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Characterization of eight types of 17β-hydroxysteroid dehydrogenases from the Japanese sardine *Sardinops melanostictus*: the probable role of type 12a in ovarian estradiol synthesis

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Abstract

17β-hydroxysteroid dehydrogenases (Hsd17bs) play a critical role in sex steroid biosynthesis. Although multiple types of Hsd17b have been found in fish, there is limited research on their expression and function. Recently, we succeeded in identifying eight types of Hsd17b (types 3, 4, 7, 8, 10, 12a, 12b, and 14) by RNA sequencing in the Japanese sardine *Sardinops melanostictus*, a commercially important clupeoid fish; however, a homologous sequence of Hsd17b1, which catalyzes the key reaction of estradiol-17β (E2) synthesis, was absent. Here, we aimed to identify the Hsd17b type that plays a major role in E2 synthesis during ovarian development in Japanese sardine. The cDNAs encoding those eight types of Hsd17b were cloned and sequenced. The expressions of *hsd17b3*, *hsd17b12a*, and *hsd17b12b* were higher in ovary than in testis. In particular, *hsd17b12a* was predominantly expressed in the ovary. Expression of *hsd17b3*, *hsd17b4*, *hsd17b12a*, and *hsd17b12b* in the ovary increased during ovarian development. The enzymatic activities of Hsd17b3, Hsd17b12a, and Hd17b12b were evaluated by expressing their recombinants in human embryonic kidney 293T cells. Hsd17b12a and Hsd17b12b catalyzed the conversion of androstenedione (AD) to testosterone (T) and estrone (E1) to E2. The results of *in vitro* bioassays using sardine ovaries indicated that E2 is synthesized from pregnenolone via AD and T, but not E1. These results suggest that Hsd17b12a plays a major role in E2 synthesis in sardine ovary by catalyzing the conversion of AD to T.

Keywords: Hsd17b, Estradiol-17β, Steroidogenesis, Ovary, Teleost

1. Introduction

Sex steroids, such as androgens (C_{19} steroid) and estrogens (C_{18} steroid), are synthesized from cholesterol, a common precursor of steroid hormones, in a series of reactions that are catalyzed by enzymes belonging to the cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) families. 17β-hydroxysteroid dehydrogenase (Hsd17b) is a multifunctional enzyme that catalyzes the oxidation/reduction reactions at position C17 of androgens and estrogens (Moeller and Adamski, 2006, 2009). Hsd17b acts downstream of the steroidogenic pathway and catalyzes the interconversion of 17-ketosteroids to 17β-hydroxysteroids, such as androstenedione (AD) to testosterone (T) and estrone (E1) to estradiol-17β (E2). In mammals, 15 types of HSD17B have been identified (Moeller and Adamski, 2006; Luu-The & Labrie 2010). Except for type 5, they all belong to the short-chain dehydrogenase/reductase (SDR) superfamily, which are NAD- or NADP-dependent oxidoreductase enzymes (Kallberg et al., 2002). Among them, types 1–3 are critical enzymes involved in sex steroid metabolism. HSD17B1 catalyzes the reduction of E1 to E2 and is highly expressed in the ovary and placenta (Luu-The et al., 1989); HSD17B2 inactivates estrogen and androgen by oxidizing E2 to E1 and T to AD (Wu et al., 1993); and HSD17B3 is involved in the reduction of AD to T and is highly expressed in testis (Geissler et al., 1994). Among the other types of HSD17B, HSD17B12 has been suggested to play a role in E2 synthesis because of its catalytic activity of converting E1 to E2 and high expression in the ovary (Luu-The et al., 2006).

In teleosts, E2 is synthesized in the oocyte-surrounding follicle cells and regulates ovarian development by inducing hepatic vitellogenin production (Lubzens et al., 2010).

Of the types of HSD17B found in mammals, nine sequence homologs (types 1, 2, 3, 4, 7, 8, 10, 12, and 14) were identified in zebrafish *Danio rerio* (Mindnich and Adamski, 2009), and ten sequence homologs (types 1, 3, 4, 7, 8, 9, 10, 12, 14, and 15) were identified in olive flounder *Paralichthys olivaceus* (Zou et al., 2020). Furthermore, both species have two paralogs of Hsd17b12: Hsd17b12a and Hsd17b12b. In zebrafish, Hsd17b1 catalyzed the interconversion of E1 to E2, whereas Hsd17b3 catalyzed the reduction of AD to T (Mindnich et al., 2004, 2005; Mindnich and Adamski, 2009). In Nile tilapia *Oreochromis niloticus*, Hsd17b1 catalyzed the interconversion of E1 to E2 and AD to T (Zhou et al., 2005). In Japanese eel, Hsd17b1 catalyzed the conversion of E1 to E2, but not AD to T (Kazeto et al., 2000). In the flounder and catfish *Clarias barachus*, ovarian expression of *hsd17b1* was upregulated by administration of gonadotropin (flounder; Zou et al., 2020) or antiandrogen flutamide (catfish; Rajakumar et al., 2014). In *Seriola* species, a missense single nucleotide polymorphism (SNP) in the gene encoding Hsd17b1 led to lower production of E2 (Koyama et al., 2009). Thus, Hsd17b1 has been suggested to have a critical role in E2 synthesis and the regulation of ovarian development in several fish species, as likewise purported in mammals. Yet, limited research has examined the expression and function of Hsd17b types in teleost fish.

Clupeoids, such as anchovy, herring, and sardine, are widely distributed in the world's oceans and include numerous species of interest to fisheries. These species are classified as evolutionarily old teleost orders (Near et al., 2012). The Japanese sardine *Sardinops melanostictus* supports the largest small pelagic fishery in Japan (Takasuka et al., 2019). Studies on the reproductive biology of this species have focused on

89 understanding its reproductive potential, of which fecundity, sexual maturity, and egg
90 yolk volume can vary in relation to the stock biomass (Morimoto 1998, 2003; Takasuka
91 et al., 2019; Furuichi et al., 2020). Analyses of the physiological and molecular
92 mechanisms of ovarian development are still needed to better understand the variation in
93 the species' reproductive potential. The reproductive system is regulated by the endocrine
94 system along the brain–pituitary–gonad (BPG) axis. We recently performed RNA
95 sequencing (RNA-seq) on the transcripts expressed in the sardine brain, pituitary, and
96 gonad to characterize transcriptome profiles of the entire sardine BPG axis (Nyuji et al.,
97 2020). That study showed that ovarian gene expression of CYPs (*cyp11a1*, *cyp17a1*, and
98 *cyp19a1a*) was upregulated during ovarian development. Meanwhile, we identified eight
99 sequence homologs of Hsd17b genes (types 3, 4, 7, 8, 10, 12a, 12b, and 14) in the sardine
100 transcripts; however, a homologous sequence of Hsd17b1 was not included. Moreover,
101 through homology searches we could not find this sequence in the genome of Atlantic
102 herring *Clupea harengus* (GCF_900700415), even though the most-detailed genome
103 information has been provided for this herring among the clupeoids (Martinez Barrio et
104 al., 2016; Kongsstovu et al., 2019). Therefore, it is likely that the Hsd17b1 gene is missing
105 in the genome of clupeoid species. This possibility needs to be confirmed with other
106 studies involving genome analyses of the Japanese sardine and other clupeoids. If
107 confirmed, this raises the question of which type of Hsd17b plays a prominent role in E2
108 synthesis in the sardine ovary.

109 Previous studies of steroid hormone biosynthesis in ovarian follicles of fish revealed
110 that during vitellogenesis, E2 is synthesized through two different pathways in teleosts

111 (Fig. 1). In chub mackerel *Scomber japonicus* and Japanese amberjack *Seriola*
112 *quinqueradiata*, E2 is synthesized from a major steroid precursor pregnenolone (P5) via
113 17-hydroxypregnenolone (17OH-P5), 17-hydroxyprogesterone (17OH-P4; chub
114 mackerel) or dehydroepiandrosterone (DHEA; Japanese amberjack), AD, and T
115 (Matsuyama et al., 2005; Rahman et al., 2002). In the bambooleaf wrasse *Pseudolabrus*
116 *sieboldi* and red seabream *Pagrus major*, E2 is synthesized from P5 via 17OH-P5, DHEA,
117 AD, and E1 (Ohta et al., 2001, 2002). In this pathway in the wrasse, Hsd17b1 is suggested
118 to act on the conversion of E1 to E2 (Ohta et al., 2003). There is no available information
119 on such pathways depicting the biosynthesis of ovarian E2 in clupeoids. To determine the
120 enzymatic functions of Hsd17b in E2 synthesis in sardine ovary, it is necessary to reveal
121 which pathway is activated between AD and E2 during ovarian development.
122 The present study was designed to isolate eight genes encoding sardine Hsd17b,
123 characterize their expression, and identify the type of Hsd17b involved in ovarian E2
124 synthesis. We first selected candidate types showing ovary-specific and developmental
125 stage-dependent gene expression, and then tested their catalytic activities toward the
126 reduction of AD to T and E1 to E2. In addition, the steroidogenic pathway from the steroid
127 precursors (i.e., P5 or AD) to E2 was estimated by *in vitro* bioassays using sardine ovaries.
128
129 **2. Materials and methods**
130 2.1. Fish
131 Commercially caught or hatchery-produced Japanese sardine were reared in indoor
132 tanks at Hakatajima Field Station, Fisheries Technology Institute, Japan Fisheries

133 Research and Education Agency (FRA), Imabari, Ehime, Japan. The fish were maintained
134 at the ambient water temperature from spring to autumn, and in temperature-controlled
135 water at 13–16 °C using a heated water circulation system during winter (January–March)
136 to maintain ovarian maturation. Fish were kept under natural day length and fed a
137 commercial dry pellet daily.

138 Adult Japanese sardine (4 females and 4 males) were sampled during the breeding
139 season, on 17 February 2020. The mean size (± standard error of the mean [SEM]) of the
140 samples was 180.6 ± 2.7 mm body length (BL) and 93.6 ± 4.6 g body weight (BW). The
141 sampled pituitary, brain, liver, intestine, skin, muscle, gill, heart, spleen, adipose tissue,
142 and gonad were stored in RNAlater at 4 °C for 1 week and thereafter at −20 °C, for
143 analysis of the tissue distribution of Hsd17b genes. The ovary tissue was also used for
144 other analyses. One portion of ovary was stored in RNAlater and used to analyze the
145 developmental stage-dependent gene expression, together with sample tissues collected
146 between October 2017 and September 2018 in the previous study (Nyuji et al., 2020).
147 Another portion of ovary was fixed in Bouin's solution for histological analysis to
148 confirm the developmental stages, as described in Nyuji et al. (2020). A portion of fresh-
149 cut ovary was immersed in Ringer's solution (135 mM NaCl, 2.4 mM KCl, 1.5 mM $CaCl_2$,
150 1 mM $MgCl_2$, 1 mM $NaHCO_3$, and 0.5 mM NaH_2PO_4) and subjected to *in vitro* bioassay
151 to analyze the steroidogenic pathway.

152 In addition, 5 adult females of Japanese sardine (mean 184.0 ± 2.2 mm BL and 88.2
153 ± 3.6 g BW) were sampled on 21 December 2020. The ovaries were immersed in Ringer's
154 solution and subjected to *in vitro* bioassay.

All experimental procedures were conducted in accordance with the guidelines for animal welfare of FRA (50322001) and were approved by the animal welfare committee of the National Research Institute of Fisheries and Environment of Inland Sea, FRA (Permit Number: 2016-2 and 2016-3).

2.2. cDNA cloning and sequence analysis

The complete nucleotide sequences of cDNAs encoding Japanese sardine Hsd17b (types 3, 4, 7, 8, 10, 12a, 12b, and 14) were confirmed by the basic procedure described previously (Nyuji et al., 2016, 2020). Total RNA was extracted from sardine ovaries (sampled on 16 January 2018; Nyuji et al., 2020) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with a TURBO DNA-free Kit (Thermo Fisher Scientific, Rockford, IL). First-strand cDNA was generated with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) using random primers. The clones containing full open reading frames (ORFs) were amplified with gene-specific primers (Supplementary Table 1), which were designed based on the sequences of contigs obtained by RNA-seq (Nyuji et al., 2020). The amplified PCR products were isolated and subcloned into pGEM-T Easy vector (Promega, Madison, WI) and then sequenced.

The HSD17B/Hsd17b gene sequences of human and several fish species were acquired from GenBank. The similarity of protein-coding sequences was calculated through pairwise alignment using BioEdit software. Amino acid sequences of eight types of Hsd17b obtained by cDNA cloning were aligned using UniProt data (https://www.uniprot.org/align/) and a phylogenetic tree was constructed using MEGA X

177 software.

178

179 2.3. Quantitative real-time PCR

180 Total RNA was extracted from the pituitary, brain, and adipose tissue using the
181 RNeasy Lipid Tissue Mini Kit (Qiagen), but was extracted from gill, spleen, and ovary
182 using the RNeasy Mini Kit. For other tissues (liver, intestine, skin, muscle, heart, and
183 testis), total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) and then
184 purified using the RNeasy Mini Kit. After DNase I treatment, reverse transcription was
185 performed in a volume of 20 μl containing 250–1,000 ng of total RNA, random primers,
186 and SuperScript III Reverse Transcriptase.

187 PCR analysis was performed using gene-specific primers (Supplementary Table 2)
188 and TB Green Premix Ex Taq II (Takara Bio, Shiga, Japan) in a Thermal Cycler Dice
189 TP800 (Takara Bio) according to the manufacturer's reaction-mixing and thermal cycle
190 conditions. A standard curve based on serial dilutions of purified PCR fragments was
191 constructed for each PCR. All reactions were performed in duplicate. The expressions of
192 *hsd17b* were normalized against the expression of a reference gene (*elongation factor 1*
193 *alpha*, *ef1a*: GenBank accession no. LC767160).

194 For developmental stage-dependent gene-expression analysis, total RNA previously
195 isolated from sardine ovaries (Nyuji et al., 2020) was used together with that from the
196 present ovary samples of 17 February 2020. A total of 24 females collected in different
197 seasons were used; BL ranged from 148 to 197 mm (average 173.9 ± 2.5 mm), and BW
198 ranged from 34.5 to 100.6 g (average 67.9 ± 3.7 g).

199

200 2.4. Enzyme activity measurement

201 Measurements of the enzymatic activities were performed as described in Suzuki
202 et al. (2020). Three DNA constructs containing sardine *hsd17b3* or *hsd17b12a* or
203 *hsd17b12b* were generated using gene-specific primers (Supplementary Table 3). Each
204 was inserted into pCAGGS expression vector using the In-Fusion HD Cloning Kit
205 (Takara Bio). Human embryonic kidney 293T (HEK293T) cells were maintained under
206 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with
207 10% fetal bovine serum, and penicillin–streptomycin solution (Nacalai Tesque, Kyoto,
208 Japan). The cells (1.0×10^5/ml) were divided into 6-well plates with 2 ml per well and
209 incubated for 24 h. The vector containing each *hsd17b* or empty vector (mock) was
210 transfected using FuGENE HD Transfection Reagent (Promega), following the
211 manufacturer's instructions. After 48 h, the medium was replaced by the medium
212 containing AD or E1 (100 ng/ml; Sigma-Aldrich, St. Louis, MO). Triplicate sets of wells
213 were incubated for each vector. After incubation for 24 h, the medium was collected and
214 then centrifuged, and the supernatant was stored at −30 °C.

215

216 2.5. *In vitro* bioassay for profiling the steroidogenic pathway

217 The *in vitro* bioassay was performed twice, on 17 February and 21 December 2020.
218 The ovaries had been immersed in Ringer's solution immediately after dissection. The
219 diameters of the 10–20 largest oocytes were measured using a profile projector and digital
220 electronic calipers. For the bioassays, the ovaries were dissected into ~25-mg pieces; each

ovary piece was plated in 24-well plates with 1 ml per well of Leibovitz's L-15 medium (Thermo Fisher Scientific) containing 10 mM HEPES (Thermo Fisher Scientific) and 0.02% gentamicin sulfate (Thermo Fisher Scientific) and then cultivated at 12 °C by shaking (50 shakes/min). After 90 min, the preincubation medium was removed and replaced by 0.5 ml per well of fresh medium containing steroid precursors (P5 or AD, 100 ng/ml; Sigma-Aldrich) or no steroid (control). For each ovary from individual fish, three sets of ovary pieces were prepared, one for each of the three types of media (P5, AD, and control) and for five time points. At hours 6, 12, 24, 36, and 48, the medium was collected and then centrifuged, and the supernatant was stored at −30 °C.

2.6. Steroid hormone measurements

The T or E2 concentrations were measured from the cell culture medium with AD or E1, respectively. The AD, T, E1, and E2 concentrations were measured from the ovarian culture mediums, though AD was not measured from cultures with AD.

The concentrations of T and E2 were measured using commercial ELISA kits supplied by Cayman Chemical (Ann Arbor, MI; Estradiol ELISA Kit and Testosterone ELISA Kit), following the manufacturer's instructions. According to the user manual supplied with the kits, in the T ELISA the cross-reactivities with AD and E2 are 3.7% and <0.01%, respectively; in the E2 ELISA, those with E1 and T are 1.38% and <0.03%, respectively. The concentrations of E1 was measured using an Estrone ELISA Kit (Abnova, Taipei, Taiwan) according to the manufacturer's instructions, but with a slight modification for preparing standards and samples. To equalize the buffer contents, each

standard (0–2,400 pg/ml of E1, supplied with the kit) was diluted 2× with the culture medium, whereas the ovarian culture medium sample was diluted 2× with 0 mg/ml of the E1 standard. According to the user manual supplied with the kit, the cross-reactivity with E2 is 1.19%. The concentrations of AD was measured using the time-resolved fluoroimmunoassay procedure described in Higuchi et al. (2020). All samples were measured in duplicate.

2.5. Statistical analysis

All data are presented as mean ± SEM. All analyses were performed using GraphPad Prism (GraphPad Software 8.4.3). Results of gene expression levels in the different tissues were analyzed by two-way analysis of variance (ANOVA). Sidak's multiple comparison's test was used for pairwise comparisons of sex differences. Results of gene expression levels in ovaries at different developmental stages were analyzed by one-way ANOVA with Tukey's multiple comparisons test. Results of hormone levels by enzymatic activity measurements were analyzed by one-way ANOVA, and differences between cells transfected with empty vector (mock) and each Hsd17b expression vector were analyzed by Dunnett's test. Results of hormone levels by the *in vitro* bioassay of ovaries were compared between controls and incubation with steroids for individual time points. The unpaired *t*-test was used for AD concentrations, whereas Bonferroni's multiple comparisons was used for T and E2 concentrations.

3. Results

265 3.1. Sequence similarities and phylogenetic analysis

266 From ovary of the Japanese sardine, cDNAs encoding eight types of Hsd17b (types
267 3, 4, 7, 8, 10, 12a, 12b, and 14) were cloned (Table 1). The deduced amino acid sequences
268 of sardine Hsd17b showed high similarities to those of Atlantic herring for each (76–
269 94%). The sequence similarities between all types of Hsd17b in sardine were generally
270 below 30%, with an average of 19%, except for 37% similarity between types 3 and
271 12a/12b, 33% similarity between types 8 and 10, and 68% similarity between types 12a
272 and 12b.

273 The alignment of sardine Hsd17b showed that several motifs characteristic of the
274 SDR family are highly conserved (Fig. 2). These motifs are the cofactor binding site
275 (TGxxxGxG), the catalytic tetrad (N-S-Y-K), the structural stabilization motif (NNAG),
276 the active center motif (YxxxK), and the reaction direction motif (PGxxxT).

277 A phylogenetic tree was constructed for the eight types of Japanese sardine, Atlantic
278 herring, zebrafish, and human Hsd17bs, together with Hsd17b1 of Japanese eel, Nile
279 tilapia, zebrafish, and human (Fig. 3). Two large clusters were formed, in which one group
280 contained Hsd17b4, Hsd17b8, Hsd17b10, and Hsd17b14, and the other group contained
281 Hsd17b3 and Hsd17b12. Hsd17b1 and Hsd17b7 were not included in those clusters. Each
282 sardine Hsd17b was included in small clusters constructed by the corresponding type of
283 Hsd17b of other species.

284

285 3.2. Tissue distribution of Hsd17b mRNAs

286 The gene expression levels of each sardine Hsd17b were compared among 11 kinds

of tissues (pituitary, brain, liver, intestine, skin, muscle, gill, heart, spleen, adipose tissue, and gonad) in both sexes (Fig. 4). The highest expression of *hsd17b3* was observed in the ovary, whereas its expression was much lower in the testis. The expression of *hsd17b4* was mainly detected in the brain, liver, intestine, muscle, and heart; in the intestine, it was higher in females than in males. The expression of *hsd17b7* was detected mainly in the brain, liver, and heart; in the liver, it was higher in males than in females. The expression of *hsd17b8* and *hsd17b10* was highly detected in the muscle and heart. There were no tissues showing sex differences in these gene expressions. The expression of *hsd17b12a* was highly detected in the ovary, whereas its expression was much lower in the testis. In the brain and gill, it was higher in males than in females. The expression of *hsd17b12b* was detected mainly in the brain, liver, muscle, heart, adipose tissue, and gonad. In the ovary it was higher than in the testis, and in the liver it was higher in males than in females. The expression of *hsd17b14* was highly detected in the intestine, and in the spleen, it was higher in males than in females.

3.3. Ovarian Hsd17b gene expression at different developmental stages

The gene expression levels of eight types of Hsd17b were compared among three different stages of ovarian development: primary growth (PG), cortical alveolus (CA), and vitellogenic (VTG) stages (Fig. 5). In the PG stage, immature ovaries were occupied by perinucleolus-stage oocytes. In the CA stage, ovaries contained cortical alveoli-stage oocytes. In the VTG stage, ovaries contained oocytes during the middle and late phase of vitellogenesis. The expression of *hsd17b3* and *hsd17b4* gradually increased between the

309 PG and VTG stages. The expression of *hsd17b7* and *hsd17b8* showed no significant
310 differences between all stages. The expression of *hsd17b10* was higher in the CA stage
311 than in the PG stage, but there were no differences between the PG and VTG stages. The
312 expression of *hsd17b12a* and *hsd17b12b* increased from the PG to the CA stage,
313 maintaining high expression at the VTG stage. The expression of *hsd17b14* was higher
314 in the CA stage than in the VTG stage, but there were no differences between the PG and
315 VTG stages.
316
317 3.4. Enzymatic activity
318 HEK293T cells transfected with Hsd17b12a and Hsd17b12b expression vectors
319 converted AD to T with 19.1% and 19.0% yields, respectively (Fig. 6A). The yields of
320 this conversion in cells transfected with Hsd17b3 expression vector and empty vector
321 were <3%. HEK293T cells transfected with Hsd17b12a and Hsd17b12b expression
322 vectors converted E1 to E2 with 50.0% and 46.9% yields, respectively (Fig. 6B). The
323 yields of this conversion in cells transfected with Hsd17b3 expression vector and empty
324 vector were <8%. There were significant differences between mock cells and Hsd17b12a
325 or Hsd17b12b expressed cells for both conversion tests.
326
327 3.5. *In vitro* steroidogenesis
328 The ovaries used for bioassays were divided into two groups based on the diameters
329 of the largest oocytes: mid-vitellogenic ovaries with an average diameter of 440–450 μm
330 (n = 3), and late-vitellogenic ovaries with an average diameter of 550–650 μm (n = 6).

In mid-vitellogenic ovaries, incubation with P5 produced 20-fold higher AD concentrations than the control at 6 h post-incubation (Fig. 7A). Then, it decreased to similar levels as the control at 24 h post-incubation. There were no significant differences at every time point between the controls and incubation with P5. The T concentrations were 1,828- and 271-fold higher than the control at 6 h post-incubation for incubations with AD and P5, respectively (Fig. 7B). Thereafter, their concentrations decreased to levels similar to the controls at 48 h post-incubation. There were significant differences at 6 and 12 h post-incubation between the controls and incubation with AD. The E2 concentrations were 10-fold higher than the control at 6 h post-incubation for both incubations (Fig. 7C). Finally, their concentrations increased to 11- and 7-fold higher than the controls at 48 h post-incubation for the incubations with AD and P5, respectively. There were significant differences at 6, 12, and 48 h post-incubation between the control and incubation with AD. There were also significant differences at 6 and 12 h post-incubation between the control and incubation with P5.

In late-vitellogenic ovaries, the incubation with P5 produced 47-fold higher AD concentrations than the control at 6 h post-incubation (Fig. 7D). Then, it decreased to similar levels as the control at 36 h post-incubation. There were significant differences at 6, 12, 24, and 48 h post-incubation between the control and incubation with P5. The T concentrations were 220- and 90-fold higher than the controls at 6 h post-incubation for the incubations with AD and P5, respectively (Fig. 7E). Then, their concentrations decreased to 7–16-fold higher concentrations than the controls at 48 h post-incubation. There were significant differences at every time point between the controls and incubation

353 with AD or P5, except with P5 at 12 h post-incubation. The E2 concentrations were 3–4-
354 fold higher than the control throughout 48 h of both incubations, but there were no
355 significant differences compared with the controls (Fig. 7F).

356 E1 concentration was measured for samples collected at 6, 24, and 48 h post-
357 incubation. In all control samples E1 levels were below the assay detection limit. The E1
358 was detected in a total of 28 samples incubated with P5 or AD. The average percentage
359 of E1 from E2 concentrations was 1.10 ± 0.1% (SEM). This value was almost consistent
360 with the cross-reactivity (1.19%) of the E1 assay with E2. This indicated that detected E1
361 concentrations were derived from the cross-reactivity of the E1 kit with E2; E1, other
362 than the cross-reaction with E2, was not detected in any of the samples analyzed.

363

364 **4. Discussion**

365 In our previous study with Japanese sardine we identified eight types of Hsd17b
366 (types 3, 4, 7, 8, 10, 12a, 12b, and 14) in the gene transcripts of the BPG axis (Nyuji et
367 al., 2020). In the present study we cloned and sequenced the cDNAs encoding these
368 Hsd17b types. The SDR superfamily possess several conserved motifs (Opperman et al.,
369 2003). These motifs were found in the deduced amino acid sequences of the sardine
370 Hsd17b. In vertebrates, the homology between Hsd17b sequences is generally low (15–
371 20%) (Moeller and Adamski, 2009). The Hsd17b of Japanese sardine shared low
372 sequence similarity, of 19% on average. Among the types, Hsd17b3 and Hsd17b12a (or
373 Hsd17b12b) shared a comparatively higher sequence similarity of 37%. Similar
374 properties between HSD17B3 and HSD17B12 are seen in mammals, and the two types

375 are thought to have originated from a common ancestor (Moeller and Adamski, 2009;
376 Mindnich et al., 2005). This hypothesis has been supported by phylogenetic analyses
377 indicating that Hsd17b3 and Hsd17b12 are clustered into one group (London and Clayton,
378 2010; Zou et al., 2020), as depicted in our results. We also observed that types 4, 8, 10,
379 and 14 constituted another large cluster, whereas types 1 and 7 were not included in these
380 clusters. This phylogenetic clustering is consistent with a previous report by Zou et al.
381 (2020).

382 We found that the expression of sardine *hsd17b3*, *hsd17b12a*, and *hsd17b12b* was
383 higher in ovary than in testis. The expression of *hsd17b3* was highest in the ovary among
384 all tissues, whereas it was scarcely expressed in the testis. Similarly, zebrafish had higher
385 expression of *hsd17b3* in ovary than in testis (Mindnich et al., 2005). In contrast, gonadal
386 expression of *hsd17b3* was low in olive flounder in both sexes (Zou et al., 2020). Among
387 mammals, human *HSD17B3* was expressed predominantly in the testis, unlike in the
388 sardine and zebrafish (Geissler et al., 1994). In other tissues of the sardine, *hsd17b3* was
389 also expressed in the brain and muscle. These expression patterns differ from observations
390 of Japanese eel, where the expression of *hsd17b3* was highest in the pituitary (Suzuki et
391 al., 2020).

392 In sardine, *hsd17b12a* was predominantly expressed in the ovary, whereas
393 *hsd17b12b* expression was observed in various tissues, such as the brain, liver, and
394 adipose tissue of both sexes. Like sardine, the flounder had higher expression of
395 *hsd17b12a* and *hsd17b12b* in the ovary than in the testis (Zou et al., 2020). Conversely,
396 catfish Hsd17b12, which had high homology with type 12b, showed higher gene

397 expression in the testis than in the ovary (Rajakumar and Senthilkumaran, 2014). Eel
398 *hsd17b12a* was also highly expressed in the testis, although the expression levels in the
399 ovary were not shown (Suzuki et al., 2020). In mammals, human *HSD17B12* was broadly
400 expressed in the brain, heart, muscle, liver, kidney, adrenal gland, testis, placenta, and
401 cerebrum (Sakurai et al., 2020). High expression of human *HSD17B12* in the ovary was
402 reported by Luu-The et al. (2006), whereas Sakurai et al. (2020) showed that its
403 expression was weak in the ovary. Overall, one or both of the Hsd17b3 and Hsd17b12
404 genes, which are probably inherited from a common ancestor, are present in the gonads
405 of most vertebrates, although the expression pattern differs between species or specimens.
406 Thus, it is possible that these Hsd17b types could play a role in gonadal steroidogenesis
407 in vertebrates.

408 Among the other five types of Hsd17b analyzed in Japanese sardine, *hsd17b4* and
409 *hsd17b7* were broadly expressed in the brain, liver, intestine, muscle, and heart. Here, sex
410 differences in expression levels were seen in the intestine for *hsd17b4* and in the liver for
411 *hsd17b7*. In mammals, HSD17B4 is responsible for the metabolism of fatty acids and
412 sterols, whereas HSD17B7 is highly expressed in the ovary and placenta, suggesting a
413 role in pregnancy (Moeller and Adamski, 2009; Nokelainen et al., 2000). The expression
414 of sardine *hsd17b8* and *hsd17b10* showed a similar tissue distribution, whereby both
415 genes were highly expressed in the muscle and heart in both sexes. In mammals, the role
416 of HSD17B8 is not clear and HSD17B10 shows the broadest substrate specificity
417 (Moeller and Adamski, 2009). The highest expression of sardine *hsd17b14* was observed
418 in the intestine, and its expression in spleen was higher in males than in females.

419 HSD17B14 proteins are widely distributed in various human tissues, but its function is
420 still unclear (Sivik et al., 2012; Badran et al., 2019). The catalytic activities of Hsd17b8
421 toward the conversion from T to AD and interconversion of E1 to E2 have been found in
422 Nile tilapia (Zhou et al., 2005), yet the biological roles of five types of Hsd17b are still
423 poorly understood in teleosts. Considering the biological functions of these enzymes in
424 mammals, our results suggest that they are unlikely to play critical roles in steroidogenesis
425 in sardine gonad.

426 In our rearing system of the sardine, the onset of ovarian development was
427 recognized histologically by the appearance of cortical alveolus-stage oocytes during
428 previtellogenic growth in October; after that, vitellogenesis proceeded from November to
429 January, with the breeding season occurring in January to March (Nyuji et al., 2020). We
430 previously demonstrated that increases in ovarian gene expression of three CYP genes
431 (*cyp11a1*, *cyp17a1*, and *cyp19a1a*) were accompanied by ovarian development in the
432 sardine (Nyuji et al., 2020). The enzymes encoded by these genes catalyze key reaction
433 steps of the teleost ovarian steroidogenic pathway (Fig. 1). These imply that gene
434 expression in the sardine ovary of any Hsd17b type involved in E2 synthesis should be
435 similarly upregulated during ovarian development. In the present study, the expression of
436 *hsd17b3*, *hsd17b4*, *hsd17b12a*, and *hsd17b12b* increased during ovarian development.
437 Here, the expression of *hsd17b3* and *hsd17b4* gradually increased from immature to
438 vitellogenesis, whereas the expression of *hsd17b12a* and *hsd17b12b* increased during
439 previtellogenic growth and was maintained at a high level during vitellogenesis.
440 Consistent with the expression profile we observed, the expression of *hsd17b12a* and

441 *hsd17b12b* in the ovary of flounder was upregulated during ovarian development (Zou et
442 al., 2020). Taken together with findings of an ovary-specific distribution, Hsd17b3,
443 Hsd17b12a, and Hsd17b12b were selected as candidate types for having a significant role
444 in E2 synthesis in sardine ovary. While *hsd17b4* expression also showed a developmental
445 stage-dependent increase, its expression levels were low in the ovary, and this type is less
446 involved in sex steroid biosynthesis in vertebrates, as mentioned above. Hence, Hsd17b4
447 was not subject to further analysis in the present study.

448 Previous studies have assessed the catalytic activities of Hsd17bs using mammalian
449 cells, such as HEK cells and monkey kidney COS cells, by expressing these recombinants.
450 In mammals, rat HSD17B3 converted AD to T, but not E1 to E2 (Tsai-Morris et al., 1999).
451 Human and monkey HSD17B12 specifically converted E1 to E2, whereas mouse
452 HSD17B12 converted both AD to T and E1 to E2 (Luu-The et al., 2006; Blanchard and
453 Luu-The, 2007; Liu et al., 2007). In teleosts, it has been described that E1 was converted
454 to E2 by the enzymatic activities of eel Hsd17b12a (Suzuki et al., 2020), and zebrafish
455 Hsd17b12a and Hsd17b12b (Mindnich and Adamski, 2009), although these data were not
456 shown. In contrast, tilapia Hsd17b12 did not convert AD to T nor E1 to E2 (Zhou et al.,
457 2005). In the present study the *in vitro* conversion of AD to T and E1 to E2 by sardine
458 Hsd17b3, Hsd17b12a, and Hsd17b12b was tested in HEK293T cells. We discovered that
459 sardine Hsd17b12a and Hsd17b12b catalyze both conversions, of AD to T and E1 to E2.

460 The *in vitro* cultivation of ovarian follicles with radiolabeled steroid precursors
461 revealed that teleosts have two major pathways of E2 synthesis (Fig. 1; Matsuyama et al.,
462 2005; Rahman et al., 2002; Ohta et al., 2001, 2002). One pathway is conversion of AD to

E2 via T, and the other via E1. The present *in vitro* bioassays showed that in mid-vitellogenic ovaries, incubation with P5 transiently elevated the production of AD and T, followed by an increase in E2 production. Incubation with AD also transiently elevated T production, followed by an increase in E2 production. These results suggest that P5 was first converted to T via AD and subsequently converted to E2 in the sardine ovary. We previously showed that the serum concentration of T was one-fifteenth that of E2 during the breeding season (Nyuji et al, 2020). This may be due to a rapid metabolism of circulating T to E2 or other steroids in the living body of female sardines, as demonstrated by the ovarian culture. Our results further showed that in late-vitellogenic ovaries, incubation with either P5 or AD did not accelerate E2 production despite facilitated production of AD and T. This observation could be explained by a shift of the steroidogenic pathway. In the teleost ovary, after completion of vitellogenesis, the pathway shifts from the biosynthesis of E2 to maturation-inducing hormones such as 17,20β-dihydroxy-4-pregnen-3-one and 17,20β,21-trihydroxy-4-pregnen-3-one (Matsuyama et al., 2005; Ohta et al., 2001). Therefore, our results suggest that in sardine ovary, AD and T are metabolized to steroids other than E2 at the late phase of vitellogenesis, in preparation of maturation. Meanwhile, we could not detect E1 in the cultivation medium of sardine ovary. We also recently tried to measure the serum concentration of E1 in the sardine, but it was not detected throughout the annual reproductive cycle (Nyuji et al., 2020). These results indicate that E1 is not synthesized in the sardine ovary. Collectively, our results indicate that in the sardine ovary, E2 is synthesized from P5 via AD and T, and not via E1, and E2 production is suppressed after

485 the completion of vitellogenesis.

486 The present study revealed that sardine Hsd17b12a and Hsd17b12b catalyze the
487 reduction of AD to T and E1 to E2. Furthermore, E1 did not appear in circulation (Nyuji
488 et al., 2020) and was not synthesized in the sardine ovary (present study). This suggests
489 that the two enzymes can function in the metabolism of AD to T in the course of E2
490 synthesis in sardine ovary. We also found that the expression of *hsd17b12a* and
491 *hsd17b12b* was higher in ovary than in testis and increased during ovarian development.
492 These findings support the possibility that Hsd17b12a and Hsd17b12b have roles in
493 ovarian development in this species. Importantly, *hsd17b12a* was expressed
494 predominantly in the ovary, unlike *hsd17b12b*, which was broadly expressed in various
495 tissues. Also, the expression levels of *hsd17b12a* were 3- to 7-times higher than those of
496 *hsd17b12b* (Nyuji et al., 2020; present study). Accordingly, we conclude that Hsd17b12a,
497 rather than Hsd17b12b, mainly functions in E2 synthesis in the ovary of Japanese sardine.
498 Unlike in sardine, Hsd17b12 did not catalyze the conversion of AD to T in tilapia (Zhou
499 et al., 2005), and *hsd17b12a* was highly expressed in the testis of Japanese eel (Suzuki et
500 al., 2020). Thus, the patterns of enzymatic activity and gene expression of Hsd17b appear
501 to be species-specific. Therefore, the current role of Hsd17b12 in sardine cannot be
502 simply inferred for other species of teleosts. Beyond this caveat, the present study
503 provides important insight into the physiological role of Hsd17b12 in vertebrates and the
504 molecular mechanisms regulating ovarian E2 synthesis in teleosts.

505

506 **CRediT authorship contribution statement**

Mitsuo Nyuji: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft, Funding acquisition. **Yuki Hongo**: Investigation, Writing – review & editing. **Yukinori Kazeto**: Conceptualization, Writing – review & editing. **Michio Yoneda**: Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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682 Figure legends

683

684 Figure 1. Steroidogenic pathway of the biosynthesis of estradiol-17β (E2) in teleost
685 ovarian follicles. During vitellogenesis, E2 is synthesized from pregnenolone (P5) via
686 17-hydroxypregnenolone (17OH-P5), 17-hydroxyprogesterone (17OH-P4) or
687 dehydroepiandrosterone (DHEA), androstenedione (AD), and testosterone (T) in chub
688 mackerel *Scomber japonicus* (Matsuyama et al., 2005) and Japanese amberjack *Seriola*
689 *quinqueradiata* (Rahman et al., 2002), whereas it is synthesized via AD and estrone
690 (E1) in bambooleaf wrasse *Pseudolabrus sieboldi* (Ohta et al., 2001) and red seabream
691 *Pagrus major* (Ohta et al., 2002). Steroid conversions are catalyzed by enzymes
692 belonging to the cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD)
693 families.

694

695 Figure 2. Sequence alignment of the amino acid sequences of eight types of 17β-
696 hydroxysteroid dehydrogenases (Hsd17b) in the Japanese sardine *Sardinops*
697 *melanostictus*. Identical residues are shaded blue; several highly conserved motifs
698 characteristic of the short-chain dehydrogenase/reductase (SDR) superfamily are
699 labeled and indicated by black boxes. Asterisks mark the catalytic tetrad (N-S-Y-K).
700 Numbers specify the number of amino acids.

701

702 Figure 3. Phylogenetic tree of the amino acid sequences of Hsd17b. Bootstrap analysis
703 was performed using the neighbor-joining method (1,000 replications). GenBank

accession numbers of Japanese sardine and Atlantic herring *Clupea harengus* Hsd17b types are shown in Table 1. GenBank accession numbers of zebrafish *Danio rerio* and human Hsd17b/HSD17B types 1, 3, 4, 7, 8, 10, 12, and 14 are as follows: zebrafish, NM_205584, AY551081, AF241285, NM_001077328, NM_001005292, NM_001006098, NM_200881, NM_199613, NM_001003521; human, BC111935, NM_000197, BC003098, BT007075, BC008185, BC000372, BC012043, NM_016246. GenBank accession numbers of Japanese eel *Anguilla japonica* and Nile tilapia *Oreochromis niloticus* Hsd17b1 are AY498620 and NP_001266724, respectively.

Figure 4. Relative mRNA levels of eight types of Hsd17b distributed in various tissues of the Japanese sardine. Values are the mean ± SEM ($n = 4$). Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.0001$).

Figure 5. Ovarian Hsd17b gene expression at different developmental stages in the Japanese sardine. (A) Histological micrographs of ovaries at different developmental stages: primary growth (PG), cortical alveolus (CA), and vitellogenic (VTG) stages. (B) Relative mRNA levels of Hsd17b types in sardine ovaries. Values are the mean ± SEM (PG and VTG, $n = 10$; CA, $n = 8$). Asterisks indicate significant differences ($p < 0.05$).

Figure 6. Enzymatic activities of Japanese sardine Hsd17b3, Hsd17b12a, and Hsd17b12b. The conversions of AD to T (A), and E1 to E2 (B), were measured in human embryonic kidney 293T (HEK293T) cells transfected with each Hsd17b expression vector or an empty vector (mock). Values are the mean ± SEM of three replicates. Asterisks indicate significant differences ($p < 0.05$) between cells transfected with empty vector (mock) and each Hsd17b expression vector.

Figure 7. *In vitro* steroid production of AD, T, and E2 in Japanese sardine ovaries cultured with the steroid precursors, P5 or AD. The ovaries were divided into two groups: mid-vitellogenic ovaries ($n = 3$; A–C) and late-vitellogenic ovaries ($n = 6$; D–F). The concentrations of AD (A and D), T (B and E), and E2 (C and F) in the ovary cell culture medium were measured at each time point (6, 12, 24, 36, and 48 h post-incubation). Asterisks indicate significant differences ($p < 0.05$) between the mediums with and without (control) steroid precursors at each sampling point.

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Table 1 Sequence information on Japanese sardine *Sardinops melanostictus* Hsd17b genes revealed by cDNA cloning

Gene symbol	Gene accession number	Sequence length of ORF		Amino acid sequence similarity (%)							
		Nucleotides (base pairs)	Amino acids	Atlantic herring*	Japanese sardine						
					hsd17b4	*hsd17b7*	*hsd17b8*	*hsd17b10*	*hsd17b12a*	*hsd17b12b*	*hsd17b14*
hsd17b3	LC767152	936	311	75.6	7.6	11.4	20.5	14.8	37.2	36.9	18.1
hsd17b4	LC767153	2172	723	86.5	–	9.2	10.3	10.6	7.8	8.3	8.3
hsd17b7	LC767154	1011	336	92.3	–	–	15.7	16.7	11.8	14.7	16.1
hsd17b8	LC767155	777	258	91.5	–	–	–	33.0	18.7	18.1	22.9
hsd17b10	LC767156	783	260	94.2	–	–	–	–	15.0	14.6	25.3
hsd17b12a	LC767157	948	315	86.8	–	–	–	–	–	67.7	14.8
hsd17b12b	LC767158	948	315	88.1	–	–	–	–	–	–	18.0
hsd17b14	LC767159	810	269	90.3	–	–	–	–	–	–	–

*GenBank accession numbers of Atlantic herring Clupea harengus Hsd17bs are as follows: Hsd17b3, XM_012820628; Hsd17b4, XM_031570100; Hsd17b7, XM_012834158; Hsd17b8, XM_012837609; Hsd17b10, XM_012820603; Hsd17b12a, XM_012842446; Hsd17b12b, XM_012814965; Hsd17b14, XM_031559452.

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