

Effects of 17 α -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*)

Humble, Joseph; Saaristo, Minna; Lindstrom, Kai; Lehtonen, Kari; Craft, John

Published in:
Marine Environmental Research

DOI:
[10.1016/j.marenvres.2014.01.006](https://doi.org/10.1016/j.marenvres.2014.01.006)

Publication date:
2014

Document Version
Author accepted manuscript

[Link to publication in ResearchOnline](#)

Citation for published version (Harvard):

Humble, J, Saaristo, M, Lindstrom, K, Lehtonen, K & Craft, J 2014, 'Effects of 17 α -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*)', *Marine Environmental Research*, vol. 96, pp. 12-18.
<https://doi.org/10.1016/j.marenvres.2014.01.006>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

If you believe that this document breaches copyright please view our takedown policy at <https://edshare.gcu.ac.uk/id/eprint/5179> for details of how to contact us.

Effects of 17 α -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*)

Joseph L. Humble ^{a,+}, Minna Saaristo ^{b,**}, Kai Lindström ^c, Kari K. Lehtonen ^d and John A. Craft ^{a,*}

a School of Health and Life Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow, G4 0BA, United Kingdom

b Department of Bio and Environmental Sciences, University of Helsinki, Finland

c Department of Environmental and Marine Biology, Åbo Akademi University, Finland

d Marine Research Centre, Finnish Environment Institute, P.O.B. 140 00251 Helsinki, Finland

* corresponding author: John A Craft, +44 141 331 3220, j.a.craft@gcu.ac.uk

+ Current Address: City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China

** Current address: School of Biological Sciences, Monash University, Clayton campus, 3800 Victoria, Australia

1 **Abstract**

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

12

13 **1.1 Introduction**

14 Estrogenic endocrine disruptors (EED) interact with the endocrine systems of animals by
15 engaging with the estrogen signal transduction pathways, resulting in estrogenic toxicity with a myriad
16 of detrimental and adverse effects (Hiramatsu et al., 2005). 17 α -ethinyl estradiol (EE2) is a model
17 EED and is found to contaminate European coastal waters with concentrations fluctuating as high as
18 125 ng/L (Pojana et al., 2004). The sand goby (*Pomatoschistus minutus*) is a small benthic fish that
19 inhabits European coastal and estuarine environments. Sand goby have a one-year life cycle with
20 distinctive well-characterized reproductive behaviours, for example sand goby males build nests into
21 which females are lured to lay their eggs (Healey, 1971). In earlier works, sand goby has been utilized
22 as a model in ecotoxicology for the study of endocrine disruption in both controlled exposure studies
23 (Saaristo et al., 2009) and environmental monitoring (Kirby et al., 2003). Exposure of male and female
24 animals to EE2 was shown to have adverse effects on reproductive output and mating behaviours
25 (Robinson et al., 2003; Saaristo et al., 2010a, 2010b). The molecular mechanisms by which female
26 egg production is impaired are poorly understood. In these studies it was difficult to contextualize the

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

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

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

52 ***2 Materials and Methods***

53 ***2.1. The exposure scheme***

54 The sand gobies used in the exposure experiments were caught using a hand trawl at natural
55 breeding sites near the Tvärminne Zoological Station (University of Helsinki) on the southern coast of
56 Finland. Trawling was conducted during the main breeding season (May-June). Only sexually mature
57 fish were chosen to this study and they were separated by sex before introduction to the holding
58 tanks. Fish were acclimated to the laboratory conditions for 2 weeks. From holding tanks fish were
59 randomly assigned to six different exposure glass aquaria (80 x 80 x 40). Males were kept at a
60 density of 45 males and females were kept at a density of 15 per tank. Tanks had a 3 cm layer of fine
61 sand on the bottom and were equipped with a flow-through of seawater (see Saaristo et al., 2009,
62 2010a,b). Fish were fed twice a day during the exposure period.

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

70 ***2.2 Cloning of estrogen receptors***

71 **2.2.1 Targeting unknown sand goby *er* sequence**

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

80 **2.2.2 RNA isolation and Reverse transcription**

81 Total RNA was isolated from EE2 exposed male and control female liver tissue samples (100 mg)
82 using NucleoSpin® RNA II kit (ABGene, Epsom, UK) following the manufacturer's protocol and
83 quantified by Nanodrop™ ND-1000 spectrophotometer. Superscript III reverse transcriptase
84 (Invitrogen, Paisley, UK) was used to convert 2 µg RNA into cDNA, again according to the
85 manufacturer's instructions. The reverse transcription reactions were primed using random hexamers
86 at a reaction concentration of 1.5 µM and oligodT at a reaction concentration of 3 µM. The reaction
87 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
88 PCR amplification, cloning and sequencing

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

106 ***2.3 Quantification of Transcripts by Relative RT-qPCR.***

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

108 **2.3.2 qPCR assays**

109 The qPCR reactions were primed with transcript-specific primers. Primers for *vtga*, *vtgb*, *vtgc* and
110 28S ribosomal RNA (*rs28*) were presented previously (Humble et al., 2013). Primers for *era* and *erb1*
111 were designed using Primer3 (Rozen and Skaletsky, 2000) and primer pairs were screened against
112 potential to form secondary structures using Netprimer (Premier Biosoft 2002). The reverse
113 transcription, quantitative, PCR (RT-qPCR) primers were validated using end-point RT-PCR to amplify
114 cDNA from female control fish (data not shown) to demonstrate single fragments of expected size and
115 RT-qPCR standard curves constructed to assess the efficiency for each primer set (Table 1). *rs28*
116 shows little variation in hepatic expression in different genders or response to EE2 when a fixed
117 amount of RNA is used (CT standard deviation ± 0.55) was therefore used as reference gene. All RT-
118 qPCR reactions were carried out in triplicate in 96 clear-well plates using Platinum® SYBR® green
119 qPCR Supermix-UDG (Invitrogen, Paisley, UK) as previously described (Humble et al., 2013). After
120 amplification a melting curve analysis (60 °C to 95 °C) was completed for each reaction to
121 demonstrate a single product melting at the correct temperature.

122 ***2.4 Mathematical and statistical analysis of RTqPCR data***

123 The RTqPCR mathematical calculation was performed separately for each target gene. First, the
124 mean CT values for each gene (target gene and reference gene) and each biological sample was
125 calculated using the three technical replicates. Second, a relative expression ratio (R) was generated
126 for the gene of interest (relative to the reference gene) for each individual biological sample using the
127 following equation presented by Pfaffl (2001) $R = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}(\text{control-sample})}}{(E_{\text{reference}})^{\Delta CT_{\text{ref}}(\text{control-}$
128 $\text{sample})}}$ with reference to assay efficiencies (E) to compensate for inter-assay efficiency variation. *RS28*
129 was used as reference gene (ref) and the mean of the control samples was used as “control”. Third,
130 the R values were Log10 transformed to fit approximately normal distributions as determined by
131 Shapiro-Wilk test and show homogeneity of variance as determined by Levene’s test.

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

137 **3. Results**

138 **3.1. Partial sequencing of two estrogen receptors in sand goby**

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

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

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

163 **3.2 EE2 induced male *er* gene expression compared with female**

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

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

176 ***3.3 Vtg-a, -b, -c & era expression over a month-long exposure to EE2***

177 Relative RT-qPCR was used to analyse *vtga*, *vtgb*, *vtgc* and *era* mRNA levels in control and
178 EE2-exposed (11 ng/L) male sand goby liver at 6 time points throughout 29 days (Fig. 3). Unexposed
179 males had very low levels of mRNA for *era* and all *vtg* subtypes throughout the 29 day exposure
180 period. This was detectable by highly significantly, lower C_T values compared with non-template
181 control (NTC). For instance, the mean CT values for the unexposed males at day 29 were 26.7, 29.4,
182 29.9 and 26.7 for *vtga*, *vtgb*, *vtgc* and *era* respectively while the respective NTC values were
183 undetermined, 35.1, 36.4, 38.2 and 37.9 for the respective genes.

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

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

191 and reveal distinct temporal and transcript specific changes in mRNA upregulation. For *vtga*, there
192 was a sharp rise at the beginning of the time course shown by a statistically significant increase
193 between day 8 and all other time points. Day 29 was also significantly greater than day 8, 13, 16 and
194 24 showing that *vtga* continued to rise at the later stages. *Vtgb* on the other hand showed no
195 significant difference between day 8 and day 13, 16 or 20 but here was a difference between day 8
196 and day 24 or 29. *Vtgb* expression reached a plateau indicated by a lack of statistically significant
197 differences between day 16, 20, 24 and 29. For *vtgc*, day 8 was found to be significantly different
198 from all other time points measured. Day 29 was found to be significantly higher than day 8, 13 and
199 16, but not significantly different to day 20 or 24. For *erα* there was a significant difference between
200 day 29 and days 8, 13 or 20 however there was no significant difference comparing day 13 or day 20
201 with any other time point.

202 A comparison was also carried out between relative mRNA expression levels of *vtg* target genes
203 over the time course. At day 8 of exposure the relative expression levels of the *vtgs* was in the order
204 *vtga* > *vtgc* > *vtgb* (ratio of relative gene expression *vtga* : *vtgc* : *vtgb*, for exposed male 1.53 : 1.13 :
205 1) though this was found not to be significantly different. However at day 29 there was a significant
206 difference (with *vtga* > *vtgc* > *vtgb*) and the respective abundance ratio for *vtga* : *vtgc* : *vtgb* was 6.94 :
207 2.34 : 1 indicating a divergence in the expression profiles for these three genes over time.

208 **4. Discussion**

209 **4.1 Analysis of novel *erα* and *erβ1* sequences**

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

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

228 **4.2 Expression of estrogen receptor genes**

229 Our study shows that the sand goby has gender-specific patterns of hepatic *er* expression and by
230 comparison, transcript levels of both *ers* were low in male with *erβ1* marginally higher than that of *erα*.
231 In contrast *ers* are reported as absent from the liver of male Japanese common goby but this may be
232 the consequence of an insensitive end-point PCR technique used in that investigation (Ito et al.,
233 2007). Work on zebrafish (*Danio rerio*) has indicated that endogenous levels of *erβ1* are higher than
234 that of *erα* or *erβ2* (Menuet et al., 2004) but it was unclear which gender of fish were used in that
235 study. Meng et al., (2010) reported gender differences in *er* transcript levels in zebrafish liver with *erα*
236 and *erβ2* in females being at higher levels than *erβ1* while in males *erβ1* and *erα* were observed at
237 very low levels and *erβ2* was higher. The results reported here indicate that sand goby is similar to
238 zebrafish in respect of gender differences in hepatic expression of *erα* and *erβ1* but differ because in
239 sand goby, no *erβ2* has been found.

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

247 for EED exposure-monitoring in male sand goby with high mRNA levels in particular signaling
248 prolonged EED exposure. In zebrafish short term exposure (48 hours) to 17 β -oestradiol has been
249 reported to cause disparate effects upon the hepatic transcript levels of the *er*, with *er α* increasing
250 *er β 1* decreasing (Menuet et al., 2002), which suggests zebrafish is dissimilar to sand goby regarding
251 its downregulation of *er β 1* in response to estrogens. The results reported here show similarity with
252 those reported in largemouth bass where the three *er* subtypes are classified as α , β (*er β 2*) and γ
253 (*er β 1*). Sand goby β 1 showed greatest similarity to largemouth bass γ type. Similar to the sand goby,
254 the liver of largemouth bass females has higher endogenous levels of *er α* than *er γ* , and the injection
255 of males with E2 causes a large increase in *er α* and a moderate increase in *er γ* (Sabo-Attwood et al.,
256 2004). The increase in *er α* expression found in EED exposed sand goby may act as a positive
257 feedback, compounding the feminization process and further sensitizing the males to estrogen
258 exposure. Here we have for the first time in a teleost species demonstrated the induction of *er α* and
259 *er β 1* by exposure to environmentally relevant concentrations of EED.

260 ***4.3 Expression of vitellogenin genes***

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

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

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

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

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

295 ***4.4 Conclusions***

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

303 abundance of all three *vtgs* only occurred after 29 days of exposure and may be considered an
304 indicator of prolonged exposure.

305 **5. Acknowledgement.**

306 JLH thanks the Natural Environment Research Council (UK) for a Doctoral Training Grant
307 (NE/G523398/1). MS, KL and KKL thank the Onni Talas Foundation (Finland) for funding.

308 **6. References**

- 309 Aranda, A., Pascual, A., 2001. Nuclear hormone receptors and gene expression. *Physiol. Rev.* 81,
310 1269–304.
- 311 Hawkins, M.B., Thomas, P., 2004. The unusual binding properties of the third distinct teleost estrogen
312 receptor subtype ERbeta are accompanied by highly conserved amino acid changes in the
313 ligand binding domain. *Endocrinology* 145, 2968–77.
- 314 Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, a, Thomas, P., 2000. Identification of
315 a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proc. Natl.*
316 *Acad. Sci. U. S. A.* 97, 10751–6.
- 317 Healey, M.C., 1971. The distribution and abundance of sand gobies, *Gobius minutus*, in the Ythan
318 estuary. *J. Zool.* 163, 177–228.
- 319 Hiramatsu, N., Cheek, A.O., Sullivan, C. V, Matsubara, T., Hara, A., 2005. Chapter 16 Vitellogenesis
320 and endocrine disruption, in: *Biochemistry and Molecular Biology of Fishes*. Elsevier, pp. 431–
321 471.
- 322 Humble, J.L., Hands, E., Saaristo, M., Lindström, K., Lehtonen, K.K., Diaz, O., Cerio, D., Cancio, I.,
323 Wilson, G., Craft, J.A., 2013. Characterisation of genes transcriptionally upregulated in the liver
324 of sand goby (*Pomatoschistus minutus*) by 17 alpha-ethinyloestradiol : Identification of distinct
325 vitellogenin and zona radiata protein transcripts. *Chemosphere* 90, 2722–2729.
- 326 Ito, K., Mochida, K., Fujii, K., 2007. Molecular cloning of two estrogen receptors expressed in the
327 testis of the Japanese common goby, *Acanthogobius flavimanus*. *Zoolog. Sci.* 24, 986–996.
- 328 Katsiadaki, I., Scott, A.P., Hurst, M.R., Matthiessen, P., Mayer, I., 2002. Detection of environmental
329 androgens: a novel method based on enzyme-linked immunosorbent assay of spiggin, the
330 stickleback (*Gasterosteus aculeatus*) glue protein. *Environ. Toxicol. Chem.* 21, 1946–54.
- 331 Kirby, M.F., Bignell, J., Brown, E., Craft, J.A., Davies, I., Dyer, R.A., Feist, S.W., Jones, G.,
332 Matthiessen, P., Megginson, C., Robertson, F.E., Robinson, C., 2003. The presence of
333 morphologically intermediate papilla syndrome in United Kingdom populations of sand goby
334 (*Pomatoschistus* spp.): Endocrine disruption? *Environ. Toxicol. Chem.* 22, 239–251.
- 335 Meng, X., Bartholomew, C., Craft, J.A., 2010. Differential expression of vitellogenin and oestrogen
336 receptor genes in the liver of zebrafish, *Danio rerio*. *Anal. Bioanal. Chem.* 396, 625–630.

- 337 Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., Pakdel, F., 2004. Analysis of the estrogen
338 regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1
339 and ERbeta2. *J. Mol. Endocrinol.* 32, 975–86.
- 340 Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular
341 characterization of three estrogen receptor forms in zebrafish: binding characteristics,
342 transactivation properties, and tissue distributions. *Biol. Reprod.* 66, 1881–92.
- 343 Murphy, C.A., Rose, K.A., Rahman, M.S., Thomas, P., 2009. Testing and applying a fish
344 vitellogenesis model to evaluate laboratory and field biomarkers of endocrine disruption in
345 Atlantic croaker (*Micropogonias undulatus*) exposed to hypoxia. *Environ. Toxicol. Chem.* 28,
346 1288–1303.
- 347 Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other
348 vertebrates. *Gen. Comp. Endocrinol.* 192, 15–24.
- 349 Ohkubo, N., Andoh, T., Mochida, K., Adachi, S., Hara, A., Matsubara, T., 2004. Deduced primary
350 structure of two forms of vitellogenin in Japanese common goby (*Acanthogobius flavimanus*).
351 *Gen. Comp. Endocrinol.* 137, 19–28.
- 352 Pojana, G., Bonfà, A., Buseti, F., Collarin, A., Marcomini, A., 2004. Estrogenic potential of the Venice,
353 Italy, lagoon waters. *Environ. Toxicol. Chem.* 23, 1874–1880.
- 354 Robinson, C.D., Brown, E., Craft, J.A., Davies, I.M., Moffat, C.F., Pirie, D., Robertson, F., Stagg, R.M.,
355 Struthers, S., 2003. Effects of sewage effluent and ethynyl oestradiol upon molecular markers of
356 oestrogenic exposure, maturation and reproductive success in the sand goby (*Pomatoschistus*
357 *minutus*, Pallas). *Aquat. Toxicol.* 62, 119–134.
- 358 Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers.
359 *Methods Mol. Biol.*
- 360 Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2009. Sand goby (*Pomatoschistus minutus*)
361 males exposed to an endocrine disrupting chemical fail in nest and mate competition. *Horm.*
362 *Behav.* 56, 315–321.
- 363 Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2010a. Exposure to 17 α -ethynyl estradiol
364 impairs courtship and aggressive behaviour of male sand gobies (*Pomatoschistus minutus*).
365 *Chemosphere* 79, 541–546.
- 366 Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2010b. An endocrine disrupting chemical
367 changes courtship and parental care in the sand goby. *Aquat. Toxicol.* 97, 285–292.
- 368 Sabo-Attwood, T., Kroll, K.J., Denslow, N.D., 2004. Differential expression of largemouth bass
369 (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. *Mol.*
370 *Cell. Endocrinol.* 218, 107–18.
- 371 Schulz, R.W., de França, L.R., Lareyre, J.-J., Le Gac, F., LeGac, F., Chiarini-Garcia, H., Nobrega,
372 R.H., Miura, T., 2010. Spermatogenesis in fish. *Gen. Comp. Endocrinol.* 165, 390–411.
- 373 Söffker, M., Stevens, J.R., Tyler, C.R., 2012. Comparative Breeding and Behavioral Responses to
374 Ethinylestradiol Exposure in Wild and Laboratory Maintained Zebrafish (*Danio rerio*) Populations.
375 *Environ. Sci. Technol.* 46, 11377–11383.
- 376 Sun, L., Shao, X., Wu, Y., Li, J., Zhou, Q., Lin, B., Bao, S., Fu, Z., 2011. Ontogenetic expression and
377 17 β -estradiol regulation of immune-related genes in early life stages of Japanese medaka
378 (*Oryzias latipes*). *Fish Shellfish Immunol.* 30, 1131–7.

379 Thacker, C., 2009. Phylogeny of Gobioidae and Placement within Acanthomorpha, with a New
380 Classification and Investigation of Diversification and Character Evolution. *Copeia* 2009, 93–104.

381 Woods, M., Kumar, A., 2011. Vitellogenin induction by 17 β -estradiol and 17 α -ethynylestradiol in male
382 Murray rainbowfish (*Melanotaenia fluviatilis*). *Environ. Toxicol. Chem.* 30, 2620–7.

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 *er β 1* (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 *era* and *er β 1* 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 significance 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 *era* relative mRNA expression in EE2 exposed (11
398 ng/L) (solid line) and control (dashed line) male sand goby. Data represent mean expression values \pm
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

406 A)

407 ER α _A._flavimanus MYPEESRSGSGVATVDFLDGTYDYTAPTAPAPLYSHSSTGYFSAPLVDVHGPPSDGSLQSL 60

408 ER α _P._minutus -----

409

410

411 ER α _A._flavimanus GSGPNSPLMFVPSSPHLSPFMHPPSHHYLETSSTPIYRSGVPSTQQLSREEHNGAEEAFR 120

412 ER α _P._minutus -----

413

414

415 ER α _A._flavimanus VSESGSGTGVGPGGFEMAKETRFCAVCSDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMC 180

416 ER α _P._minutus -----HYGVWSCEGCKAFFKRSIQGHNDYMC 26

417 *****

418

419 ER α _A._flavimanus PATNQCTIDRNRKSCQACRLRKCCEYEVGMKGGIRKDRGRVLRDRKRRTRDRKSSKSDSCQ 240

420 ER α _P._minutus PATNQCTIDRNRKSCQACRLRKCCEYEVGMKGGIRKDRGRVVRDRKRPDKDKNSKGSHP 86

421 *****:****:.*:*.**.*

422

423 ER α _A._flavimanus KTAPPQDNKKHYSSNAGGGAKFAVSGMSPDQVLQLLQGAEPPICSRQKLNQPYTEGTMM 300

424 ER α _P._minutus KTAPLQD-KRQYVSSSGGQAKLSITGMSPDQVLQLLQGAEPPICSRQKLSGPYTEITMM 145

425 **** * *:.* *.:** **:::*****.***** **

426

427 ER α _A._flavimanus SLLTSMADKELVHMIAWAKKLPGFQLSLHDQVLLLESSWLEVLMIISLIWRSIHCPGKLI 360

428 ER α _P._minutus TLLTSMADKELVHMIAWAKKLPGFQLSLHDQVLLLESSWLEVLMIISLIWRSIHCPGKLI 205

429 :*****

430

431 ER α _A._flavimanus FARDLILDRDEGECVEGMAEIFDMLLATASRFMLKLRPEEFICLKAIILPNSGAFSFACT 420

432 ER α _P._minutus FAQDLILDRSEGDCEGMAEIFDMLLATASRFMLKLRPEEFICLKAIILLNSGAFSFACT 265

433 **:*****.*:*****.*****.*****.*****.*****

434

435 ER α _A._flavimanus GTMEPLHDSAQVQNILDITDALIHHSIQSGYSAQQSRRQAQLLLLLSHIRHMSNKGME 480

436 ER α _P._minutus GTMEPLHDAAAVQSILDTITDALIYHISQSGYSGQQARRQAQLLLLLSHIRHMSNKGMI 325

437 *****:****.*****:*****.*:*****.*****

438

439 ER α _A._flavimanus HLYNMKCKNKVPLYDLLLEMLDAHHLHPVRTNQASSLNSDPVYGSSSSLSSDPRGTST 540

440 ER α _P._minutus -----

441

442

443 ER α _A._flavimanus GGGKMSSPSVLQFGGSPGNCTHIA 564

444 ER α _P._minutus -----

445

446

447 B)

448 ERβ_P._minutus -----

449 ERβ_A._flavimanus MAAASPEKDKPPLLQLQEVDSRAASRVLTPIILGSSSPALSIEAAPPICIPSPYTELGPDY 60

450

451

452 ERβ_P._minutus -----

453 ERβ_A._flavimanus APLPFYSPSIFSYNSTGLSECSTVHQPLSPSLFWPGHRHVGSSSLPMHRSQARPAHTQPTP 120

454

455

456 ERβ_P._minutus -----

457 ERβ_A._flavimanus SPWVEIQPRDSVLMTCRRRSQESDEAVVSSGGKSDLHYCAVCHDYASGYHYGVWSCEGC 180

458

459

460 ERβ_P._minutus -----VYEVGMTKCGMRKERGPLRSA 21

461 ERβ_A._flavimanus KAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMTKCGMRKERGTLRSP 240

462 *****.*.*. :.:** *****.:**.*:*****:***

463

464 ERβ_P._minutus QASRRMTRLSTQGRGAVSRLIPVPSVVRPETHPPTLTPEQLIGRIMEAEPPEIYLIKDM 81

465 ERβ_A._flavimanus QASRRLTRLSSQSRSTGAKLLPVP-VVPRPEPQPPALSPEQLIGRIMEAEPPEIYLMKDM 299

466 *****:*****.*.*.: :.:** *****.:**.*:*****:***

467

468 ERβ_P._minutus KRPLTEANVMMSLTNLADKELVHMITWAKKIPGFVDLSLVDQVHLLLECCWLEVLMIGLMW 141

469 ERβ_A._flavimanus KRPLTEANVMMSLTNLADKELVHMITWAKKIPGFVELSLGDQVHLLLECCWLEVLMIGLMW 359

470 *****:*****:*** *****:***

471

472 ERβ_P._minutus RSVEHPGKLI FSPDLSLSREEGSCVQGFVEIFDMLVAATSRVRELKLRQREYVCLKAMI- 200

473 ERβ_A._flavimanus RSVDPHGKLI FSPDLSLSREEGSCVQGFVEIFDMLLAATSRVRELKLRQREYVCLKAMIL 419

474 ***:*****:***

475

476 ERβ_P._minutus -----

477 ERβ_A._flavimanus LNSNMCLSSSEGSEEVQSRSKLLCLLDVTVDALVWAI AKTGLSFRQQYTRLAHLLMLLSH 479

478

479

480 ERβ_P._minutus -----

481 ERβ_A._flavimanus IRHASNKGMDDLHLCMKMNMVPLYDLLLEMLDAHIMHNSRLPCRPTQQEPRDPMEPQERP 539

482

483

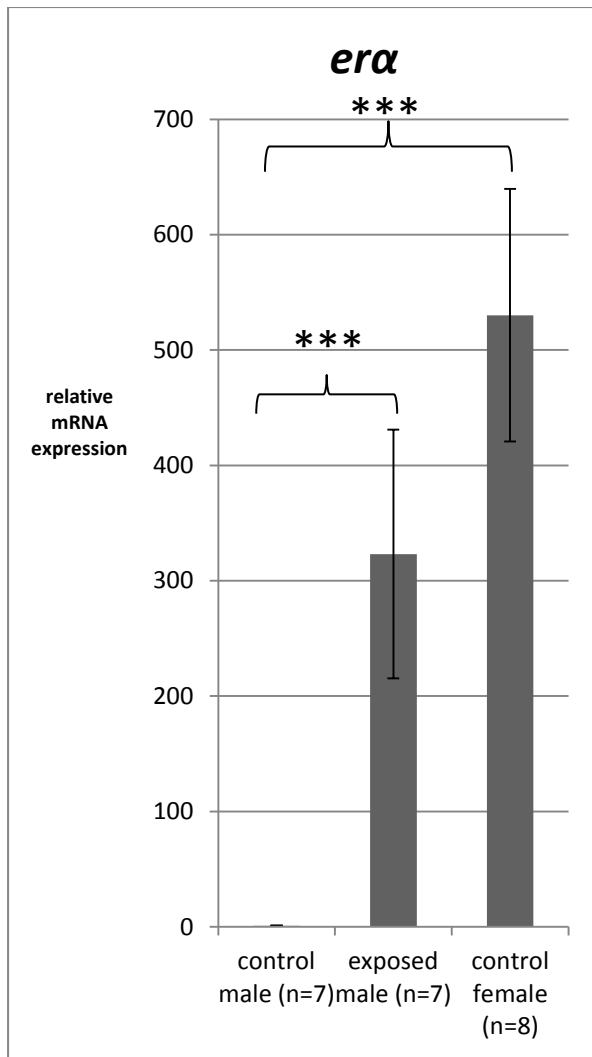
484 ERβ_P._minutus -----

485 ERβ_A._flavimanus HISPSGPNCTCTPSEDENQPSETIKTPQ 567

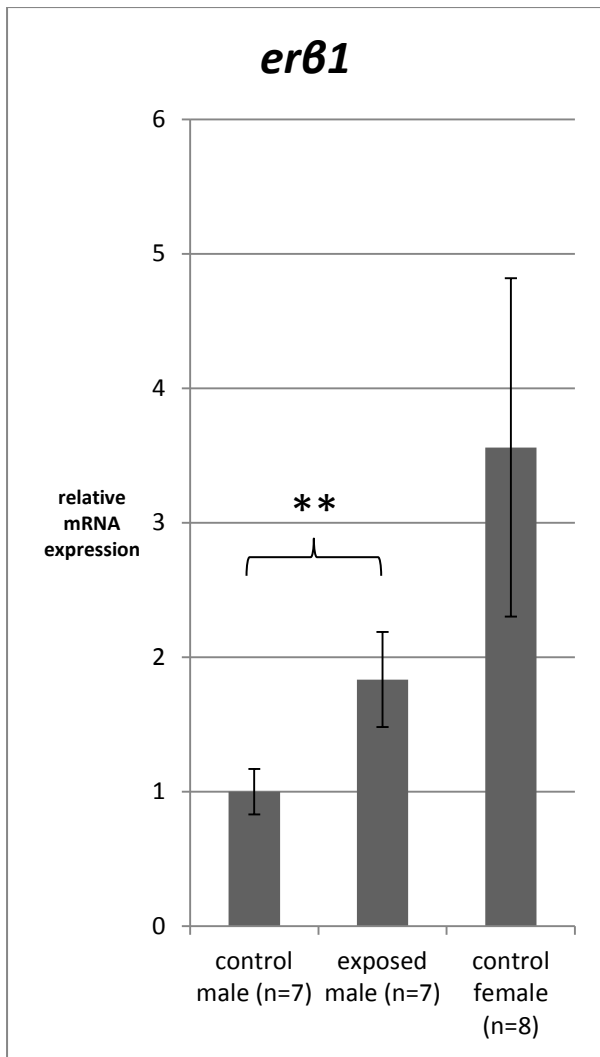
486

487 Fig 1

488



489

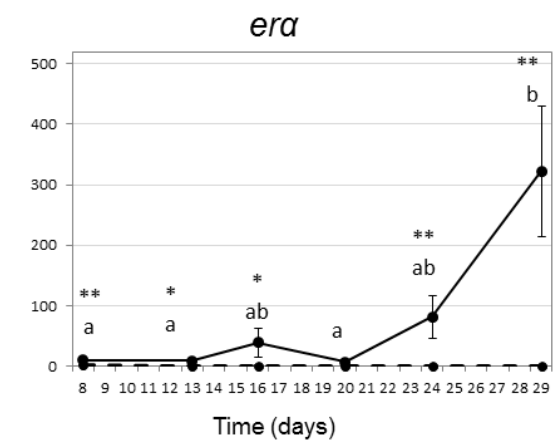
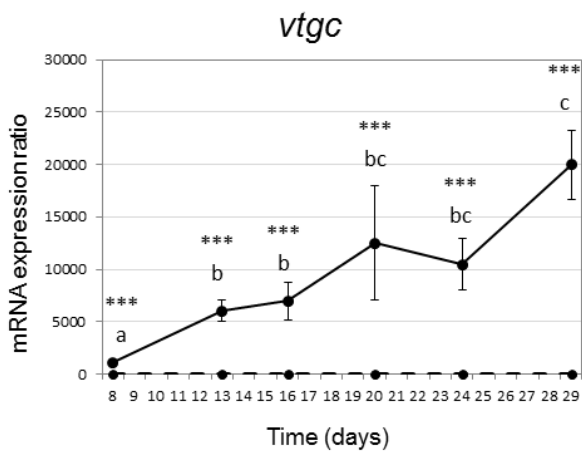
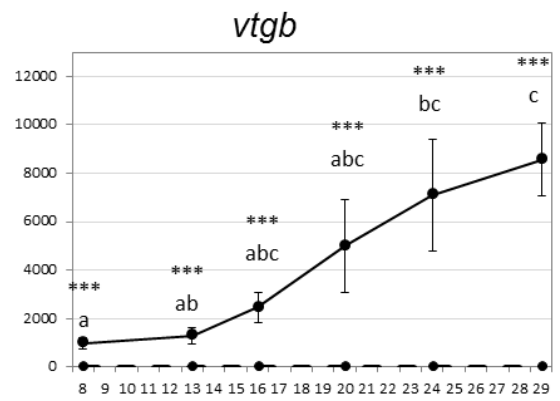
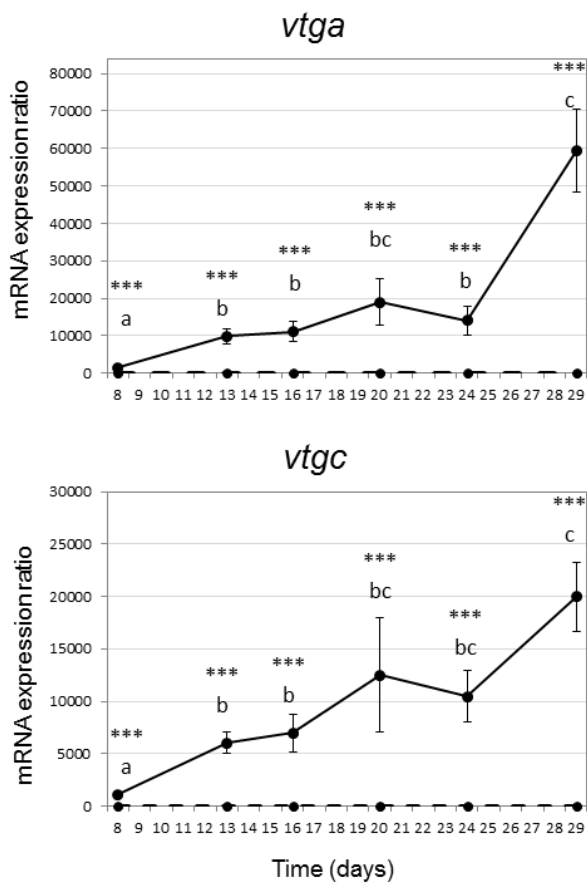


490

491 Fig 2

492

493



494

495

496