

1 Tide-related changes in mRNA abundance of aromatase and estrogen receptors in the ovary  
2 and brain of the threespot wrasse *Halichoeres trimaculatus*

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28

29 **ABSTRACT**

30

31 The threespot wrasse (*Halichoeres trimaculatus*; Family Labridae) is a common coral reef  
32 species of the Indo-Pacific Ocean. Given that this species spawns daily at high tide (HT), we  
33 hypothesized that endocrine changes in relation to gonadal development are synchronized  
34 with the tidal cycle. To test this, we examined the transcript abundance of two cytochrome  
35 P450 aromatases (*cyp19a* and *cyp19b*) and two estrogen receptors (*er $\alpha$*  and *er $\beta$* ) in the ovary  
36 and brain of this species in response to tidal change. When fish were collected around four  
37 tidal points [low tide (LT), flood tide (FT), high tide (HT), and ebb tide (ET)], gonadosomatic  
38 index and oocyte diameter increased around HT and FT, respectively. Ovulatory follicles were  
39 observed in ovaries around HT. Real-time quantitative polymerase-chain reaction revealed  
40 that mRNA abundance of *cyp19a* and *er $\alpha$* , but not *er $\beta$* , in the ovary increased around ET and  
41 HT, respectively. On the other hand, mRNA levels of *cyp19b* in the forebrain were  
42 significantly higher around FT. Increases of *er $\alpha$*  and *er $\beta$*  mRNA abundance around FT were  
43 observed in all areas of the brain and the midbrain, respectively. The changes in mRNA  
44 abundance of key genes involved in reproduction at specific tidal cycles, along with the  
45 development of the vitellogenic oocytes in the ovary, support our hypothesis that  
46 synchronization of endocrine changes to the tidal periodicity plays a role in the gonadal  
47 development of this species. We hypothesize that conversion of testosterone to E2 in the brain  
48 may be associated with the spawning behavior given that the wrasse exhibits group spawning  
49 with a territory-holding male around HT.

50

51 Keywords: aromatase, brain, estrogen receptor, ovary, tide, wrasse

52

53 **Introduction**

54

55 Estradiol-17 $\beta$  (E2) plays an important role in reproductive processes including oogenesis,  
56 vitellogenesis, and gonadotropin regulation in female fish (Nelson and Habibi 2013). This sex  
57 steroid is converted mainly from testosterone through catalytic activity by aromatases  
58 (P450arom) that belong to the cytochrome P450 (CYP19) superfamily and are differentially  
59 expressed in the ovary (named CYP19A1 or P450aromA) and the brain (named  
60 CYP19A2/P450aromBb). They are derived from the separated gene loci of *CYP19a1a* and  
61 *CYP19a1b*, respectively (Blázquez and Piferrer, 2004; Tchoudakova and Callard, 1998;  
62 Tchoudakova et al., 2001). It was reported in certain fishes that mRNA transcript abundance  
63 of *cyp19a1a* and *cyp19a1b* are positively correlated with aromatase activity and plasma E2  
64 levels during ovarian recrudescence, suggesting that these genes are involved in female  
65 reproduction through E2 synthesis in the brain and ovary during the breeding season (Chaube  
66 et al., 2015; Li et al., 2007). The brain-derived E2 is considered to play a role in neuronal  
67 and/or glial signaling in relation to reproduction (Hojo et al., 2003; Holloway and Clayton,  
68 2001; Zwain and Yen, 1999).

69 E2 action in target tissues is mediated by cytosolic estrogen receptors (ERs), which  
70 belong to the nuclear receptor superfamily and are made up of distinct subtypes including  
71 ER $\alpha$  and ER $\beta$  (Hawkins et al. 2000). An additional ER subtype (named ER $\beta$ 2 or ER $\gamma$ ) was  
72 also reported in ray-finned fish species (Choi and Habibi, 2003; Hawkins et al., 2000; Ma et  
73 al. 2000; Menuet et al., 2002; Nagler et al., 2007). In some fish species including the Korean  
74 rockfish (*Sebastes schlegeli*), the rainbow trout (*Oncorhynchus mykiss*), and the orange-  
75 spotted grouper (*Epinephelus coioides*), the mRNA abundance of *er $\alpha$ 1*, but not *er $\beta$ 1* and  
76 *er $\beta$ 2*, is highly expressed in the liver and positively correlated with plasma levels of E2 and  
77 vitellogenin (Chen et al., 2011; Mu et al., 2013; Nagler et al., 2012). On the other hand, E2

78 treatment resulted in up-regulation of three ER subtypes (*era*, *erβ1*, and *erβ2*) in the ovary of  
79 goldfish *Carassius auratus* (Nelson et al., 2007). Down-regulation of *eral* and *er2b* was  
80 observed in the ovary of the fathead minnow (*Pimephales promelas*) in relation to exposure to  
81 exogenous E2 (Filby et al., 2006). It, therefore, appears that ERs are differently expressed  
82 among tissues in accordance with gonadal development.

83 Wrasses belong to the Family Labridae and are largely distributed in shallow waters with  
84 rocky and sandy bottoms from tropical to temperate waters. Most wrasses are known to be  
85 protogynous hermaphrodites with a harem mating system and to spawn daily during the  
86 breeding season (Ross, 1983; Warner, 1982). A diurnal pattern of ovarian development is  
87 reported in certain wrasses (Matsuyama et al., 1998, 2002; Takemura et al., 2008).  
88 Interestingly, tidal cycle seems to be superimposed on daily spawning in tropical wrasses  
89 (Colin and Bell, 1991; Hoffman and Grau, 1989; Ross, 1983; Takemura et al., 2008; Warner,  
90 1982). For instance, in the ovary of the threespot wrasse (*Halichoeres trimaculatus*),  
91 vitellogenic oocyte development is synchronized to the tidal cycle and ovulation/spawning  
92 occurs around daytime high tide (Takemura et al., 2008). Since E2 synthesis is also related to  
93 the developmental pattern of vitellogenic oocytes in the ovary (Takemura et al., 2008), we  
94 hypothesized that the expression of steroidogenic enzymes and steroid hormone receptors will  
95 also change in relation to the tidal cycle. The aim of the present study was to investigate  
96 changes in the mRNA abundance of *ER* and *CYP19* genes in the ovary and brain of the  
97 threespot wrasse during the tidal cycle in Okinawan waters. Mature females were collected  
98 around four tidal points [low tide (LT), flood tide (FT), high tide (HT), and ebb tide (ET)] and  
99 mRNA transcript abundance of *CYP19* paralogs (*cyp19a* and *cyp19b*) and *ER* paralogs (*era*  
100 and *erβ*) were assessed using real-time quantitative polymerase-chain reaction (qPCR).

101

102 **Materials and methods**

103

104 Animals

105

106 Mature females were collected in coral reefs around Sesoko Island, Okinawa, Japan, during  
107 the low tide (LT), flood tide (FT), high tide (HT), and ebb tide (ET) in July. Fish were  
108 anesthetized with 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan). After recording body  
109 mass and total length, blood was taken from the caudal vein using a 1-ml heparinized syringe,  
110 centrifuged at 10,000 g for 10 min to obtain plasma, and then frozen at  $-30^{\circ}\text{C}$  until analyzed.  
111 Fish were then euthanized by decapitation. The whole brain was removed, divided into three  
112 parts – forebrain, midbrain and hindbrain (Fig. 1) – and kept frozen at  $-80^{\circ}\text{C}$  until RNA  
113 extraction. Ovary was also removed from the body cavity and weighed and pieces of this  
114 tissue were frozen at  $-80^{\circ}\text{C}$  for RNA extraction or fixed in Bouin's solution for histological  
115 observation. Gonadosomatic index (GSI) was calculated using the following equation;  $\text{GSI} =$   
116  $(\text{gonad mass}/\text{body mass}) \times 100$ .

117 All experiments were conducted in compliance with both the Animal Care and Use  
118 Committee guidelines of the University of the Ryukyus and the Regulations for the Care and  
119 Use of Laboratory Animals in Japan.

120

121 Histological observation and oocyte diameter measurement

122

123 The fixed ovary samples were dehydrated with an ethanol series, permeated with xylene, and  
124 then embedded in histoparaffin (Paraplast plus; Sigma-Aldrich, St. Louis, MO, USA). The  
125 embedded ovaries were serially sectioned at  $5\ \mu\text{m}$  thickness and stained with Delafield's  
126 hematoxylin-eosin for microscopic observation. Development of vitellogenic oocytes was  
127 determined by measuring oocyte diameter of the most developed oocytes ( $n = 30$ ) in each

128 ovary. Oocytes were classified according to Hoque et al. (1998) into the following stages:  
129 peri-nucleolus stage (PNS), oil-droplet stage (ODS), primary yolk stage (PYS), secondary  
130 yolk stage (SYS), tertiary yolk stage (TYS), migratory nucleus stage (MNS), and maturation  
131 stage (MS). Post-ovulatory follicle (POF) were classified as described previously (Matsuyama  
132 et al., 1990).

133

134 Measurement of steroid hormone levels

135

136 Plasma levels of E2 were measured by enzyme immunosorbent assay (EIA), according to the  
137 method of Asahina et al. (1995). Briefly, plasma samples (30  $\mu$ l) were extracted three times  
138 with 1 ml diethyl ether (Kanto Chemical) and vortexed for 1 min. Diethyl ether fractions  
139 containing steroid hormones were transferred to a clean assay tube and subjected to  
140 centrifugal evaporation (VEC-310, EYELA, Tokyo, Japan). Then, 120  $\mu$ l 50 mM borate  
141 buffer (pH 7.8, containing 0.5% bovine serum albumin) was added to each tube and vortexed  
142 for 1 min.

143 Each well of a 96-well plate (AFC Techno Glass, Funabashi, Japan) was coated with 100  
144  $\mu$ l (4.6  $\mu$ g/ml) of goat anti-rabbit IgG (Jackson ImmunoResearch, PA, USA) in 50 mM  
145 carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The well were washed three times  
146 with 10 mM phosphate-buffer saline (PBS) containing 0.05% Tween (PBS-Tween) using an  
147 ImmunoWash 1575 microplate washer (Bio-Rad Laboratories, Hercules, CA, USA). The  
148 assay was performed in a total volume of 120  $\mu$ l, which consisted of 40  $\mu$ l E2 standards  
149 (Sigma-Aldrich; 12.8 to 0.025 ng/ml) or plasma samples, 40  $\mu$ l diluted steroid labeled with  
150 horseradish peroxidase (Cosmo-Bio, Tokyo, Japan), and 40  $\mu$ l rabbit anti-E2 antibody  
151 (Cosmo-Bio).

152 Incubation was done overnight at 4 °C. After washing three times with PBS-Tween, 100

153  $\mu$ l 100 mM citrate buffer (pH 4.5) containing 0.01% *o*-phenylenediamine dihydrochloride  
154 (Sigma-Aldrich) and 0.04% H<sub>2</sub>O<sub>2</sub> was added to each well. After leaving the plate at room  
155 temperature for 30 min, 25  $\mu$ l 4 N H<sub>2</sub>SO<sub>4</sub> was added to each well to stop the reaction.  
156 Absorbance of each well was measured at 490 nm using a 550 microplate reader (BioRad).  
157 The intra- and inter-assay coefficients of variations at the 50% binding point were 5% (n = 4,  
158 duplicate) and 8% (n = 4, duplicate), respectively, for E2.

159

160 RNA extraction and cDNA synthesis

161

162 Total RNA was extracted using TRI-reagent (Molecular Research Center, Cincinnati, OH,  
163 USA), according to the manufacturer's instructions. After ovarian or brain samples (50 to 100  
164 mg) were homogenized in 1 ml TRI-reagent, 0.2 ml chloroform was added to the homogenate  
165 and mixed vigorously. The mixture was stored at room temperature for 15 min and  
166 centrifuged at 12,000 g for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube  
167 and RNA was precipitated from the aqueous phase by mixing in 0.5 ml isopropanol. Samples  
168 were stored at room temperature for 10 min and centrifuged at 12,000 g for 8 min at 4 °C.  
169 After removal of the supernatant, RNA pellets were washed twice with 80%  
170 diethylpyrocarbonate (DEPC)-ethanol and then centrifuged at 7,500 g for 5 min at 4 °C. After  
171 the ethanol was removed, RNA pellets were briefly air-dried for 5 min at room temperature  
172 and dissolved in DEPC-H<sub>2</sub>O. Absorbance was measured at 260 nm and 280 nm to calculate  
173 the quantity and purity of RNA.

174 The first strand cDNAs were synthesized from 1  $\mu$ g total RNA using ImProm-II™  
175 Reverse Transcription System (Promega, Madison, WI, USA) according to manufacturer's  
176 instructions. After incubation at 70 °C for 5 min, 25 °C for 5 min, and 42 °C for 60 min,  
177 enzymatic activity was inactivated by heating at 72 °C for 15 min. After cDNA synthesis, the

178 reaction mixture was diluted to a final volume of 100  $\mu$ l by adding 80  $\mu$ l of nuclease-free  
179 water.

180

181 mRNA transcript abundance of ER and aromatase genes

182

183 The *cyp19a*, *cyp19b*, *era*, and *er $\beta$*  cDNAs of the threespot wrasse were amplified by PCR  
184 reaction with primer sets (Table 1) designed from those of the bambooleaf wrasse  
185 (*Pseudolabrus japonicus*) (GenBank Accession Numbers: DQ298134, DQ298133, DQ298135,  
186 and DQ298136, respectively). PCR was performed using 30 cycles each of denaturation (45 s  
187 at 94  $^{\circ}$ C), annealing (45 s at 53  $^{\circ}$ C), and extension (1 min at 72  $^{\circ}$ C). PCR products were  
188 cloned into the pGEM-T easy vector (Promega) and sequenced. A phylogenetic tree was  
189 constructed by the JTT method with the PRODIST program from the PHYLIP package (ver.  
190 3.63, J. Felsenstein, University of Washington, Seattle, WA, USA). One thousand bootstrap  
191 trials were run using the neighbor-joining method. The CONSENSE program of PHYLIP was  
192 used to construct a strict consensus tree.

193 The mRNA abundance of *ers* and *cyp19s* in tissues were analyzed using the ABI Prism  
194 7000 (Thermo Fisher Scientific, Waltham, MA, USA). Primer sets of *ers*, *cyp19s*, and  *$\beta$ -actin*  
195 for qPCR analysis were designed to anneal to a region that included the exon/intron  
196 boundaries of each gene to eliminate amplification from genomic DNA (Table 1). The length  
197 of the amplicon was kept as close as possible to 100-200 bp and the melting temperature of  
198 the primers was set at 57-60  $^{\circ}$ C. Each PCR reaction mix contained 10  $\mu$ l SYBR *Premix Ex taq*  
199 (Takara, Otsu, Japan), 0.4  $\mu$ l forward primer and 0.4  $\mu$ l reverse primer (10  $\mu$ M) and 2  $\mu$ l  
200 cDNA template, which was adjusted to a total volume of 20  $\mu$ l by adding distilled water. The  
201 initial 1 min denaturation was followed by 40 cycles of denaturation for 5 s at 95  $^{\circ}$ C, and  
202 annealing and extension for 1 min at 60  $^{\circ}$ C. To ensure specificity, a dissociation curve



203 analysis was performed by slowly raising the temperature of the sample from 60 °C to 95 °C.  
204 A series of 10-fold dilutions of plasmid DNA encoding *ers*, *cyp19s*, and *β-actin* were prepared  
205 and included in each amplification reaction to generate a standard curve. These curves  
206 showed a single amplified product and the absence of primer-dimer formation (data not  
207 shown). The abundance level of each transcript was calculated relative to the internal control  
208 (*β-actin*).

209

210 Statistics

211

212 All the data are expressed as means ± standard error of the mean (SEM). Differences between  
213 the mRNA transcript levels of *cyp19a*, *cyp19b*, *erα*, and *erβ*, plasma levels of E2, as well as  
214 GSI and oocyte diameters with tidal change were determined by one-way analysis of variance  
215 (ANOVA), followed by LSD-Duncan test, using SPSS for windows software. A significant  
216 level at  $P < 0.05$  was accepted.

217

218 **Results**

219

220 The deduced amino acid sequences of CYP19s (GenBank accession No. LR35003 for  
221 CYP19a, LR35004 for CYP19b) and ERs (KT210387 for ERα , and KT210388 for ERβ) of  
222 the threespot wrasse were compared with those of both teleost and other vertebrate CYP19s  
223 and ERs. A phylogenetic analysis clearly clusters the CYP19a and CYP19b of the threespot  
224 wrasse with those of other seawater teleost fish and with the appropriate teleost CYP19a and  
225 teleost CYP19b orthologs, respectively (Fig. 2). Similarly, ERα and ERβ of this species were  
226 orthologous to teleost ERs (Fig. 3).

227 Reproductive parameters of the female threespot wrasse were compared among the four

228 tidal points (Fig. 4). High values of GSI and oocyte diameter (vitellogenic oocytes) were  
229 recorded around HT (Fig. 4a) and FT (Fig. 4b), respectively. Plasma E2 levels significantly  
230 increased around LT (Fig. 4c). Oocytes at vitellogenic stages were observed in all the ovaries  
231 collected around four tidal points. Ovaries around HT, but not at other tidal points, contained  
232 POF and ovulated eggs (data not shown).

233 Transcript levels of *cyp19a* (ovary) or *cyp19b* (brain), *era*, and *erβ* genes were measured  
234 in the ovary (Fig. 5) and brain (Fig. 6). In the ovary, mRNA abundance of *cyp19a*  
235 significantly increased from LT to ET (Fig. 5a), while that of *era*, but not that of *erβ*,  
236 increased significantly around HT (Fig. 5b). Abundance of *cyp19b* mRNA significantly  
237 increased around FT in the forebrain, but not in the midbrain and hindbrain (Fig. 6).  
238 Abundance of *era* mRNA was significantly higher around FT in three parts of the brain, while  
239 the *erβ* mRNA level was significantly higher at FT but only in the midbrain.

240

## 241 **Discussion**

242

243 In the present study, we cloned and sequenced cDNAs of two cytochrome P450 aromatases  
244 and two estrogen receptors of the threespot wrasse, and showed that they were  
245 phylogenetically related to those of other fishes. It was reported that ray-finned fish species  
246 (Actinopterygii) has at least three distinct subtypes, including ER $\alpha$ , ER $\beta$ -I (ER $\gamma$ ), and ER $\beta$ -II  
247 (Choi and Habibi, 2003; Halm et al., 2004; Hawkins et al., 2000; Ma et al., 2000; Menuet et  
248 al., 2004; Nagler et al., 2007; Tchoudakova et al., 1999). ER $\beta$  cloned in the present study  
249 seems to share identity with ER $\beta$ 2 (ER $\beta$ -II) because it is included in the subclade of ER $\beta$ 2,  
250 but not ER $\beta$ 1, of the European sea bass (*Dicentrarchus labrax*) (Halm et al., 2004).

251 Our results demonstrate that the spawning of the threespot wrasse occurs at HT as  
252 evidenced by the higher GSI at this time and the presence of POF in the ovary during ET. This

253 supports the hypothesis that this wrasse species is a tidal spawner with high tide preference as  
254 we previously reported (Takemura et al., 2008). A similar spawning rhythmicity with high tide  
255 preference was also reported in many tropical wrasses (Colin and Bell, 1991; Ross, 1983;  
256 Warner, 1982). Our previous study revealed histologically that a clutch of vitellogenic oocytes  
257 at the tertiary yolk stage develops daily toward HT and completes the process of late  
258 vitellogenesis to final oocyte maturation within a short time period (3 h) between FT and HT  
259 (Takemura et al., 2008). Therefore, it is likely that endocrine changes triggering the final  
260 process of oocyte development occur rapidly in the ovary and brain in accordance with the  
261 tidal cycle, and repeated at regular tidal intervals (12.4 h). In support of this, plasma E2 levels  
262 increased at LT and this was followed by an increased oocyte diameter in a clutch of  
263 developing oocytes at FT. This result implies that vitellogenin synthesis in the liver occurs in  
264 response to elevated E2 at LT, which then gets incorporated into the developing oocytes.  
265 Since it was reported that plasma E2 levels peak around a period of ET (Takemura et al.,  
266 2008), there seems to be 3-h difference in a E2 peak between the two studies. This may be  
267 partially due to the rapid progress of oocyte development with tide, especially given that tide  
268 is a progressive change repeated at an interval of 12.4 h and there is only a 3-h difference  
269 between ET and LT.

270 Changes in P450arom enzymatic activities in the ovary play an important role in  
271 regulating the gonadal production of E2 during reproduction and development (Chang et al.,  
272 1997). Since E2 is positively correlated with stimulation of vitellogenin synthesis in  
273 hepatocytes (Nagahama, 1994), it is considered that changes in P450arom reflect the process  
274 of vitellogenesis in the ovary. Positive relationship between P450arom change and plasma E2  
275 levels were reported in fishes, including the red-spotted grouper (*E. akaara*), where aromatase  
276 activities in the ovary peak during the breeding season when plasma E2 levels were high (Li  
277 et al., 2007). This relationship was also confirmed by treatment with an aromatase inhibitor

278 Fadrozole, which decreased plasma E2 levels in the female coho salmon (*O. kisutch*) (Afonso  
279 et al., 1999). Molecular-based studies have also demonstrated a similar relationship between  
280 *cyp19a* and vitellogenesis (Chang et al., 2005; Villeneuve et al., 2006; Rasheeda et al., 2010).  
281 The present study showed a steady increase of *cyp19a* mRNA abundance in the ovary of the  
282 threespot wrasse from LT through ET. This pattern seemed to be different from the  
283 abovementioned reports (Chang et al., 2005; Villeneuve et al., 2006; Rasheeda et al., 2010)  
284 showing seasonal changes in *cyp19a* mRNA abundance in the ovary of fish species with  
285 synchronous or group-synchronous oocyte development (Wallace and Selman, 1981). Since  
286 our previous study revealed rapid development of a clutch of larger vitellogenic oocytes (the  
287 first clutch) and existence of smaller vitellogenic oocytes (the second or subsequent clutches)  
288 in the ovary (Takemura et al., 2008), the present results on the expression pattern of *cyp19a*  
289 mRNA abundance may be due to a rapid rise of aromatase activity in relation to the second  
290 clutch of vitellogenic oocytes in an ovary.

291 ERs are cytosolic transducers of the estrogen signal in cells or neurons of target tissues.  
292 Therefore, the magnitude and pattern of their mRNA abundance likely reflects potential  
293 function and regulation (Nagler et al., 2012). The present study revealed that the abundance of  
294 *erα* mRNA in the ovary increased around HT, while that of *erβ* mRNA did not change  
295 throughout the tidal cycle. This result implies that *erα*, but not *erβ*, may be playing a role in  
296 transducing the estrogen signal linked with periodical tidal change. It was reported in the  
297 largemouth bass (*Micropterus salmoides*) that abundance of *erα* mRNA in the liver increased  
298 in association with the increased abundance of vitellogenin mRNA in the liver and E2 levels  
299 in circulation, while mRNA abundance of three *ers* (*erα*, *erβ*, *erγ*) in the ovary increased  
300 during the early oocyte development and prior to the increases in plasma E2 levels (Sab-  
301 Attwood et al., 2004). In the rainbow trout, the mRNA abundance of *erα* in the liver and *erβs*  
302 in the ovary are thought to be related to the process of vitellogenin synthesis and the

303 preparation and growth of early follicles, respectively (Nagler et al., 2012). In this case,  
304 however, peak *era2* mRNA abundance was found during the late vitellogenic phase,  
305 suggesting that this ER subtype plays a role in the final phase of ovarian growth in this  
306 species (Nagler et al., 2012). In this regard, Nagler et al. (2012) proposed that in response to  
307 an increase in E2 toward the final phase of ovarian growth, *era2* plays a signaling role in the  
308 upregulation of gonadotropin receptors in the granulosa cells, and this is responsible for  
309 shutting down vitellogenin uptake through a reduction in the cycling of vitellogenin receptors  
310 to the oocyte plasma membrane. Unlike yearly and synchronous ovarian growth seen in trout  
311 (Wallace and Selman, 1981), a clutch of vitellogenic oocytes develops toward HT in the  
312 threespot wrasse. Consequently, *era* may be related to a rapid growth of early follicles  
313 following ovulation and spawning around HT. Conversely, it is more likely that this increase  
314 in *era* accelerates the process of vitellogenesis to final oocyte maturation in a clutch of  
315 oocytes facing ovulation during HT, but this remains to be tested.

316 Several reports have demonstrated reproduction-related changes in mRNA abundance of  
317 *cyp19a1b* in the brain of teleost fishes (Chaube et al., 2015; Hoffman et al., 2013; Kazeto and  
318 Trant, 2005; Li et al., 2007; Rasheeda et al., 2010). In the brain of the female stinging catfish  
319 (*Heteropneustes fossilis*), *cyp19a1b* increased during the resting phase and preparatory phase  
320 and subsequently decreased during the prespawning and spawning phase (Chaube et al., 2015;  
321 Rasheeda et al., 2010). On the contrary, aromatase activity increased in the brain of the red-  
322 spotted grouper during the breeding season (Li et al., 2007). Also, an increase in the transcript  
323 abundance of *cyp19a2* was found in the brain and pituitary of the channel catfish (*Ictalurus*  
324 *punctatus*) prior to spawning (Kazeto and Trant, 2005). These results suggest that *cyp19a1b*  
325 mRNA expressions in the brain of fish show diverse patterns among species and this may be  
326 partially related to their respective reproductive strategies. The present study revealed an  
327 increase in *cyp19b* mRNA abundance in the forebrain, but not in the midbrain or hindbrain, of

328 the threespot wrasse during FT. Higher abundance of *cyp19b* mRNA in the telencephalon has  
329 been reported in the channel catfish (Kazeto and Trant, 2005). *In situ* hybridization analysis  
330 has also demonstrated a strong signal for *cyp19b* mRNA in the telencephalon of stinging  
331 catfish, although high transcript levels were also observed in the lateral hypothalamus and  
332 medulla oblongata (Chaube et al., 2015). The brain regions, including the ventral  
333 telencephalon and hypothalamus, are involved in reproductive activity and sexual behavior,  
334 and hormones, including gonadotropin-releasing hormones, vasotocin and dopamine play a  
335 role in modulating these responses (Chaube et al., 2015). Since the forebrain mainly contains  
336 the telencephalon (Fig. 1), this part of the wrasse brain may be involved in regulating the  
337 tidal-related reproduction of this species based on changes in aromatase activity. In the  
338 African cichlid fish (*Astatotilapia burtoni*), treatment with E2 increased aggression  
339 (O'Connell and Hofmann, 2012), while an aromatase inhibitor abolished this aggressive  
340 behaviour (Huffman et al., 2013), suggesting that local conversion from testosterone to E2 by  
341 aromatase in the brain is necessary for displaying such behavior (McEwen, 1981). A similar  
342 action of aromatase may occur in the brain of the threespot wrasse, since this hermaphroditic  
343 labrid species exhibits group spawning with a territory-holding male (Suzuki et al., 2010).  
344 Concomitant with an increase in *cyp19b*, the mRNA abundance of *ers* in the brain also  
345 increased around FT. Since *era* was highly expressed in the forebrain, midbrain, and  
346 hindbrain as well as *erb* in the midbrain, we propose that targets of E2 in the brain are likely  
347 to be located to neurons and cells in these areas.

348 It is concluded that fundamental roles of these genes in the ovary and brain of the  
349 threespot wrasse are equivalent to those of teleost fishes studied so far and tidal cycle is  
350 superimposed on processes of gonadal development. In this regard, it was reported that  
351 hydrostatic pressure is an important cue to stimulate the hypothalamic-pituitary-gonadal

352 (HPG) axis (Takemura et al., 2012). Further studies would be needed to clarify how the HPG  
353 axis is influenced by tidal stimuli in fish.

354

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362

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490



Table 1. Primer sets used for PCR amplification of three-pot wrasse transcripts

Name	Sequence
Cloning for <i>era</i>	
Forward	5'-TGCAGTGA CTATGCCTCTGG-3'
Reverse	5'-ATCAGAACCTCAAGCCAGGA-3'
Cloning for <i>erb</i>	
Forward	5'-TCTACATCCCCTCGCCATAC-3'
Reverse	5'-CTTTTACGCCGGTTCTTGTC-3'
Cloning for <i>cyp19a</i>	
Forward	5'-AGGCAGTATGTGTTGGAGATGG-3'
Reverse	5'-ACCAGGATGGATTTCTCATCA-3'
Cloning for <i>cyp19b</i>	
Forward	5'-GACATGTGGATGCCCTAAATCT-3'
Reverse	5'-AAAGGCTGGAAGAAGCGACT-3'
Cloning for <i>era</i>	
Forward	5'-TCGTGCGCCTCAGGAAGTGTTA-3'
Reverse	5'-TCGTACAAGTCCGCCTTTTGT-3'
qPCR for <i>erb</i>	
Forward	5'-AGTCCAAACCCAACAGCATCAG-3'
Reverse	5'-ACCACAGAAGAGCACAACGAGG-3'
qPCR for <i>cyp19a</i>	
Forward	5'-TTCTGAACACAGGCCACATGC-3'
Reverse	5'-AAACGGCTGGAAGTAACGACG-3'
qPCR for <i>cyp19b</i>	
Forward	5'-TGAAACATGGCAGACGGTTCT-3'
Reverse	5'-ATCACGTCTTGCAGCTCTTGG-3'
qPCR for $\beta$ - <i>actin</i>	
Forward	5'-TACCACCATGTACCCTGGCATC-3'
Reverse	5'-TACGCTCAGGTGGAGCAATGA-3'

## Figure legends

Figure 1. Representative view of a brain of the threespot wrasse. The brain of threespot wrasse is divided into three parts: forebrain including telencephalon (TE); midbrain (MB) including optic tectum (OT) and diencephalon (DE); hindbrain (HB) including cerebellum (CE) and medulla oblongata (MO).

Figure 2. Phylogenetic tree of CYP19a and CYP19b in vertebrates. One thousand bootstrap repetitions were performed and values are shown under the nodes. The scale bar is calibrated in substitutions per site. The accession numbers for CYP19a and CYP19b proteins used in the phylogenetic tree analysis are as follows: threespot wrasse *Halichoeres trimaculatus* CYP19a (GenBank accession No. LR35003) and CYP19b (LR35004); Chinese wrasse *H. tenuispinis* CYP19a (AR37048) and CYP19b (AR37047); European seabass *Dicentrarchus labrax* CYP19a (DQ177458) and CYP19b (AY138522); gilthead seabream *Sparus aurata* CYP19a (AF399824); goldfish *Carassius auratus* CYP19a (AB009336) and CYP19b (AB009335); zebrafish *Danio rerio* CYP19a (AF226620) and CYP19b (AF226619); chicken *Gallus gallus* CYP19 (D50335); human *Homo sapiens* CYP19 (AF419338); mouse *Mus musculus* CYP19 (AJ437576); rat *Rattus rattus* CYP19 (EU025135); sheep *Ovis aries* CYP19 (NM001123000).

Figure 3. Phylogenetic tree of ER $\alpha$  and ER $\beta$  in vertebrates. One thousand bootstrap repetitions were performed and values are shown on the nodes. The scale bar is calibrated in substitutions per site. The accession numbers for ER $\alpha$  and ER $\beta$  proteins used in the phylogenetic tree analysis are as follows: threespot wrasse *Halichoeres trimaculatus* ER $\alpha$  (KT210387) and ER $\beta$  (KT210388); Chinese wrasse *H. tenuispinis* ER $\alpha$  (AP72178) and ER $\beta$  (AP72179); European seabass *Dicentrarchus labrax* ER $\alpha$  (AJ505009), ER $\beta$ 1 (AD33851), and ER $\beta$ 2 (AD33882); gilthead seabream

*Sparus aurata* ER $\alpha$  (AF136979) and ER $\beta$  (AF136980); goldfish *Carassius auratus* ER $\alpha$  (AY055725) and ER $\beta$  (AF061269); olive flounder *Paralichthys olivaceus* ER $\alpha$  (AB070629) and ER $\beta$  (AB070630); zebrafish *Danio rerio* ER $\alpha$  (AF349412) and ER $\beta$  (AF349414); human *Homo sapiens* ER $\alpha$  (NM001122741) and ER $\beta$  (AF051427); mouse *Mus musculus* ER $\alpha$  (AB560752) and ER $\beta$  (U81451); rat *Rattus rattus* ER $\alpha$  (NM012689) and ER $\beta$  (NM012754); sheep *Ovis aries* ER $\alpha$  (AY033393) and ER $\beta$  (AF177936).

Figure 4. Changes of gonadosomatic index (a), oocyte diameter (b), and plasma estradiol-17 $\beta$  levels (c) with tidal cycle. Fish (n = 6 – 8 per each point) were collected around points of low tide (LT), flood tide (FT), high tide (HT), and ebb tide (ET). Oocyte diameter of the most developed oocytes (n = 30) on histological slides was calculated in each ovary. Plasma levels of estradiol-17 $\beta$  were measured with enzyme-immunoassay. Each point is expressed as mean  $\pm$  standard error of the means (SEM). Different letters indicate statistical significance at  $P < 0.05$ .

Figure 5. Changes in relative mRNA abundance of aromatases (*cyp19a*) and estrogen receptors (*er $\alpha$*  and *er $\beta$* ) in the ovary of the threespot wrasse with tidal cycle. Fish (n = 6 – 8 per each point) were collected around points of low tide (LT), flood tide (FT), high tide (HT), and ebb tide (ET). The mRNA abundance of aromatase (a) and estrogen receptors (b) in the ovary was measured qPCR and reported as a ratio with respect to  *$\beta$ -actin*. Each point is expressed as mean  $\pm$  standard error of the means (SEM). Different letters indicate statistical significance at  $P < 0.05$  using a one-way ANOVA followed by LSD-Duncan test.

Figure 6. Changes in relative mRNA abundance of aromatases (*cyp19b*) and estrogen receptors (*er $\alpha$*  and *er $\beta$* ) in the brain of the threespot wrasse with tidal cycle. Fish (n = 6 – 8 per each point) were collected around points of low tide (LT), flood tide (FT),



high tide (HT), and ebb tide (ET). The mRNA abundance of *cyp19b* (a, b, and c), *er $\alpha$*  (d, e, and f), and *er $\beta$*  (g, h, and i) in forebrain (a, d, and g), midbrain (b, e, and h), and hindbrain (c, f, and i) was measured qPCR and reported as a ratio with respect to  *$\beta$ -actin*. Each point is expressed as mean  $\pm$  standard error of the means (SEM). Different letters indicate statistical significance at  $P < 0.05$  using a one-way ANOVA followed by LSD-Duncan test.

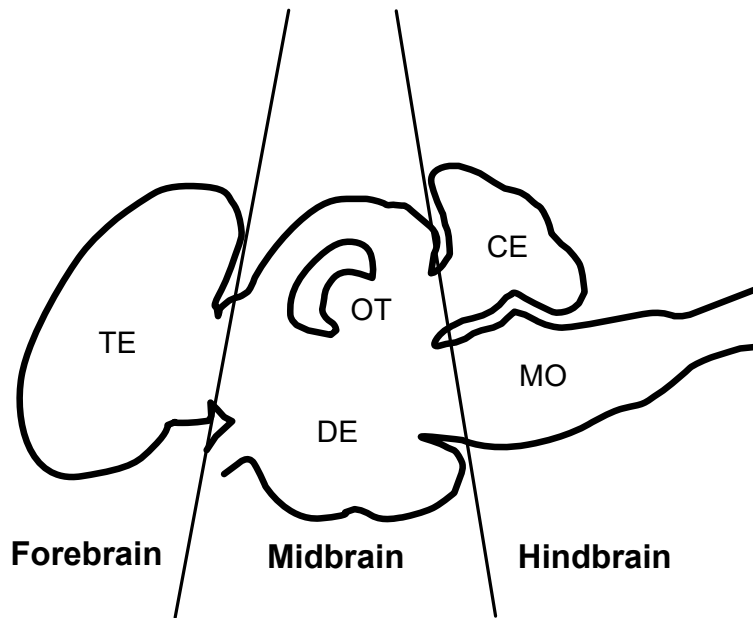


Figure 1

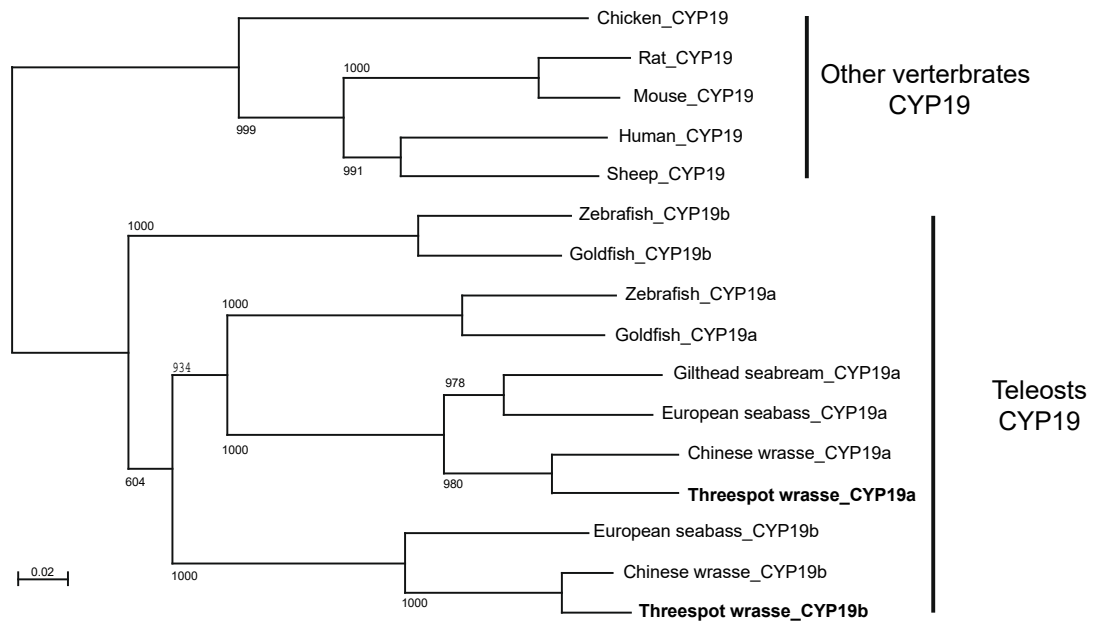
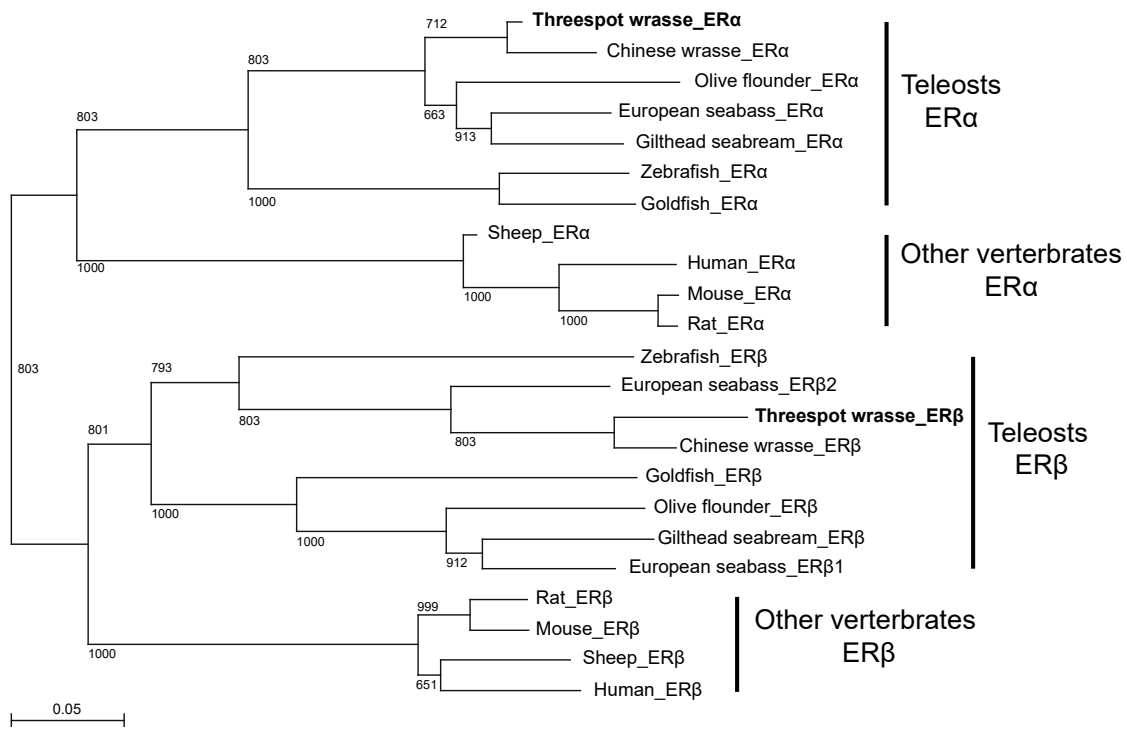


Figure 2



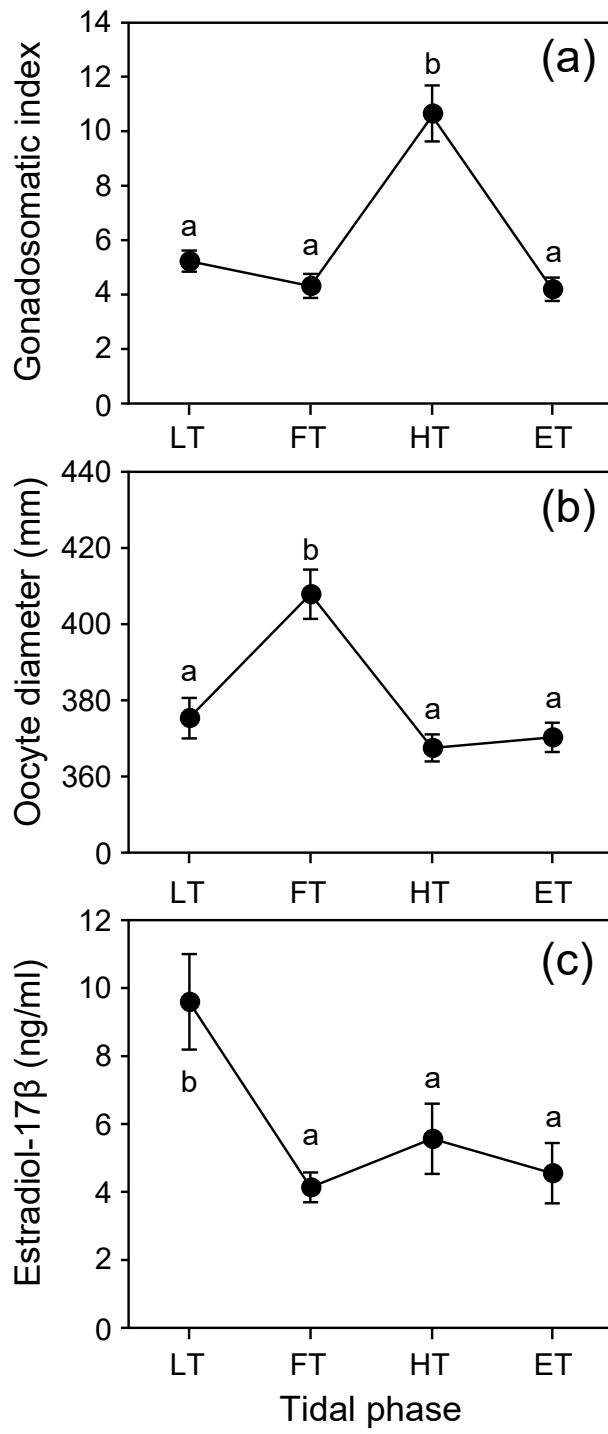


Figure 4

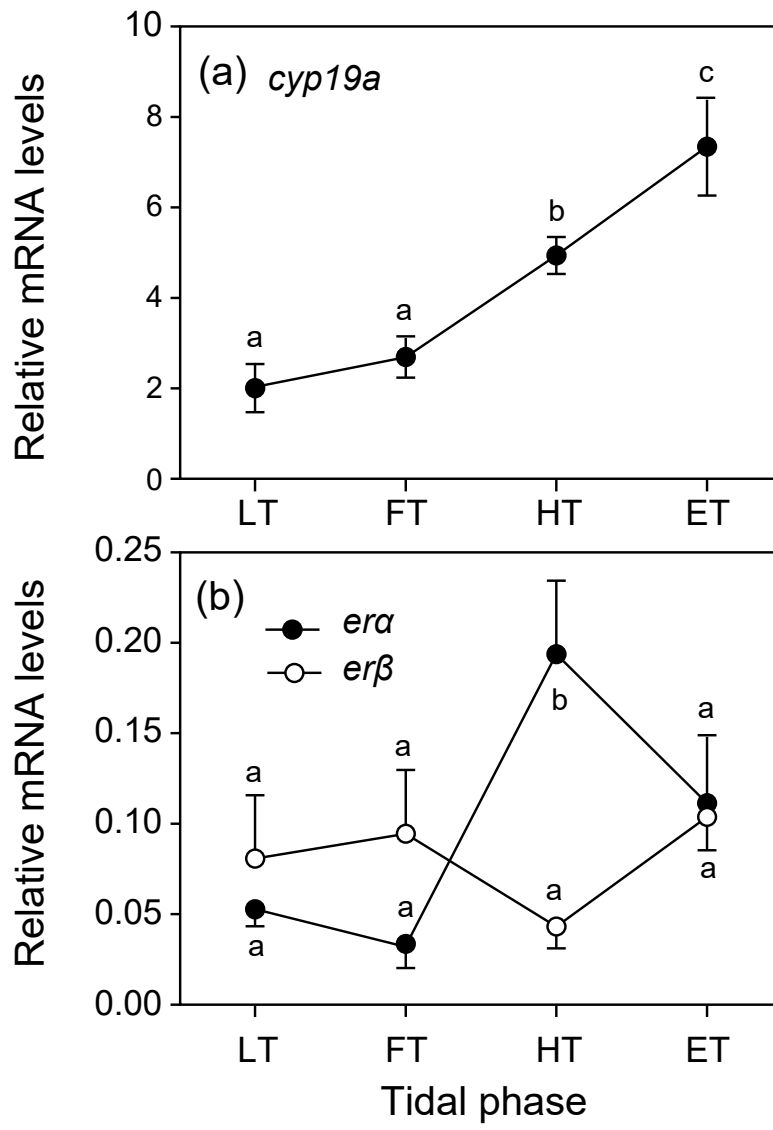


Figure 5

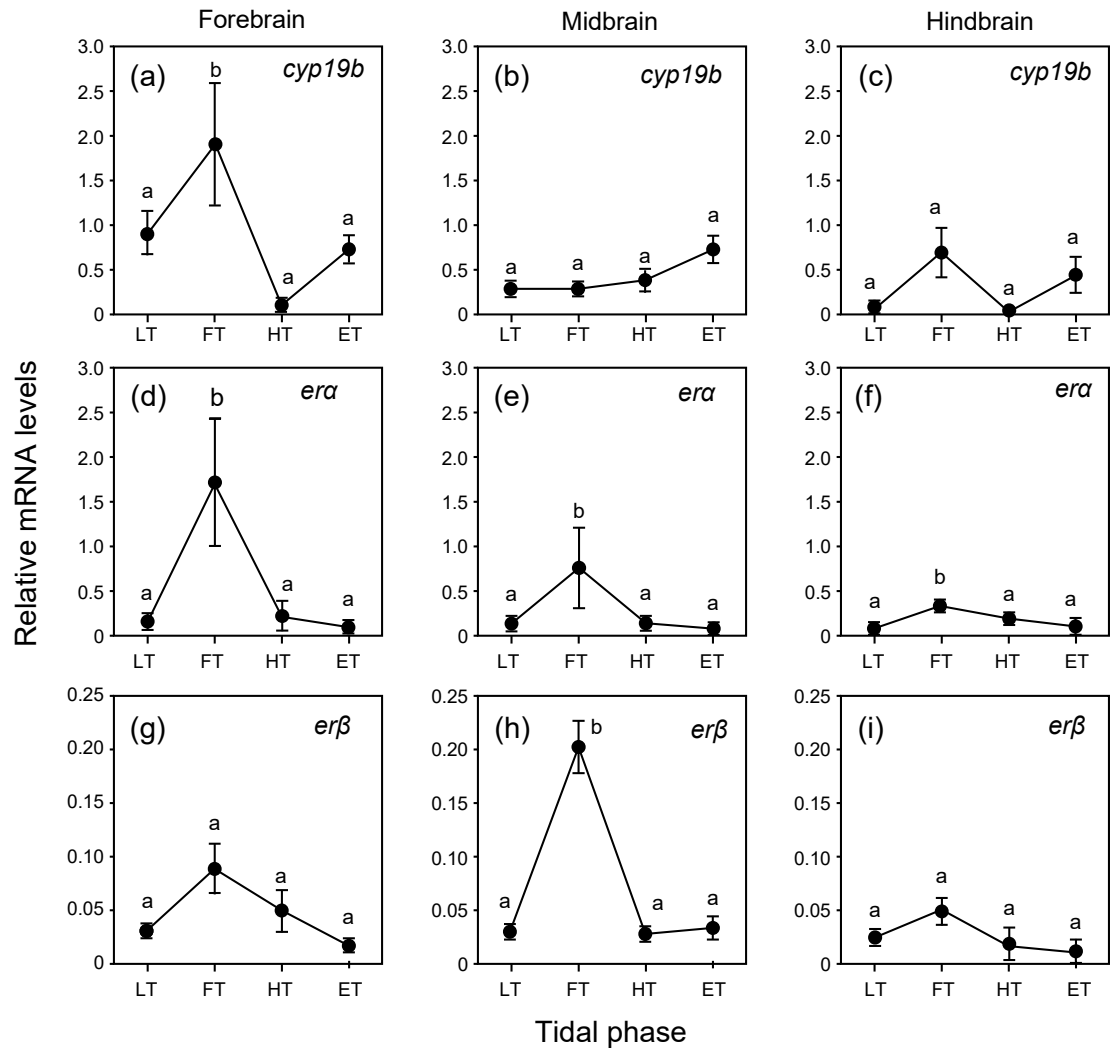


Figure 6