

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 α* and *er β*) 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 α* , but not *er β* , 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 α* and *er β* 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 β (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 α and ER β (Hawkins et al. 2000). An additional ER subtype (named ER β 2 or ER γ) 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 α 1*, but not *er β 1* and
76 *er β 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 *era1* 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°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°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°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 μ 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 μ 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 μ l (4.6 μ 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 μ l, which consisted of 40 μ l E2 standards
149 (Sigma-Aldrich; 12.8 to 0.025 ng/ml) or plasma samples, 40 μ l diluted steroid labeled with
150 horseradish peroxidase (Cosmo-Bio, Tokyo, Japan), and 40 μ 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 μ l 100 mM citrate buffer (pH 4.5) containing 0.01% *o*-phenylenediamine dihydrochloride
154 (Sigma-Aldrich) and 0.04% H₂O₂ was added to each well. After leaving the plate at room
155 temperature for 30 min, 25 μ l 4 N H₂SO₄ 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-H₂O. 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 μ 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 μ l by adding 80 μ l of nuclease-free
179 water.

180

181 mRNA transcript abundance of ER and aromatase genes

182

183 The *cyp19a*, *cyp19b*, *era*, and *er β* 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 *β -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 μ l SYBR *Premix Ex taq*
199 (Takara, Otsu, Japan), 0.4 μ l forward primer and 0.4 μ l reverse primer (10 μ M) and 2 μ l
200 cDNA template, which was adjusted to a total volume of 20 μ 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 α , ER β -I (ER γ), and ER β -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 β cloned in the present study
249 seems to share identity with ER β 2 (ER β -II) because it is included in the subclade of ER β 2,
250 but not ER β 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 (Sabo-
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'-ACCACAGAAGAGCACAAACGAGG-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 <i>β-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 α and ER β 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 α and ER β proteins used in the phylogenetic tree analysis are as follows: threespot wrasse *Halichoeres trimaculatus* ER α (KT210387) and ER β (KT210388); Chinese wrasse *H. tenuispinis* ER α (AP72178) and ER β (AP72179); European seabass *Dicentrarchus labrax* ER α (AJ505009), ER β 1 (AD33851), and ER β 2 (AD33882); gilthead seabream

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

Figure 4. Changes of gonadosomatic index (a), oocyte diameter (b), and plasma estradiol-17 β 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 β 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 α* and *er β*) 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 *β -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 α* and *er β*) 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 α* (d, e, and f), and *er β* (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 *β -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.

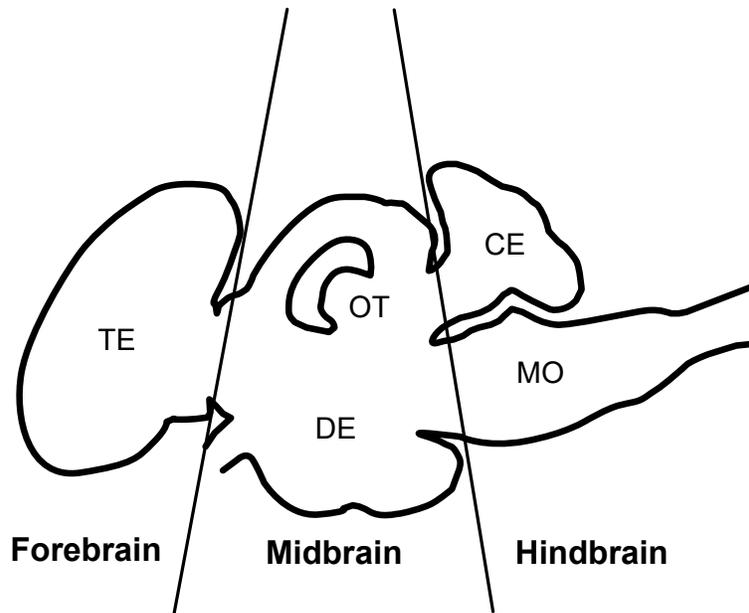


Figure 1

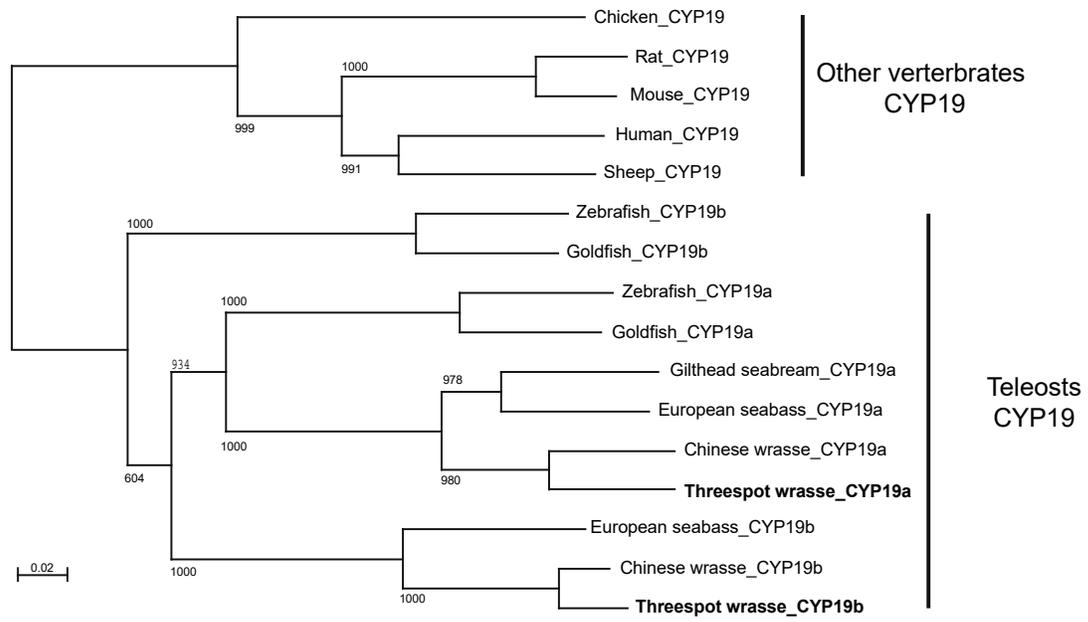
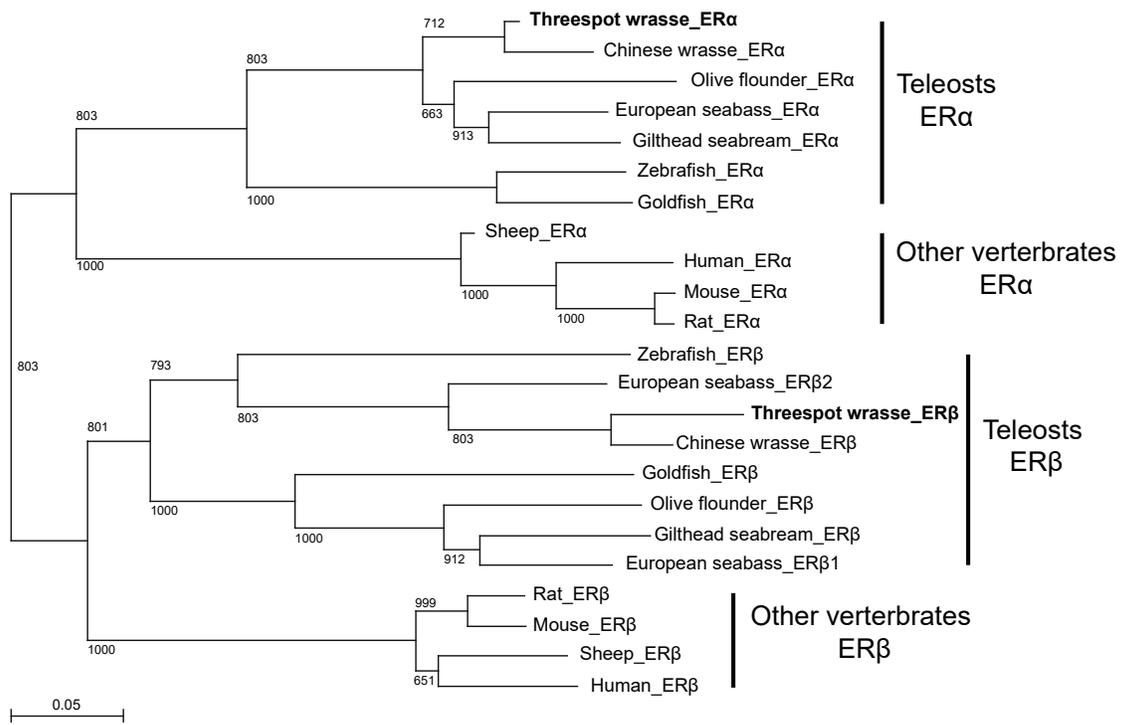


Figure 2



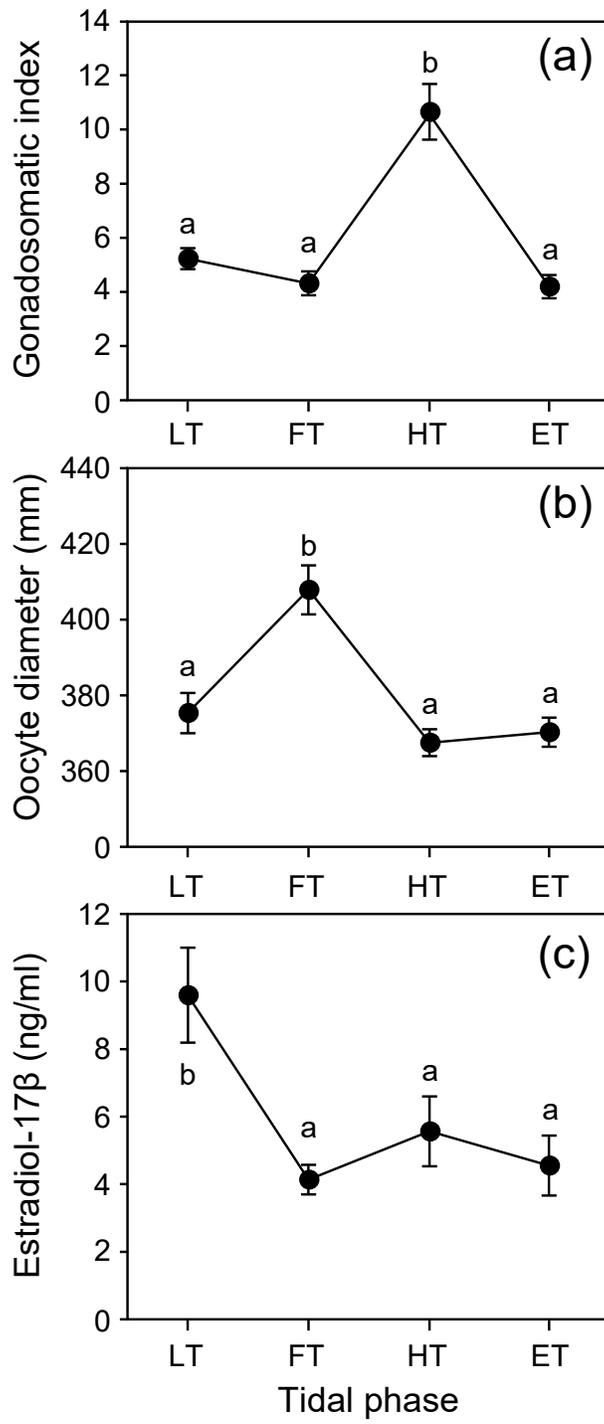


Figure 4

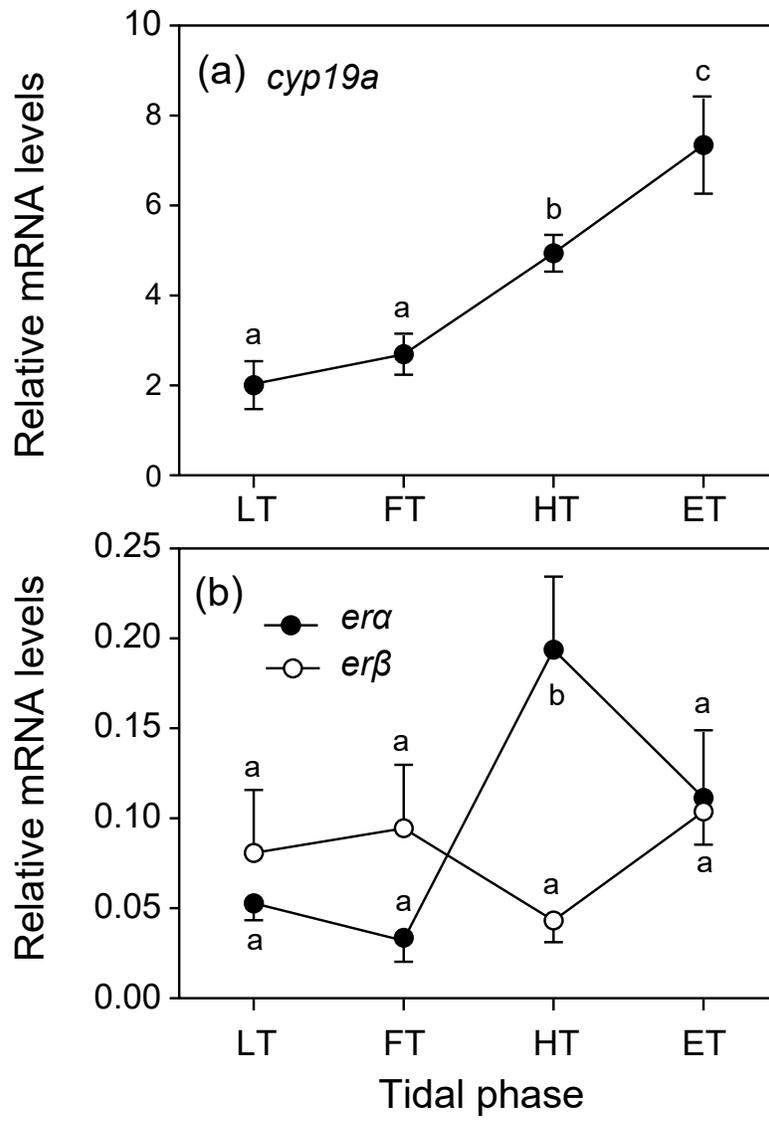


Figure 5

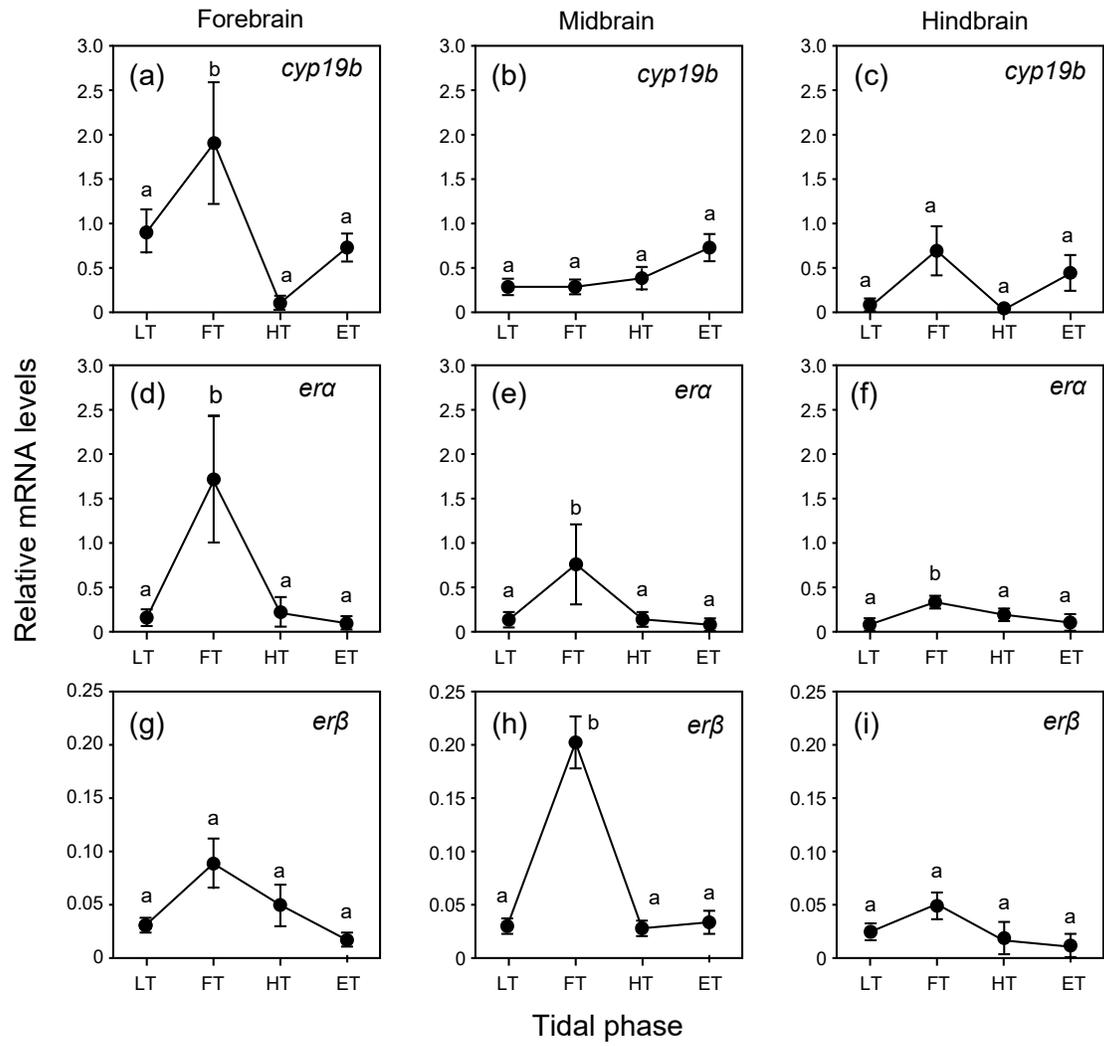


Figure 6