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Effects of 17α-ethinyl estradiol exposure on estrogen receptors α and β and vitellogenins A, B and C mRNA expression in the liver of sand goby (*Pomatoschistus minutus*)

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Abstract

This study aims to characterize the estrogen receptor (*er)* in sand goby (*Pomatoschistus minutus*) and determine the temporal effects of 17α-ethinyl estradiol (EE2) on *er*α and vitellogenin (*vtg)* gene expression in males. Two partial cDNA sequences (*er*α and *er*β*1*) are presented showing conserved structural features with *er*s of other species. Transcript levels for both ers were low in control fish but EE2 exposure (11 ng/L, for 29 days) increased both to a pattern similar to vitellogenic females. The relative expression of three *vtg* genes (*vtga*, *vtgb* and *vtgc*) along with *er*α was determined in control and male fish exposed to EE2 (11 ng/L) at multiple time-points over 29 days. All four transcripts were significantly induced due to exposure and expression rose during the time course with distinct temporal patterns and *vtga* reached a substantially higher level at the end of the time course coinciding with rapid elevation in *erα* expression.

1.1 Introduction

Estrogenic endocrine disruptors (EED) interact with the endocrine systems of animals by engaging with the estrogen signal transduction pathways, resulting in estrogenic toxicity with a myriad of detrimental and adverse effects (Hiramatsu et al., 2005). 17α-ethinyl estradiol (EE2) is a model EED and is found to contaminate European coastal waters with concentrations fluctuating as high as 125 ng/L (Pojana et al., 2004). The sand goby (*Pomatoschistus minutus*) is a small benthic fish that inhabits European coastal and estuarine environments. Sand goby have a one-year life cycle with distinctive well-characterized reproductive behaviours, for example sand goby males build nests into which females are lured to lay their eggs (Healey, 1971). In earlier works, sand goby has been utilized as a model in ecotoxicology for the study of endocrine disruption in both controlled exposure studies (Saaristo et al., 2009) and environmental monitoring (Kirby et al., 2003). Exposure of male and female animals to EE2 was shown to have adverse effects on reproductive output and mating behaviours (Robinson et al., 2003; Saaristo et al., 2010a, 2010b). The molecular mechanisms by which female egg production is impaired are poorly understood. In these studies it was difficult to contextualize the

apical endpoints with the classic molecular biomarkers of estrogenic exposure, such as expression of vitellogenin (*vtg*), partially because at that time, only a small fragment a single *vtg* was available. Recently this situation has been improved when three *vtg*s transcripts were identified and shown to be inducible by EE2 exposure in male sand goby (Humble et al., 2013). The three *vtg* complete cDNA sequences have now been fully sequenced (accession AGO64301.1 AGO64302.1 and AGO64303.1). Although male hepatic *vtg* is known to be inducible by exposure to EE2, the EED induced expression patterns of multiple *vtg* genes over time are unknown.

The *er* is central to the estrogen transduction pathway that is both crucial to vitellogenesis and EED mediated toxicity. Typically teleost fish have three subtypes of *er* (*er*α, *er*β*1* and *er*β*2*) (Hawkins et al., 2000; Sabo-Attwood et al., 2004). *er*α is known to be up-regulated by high level EED exposure in male fish (Katsiadaki et al., 2002) and *erβ1* is known to be inducible by injection of estradiol (Sabo-Attwood et al., 2004). However, the estrogen receptors (*er*s) have not been characterized in sand goby and it is uncertain if *er*s are suitable as biomarkers as their sensitivity to environmentally relevant concentrations of EED is questionable.

Our hypothesis is that sand goby possesses three *er* with conserved domain structures and exposure to environmentally relevant concentrations of EE2 induces time-dependent expression patterns in the hepatic expression of these *er* genes as well as in the multiple *vtg* genes already sequenced. A comparison of the temporal expression patterns of these estrogen sensitive genes will be useful for biomarker evaluation and risk assessment. The objectives are 1) to sequence multiple *er*s of the sand goby (at the mRNA and predicted protein level), categorize the *er*s by subtype and characterize the domain structures and evolutionarily conserved regions; 2) To quantify relative hepatic gene expression of *er*s in males EE2 exposure at environmentally relevant concentrations of EED and compare this to control females and control males; 3) characterize the temporal mRNA expression pattern of *er*s and *vtg*s throughout a long term exposure period to an EED enabling comparison of estrogen responsive genes to evaluate the sensitivity of these potential biomarkers.

2 Materials and Methods

2.1. The exposure scheme

The sand gobies used in the exposure experiments were caught using a hand trawl at natural breeding sites near the Tvärminne Zoological Station (University of Helsinki) on the southern coast of Finland. Trawling was conducted during the main breeding season (May-June). Only sexually mature fish were chosen to this study and they were separated by sex before introduction to the holding tanks. Fish were acclimated to the laboratory conditions for 2 weeks. From holding tanks fish were randomly assigned to six different exposure glass aquaria (80 x 80 x 40). Males were kept at a density of 45 males and females were kept at a density of 15 per tank. Tanks had a 3 cm layer of fine sand on the bottom and were equipped with a flow-through of seawater (see Saaristo et al., 2009, 2010a,b). Fish were fed twice a day during the exposure period.

The treatment was as follows: EE2 exposure (males), with nominal concentration of 20ng/L (measured concentration 11ng/L, standard deviation (SD) = 3.7, n = 10), During preparation of chemicals, EE2 (Sigma-Aldrich, Finland) powder was dissolved in acetone, which was evaporated using a stream of nitrogen thus eliminating the presence of solvent (Saaristo et al., 2010a,b). The EE2 concentration in the male exposure aquaria was measured by liquid chromatograph-mass spectrometer (LC-MS; HS 1100-Water Quattro II) using methods described in Saaristo et al., 2009). The study was approved by the Finnish National Board of Laboratory Animals.

2.2 Cloning of estrogen receptors

2.2.1 Targeting unknown sand goby *er* sequence

Deduced amino acid sequences for complete cDNAs of *er*s were sourced from the GenBank website for a variety of teleost species and aligned using CLUSTALW2 to identify conserved subtype-specific sequences (list of teleost species, protein IDs and accession numbers shown in Supplement 1). These were used to design the primers as follows: *erα* forward primer (FP) 5' ACCACTATGGGGTGTGGTC 3' and reverse primer (RP) 5' CATGCCTTTGTTGCTCATGT 3', *erβ1* FP 5' GCTATGAAGTCGGCATGACC and RP 5' GATCATGGCTTTGAGGCAGA 3' and *erβ2* FP 5' GTGTGAGGCGTGAACGCTGC, RP 5' GCTGGCTGGAGATCCTGATG 3' for reverse transcription polymerase chain reaction (RT-PCR).

2.2.2 RNA isolation and Reverse transcription

Total RNA was isolated from EE2 exposed male and control female liver tissue samples (100 mg) using NucleoSpin® RNA II kit (ABGene, Epsom, UK) following the manufacturer's protocol and quantified by Nanodrop™ ND-1000 spectrophotometer. Superscript III reverse transcriptase (Invitrogen, Paisley, UK) was used to convert 2 µg RNA into cDNA, again according to the manufacturer's instructions. The reverse transcription reactions were primed using random hexamers at a reaction concentration of 1.5 µM and oligodT at a reaction concentration of 3 µM. The reaction was incubated at 50 ˚C for 60 min and then at 70 ˚C for 15 min. The cDNA was stored at -20 ˚C.2.2.3 PCR amplification, cloning and sequencing

RTPCR was used to amplify *er* fragments from livers of EE2-exposed male and control female sand goby. Reddymix™ PCR Master Mix (1.1X) (Thermo Fisher Scientific., USA) was used with 0.2 µM reaction concentration for each primer and 1 µL sand goby cDNA and thermo- cycled following the manufacture's recommendations. The PCR products were analysed by agarose gel electrophoresis (data not shown) and purified fragments were cloned into pJET1.2 vector and DH5α *E. coli* host using the CloneJET™ PCR cloning kit (Fermentas, UK) and the Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen, UK). Plasmids were purified from recombinant colonies (using GeneJET Plasmid Miniprep Kit, Fermentas, UK) and sequenced using GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman UK) using the manufacturers' protocol on the CEQ-8000 (Beckman Coulter Inc., Fullerton, USA) and processed using Long Fast Read program 1 (LFR1), a standard setting for DNA sequencing. The sequences generated were pair aligned with *er* sequences from *Micropogonias undulatus* (*erα* accession: AAG16713.1, *erβ* accession AAG16711.1 and *erγ* AAG16712.1)*, Gambusia affinis* (*erα* accession BAF76770.1, *erβ1* accession: BAF76771.1 and *erβ2* accession: BAF76772.1)*, Acanthogobius flavimanus* (*erα* accession: BAF46102.1 *erβ* accession:BAF46103.1*, Oryzias latipes* (*erα* accession BAA86925.1, *erβ* accession NM_001104702.1 and *erβ2* accession NM_001128512.1) and *Acanthopagrus schlegelii* (*erα* accession AY074780.1, accession *erβ* AY074779.1 and *er*β2 accession EU346949).

2.3 Quantification of Transcripts by Relative RT-qPCR.

RNA extraction from sand goby liver as described in section 2.2.2.

2.3.2 qPCR assays

The qPCR reactions were primed with transcript-specific primers. Primers for *vtga*, *vtgb*, *vtgc* and 28S ribosomal RNA (*rs*28) were presented previously (Humble et al., 2013). Primers for *erα* and *erβ1* were designed using Primer3 (Rozen and Skaletsky, 2000) and primer pairs were screened against potential to form secondary structures using Netprimer (Premier Biosoft 2002). The reverse transcription, quantitative, PCR (RT-qPCR) primers were validated using end-point RT-PCR to amplify cDNA from female control fish (data not shown) to demonstrate single fragments of expected size and RT-qPCR standard curves constructed to assess the efficiency for each primer set (Table 1). *rs28* shows little variation in hepatic expression in different genders or response to EE2 when a fixed amount of RNA is used (CT standard deviation ±0.55) was therefore used as reference gene. All RT-qPCR reactions were carried out in triplicate in 96 clear-well plates using Platinum® SYBR® green qPCR Supermix-UDG (Invitrogen, Paisley, UK) as previously described (Humble et al., 2013). After amplification a melting curve analysis (60 ˚C to 95 ˚C) was completed for each reaction to demonstrate a single product melting at the correct temperature.

2.4 Mathematical and statistical analysis of RTqPCR data

The RTqPCR mathematical calculation was performed separately for each target gene. First, the mean CT values for each gene (target gene and reference gene) and each biological sample was calculated using the three technical replicates. Second, a relative expression ratio (R) was generated for the gene of interest (relative to the reference gene) for each individual biological sample using the following equation presented by Pfaffl (2001) $R=((E_{target})^{\Delta CTtarget(control-sample)}) / ((E_{reference})^{\Delta CTref(control-sample)})$ with reference to assay efficiencies (E) to compensate for inter-assay efficiency variation. RS28 was used as reference gene (ref) and the mean of the control samples was used as “control”. Third, the R values were Log10 transformed to fit approximately normal distributions as determined by Shapiro-Wilk test and show homogeneity of variance as determined by Levene’s test.

Statistically significant differences in mRNA expression between control and exposed (or male and control female) samples were tested using Student’s unpaired t-test. For each gene of interest, significant differences between time points were calculated using one-way ANOVA with Tukey’s HSD post-hoc test. Between target gene comparisons were made using MANOVA with Tukey’s HSD post-hoc test. All statistical analyses were performed using the SPSS package (IBM SPSS Statistics 19).

3. Results

3.1. Partial sequencing of two estrogen receptors in sand goby

RTPCR products of anticipated size were produced for a putative *erα* and *erβ1* from EE2-exposed male and control female sand goby liver, but no product for *erβ2* was formed even after using alternative tissues and primers (data not shown). The PCR products were cloned and sequenced resulting in contigs 977 bp (*erα*) and 600 bp (*erβ1*) in length that were used as queries for BLASTx search and showed highest homology to *erα* (accession: BAF46102.1, E-value: $8e^{-121}$) and *erβ* (accession: BAF46103.1, E-value: $7e^{-120}$) of Japanese common goby (*Acanthogobius flavimanus*). The *erβ* of the Japanese common goby has not been categorized as subtype *erβ1* or *erβ2* yet the sand goby cDNA fragment has high similarity with *erβ1* of other species (such as estrogen receptor beta1, partial [*Acanthopagrus latus*] accession: gb|AEX68678.1) to suggest this novel sand goby fragment is subtype *erβ1*.

To confirm the identity of the cDNA fragments, the sequences were translated, to generate 325 aa for *erα* and 200 aa for *erβ1,* and pair-aligned using water alignment with full length protein sequences of *er*s from other teleosts species (*Micropogonias undulatus, Gambusia affinis, Acanthogobius flavimanus, Oryzias latipes* and *Acanthopagrus schlegelii*). Sand goby *erα* (accession: KC782769) showed highest identity with Japanese common goby *erα* (90.2%) while sand goby *erβ1* (accession: KC782770) shows greatest homology with *erβ* of the Japanese common goby (89.4%). The partial sand goby *er*α and *erβ1* deduced protein sequences pair-alignments with full length Japanese common goby *er*α and *erβ* protein sequence are shown in Fig. 1A and Fig. 1B respectively.

For *er*α, the sand goby sequence covers 60 amino acids (aa) of the 76 aa-long DNA binding domain, all the hinge domain and 201 aa of the 238 aa ligand-binding domain. There was a 100% match for the DNA binding domain, a 61.5% match for the hinge domain and a 93.9% match for the ligand binding domain. For *erβ1* the sand goby sequence covered 15 aa of the 79 aa-long DNA binding domain, (93.3% match), all of the 41 aa hinge domain, (65.9%), and covered 143 of the 238 aa-long ligand binding domain (95.8%).

3.2 EE2 induced male er gene expression compared with female

Primers (shown in Table 1) were designed for RT-qPCR to amplify fragments of 158 bp long for both sand goby *erα* and *erβ1*. The endogenous mRNA levels of *erα* and *erβ1* in male and female sand goby were analyzed using RT-qPCR. Very low mRNA levels were found for both *er*s in control males (mean CT for *erα* was 26.7 and for *erβ1* was 25.2). Females had higher endogenous levels of *erα* (mean CT: 20.02) than *erβ1* (mean CT: 26.2).

Relative RT-qPCR was used to analyse the fold change in *erα* mRNA in male sand goby exposed to 11 ng/L EE2 for 29 days to show a highly significant increase in *erα* mRNA. A significant difference in *erα* was found between control males and females but no significant difference was found between females and EE2 exposed males indicating this exposure induced hepatic *erα* expression in males similar to that of females. A small but significant difference in *erβ1* was found between control males and males exposed to *EE2* but no significant difference was found comparing control males with females.

3.3 Vtg-a, -b, -c & erα expression over a month-long exposure to EE2

Relative RT-qPCR was used to analyse *vtga*, *vtgb*, *vtgc* and *erα* mRNA levels in control and EE2-exposed (11 ng/L) male sand goby liver at 6 time points throughout 29 days (Fig. 3). Unexposed males had very low levels of mRNA for *erα* and all *vtg* subtypes throughout the 29 day exposure period. This was detectable by highly significantly, lower C_T values compared with non-template control (NTC). For instance, the mean CT values for the unexposed males at day 29 were 26.7, 29.4, 29.9 and 26.7 for *vtga, vtgb, vtgc* and *erα* respectively while the respective NTC values were undetermined, 35.1, 36.4, 38.2 and 37.9 for the respective genes.

Samples from exposed males had a highly significant ($p < 0.0001$) increase in levels of *vtg-a –b* and *-c* mRNA expression compared with controls at all time-points measured. On the other hand, *erα* showed significant differences ($p < 0.05$) at day 13 and 16, very significant differences at day 8, 24 and 29 ($p < 0.01$) but no significant difference at day 20 when comparing exposed and control samples.

Expression of mRNA in males exposed to 11 ng/L over 29 days EE2 relative to controls was used for comparison of multiple time-points to show increases in expression ratios for *vtg-a, -b, -c* and *erα*

and reveal distinct temporal and transcript specific changes in mRNA upregulation. For *vtga*, there was a sharp rise at the beginning of the time course shown by a statistically significant increase between day 8 and all other time points. Day 29 was also significantly greater than day 8, 13, 16 and 24 showing that *vtga* continued to rise at the later stages. *Vtgb* on the other hand showed no significant difference between day 8 and day 13, 16 or 20 but here was a difference between day 8 and day 24 or 29. *Vtgb* expression reached a plateau indicated by a lack of statistically significant differences between day 16, 20, 24 and 29. For *vtgc,* day 8 was found to be significantly different from all other time points measured. Day 29 was found to be significantly higher than day 8, 13 and 16, but not significantly different to day 20 or 24. For *erα* there was a significant difference between day 29 and days 8, 13 or 20 however there was no significant difference comparing day 13 or day 20 with any other time point.

A comparison was also carried out between relative mRNA expression levels of *vtg* target genes over the time course. At day 8 of exposure the relative expression levels of the *vtgs* was in the order *vtga* > *vtgc* > *vtgb* (ratio of relative gene expression *vtga* : *vtgc* : *vtgb*, for exposed male 1.53 : 1.13 : 1) though this was found not to be significantly different. However at day 29 there was a significant difference (with *vtga* > *vtgc* > *vtgb*) and the respective abundance ratio for *vtga* : *vtgc* : *vtgb* was 6.94 : 2.34 : 1 indicating a divergence in the expression profiles for these three genes over time.

4. Discussion

4.1 Analysis of novel erα and erβ1 sequences

We successfully cloned and sequenced two cDNA fragments from liver of sand goby which show high homology to *erα* and *erβ1* in other fish species. These sequences were translated to gain partial deduced protein sequences which were aligned with Japanese common goby (*Acanthogobius flavimanus*) deduced proteins to show they had higher similarity at the DNA binding domains and ligand domains than the hinge domains. This is consistent with the functional roles of these domains reportedly conserved during evolution (Aranda and Pascual, 2001). Ray-finned fish (Actinopteriygii) contain multiple *er*s due to gene and genome duplication. Typically there are three *er* genes in teleosts as described in the Atlantic croaker (*Micropogonias undulates)* and largemouth bass

(*Micropterus salmoides*) (Hawkins et al., 2000; Sabo-Attwood et al., 2004). It is possible that our efforts to clone *erβ2* failed for technical reasons but it is of interest that only two *er*s (α and β) are found in the closely related Japanese common goby (Ito et al., 2007). Phylogenetic analysis suggests that *erβ1* and *erβ2* are the result of duplication in an ancestor that was shared with higher vertebrates in which only a single *er*β is present (Nelson and Habibi, 2013). It is unlikely that the goby lineage were not subject to the same duplication event considering *er*β of mammals shares more identities with *erβ2* of fish than with *erβ1* (Hawkins and Thomas, 2004). It is more likely that *erβ2* was redundant and was lost in the goby lineage and all *er*β functions are maintained by *erβ1*. Phylogenetic analysis of VTGs also suggests a distinct evolutionary pathway in the gobies compared to other ray-finned fish (Thacker, 2009).

4.2 Expression of estrogen receptor genes

Our study shows that the sand goby has gender-specific patterns of hepatic *er* expression and by comparison, transcript levels of both *er*s were low in male with *erβ1* marginally higher than that of *erα*. In contrast *er*s are reported as absent from the liver of male Japanese common goby but this may be the consequence of an insensitive end-point PCR technique used in that investigation (Ito et al., 2007). Work on zebrafish (*Danio rerio*) has indicated that endogenous levels of *erβ1* are higher than that of *erα* or *erβ2* (Menuet et al., 2004) but it was unclear which gender of fish were used in that study. Meng et al., (2010) reported gender differences in *er* transcript levels in zebrafish liver with *erα* and *erβ2* in females being at higher levels than *erβ1* while in males *erβ1* and *erα* were observed at very low levels and *erβ2* was higher. The results reported here indicate that sand goby is similar to zebrafish in respect of gender differences in hepatic expression of *erα* and *erβ1* but differ because in sand goby, no *erβ2* has been found.

In this study we demonstrated a marked increase in transcripts for *erα*, up to levels comparable to those seen in mature females, and a modest increase in *erβ1* in response to EE2 exposure. Exposure of male Japanese common goby to xeno-estrogens has been reported to result in the induced hepatic expression of estrogen-dependent genes implying the presence of *er*s (Ohkubo et al., 2004). Here, temporal variation in transcription for *erα* in male liver was studied over a 29 day EE2 exposure, and a very significant increase in *erα* relative to the control group was seen at day 24 and expression continued to rise at day 29. This observation promotes the idea that *erα* may be a suitable biomarker

for EED exposure-monitoring in male sand goby with high mRNA levels in particular signaling prolonged EED exposure. In zebrafish short term exposure (48 hours) to 17β-oestradiol has been reported to cause disparate effects upon the hepatic transcript levels of the *er*, with *erα* increasing *erβ1* decreasing (Menuet et al., 2002), which suggests zebrafish is dissimilar to sand goby regarding its downregulation of *erβ1* in response to estrogens. The results reported here show similarity with those reported in largemouth bass where the three *er* subtypes are classified as *α*, β (*erβ2*) and *γ* (*erβ1*). Sand goby *β1* showed greatest similarity to largemouth bass *γ* type. Similar to the sand goby, the liver of largemouth bass females has higher endogenous levels of *erα* than *erγ*, and the injection of males with E2 causes a large increase in *erα* and a moderate increase in *erγ* (Sabo-Attwood et al., 2004). The increase in *erα* expression found in EED exposed sand goby may act as a positive feedback, compounding the feminization process and further sensitizing the males to estrogen exposure. Here we have for the first time in a teleost species demonstrated the induction of *erα* and *erβ1* by exposure to environmentally relevant concentrations of EED.

4.3 Expression of vitellogenin genes

The determination of complete sequences for three VTGs (accessions: JQ511252.1, JQ511253.1 and JQ511254.1) and the development of *vtg* type specific RT-qPCR assays (Humble et al., 2013) opened the door for a study of the temporal effects of EE2 on the abundance of these transcripts. Low levels of transcripts for all of the *vtg* types were found in liver of non-exposed males, arguably the result of low level exposure to an estrogenic chemical during the maintenance and treatment periods. We can discount that these low *vtg* levels in males were caused by EE2 since in control tanks EE2 was below detection level by LC-MS-MS quantification (Saaristo et al., 2010a). Other researchers have also found basal level of VTG mRNA in untreated males in Murray rainbowfish (*Melanotonia fluviatilis*) (Woods and Kumar, 2011) Japanese medaka (*Oryzias latipes*) (Sun et al., 2011) and zebrafish (Söffker et al., 2012). Endogenous male estrogen synthesis is essential for testicular development and sperm formation in vertebrates including fish (Schulz et al., 2010) thus could be responsible for basal *vtg* mRNA expression. However, other factors such as hypoxia and parasite infection are also known to stimulate vitellogenin expression in males (Murphy et al., 2009).

Exposure of male sand goby to EE2 (11 ng/L) resulted in large increases of each *vtg* type with *vtga* > *vtgc* > *vtgb* at all-time points although the differences in gene expression were only found to be

significant at day 29. Considering a significant difference between the relative levels of *vtga*, *vtgb* and *vtgc* was only found at later time-points of the EE2 exposure, statistical comparison of the hepatic expression of these *vtg* genes may be used as an indicator for the duration of estrogen contamination of coastal environments prior to sampling thus providing information useful to risk assessment in marine ecotoxicology.

At early time-points the level of each transcript was similar to that seen in vitellogenic females (Humble et al., 2013). In many other fish species induction of *vtg* mRNA and protein is observed with LOECs for EE2 in the range 5-10 ng/L suggesting that the sand goby is as sensitive to estrogenic endocrine disruption as the other species investigated (Woods and Kumar, (2011)). However, clear temporal differences between *vtg* types became apparent after 20 days of exposure with *vtgb* reaching a plateau while the rate of increase for *vtgc* slowed and *vtga* continued to increase. It is notable that the continued increase in *vtga* occurs at the same time that *er*α increases markedly. This makes it plausible that the EED-induced temporal rise of *erα* promotes a continued increase in *vtg* expression. Future work will test this hypothesis by cloning the promoter regions of *vtg* genes and studying their *erα*-dependent control of transcription.

Future work will also apply these assays to study the natural seasonal variation in the production of multiple *vtg*s in females and the effects that EEDs on vitellogenesis. It is conceivable that the normal pattern of *vtg* production in females is altered by such exposure and that this may not produce an optimum balance of nutrient for embryonic development.

4.4 Conclusions

Unlike the situation in many other teleosts only two *ers* are evident in the sand goby. Both can be induced in males by EE2 exposure, a consequence of which might be to exacerbate the adverse effects of EED exposure. Supporting evidence is provided by the observation that the temporal increase in *er* expression occurs coincidentally with an increase in expression of *vtga*, *vtgb* and *vtgc* with *vtga* demonstrating the greatest temporal and total increase. The temporal change in *erα* and *vtgs* transcript abundance reveals variation in the sensitivity of each of these potential biomarkers which is helpful for assessing their potential as biomarkers. The significant difference between the

abundance of all three *vtg*s only occurred after 29 days of exposure and may be considered an indicator of prolonged exposure.

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383

384 *Legends to Figures*

385

386 Fig.1. Alignment of sand goby partial protein sequences with full length protein sequences of
387 Japanese goby *era* (A) and *erβ1* (B) by ClustalW2. Green text represents the N-terminal domain, red
388 text the DNA binding domain, yellow text the hinge domain and blue text the ligand binding domain.
389 Numbers represent the amino acid residues, -=gap, * = fully conserved residue, : = strongly similar
390 residue . = weakly similar residue.

391 Fig. 2. Hepatic *erα* and *erβ1* relative mRNA expression in livers of control female and EE2-
392 treated male sand goby relative to control males determined by relative RTqPCR. EE2 treated males
393 were exposed to EE2 at 11ng/L for 29 days. Numbers of individuals are as follows: control males n =
394 7, exposed males n = 7 and control females n = 8. Error bars represent standard error for the mean
395 (SEM) and statistical signicance between control males and control females or exposed males were
396 determined by Student's unpaired t-test test ** = $P < 0.01$ *** = $P < 0.001$.

397 Fig. 3. Temporal, hepatic *vtga, vtgb, vtgc* and *erα* relative mRNA expression in EE2 exposed (11
398 ng/L) (solid line) and control (dashed line) male sand goby. Data represent mean expression values ±
399 SEM normalized using RS28 reference gene and relative to control fish sampled at each time point.
400 Unpaired student t-test was used to test for significant difference between exposed and control
401 samples (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Separately for each gene of interest, one-way
402 ANOVA with Tukey HSD post-hoc test was used to test for significant differences between time points
403 in exposed samples, The same letter (a, b, c) indicate no significant difference between time points
404 whereas different letters indicate significant differences ($p < 0.05$) between time points.

405

A)

```
ERα_A._flavimanus      MYPEESRGSGGVATVDFLDGTYDYTAPTPAPTLYSHSSTGYFSAPLDVHGPPSDGSLQSL 60
ERα_P._minutus         ------------------------------------------------------------


ERα_A._flavimanus      GSGPNSPLMFVPSSPHLSPFMHPPSHHYLETSSTPIYRSGVPSTQQLSREEHNGAEEAFR 120
ERα_P._minutus         ------------------------------------------------------------


ERα_A._flavimanus      VSESGSGTGVGPGGFEMAKETRFCAVCSDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMC 180
ERα_P._minutus         ----------------------------------HYGVWSCEGCKAFFKRSIQGHNDYMC 26
                                                          **************************


ERα_A._flavimanus      PATNQCTIDRNRRKSCQACRLRKCYEVGMMKGGIRKDRGRVLRRDKRRTDRDKSSKDSCQ 240
ERα_P._minutus         PATNQCTIDRNRRKSCQACRLRKCYEVGMMKGGIRKDRGRVVRRDKRKPDKDKNSKGSHP 86
                       ****************************************:*****:.*:**.**.*


ERα_A._flavimanus      KTAPPQDNKKHYSSNAGGGAKFAVSGMSPDQVLQLLQGAEPPILCSRQKLNGPYTEGTMM 300
ERα_P._minutus         KTAPLQD-KRQYVSSSGGQAKLSITGMSPDQVLQLLQGAEPPILCSRQKLSGPYTEITMM 145
                       **** ** *::* *.:** **::::*************************.***** ***


ERα_A._flavimanus      SLLTSMADKELVHMIAWAKKLPGFLQLSLHDQVLLLESSWLEVLMISLIWRSIHCPGKLI 360
ERα_P._minutus         TLLTSMADKELVHMIAWAKKLPGFLQLSLHDQVLLLESSWLEVLMISLIWRSIHCPGKLI 205
                       :************************************************************


ERα_A._flavimanus      FARDLILDRDEGECVEGMAEIFDMLLATASRFRMLKLRPEEFICLKAIILPNSGAFSFCT 420
ERα_P._minutus         FAQDLILDRSEGDCVEGMAEIFDMLLATASRFRMLKLRPEEFICLKAIILLNSGAFSFCT 265
                       **:******.**:************************************ *********


ERα_A._flavimanus      GTMEPLHDSAAVQNILDTITDALIHHISQSGYSAQQQSRRQAQLLLLLSHIRHMSNKGME 480
ERα_P._minutus         GTMEPLHDAAAVQSILDTITDALIYHISQSGYSGQQQARRQAQLLLLLSHIRHMSNKGMI 325
                       ********:****.**********:********.***:**********************


ERα_A._flavimanus      HLYNMKCKNKVPLYDLLLEMLDAHHLHHPVRTNQASSLNNSDPVYGSSSSLSSDPRGTST 540
ERα_P._minutus         ------------------------------------------------------------


ERα_A._flavimanus      GGGKMSSPSVLQFGGSPGNCTHIA 564
ERα_P._minutus         ------------------------
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B)

```
ERβ_P._minutus      ------------------------------------------------------------
ERβ_A._flavimanus   MAAASPEKDKPLLQLQEVDSSRAASRVLTPILGSSSPALSIEAAPPICIPSPYTELGPDY 60

ERβ_P._minutus      ------------------------------------------------------------
ERβ_A._flavimanus   APLPFYSPSIFSYNSTGLSECSTVHQPLSPSLFWPGHRHVGSSLPMHRSQARPAHTQPTP 120

ERβ_P._minutus      ------------------------------------------------------------
ERβ_A._flavimanus   SPWVEIQPRDSVLMTCKRRRSQESDEAVVSSGGKSDLHYCAVCHDYASGYHYGVWSCEGC 180

ERβ_P._minutus      ----------------------------------------VYEVGMTKCGMRKERGPLRSA 21
ERβ_A._flavimanus   KAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMTKCGMRKERGTLRSP 240
                                                           ***************.***.

ERβ_P._minutus      QASRRMTRLSTQGRGAVSRLIPVPSVVPRPETHPPTLTPEQLIGRIMEAEPPEIYLIKDM 81
ERβ_A._flavimanus   QASRRLTRLSSQSRSTGAKLLPVP-VVPRPEPQPPALSPEQLIGRIMEAEPPEIYLMKDM 299
                    *****:****:*.*.: ::*:*** ******.:**:*:******************:***

ERβ_P._minutus      KRPLTEANVMMSLTNLADKELVHMITWAKKIPGFVDLSLVDQVHLLECCWLEVLMIGLMW 141
ERβ_A._flavimanus   KRPLTEANVMMSLTNLADKELVHMITWAKKIPGFVELSLGDQVHLLECCWLEVLMIGLMW 359
                    ************************************:*** ********************

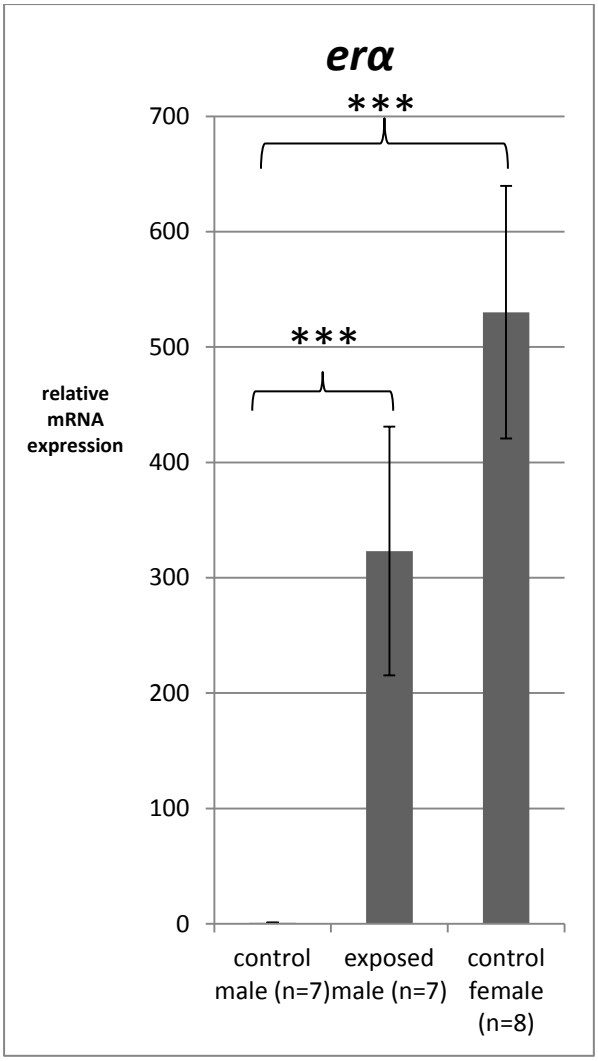
ERβ_P._minutus      RSVEHPGKLIFSPDLSLSREEGSCVQGFVEIFDMLVAATSRVRELKLQREEYVCLKAMI- 200
ERβ_A._flavimanus   RSVDHPGKLIFSPDLSLSREEGSCVQGFVEIFDMLLAATSRVRELKLQREEYVCLKAMIL 419
                    ***:*********************************:**********************

ERβ_P._minutus      ------------------------------------------------------------
ERβ_A._flavimanus   LNSNMCLSSSEGSEEVQSRSKLLCLLDTVTDALVWAIAKTGLSFRQQYTRLAHLLMLLSH 479

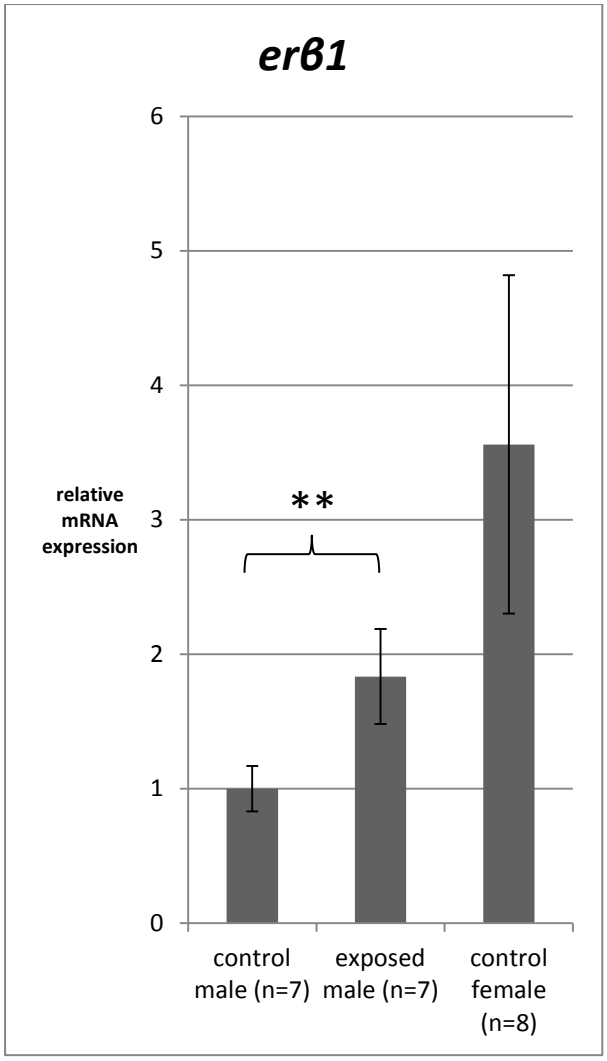
ERβ_P._minutus      ------------------------------------------------------------
ERβ_A._flavimanus   IRHASNKGMDHLHCMKMKNMVPLYDLLLEMLDAHIMHNSRLPCRPTQQEPRDPMEPQERP 539

ERβ_P._minutus      ----------------------------
ERβ_A._flavimanus   HISPSGPSNTCTPSEDENQPSETIKTPQ 567
```

Fig 1


erα
***
***
700
600
500
400
300
200
100
0
relative mRNA expression
control male (n=7)
exposed male (n=7)
control female (n=8)

489



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491 Fig 2

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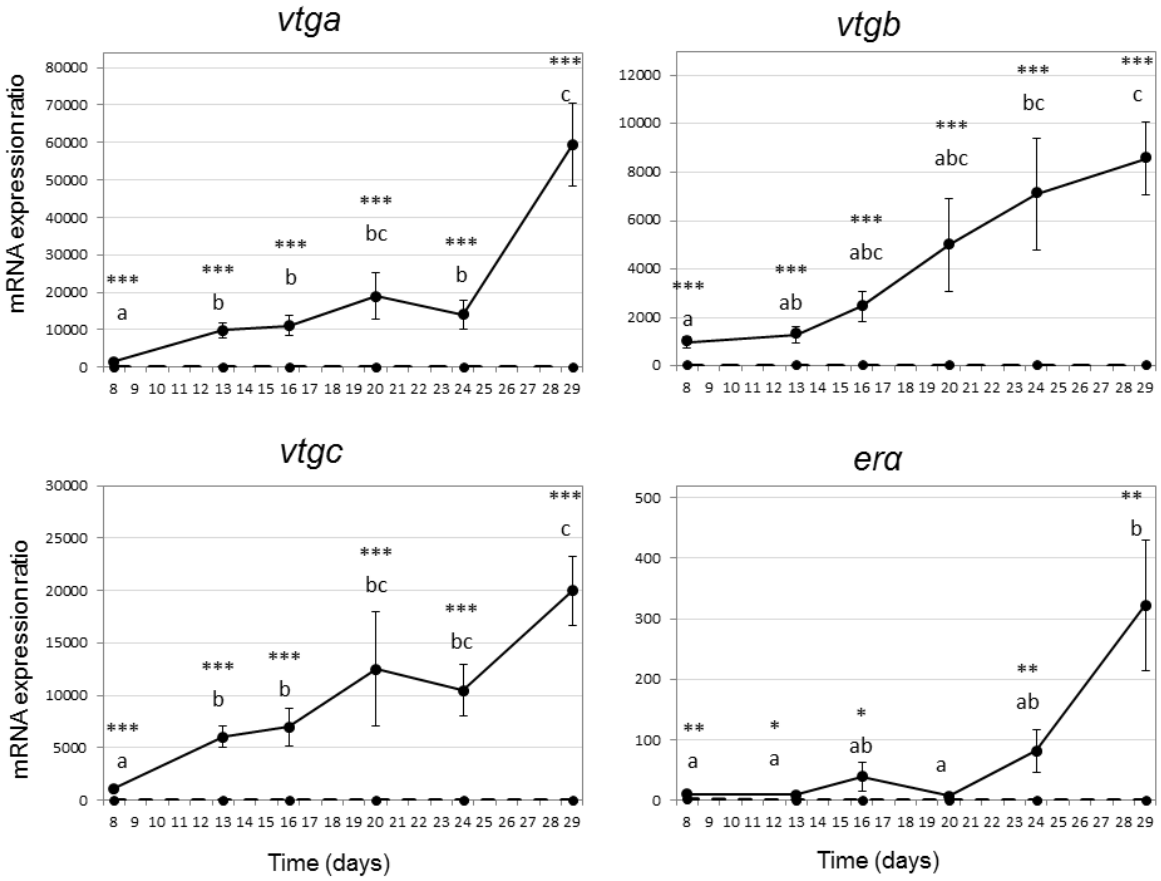

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