime F	AGTTGATGCACATGTAAC	
mVTGR-RealtimeR	CAGCGTAGCTCATGACC	
*mrps*18 Realtime F	ACAACATGGGCGTAACGTC	Real time PCR for house keeping gene
mrps 18 Realtime R	ATCCAAGATGTCTCTGGTAATC	
ERα ISH F	CATGTACCCTGAAGAGAGCCG	*In situ* probe preparation
ERα ISH R	GCTGCGTTTCCTTCCATCA	
ERβ1 ISH F	ACCAGCAAGGGTGTGAGGAG	
ERβ1 ISH R	AAACCCTGGAATCTTCTTGGC	
ERβ2 ISH F	TGACTATGGGAACGGGCCT	
ERβ2 ISH R	GGACGGACGATGAGTGTGGT	

2.5.3. Quantification of ERs by real-time PCR

Changes in gene expression were quantified using the ABI prism 7500 sequence detection system (Applied Biosystems, Foster City, USA). Total RNA was isolated with RNeasy kit (Qiagen, Chatsworth, CA, USA) following manufacturer's protocol. cDNA synthesis was carried out using QuantiTect reverse transcription kit (Qiagen) from 100 ng of total RNA. The first strand cDNAs were diluted to 100 μl for subsequent use. Gene specific RT-PCR was performed using SYBR green master mix (Applied Biosystems) and 5 ng cDNA according to manufacturer's instruction. The PCR conditions were as follows: initial denaturation at 94 °C (2 min) followed by 40 cycles at 94 °C (30 s) and 60 °C (1 min). The relative expression was calculated using RPS18 expression (house keeping gene). Sequences of gene specific primers are listed in Table 1. Average relative expressions derived from triplicates were used for further analysis. Specificity of primer sets throughout this range of detection was confirmed by the observation of single amplification products of the expected size and dissociation curve. All assays were quantified with standard curve (mean Ct vs log cDNA dilution) slopes between −2.99 and −3.34, and the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was >0.985 in each case.

2.5.4. Data analysis

Statistical differences in relative mRNA expression between experimental groups were assessed using One Way ANOVA, followed by Student's *t*-test. All *in vivo* data were statistically analyzed using Stat View, version 5 software (SAS Institute, Cary, NC, USA). All experimental data are shown as the mean ± SEM. Differences were considered statistically significant at $p < 0.05$. Regression and correlation analyses were also performed using descriptive statistics of Stat View 5.

3. Results

3.1. Some pesticides can act through estrogen receptors

To investigate the estrogenic activity of common pesticides through mERs, luciferase reporter assay was performed using three mER subtypes with 7 pesticides and E_2 as a positive control (Fig. 1). All three subtypes of mERs were activated by E_2 and *o,p′*-DDT. *ERβ2*-dependent induction was found to be several fold higher than *ERα* and *ERβ1* specifically for E_2 and *o,p′*-DDT. Among other pesticides, endosulfan showed estrogenicity with *ERα* only. However, endosulfan failed to trigger *ERβ1* and *ERβ2* responsive luciferase activity. But other pesticides like amitraz, dimethoate and heptachlor, deltamethrin, acephate and carbendazim were found to have little/no induction capacity.

3.2. Changes in expression of ER subtypes in various tissues

Excessive estrogen responsive changes in mERs expression was analyzed by exposing to different concentration of E_2 followed by measuring the expression of ERs in different tissues both in 140 dph adult male and female medaka.

ERα expression was reduced significantly in ovary even at lowest concentration (1 ng/ml E_2) of E_2. Testicular expression of *ERα* was suppressed at higher concentration (10 ng/ml E_2) (Fig. 2A). However, at higher concentration, *ERα* was found to decrease in both sexes. Interestingly *ERβ1* depicted a E_2 responsive up regula-

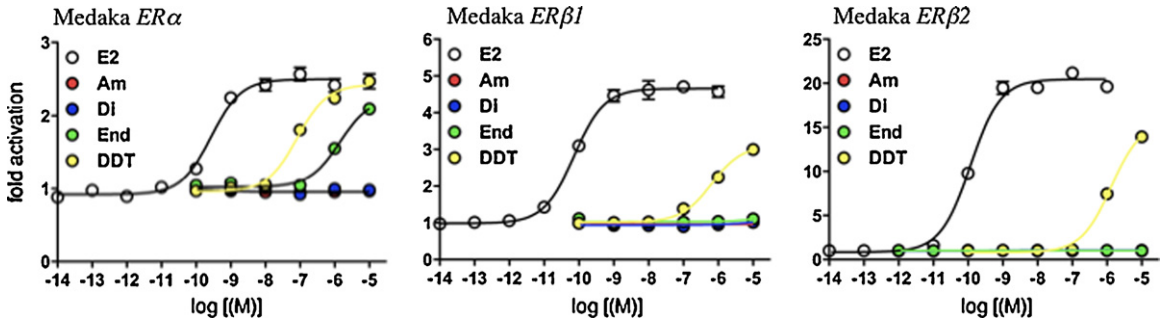

Fig. 1. Estrogen and estrogenic pesticide regulated *in vitro* transcriptional activity of different medaka ER subtypes. Transcriptional activity profiles of *mERα* (A), *mERβ1* (B) and *mERβ2* (C) generated using 17β-estradiol (E_2), *o,p′*-DDT, amitraz, dimethoate, and endosulfan depicts differential activation pattern. Acephate, heptachlor, deltamethrin and carbendazim showed no estrogenic activity in all three *mER* subtypes in this transcriptional assay (data not shown).

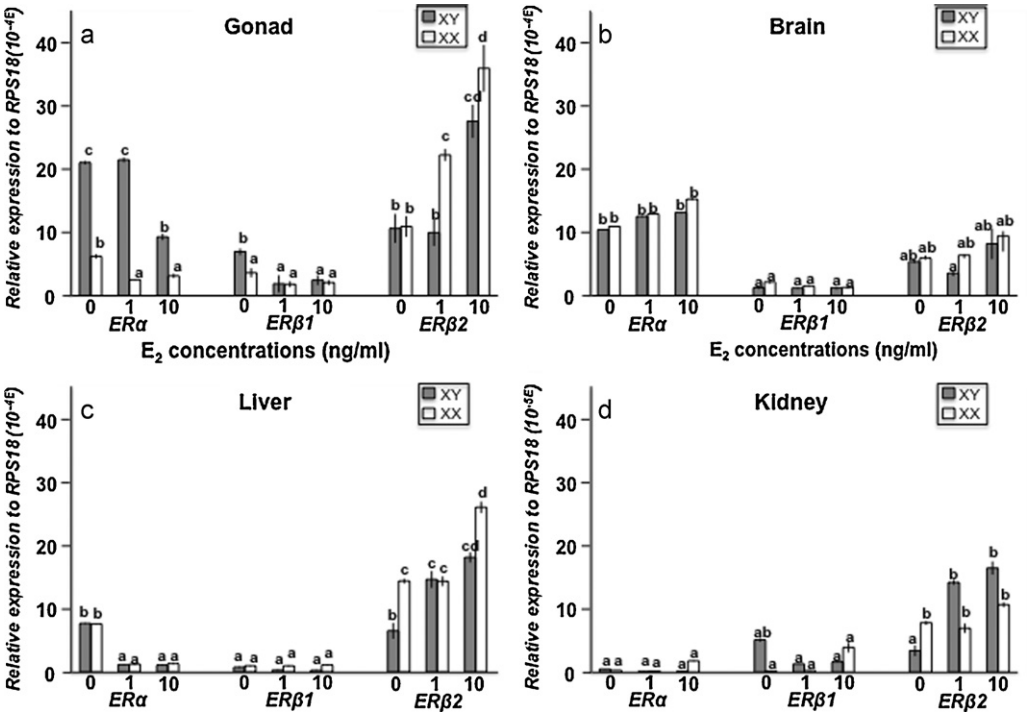

Fig. 2. External estrogen responsive ER subtypes in different tissues of medaka. Realtime PCR based modulation of three ER subtypes in gonad, liver, brain and kidney upon short term (7 days) E_2 (0, 1 and 10 ng/ml) exposure demonstrates differential pattern of expression specific to sex. In graphs, relative expressions corrected for *RPS-18* are plotted on *Y*-axis against concentration (ng) in *X*-axis. Different letters (a–d) denotes a significant difference at $p < 0.05$ (using Duncan's Multiple Range Test).

tion in male only. *ERβ2* expression was initially different between sexes, at 0 and 1 ng/ml E_2 treatment group, but became insignificant at higher concentration (10 ng/ml E_2) group. 10 ng/ml E_2 treatment group showed a significant induction in *ERβ2* expression in both sexes.

In both sexes, *ERα* and *ERβ1* expression in the brain was unaffected by E_2 treatment (Fig. 2B). However, similar to gonad, *ERβ2* expression changed significantly between sexes at 1 ng/ml E_2 group but became insignificant in 10 ng/ml E_2 group in the brain. No sexual dimorphism in expression of ERs in the brain was observed within treatment groups.

In agreement with gonad, *ERα* expression reduced significantly in livers of both sexes. Interestingly, no significant changes were observed in case of *ERβ1*. However, opposite pattern of *ERβ2* expression was encountered in the liver compared to *ERα* expression. *ERβ2* expression increased significantly along with elevated concentration in males, but it in females only increased at the higher concentration (10 ng/ml E_2) (Fig. 2C).

ERα had no differential expression in kidney, which can be correlated with E_2 exposure (Fig. 2D). But the other two receptor subtypes had changed their expression profile with E_2 treatment. *ERβ1* showed significant decrease in expression in male fish only. However, the expression remained higher in males than females. In case of *ERβ2*, expression increased in male kidney and became indifferent from females even at lower concentration group (Fig. 2D).

Effects of E_2 treatment on expression of different ER subtypes were analyzed using *in situ* hybridization. Expressions remained unchanged in the ovary, while in the testis, elevated expression was observed for all ER subtypes (Fig. 3). Expression of ERs in liver and brain (data not shown) were in-compliance with real-time PCR data (Fig. 2).

Vitellogenin (VTG) is a biomarker for estrogen response [17]. On the other hand, VTG receptor (VTGR) is the prime transporter of VTG into oocytes [18]. In order to shed some light on possible correlation between VTG production and ER-mediated response, the expression of medaka *VTG1* (*mVTG1*) and *mVTGR* mRNA was measured in the E_2-treated adult (147 dph) medaka liver and gonad in both sexes. *mVTG1* depicted a similar expression pattern of *ERβ2* in liver, i.e. increased significantly with E_2 treatment in both sexes (Fig. 4A, Table 2). But in gonad, *mVTGR* expression showed sexual dimorphic pattern. The expressions increased and decreased with elevating concentration of E_2 in males and females, respectively (Fig. 4B). Furthermore, the correlation between *mVTG1* and *ERβ2* was found to highest (0.509) followed by *ERβ1* and *ERα* (Table 2). Regression analysis also showed higher R^2 value for *ERβ2* in both sexes (Table 2).

4. Discussion

ERα and *ERβ* were found in almost all organs of the body, yet, relatively few tissues are considered target for estrogen action in mammals. In contrast to mammals, which have two ERs, teleost fish have three known ER subtypes [19]. These include *ERα* and two isoforms of *ERβ*, *ERβ1* and *ERβ2* [20,21]. To develop a more complete understanding of the roles of different ERs, in line of estrogen biology, we hereby begin with the measurement of the transcriptional activity of different ERs. The *in vitro* transcriptional activation assay allows simultaneous identification of promoter and enhancers, with a correlative assessment of their activities via reporter protein.

In one study, transfection assays showed that all zebrafish (zf) ER subtypes are able to induce a reporter gene driven by an estrogen-response element (ERE) in a strictly estrogen-dependent manner [8]. Dose-effect experiments revealed that the zf*ERβ2* is highly sensitive to E_2 and is able to induce a reporter gene at a minimal concentration of 10^{-11} M E_2. This high sensitivity could be due to its higher affinity for E_2 and/or a better recruitment of co-activators [8]. Despite dissimilarities between zebrafish and medaka ER sequences, our results also suggest that m*ERβ2* is highly sensitive to E_2 followed by *mERβ1* and *mERα*. The estrogenic response was further confirmed by similar experiment with. *o,p′*-

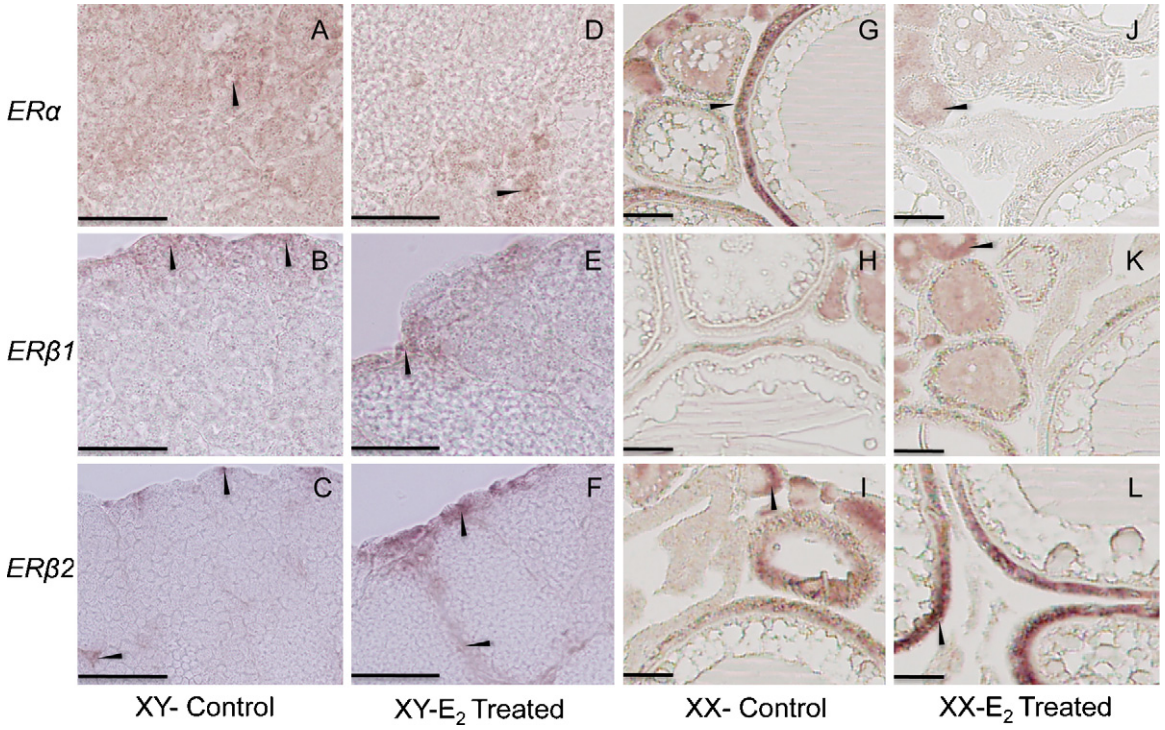

Fig. 3. Comparative expression analysis of different ER subtypes in gonad by *in situ* hybridization. *In situ* hybridization shows differential abundance of three ER subtypes in both E_2-treated (10 ng/ml) and control gonads of adult (147 days post hatching) female (XX) and male (XY) medaka. Arrowhead indicates the representative signal. Bar length = 50 μM.

DDT, a xenoestrogen [22], however, the induction was several fold lower then E_2. Endosulfan mimics estrogen [23], caused testicular atrophy in male rats (fed with a diet containing 10 ppm endosulfan) [24,25]; showed lowered plasma levels of gonadotrophin and testosterone [26]. These results are consistent with its estrogenicity revealed by the E-screen assay [27]. The estrogenic potency of endosulfan as measured by the E-screen assay was comparable to that of *o,p′*-DDT while lower to that of E_2 [27], are in compliance with our results. Although all pesticides used in the present study are directly or indirectly related to estrogen action [28], but little or no activation may be due to lower affinity to different medaka ERs or less ability to recruit the co-activators. It should be noted that the estrogenic activity of chemicals cannot be deduced solely from their molecular structure but need more understanding on their mechanism of action [27]. For example, dimethoate posseses reproductive toxicity, but it acts through *star* [29], which is the master regulator of cholesterol bio-synthesis. So, there may be less possibility for it to directly act upon ERs [29].

Interestingly, exposure of medaka to the natural ER ligand, E_2, invoked differential responses of medaka ER genes. Our data indicates that *ERα* may act through a negative feedback regulation because the expression goes down significantly in both sexes along with E_2 treatment. Gender specific expression pattern of *ERβ1* also supports the above hypothesis at least in males. Similar negative feedback regulation, or after effect of excessive estrogen, may explain the minified expression of *ERβ1* in females too. Furthermore, *ERβ2* appears to be the most potent ER found in germ cells, indicating a more fundamental role for *ERβ* in germ cell development/differentiation [30].

Germ cell development or gonad maintenance is the outcome of gene regulation in many tissues. Although in the present work, no interesting changes in expression of ERs were observed in the brain and kidney but significant changes were detected in the liver. Unlike zebrafish, instead of E_2-dependent *ERα* up-regulation [31], our finding indicates the estrogen-responsive induction of *ERβ2* expression in livers of both male and female medaka. However, these data contradict the theory that *ERβ* may not be involved in the regulation of vitellogenesis [32]. Specific knockdown of each receptor in livers of gold fish (*Carassius auratus*) suggest that *ERα* is induced by E2 through activation of the *ERβ* subtypes [33]. But in the present study, we observed opposite regulation for *ERα* and *ERβ2*, i.e. suppression for the former and up-regulation of the latter. Contrast to Nelson and Habibi [33], present results suggest that *ERβ2* has an obvious and compensatory role in E_2 regulation even in liver. Moreover, tissue specific expression of mERs coupled with our previous report (Chakraborty et al., Communicated elsewhere) indicates species differences in the expression of mER subtypes in different tissues and emphasizes the importance of precise staging of the fish while reporting and assessing the functional relationships for the expression of specific genes. Increasing expression of

Table 2
Relationship between estrogen receptor subtypes and *VTG-1* mRNA expression in medaka liver.

Group	R^2 (p-values)		Correlation	
	Female	Male	Female	Male
ERa vs *VTG1* in liver	0.058 ($p < 0.001$)	0.087 (NS)	−0.156	−0.163
ERb1 vs *VTG1* in liver	0.078 ($p < 0.0001$)	0.045 (NS)	−0.229	−0.178
ERb2 vs *VTG1* in liver	0.18 ($p < 0.0001$)	0.132 ($p < 0.0001$)	0.509	0.678

Using descriptive statistics from Stat View.

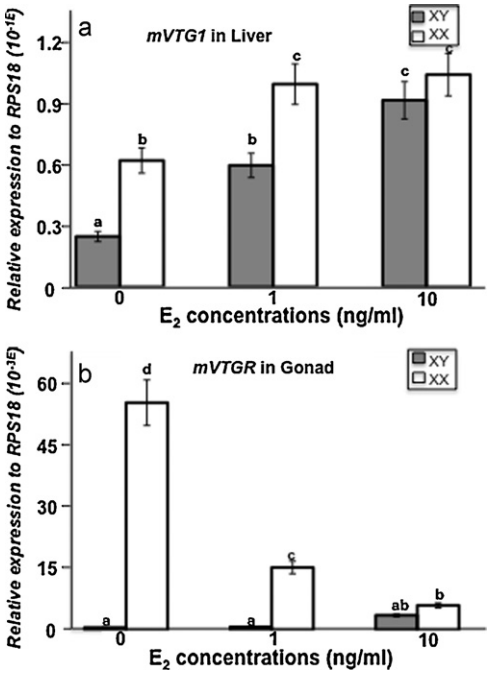

Fig. 4. Effect of external estrogen on vitellogenin production and transport in medaka. Real time PCR analysis of E_2 (0, 1 and 10 ng/ml) treated medaka depicts a sex specific changes of *VTG1* mRNA in liver (A) and VTGR mRNA in gonad (B). Relative expressions corrected for *RPS-18* are plotted on *Y*-axis against concentration (ng) in *X*-axis. Different letters (a–d) denote a significant difference at $p < 0.05$ (using Duncan's Multiple Range Test).

ERβ2 along with E_2 treatment suggests a highly likely role in ovarian maintenance or germ cell development/differentiation in medaka.

E_2 responsive VTG induction in liver is mediated through ERs in fish [32]. However, individual/correlative role of ER subtypes on VTG gene expression is largely unknown. In the present study, expression profile of *mVTG1* and *choriogenin* (data not shown) are maximally correlated with *ERβ2* abundance. Moreover, VTG is essential for oocyte maturity. Although E_2 can induce VTG production in medaka liver [34], but uptake of VTG may be reduced due to suppression of VTGR expression in the ovary, following a decreased fecundity and unimpaired fertility in female medaka [35]. However, in case of males, increase in *VTG* and *VTGR* expression indicates the female directive gonadal changes. Together with transactivation data and changes in expression profile of gonad, brain and liver upon E_2 exposure, it can be speculated that *ERβ2* has a significant role in estrogen-dependent ovarian maintenance in medaka. However, sex specific regulation of *VTGR* expression in medaka gonads and expression pattern of *ERα* and *ERβ1* support the feedback regulation hypothesis.

In this investigation, we focused on characterization of each subtype of medaka ER via documenting trans-activation capacity of different ERs to various chemicals and effect of E_2 treatment during adulthood. The data generated here may provide insight into the differential physiological roles of the ER subtypes in fish and provide a foundation for studies on the molecular basis of estrogenic disruption in the medaka. This also opens future avenue for *ERβ2* to be used as a potential biomarker for assessing estrogenic contamination in medaka.

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