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Title	17 beta-HSD Type 12-Like Is Responsible for MaturationInducing Hormone Synthesis During Oocyte Maturation in Masu Salmon
Author(s)	Ijiri, Shigeo; Shibata, Yasushi; Takezawa, Nonoha et al.
Citation	Endocrinology, 158(3), 627-639 https://doi.org/10.1210/en.2016-1349
Issue Date	2017-03
Doc URL	https://hdl.handle.net/2115/66278
Type	journal article
File Information	Ijiri HUSCUP.pdf



17 β -HSD type 12-like is responsible for maturation-inducing hormone synthesis during oocyte maturation in masu salmon^{1,2}

¹Supported by JSPS (Japan Society for the Promotion of Science) KAKENHI Grant #21228005 (Sequencing part), #24658165 and #26252030 (Isolation and characterization of 20 β -HSD).

²Presented in part at the 17th International Congress of Comparative Endocrinology, 15-19 July 2013, Universitat de Barcelona, Spain.

Shigeho Ijiri³, Yasushi Shibata⁴, Nonoha Takezawa³, Yukinori Kazeto⁵, Naoki Takatsuka³, Erika Kato³, Seishi Hagihara³, Yuichi Ozaki⁵, Shinji Adachi^{3*}, Kohei Yamauchi⁶, and Yoshitaka Nagahama^{6*}.

Endocrinology (2017) 158(3): 627-639. DOI: <https://doi.org/10.1210/en.2016-1349>

Published: 14 December 2016

³*Division of Marine Life Sciences, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*

⁴*School of Aquatic and Fishery Science, University of Washington, Seattle, Washington*

⁵*National Research Institute of Aquaculture, Fisheries Research Agency, Watarai, Mie, Japan*

⁶ *South Ehime Fisheries Research Center, Ehime University, Matsuyama, Ehime, Japan*

Abbreviated title: 17 β -HSD12-like responsible for DHP production

Key terms: 17 α ,20 β -dihydroxy-4-pregnen-3-one, 20 β -hydroxysteroid dehydrogenase, 17 β -hydroxysteroid dehydrogenase, Oocyte maturation, Granulosa, Masu salmon, *Oncorhynchus masou*

Word count: 5347

Number of figures and tables: 7 figures

Corresponding authors and people to who reprint requests should be addressed:

Yoshitaka Nagahama

Institution for Collaborative Relations

Ehime University

Bunkyo-cho 3, Matsuyama, Ehime 790-5877, Japan

Phone: 81 89 927 8513

Fax: 81 89 927 8820

e-mail: nagahama.yoshitaka.mh@ehime-u.ac.jp

Shinji Adachi

Division of Marine Life Sciences, Graduate School of Fisheries Sciences

Hokkaido University

Minato-cho 3-1-1, Hakodate, Hokkaido 041-8611, Japan

Phone: 81 138 40 5545

Fax: 81 138 40 5545

e-mail: s-adachi@fish.hokudai.ac.jp

Disclosure statement: The authors have nothing to disclose.

Abstract

The maturation-inducing hormone $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) was first identified in the amago salmon. However, although carbonyl reductase-like 20β -hydroxysteroid dehydrogenase (CR/ 20β -HSD) was reported to convert 17α -hydroxyprogesterone (17OHP) to DHP in rainbow trout, we previously found that CR/ 20β -HSD mRNA was not up-regulated in stimulated granulosa cells from masu salmon, which suggested that DHP is synthesized by a different enzyme. Accordingly, the present study aimed to identify the specific 20β -hydroxysteroid dehydrogenase (20β -HSD) responsible for DHP production by granulosa cells during final oocyte maturation in masu salmon. RNA-sequencing was performed on granulosa layers that were isolated from ovarian follicles at one month before ovulation and incubated with or without forskolin, which was used to mimic luteinizing hormone, and ~12 million reads were obtained, which yielded 71,062 contigs of > 100 bp. tBlastx analysis identified one contig (#f103496) as similar to 17β -hydroxysteroid dehydrogenase type 12 (*hsd17 β 12*); however, because the full-length #f103496 sequence was different from *hsd17 β 12*, it was termed to *hsd17 β 12*-like (*hsd17 β 12l*). We found that mammalian cells transfected with full-length *hsd17 β 12l* exhibited considerable 20β -HSD activity, as indicated by efficient conversion of exogenous 17OHP to DHP. In addition, we found that *hsd17 β 12l* mRNA levels were consistently low in follicles during vitellogenic growth; however, the levels increased significantly during final oocyte maturation. The levels of *hsd17 β 12l* mRNA were also considerably increased in granulosa layers in which 20β -HSD activity was induced by salmon pituitary extract. Therefore, we suggest that *hsd17 β 12l*, not CR/ 20β -HSD, is the 20β -HSD responsible for DHP production by granulosa cells in masu salmon during final oocyte maturation.

Introduction

For the first time in any vertebrate, the maturation-inducing hormone was identified as 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) in the amago salmon in 1985 (1). Since then, many efforts have been made to identify the 20 β -hydroxysteroid dehydrogenase enzyme (20 β -HSD) responsible for synthesizing DHP from 17 α -hydroxyprogesterone (17OHP). Unexpectedly, strong 20 β -HSD activity for the substrate 17OHP was detected in the cytosol fractions of neonatal, but not mature, pig testis (2) and purified to homogeneity (3). Subsequently, partial amino acid sequences of the purified enzyme were determined, and a cDNA encoding the protein has been isolated and characterized (4). Using the pig 20 β -HSD cDNA as a probe, an ortholog was also isolated from the ovarian cDNA library of rainbow trout (5), and homologs have been isolated in other teleost fish as well (6, 7).

These homologous genes have been categorized as monomeric carbonyl reductase genes, which encode enzymes that possess wide substrate specificity for carbonyl compounds and are known as carbonyl reductase-like 20 β -HSD (CR/20 β -HSD) (5, 6). When produced in an *Escherichia coli* heterologous expression system, these enzymes exhibit weak 17OHP-to-DHP conversion activity and a much stronger affinity for non-17OHP carbonyl compounds, such as 9,10-phenanthrenequinone, cyclohexanone, and 4-nitrobenzaldehyde (6). However, the lack of strong 17OHP-to-DHP conversion activity seems inconsistent with the high concentration of DHP produced in salmonid fish during final oocyte maturation (FOM), and it also seems unlikely that CR/20 β -HSD would exhibit such a wide substrate specificity if it was a truly steroidogenic enzyme responsible for oocyte maturation, which should be strictly controlled to ensure spawning success.

In salmonid fish, including the masu salmon (*Oncorhynchus masou*), a sharp increase in serum DHP occurs around FOM and during the subsequent ovulation period (8–10), and in the 1980s, a series of *in vitro* studies using salmonid ovarian follicles demonstrated the hormonal control of DHP production (8, 11–15). Those studies reported that ovarian follicles (comprised of oocytes, granulosa layers, and theca layers) acquired the ability to produce DHP after completion of vitellogenic growth in response to the release of gonadotropin (8) or exposure to dibutyryl cAMP (13). The *in vitro* studies also demonstrated that isolated theca layers produced a substantial amount of 17OHP in response to partially purified chinook salmon gonadotropin (SG-G100; see 16) exposure, but granulosa layers did not (12). Furthermore, the studies found that both theca and granulosa layers are incapable of producing DHP by themselves; however, a co-culture of both layers can be induced to produce DHP by SG-G100, and a substantial amount of DHP can be synthesized from 17OHP by isolated granulosa layers incubated with SG-G100 (12). These findings clearly indicate that DHP is synthesized in the granulosa layer from 17OHP that is produced and delivered from the theca layer. Furthermore, these studies demonstrated that the 20 β -HSD activity-inducing role of gonadotropin could be replaced with dibutyryl cAMP and forskolin (an adenylate cyclase activator), indicating the involvement of an adenylate cyclase-cAMP-dependent step (12, 17, 18). The acquisition of 20 β -HSD activity by granulosa layers in response to SG-G100 exposure could also be inhibited by either actinomycin D, which inhibits RNA synthesis, or cycloheximide, which inhibits protein synthesis, clearly indicating that the step requires *de novo* synthesis of the enzyme from transcriptional events (19).

However, evidence that CR/20 β -HSD is responsible for the conversion of 17OHP to DHP during FOM is

yet to be reported, and it remains unclear whether changes in *CR/20 β -HSD* mRNA levels coincide with changes in 20 β -HSD activity in naturally developing ovarian follicles before and during FOM or whether *CR/20 β -HSD* mRNA levels increase in cultures of gonadotropin- or forskolin-induced granulosa cells before FOM. Moreover, studies using cell-free systems in amago salmon (our unpublished data) and Japanese eel (20) have demonstrated that the 20 β -HSD activity in ovarian follicles is mainly located in the membrane-bound fractions of mitochondria and microsomes, with lower levels detected in the cytosolic fraction, which is inconsistent with the observation of CR/20 β -HSD in the cytosol of pigs (2). Therefore, it seems premature to conclude that CR/20 β -HSD is responsible for DHP production in the ovarian follicles of salmonids during FOM. Accordingly, in the present study, we investigated whether the expression of *CR/20 β -HSD* mRNA was associated with 20 β -HSD activity in forskolin-induced granulosa cells and aimed to identify any other 20 β -HSD-encoding genes that could be responsible for producing DHP in the granulosa cells of masu salmon around FOM.

Materials and Methods

Animals and sample preparations

Masu salmon (*O. masou*) were taken from the breeding stock of the Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan. Sampling, which consisted of taking caudal vein blood samples and removing ovaries, was conducted from April to September, since FOM and ovulation normally occur between the middle and end of September. Three to five females were selected for sampling each month, between May and August, and 17 were selected during September and classified by the developmental stages of their oocytes. To isolate serum, the collected blood samples were stored at 4 C overnight, after which the serum was separated by centrifugation at 2000 $\times g$ for 5 min. Ovarian follicles were isolated from the ovaries by careful dissection in ice cold trout balanced salt solution (21), and after removing yolks, the follicles were frozen in liquid nitrogen and stored at -80 C until used for RNA extraction as *in vivo* samples. All experimental procedures complied with the National and Institutional Guidelines for Care and Use of Laboratory Animals and were approved by the Animal Research Committee of Hokkaido University.

For quantitative PCR analysis, groups of 20 isolated granulosa preparations or post-ovulation follicle layers were incubated in 4 mL Ringer medium, with or without forskolin and/or salmon pituitary extract (SPE, from the pituitaries of post-ovulation chum salmon), for 18 h in 6-well non-coated plastic culture plates. Granulosa layers and post-ovulation follicle layers were immersed in RNAlater Solution (Applied Biosystems, Foster City, CA, USA) and stored at -80 C until used for RNA extraction as *in vitro* samples. Each experiment used oocytes from a single female, in order to discount the possibility of individual variation in follicle responsiveness.

For next-generation sequence analysis, granulosa layers that were sampled from different females at one month before the spawning period were subject to five different experimental treatments. Some were independently incubated in either Ringer medium, Ringer medium with 17OHP, or Ringer medium with both forskolin and 17OHP, with three replicates each, and ~50 additional granulosa layer samples were incubated independently with or without forskolin for future RNA extraction.

Measuring 20 β -HSD activity

To measure 20 β -HSD activity, granulosa layer preparations were collected from females with undetectable germinal vesicles, during the two months before ovulation (at two months and one month before ovulation), and after ovulation, follicle layers that consisted of both granulosa and theca layers were drawn out at 1–2 d post-ovulation. Granulosa layers were separated from the follicle layers of oocytes using forceps under a dissecting microscope, which is a technique that yields granulosa layers that are not contaminated by thecal cells, as reported by Kagawa et al. (21). Afterward, groups of five granulosa preparations and post-ovulation follicle layers were independently incubated in 1 mL Ringer medium, Ringer medium with 100 ng/mL 17OHP and either 10 μ M forskolin or 100 μ g/mL SPE, or Ringer medium without 17OHP and with either 10 μ M forskolin or 100 μ g/mL SPE, in a 24-well non-coated plastic culture plate. Three replicate incubations were conducted for each treatment, and the assays were maintained in a humidified incubator at 37 C for 36 h, after which the DHP in the media was quantified.

DHP quantification

The DHP in blood sera and media from the 20 β -HSD activity assays were extracted, according to the method of Kagawa et al. (22), and DHP concentrations were measured using time-resolved fluoroimmunoassay, according to the method of Yamada et al. (23). DHP was purchased from Sigma Chemicals (St. Louis, MO, USA), and the FKA332 antibody for DHP was purchased from Cosmo-Bio Co., Ltd. (Tokyo, Japan).

Next-generation sequencing and analysis

After incubation with or without forskolin, total RNA was extracted from the granulosa layers of female #1, which exhibited the highest induction of 20 β -HSD activity by forskolin, using a column-based RNA extraction kit (RNeasy Mini Kit; Qiagen, Hilden, Germany). Complementary DNA (cDNA) libraries were prepared using an mRNA-Seq-8 Sample Prep Kit (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. Briefly, poly-A RNA was fragmented and reverse transcribed using random primers. Then, the cDNA fragments were ligated to Illumina adaptors, size selected, and amplified *via* PCR. Next-generation sequencing (RNA-seq) of the RNA from the control (Ringer alone) and forskolin-treated groups was conducted using a Genetic Analyzer IIx (Illumina) by Hokkaido System Science Co., Ltd. (Sapporo, Japan), according to Illumina's instructions.

After passing the cDNA sequence reads through the Illumina chastity filter to remove aberrant reads, the reads of both groups were independently assembled using Velvet version 0.7.55 (24). The resulting contigs from both groups were further assembled using CAP3 (25), as described by Iorizzo et al. (26), in order to remove redundancy and to generate consensus contigs, and the number of reads that mapped to each contig was calculated using Bowtie (27). To evaluate whether the number of reads actually reflected a difference between the mRNA levels of the control and forskolin-treated group, we examined 13 genes that had been previously identified as up-regulated under forskolin treatment (unpublished data). In addition, contigs that were only recovered from the

forskolin-treated group were analyzed using tBlastx, and one contig that encoded a 17 β -hydroxysteroid dehydrogenase type 12-like sequence (*omhsd17 β 12l*) was selected for further analysis.

The differential expression of *omhsd17 β 12l* was confirmed using qPCR, and the full-length *omhsd17 β 12l* cDNA sequence was determined using amplification with the SMART RACE cDNA amplification Kit (Clontech, Otsu, Japan), gene-specific primers, and Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and sequencing with a Genetic Analyzer 3100xl (Applied Biosystems). The open reading frames and predicted molecular weights of the predicted amino acid sequences were calculated using GENETYX-WIN Version 5.1.1 (GENETYX Co., Tokyo, Japan), without considering post-translational modification.

Phylogenetic analysis

A phylogenetic tree was constructed using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis (MEGA4) software (<http://www.megasoftware.net>), and the GenBank accession numbers for the aligned amino acid sequences were as follows: *Salmo salar* hsd17b12, BT045689; *S. salar* hsd17b3a, BT046393; *S. salar* hsd17b3b, BT046871.

Heterologous *omhsd17 β 12l* expression and activity analysis

The two forms of the *omhsd17 β 12l* coding region (*typeI* and *typeII*) were subcloned into pSI, an *in vitro* eukaryotic expression vector that includes an SV40 promoter (Promega Co., Madison, WI, USA), and transfections were conducted using the jetPRIME transfection reagent (Polyplus-Transfection SA, Illkirch-Graffenstaden, France), according to the manufacturer's instructions. Briefly, HEK293T cells were transfected in 12-well cell culture plates containing Dulbecco's Modified Eagles medium (Nissui Pharmaceutical, Tokyo, Japan) with 10% fetal bovine serum, 0.65 μ g *omhsd17 β 12l*-containing pSI, 0.13 μ g pAdvantage Vector (Promega), and 1.5 μ L of jetPRIME reagent at 37 C. Empty pSI was used as a control, and three independent replicates were conducted for each treatment (*omhsd17 β 12l typeI*-pSI, *omhsd17 β 12l typeII*-pSI, and empty-pSI).

After transfection, the cells were pre-cultured in Dulbecco's Modified Eagles medium without fetal bovine serum for 12 h and then separately incubated with ³H-labeled 17OHP (PerkinElmer, Waltham, MT, USA; 30 ng/mL; n = 9) or cold 17OHP (100 ng/mL; n = 9), as substrates for *omhsd17 β 12l* activity, for an additional 12 h. After incubation, DHP and other steroid metabolites were extracted from the media, using three extractions with dichloromethane, and the DHP concentrations of the extracted steroid metabolites from the tritiated and cold 17OHP-treated incubations were then measured, using thin-layer chromatography (TLC) and time-resolved fluoroimmunoassay, respectively. For TLC analysis, the extracted steroid metabolites were loaded onto TLC plates, separated using benzene-acetone (4:1, v/v), detected autoradiographically with X-ray film, and quantified using ImageJ (<http://rsb.info.nih.gov/ij/>).

Tissue-specific *omhsd17 β 12l* expression

Total RNA was isolated from brain, pituitary, head kidney, hind kidney, liver, spleen, heart, follicle layers

from ovaries in late vitellogenesis or FOM, and sperm-containing testis, using ISOGEN II (Nippon Gene, Tokyo, Japan). Total RNA (1 µg) from each tissue was reverse transcribed (RT) using random hexamer primers (Life Technologies Co., Carlsbad, CA, USA) and the ReverTra Ace RT enzyme (Toyobo Co., Ltd., Osaka, Japan) in 20 µL reactions, according to the manufacturer's instructions. The diluted RT reactions (10%, v/v; 2 µL) were then used as the template for PCR amplification (20 µL reactions) of both *omhsd17β12l* and *elongation factor 1α* (*ef1α*) as a control. The primers were designed to amplify fragments that included an intron/exon boundary (Supplemental Fig. 1), in order to distinguish the amplification of genomic contaminants: *omhsd17β12l* 1-forward, 5'-GAGTGAAATTTGCCAGACATGG-3'; *omhsd17β12l* 2-reverse, 5'-CGTCCATGTTGGCTCTCAATC-3'; *ef1α* 1-forward, 5'-AAATCTGGAGACGCCGCCA-3'; *ef1α* 3-reverse, 5'-ATGGCGGACTTGGTCACCTT-3'. Ten microliters of the PCR reactions was electrophoresed on 2% agarose gels, stained with ethidium bromide, and digitally photographed under UV light.

Quantitative PCR

Total RNA was extracted from incubated granulosa layers, yolkless follicles, or post-ovulation follicle layers (i.e., *in vitro* samples), using ISOGEN II (Nippon Gene), treated with DNase, and further cleaned, using a column-based RNA extraction kit (NucleoSpin RNA II; Macherey-Nagel GmbH & Co. KG, Düren, Germany). Subsequently, 45 or 50 ng of total RNA was amplified, using a one-step qRT-PCR system (One Step SYBR Prime Script PLUS RT-PCR Kit; TaKaRa Bio, Inc., Otsu, Japan) in a final volume of 10 µL. Total RNA (1 µg) from *in vivo* yolkless follicles was reverse transcribed as described above, and diluted RT reactions (10%, v/v; 2 µL) were used as templates for 20 µL quantitative PCR (qPCR) reactions, using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) and the StepOnePlus Real-Time PCR system (Life Technologies Co.). The qPCR primer sets included one (*ef1α*) or both (*omhsd17β12l*) primers that flanked intron–exon boundaries, in order to prevent genomic amplification: *omhsd17β12l* 3-forward, 5'-ATGGACAATGGGCAGTTGTCA-3'; *omhsd17β12l* 4-reverse, 5'-TCTGGCCAACTCATTTCGTA-3'; *ef1α* 1-forward, 5'-AAATCTGGAGACGCCGCCA-3'; *ef1α* 2-reverse, 5'-ACGGCAAAACGACCAAGGG-3'; *CR/20β-HSD typeA* forward, 5'-CCGGTGCCAATAAAGGCAT-3'; *CR/20β-HSD typeA* reverse, 5'-AGCTCCCTCACAATCGCAAG-3'; *CR/20β-HSD typeB* forward: 5'-TCATTAACAACGCTGGAATGTCC-3', *CR/20β-HSD typeB* reverse, 5'-CCAAAAGTCTCAGTCGCATCATTTT-3'. The same primers were used for the *omhsd17β12l* typeI and typeII sequences because qPCR was incapable of distinguishing the one nucleotide difference, and separate primer sets were used for *CR/20β-HSD typeA* and *typeB* (Supplemental Fig. 1).

Statistical analysis

Paired *t*-tests were used to detect significant differences, when comparing the data of two groups, and multiple comparisons were performed using Tukey-Kramer tests or ANOVA with Fisher's *PLSD* post hoc test. The data from mRNA quantification were converted to log 10 values before analyses, and statistical analyses were performed using StatView for Windows version 5.0.1 (SAS Institute Inc., Cary, NC, USA).

Results

In vitro 20 β -HSD activity and CR/20 β -HSD mRNA expression

The 20 β -HSD activity of granulosa layers was significantly higher when incubated with forskolin than without forskolin ($n = 5$, Tukey-Kramer, $P < .01$; Figure 1), as indicated by increased conversion of exogenous 17OHP to DHP, and the granulosa layers of two females (#1 and #5) exhibited especially strong 20 β -HSD activity (mean $\pm$ SE; 18.0 ± 1.1 ng/mL and 17.6 ± 2.7 ng/mL, respectively; Supplemental Fig. 2). Relatively low concentrations of DHP (6–8 ng/mL) were produced in the other three females (Supplemental Fig. 2). However, non-normalized mRNA levels [Figure 1(B) and (C)] and *ef1 α* -normalized mRNA levels (data not shown) of both CR/20 β -HSD typeA and typeB were not higher in granulosa layers incubated with forskolin than in those without forskolin ($n = 5$; paired *t*-tests).

RNA-seq analysis

A total of 12.538 and 12.273 million reads from RNA-seq of the control and forskolin-treated granulosa layers of female #1 were approved by the chastity filter, respectively, which accounted for 639,452 and 625,907 kb of data (BioSample accession no. SAMD00056133 and SAMD00056134, respectively). The average read length was 51 bp, and Velvet produced 95,091 and 80,282 contigs from the control and forskolin-treated reads, respectively. Furthermore, Velvet+CAP3 assembly yielded 40,013 mixed contigs and 35,762 and 22,150 singlets from the control and forskolin-treated Velvet contigs, respectively (mixed and singlet contig sequences are deposited in the NCBI Transcriptome Shotgun Assembly (TSA) Database, accession nos. IABA01000001– IABA01097925). Of these, ~71,000 contigs (73%) had lengths >100 bp, and the contigs had an N50 length of 181 bp, average length of 182 bp, and maximum length of 5190 bp. The number of mapped and singlet reads that were obtained from the control and forskolin-treated granulosa samples have been deposited in the NCBI Gene Expression Omnibus (GEO) Database (accession no. GSE85134).

Among the contigs, we identified 11 of the 13 genes that we had previously identified as up-regulated under forskolin treatment (unpublished data, cDNA sequence data is included in Supplemental Fig. 3), and all of those 11 genes were represented by a greater number of reads in the forskolin group (Supplemental Fig. 4), in agreement with the qPCR findings.

In addition, we also identified 953 unique contigs of >100 bp that were constructed exclusively from the forskolin-treated group reads (Supplemental data 1), and tBlastx analysis identified a 117-bp contig (*#f103496*) as a member of the 17 β -hydroxysteroid dehydrogenase type 3 (*hsd17 β 3*) family (Figure 2A). One-step qPCR analysis confirmed that the expression of *#f103496* was considerably higher in the forskolin-treated granulosa layers than in the control group ($n = 5$, females #1–5, paired *t*-tests; $P < .0005$; Figure 2C), whereas the levels of *ef1 α* mRNA were not different between the two groups, and the *ef1 α* -normalized level of *#f103496* expression was also greater in the forskolin group than in the control group ($n = 5$, paired *t*-tests; $P < .0005$; Figure 2D).

Full-length #f103496 cDNA analysis

The full-length #f103496 cDNA (1622 bp) contained an open reading frame of 984 bp, which was predicted to encode a 36.7 kDa protein of 328 residues. The sequence also contained a 40-bp 5' untranslated region, a 597-bp 3' untranslated region, and a terminal polyadenylation sequence. Furthermore, we identified two forms of cDNA open reading frame sequences (*typeI* and *typeII*), which differed by a single nucleotide (A or G at position 718; Figure 3A) that altered the Lys residue at position 226 to Arg (AC149904 and LC149905, respectively). Phylogenetic analysis, using amino acid sequences, placed the full-length #f103496 between the *S. salar* hsd17 β 3 and hsd17 β 12 clusters (Figure 3B), and the nucleotide sequence identities between the full-length #f103496 open reading frame and *S. salar* hsd17 β 12, hsd17 β 3a, and hsd17 β 3b were 60%, 61%, and 60%, respectively.

In addition, Blastn analysis identified another contig (#f168003) that matched the full-length #f103496 sequence. The number of #f168003 reads in the control and forskolin-treated groups were 0 and 5, respectively, and the combined total of both #f168003 and #f103496 reads was 0 and 17, respectively (Figure 3A). Meanwhile, three other contigs (mixed_23722 (#m23722), #m27275, and #f39886) were also found to encode partial *O. masou* hsd17 β 12 sequences. Their sequence identities against *S. salar* hsd17 β 12 were 94%, 97%, and 97%, respectively, and the total number of #m23722, #m27275, and #f39886 reads in the control and forskolin-treated groups were 569 and 655, respectively (Figure 3C). However, no hsd17 β 3-encoding cDNA was found in the contigs from incubated *O. masou* granulosa layers. Thus, based on data described above, the full-length #f103496 cDNA was designated as *omhsd17 β 12l*.

Heterologous *omhsd17 β 12l* expression and activity analysis

Thin-layer chromatography revealed that HEK293T cells transfected with *omhsd17 β 12l typeI* or *typeII* exhibited a strong ability to convert 17OHP to DHP (Figure 4A). In fact, the HEK293T cells transfected with *omhsd17 β 12l typeI* or *typeII* converted 94–97% of the ³H-17OHP added to their cultures to DHP, which was significantly higher than the 7–8% conversion observed in HEK293T cells transfected with an empty vector (mock; ANOVA with Fisher's PLSD, $F_{2,6} = 3857.1$ $P < .0001$; Figure 4B), and the *omhsd17 β 12l*-transfected cells also demonstrated higher conversion activity when exposed to 100 ng/mL (303 nmol/L) of cold 17OHP ($F_{2,6} = 19.4$, $P < .05$; Figure 4C). However, the 20 β -HSD activity of *omhsd17 β 12l typeI* or *typeII* was not significantly different.

Tissue-specific *omhsd17 β 12l* expression

RT-PCR analysis revealed that *omhsd17 β 12l* mRNA was barely present in head kidney, whereas it was strongly amplified from testis and ovarian follicles at FOM. However, no amplification was detected from the RNA of brain, pituitary, hind kidney, liver, spleen, heart, or vitellogenic ovarian follicles (Figure 5A).

Seasonal variation in serum DHP and *omhsd17 β 12l* expression

The serum concentration of DHP was consistently low during vitellogenic growth, from May to early September, as were the *ef1 α* -normalized *omhsd17 β 12l* mRNA levels of follicle layers, and this trend was also

observed for two of three females with oocytes at the migratory nucleus stage. However, the DHP serum concentration was considerably elevated in the third female with oocytes at the migratory nucleus stage (300 ng/mL), as was *ef1a*-normalized *omhsd17β12l* mRNA level in the follicle layers of the same female.

In contrast, serum DHP was highly elevated (90–250 ng/mL) in all four females with oocytes that had completed germinal vesicle breakdown (GVBD), although the *omhsd17β12l* mRNA levels were higher in the follicle layers of three of the four females than in those of the fourth female. In addition, the female whose follicle layers exhibited lower *omhsd17β12l* mRNA also exhibited a higher serum DHP concentration.

After ovulation, all five females retained DHP serum concentrations that were high, but lower than those during GVBD. However, the *omhsd17β12l* mRNA levels in the follicle layers from ovulated ovaries were low in all females (Figure 5B).

According to Tukey–Kramer analyses, the DHP concentration among the late vitellogenic, GVBD, and post-ovulation groups were significantly different from one another ($P < .05$). The *omhsd17β12l* mRNA levels of the GVBD group were also significantly different from the late vitellogenic and post-ovulation groups ($P < .05$), although there was no significant difference between the late vitellogenic and post-ovulation groups ($P > .05$).

***In vitro* 20β-HSD activity and *omhsd17β12l* expression in pre-ovulation granulosa layers**

Granulosa layers from females at two months before ovulation converted significantly more 17OHP to DHP when incubated with forskolin or SPE than when incubated without ($n = 4$, Tukey–Kramer, $P < .01$; Figure 6A), indicating higher levels of 20β-HSD. In addition, the *omhsd17β12l* mRNA levels were significantly higher in granulosa layers incubated with forskolin and SPE, than in those incubated without ($n = 4$, Tukey–Kramer, $P < .01$; Figure 6B), and the *ef1a*-normalized *omhsd17β12l* mRNA levels were also higher in the forskolin and SPE groups than in the control group ($n = 4$, Tukey–Kramer, $P < .01$; Figure 6C). The five females sampled at one month before ovulation (#1–5; Figure 1 and 2) also yielded similar results, although we only tested the effects of forskolin (i.e., not SPE).

In the one female sampled at the migratory nucleus stage, a small amount of DHP was detected in the control and 17OHP groups (9 ng/mL and 7 ng/mL, respectively), but when forskolin or SPE were combined with 17OHP, the granulosa layers exhibited much higher levels of 20β-HSD, as indicated by substantially higher DHP concentrations (20 ng/mL and 49 ng/mL, respectively; Figure 6D). Furthermore, the raw *omhsd17β12l* mRNA level was strikingly elevated when the granulosa layers were incubated with SPE (2600 times higher than without; Figure 6E), and the *ef1a*-normalized *omhsd17β12l* mRNA level was ~1900 times higher (Figure 6F).

***In vitro* 20β-HSD activity and *omhsd17β12l* expression in post-ovulation follicle layers**

The post-ovulation follicle layers from five experiments using different females produced significantly higher DHP in the presence of SPE than without (Tukey–Kramer, $P < .01$), and in the presence of 17OHP, forskolin and SPE induced higher DHP production (Tukey–Kramer, $P < .05$ and $.01$, respectively); however, the level of DHP production was not further enhanced by the presence of 17OHP (fors vs. 17OHP + fors, SPE vs. 17OHP + SPE;

Figure 7A). The raw *omhsd17β12l* mRNA levels were not higher in the follicle layers incubated with SPE than in those incubated in Ringer medium alone (Tukey–Kramer, $P < .05$; Figure 7B), and no significant difference was observed between the *ef1α*-normalized *omhsd17β12l* levels of follicle layers incubated in Ringer medium alone or in Ringer medium with SPE (Tukey–Kramer, $P < .05$; Figure 7C).

However, the *ef1α*-normalized *omhsd17β12l* level of the follicle layer control group (0.469 ± 0.094 , $n = 5$) was significantly higher than that of the granulosa layer control group of vitellogenic females (0.008 ± 0.003 , $n = 9$, Tukey–Kramer; $P < .01$) and lower than that of the SPE-treated granulosa layers of females at two months pre-ovulation (7.116 ± 3.655 , $n = 4$, Tukey–Kramer; $P < .05$).

Discussion

Since the isolation of a cDNA encoding CR/20β-HSD from pig (4), many studies have targeted CR/20β-HSD orthologs as being responsible for maturation-inducing hormone (MIH) synthesis (7, 28–32). Some studies demonstrated weak 20β-HSD activity of CR/20β-HSD towards 17OHP (7, 30), whilst others did not check its activity (28, 29, 31, 32). Moreover, several reports used the gene name 20β-HSD without even using the prefix CR (29–31). In this study, we do not find support for crediting CR/20β-HSD with MIH production but instead present compelling evidence that 17β-HSD type 12-like is the enzyme responsible for DHP production in masu salmon. Accordingly, we urge researchers who study the regulation of MIH production during FOM in other teleosts to reconsider whether CR/20β-HSD is suitable for that purpose.

Relationship between 20β-HSD activity and CR/20β-HSD mRNA levels in cultured granulosa cells

Previous studies identified two forms of *CR/20β-HSD* from rainbow trout and demonstrated that one form (*typeA*) exhibited weak 20β-HSD activity when offered 17OHP, whereas the other (*typeB*) did not have any 20β-HSD activity. (5). In the present study, we found that the mRNA levels of both *CR/20β-HSD typeA* and *typeB* in forskolin-induced granulosa were no greater than the levels observed in the control group. This result was inconsistent with a previous study that reported that the induction of 20β-HSD activity in granulosa cells requires *de novo* mRNA synthesis (19). The only other relevant *in vivo* study reported 7-fold increases in the *CR/20β-HSD* mRNA levels of follicles during sexual maturation of rainbow trout (33). However, the *CR/20β-HSD* autoradiograph band of the study's northern blot analysis (per 100 ng of poly(A) RNA) failed to indicate a substantial increase in *CR/20β-HSD* mRNA during the final maturation period. These findings, together with those from the present study, prompt us to conclude that *CR/20β-HSD* does not encode the 20β-HSD that is responsible for DHP production during the period of oocyte maturation in salmonid species.

Characterization of 17β-HSD type 12-like

In the present study, RNA-seq analysis identified one contig (#f103496) as encoding a putative hydroxysteroid dehydrogenase from among 953 contigs that were constructed exclusively from the forskolin-treated group reads. The full-length #f103496 sequence was placed between Atlantic salmon (*S. salar*) hsd17β3 and

hsd17 β 12 but did not seem orthologous to either. Rather, a substantial number of reads mapped to three contigs that matched *S. salar* hsd17 β 12, but this number was comparable in both the control and forskolin groups, suggesting that hsd17 β 12 does not play a role in 20 β -HSD activity. Although we were unable to locate a contig that sufficiently matched *S. salar* hsd17 β 3a and 3b, full-length #f103496 is obviously not an hsd17 β 3 ortholog, since full-length #f103496 and *S. salar* hsd17 β 3 exhibit low sequence identity (only 35–36% amino acid identity). Therefore, we suggest that #f103496 differs from hsd17 β 12 and hsd17 β 3 and have designated the gene as *omhsd17 β 12-like* (*omhsd17 β 12l*).

Mindnich et al. (34) recently identified a novel 17 β -HSD-like enzyme similar to hsd17 β 3 and hsd17 β 12 in the zebrafish genome. The group demonstrated that the enzyme was able to convert cortisone to 20 β -hydroxycortisone and accordingly named the gene 20 β -HSD type2 (*hsd20 β 2*) (35). Subsequently, *omhsd17 β 12l* was classified in the *hsd20 β 2* subcluster in a phylogenetic tree built by Tokarz et al. (35). Therefore, *omhsd17 β 12l* is considered a masu salmon ortholog of zebrafish *hsd20 β 2* (*drhsd20 β 2*).

The present study clearly demonstrated that both *omhsd17 β 12l* typeI and typeII exhibited considerable 20 β -HSD activity, converting 17OHP to DHP in high yields. Furthermore, the expression of *omhsd17 β 12l* was strictly limited to maturing ovaries and sperm-containing testis, which may indicate that *omhsd17 β 12l* is involved in reproduction-related steroid synthesis in masu salmon. In zebrafish, 20 β -HSD activity of *drhsd20 β 2* towards 17OHP has not yet been examined; nevertheless, the authors suggested that *drhsd20 β 2* was not likely to play a role in reproduction-related steroid biosynthesis, owing to its ubiquitous expression pattern in all the tissues examined, both in adult fish and throughout embryogenesis (35).

Changes in serum DHP and *omhsd17 β 12l* mRNA levels during sexual maturation

The serum DHP concentration and *omhsd17 β 12l* mRNA levels in the follicle layers were largely well-correlated, but the odd exception was evident; thus, although all four post-GVBD females exhibited elevated serum DHP levels, a notable increase in *omhsd17 β 12l* transcript abundance in the follicle layers was observed in only three of the fish, *omhsd17 β 12l* mRNA levels remaining relatively low in the fourth female. This inconsistency can be attributed to the degradation of *omhsd17 β 12l* transcripts, which may occur rapidly after expression. In fact, such inconsistencies between serum DHP and *omhsd17 β 12l* expression were also observed in the follicles of post-ovulation females. Serum DHP concentrations remained relatively high in all five post-ovulation females, but the *omhsd17 β 12l* transcripts were relatively low, again suggesting that the expression of *omhsd17 β 12l* is temporal and only exists for a short time span. In contrast, *omhsd17 β 12l* protein and serum DHP can persist for relatively long periods.

Relationship between induction of 20 β -HSD activity and up-regulation of *omhsd17 β 12l* mRNA expression *in vitro*

Our present *in vitro* investigation showed a close relationship between the induction of 20 β -HSD activity and up-regulation of *omhsd17 β 12l* mRNA by forskolin and SPE in granulosa layers at one or two months before

ovulation. Human chorionic gonadotropin (HCG) exhibited similar effects as the forskolin and SPE treatments (Supplemental Figure 5), making it reasonable to assume that forskolin and SPE are mimicking luteinizing hormone stimulation. After the migratory nucleus stage, in most follicles, the connection between granulosa layers and the oocyte membrane becomes loose (reduced inter-digitation), which allows granulosa layers to be manually removed along with theca layers. Therefore, granulosa layers were only successfully separated from a single female at the migratory nucleus stage. Although statistical analysis was not possible, we found that the *omhsd17β12l* mRNA level was drastically elevated in the presence of SPE, by over 10 times that of granulosa layers from one or two months before ovulation. This indicates that the responsiveness of *omhsd17β12l* transcription to induction increases further during the FOM period. However, the 20β-HSD activity of the migratory nucleus-stage granulosa layer was not elevated as drastically as the up-regulation of *omhsd17β12l*. Therefore, changes in 20β-HSD activity and *omhsd17β12l* mRNA levels of incubated granulosa cells apparently do not exhibit a linear relationship. This suggests that post-transcriptional and/or post-translational regulation may exist over and beyond the principal regulation by transcription.

In the post-ovulation follicle layers, DHP production could still be induced by SPE stimulation, whereas *omhsd17β12l* expression could not. This observation is consistent with our previous report that 20β-HSD activity becomes non-inducible in the post-ovulation granulosa layers of amago salmon (11). The increased DHP production by ovulated follicle layers is probably caused by stimulated 17OHP production in the theca rather than by induction of 20β-HSD activity; indeed, addition of exogenous 17OHP (SPE vs. SPE + 17OHP) did not further increase DHP production beyond that achieved with SPE only, as was reported from amago salmon previously (11). Both 20β-HSD activity and *omhsd17β12l* expression thus appear to have become insensitive to induction by SPE and the gene may have been silenced after its activation during FOM. Furthermore, we also found that the levels of *omhsd17β12l* mRNA in the control incubations of post-ovulation follicles remained relatively high, when compared with the *omhsd17β12l* mRNA levels of granulosa layers from females at one and two months before ovulation, which suggests that detectable levels of *omhsd17β12l* mRNA remain in the ovulated follicles. Accordingly, post-ovulation follicles retain 20β-HSD activity.

Future directions

To date, fourteen different types of 17β-HSD have been identified in mammals (36). Some of these are known to be multifunctional because of their preference for diverse substrates, extending well beyond steroids (37, 38). Moreover, human HSD17B2 was shown to have 20α-HSD (oxidase) activity using 20α-progesterone as substrate, aside from its catalytic 17β-HSD (oxidase) activity with estradiol and testosterone (39, 40). We have now demonstrated profound 20β-HSD activity by *omhsd17β12l*, resulting in DHP production, yet, we have retained the term *hsd17β* as the gene name in view of its possible multifunctionality. However, if 20β-HSD activity of *hsd17β12l* orthologs are conserved among other taxa, then it should be renamed as *hsd20β*. Such characterization of *hsd17β12l* in other species is currently in progress.

Acknowledgements

We would like to thank Etsuro Yamaha at the Nanae Fresh-Water Laboratory for providing masu salmon.

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Supplemental figure legends

Supplemental Fig. 1. qPCR primer sets used for *ef1α*, *omhsd17β12l*, and CR/20β-HSD typeA and typeB.

Supplemental Fig. 2. Production of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) by isolated granulosa layers from females #1–5. Vertical bars represents the means ± SD (n = 3).

Supplemental Fig. 3. Complementary DNA sequences and primer sets used for qPCR of 13 genes that were identified as up-regulated under forskolin treatment by cDNA subtraction analysis.

Supplemental Fig. 4. The number of mapped reads and cDNA levels in incubated granulosa layers and the mRNA levels of previously identified forskolin-induced genes in *in vivo* sampled follicle layers. The number of reads represents the total number of reads mapped to cDNA sequences from the control and forskolin-treated groups (A, B). Relative cDNA levels were measured by quantitative PCR, using 50 ng of amplified cDNA that was prepared from incubated granulosa layers (C), and the expression level of each gene was normalized using *ef1α* mRNA levels in 45 ng of total RNA from follicle layers collected from *in vivo* samples collected from a single female at each developmental stage (D).

Supplemental Fig. 5. 20β-Hydroxysteroid dehydrogenase activity and *omhsd17β12l* mRNA levels in incubated granulosa layers of a single female, at one month before ovulation. Incubations were conducted in Ringer medium with no additives (cont) or 100 ng/mL 17α-hydroxyprogesterone (17OHP), or a combination of 17OHP and human chorionic gonadotropin (HCG; 100 IU/mL; Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Fukushima, Japan). The post-incubation concentrations of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in the incubation media are represented as the mean ± SD of three replicate incubations (A). Levels of *omhsd17β12l* and *ef1α* mRNA in 45 ng of total RNA from granulosa layers incubated in Ringer medium (cont, open columns) or with HCG (gray column) (B). *ef1α*-Normalized *omhsd17β12l* mRNA levels (C).

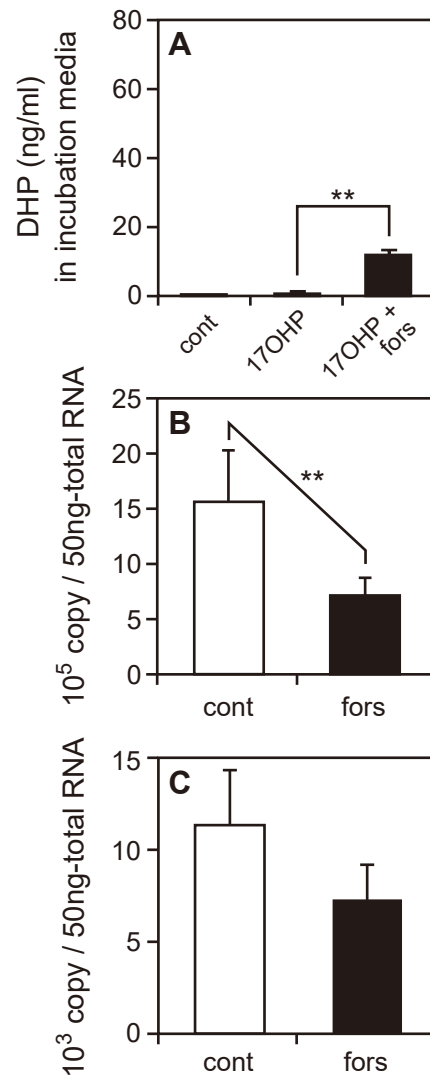


Figure 1. Production of $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) by isolated granulosa layers incubated in Ringer medium alone (cont), Ringer medium with 100 ng/mL 17α -hydroxyprogesterone (17OHP), or Ringer medium with 100 ng/mL 17OHP and 10 μ M forskolin. Each vertical bar represents the mean $\pm$ SE of three replicate incubations from five females (total 15 incubations) (A). The raw mRNA levels of CR/20 β -HSD typeA (B) and typeB (C) in granulosa layers ($n = 5$) incubated in Ringer medium alone or in Ringer medium with forskolin. The double asterisk indicates a significant difference of $P < .01$.

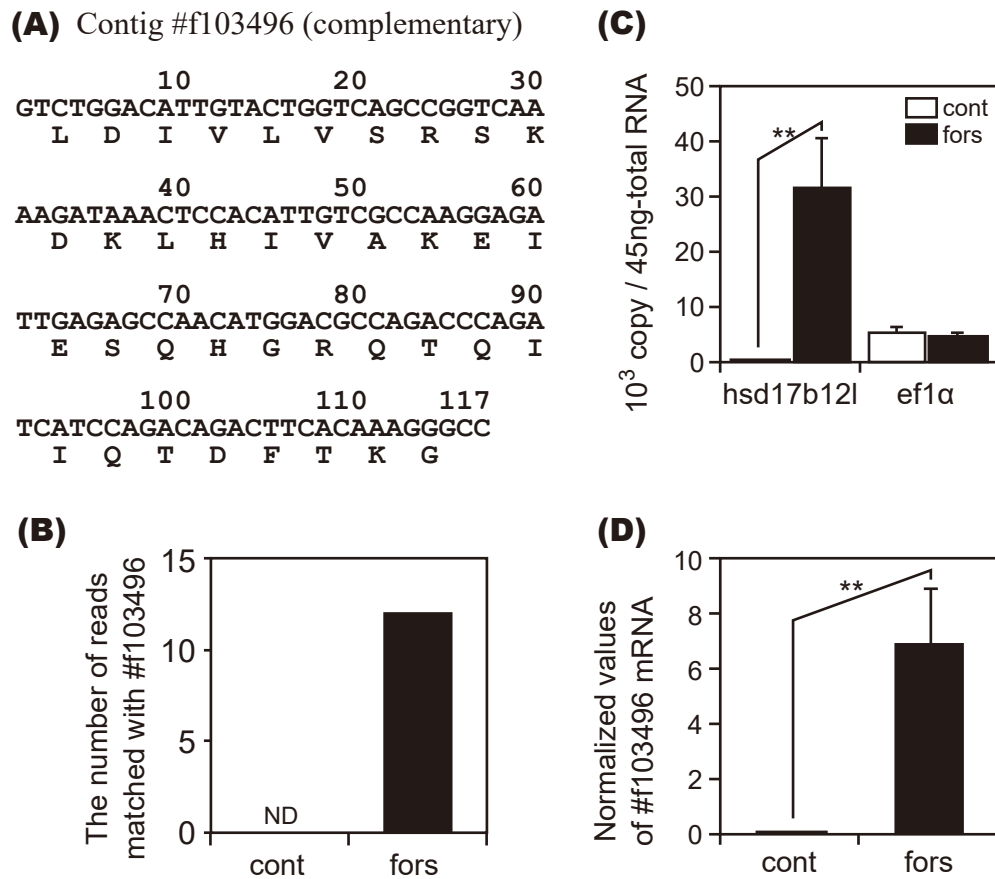
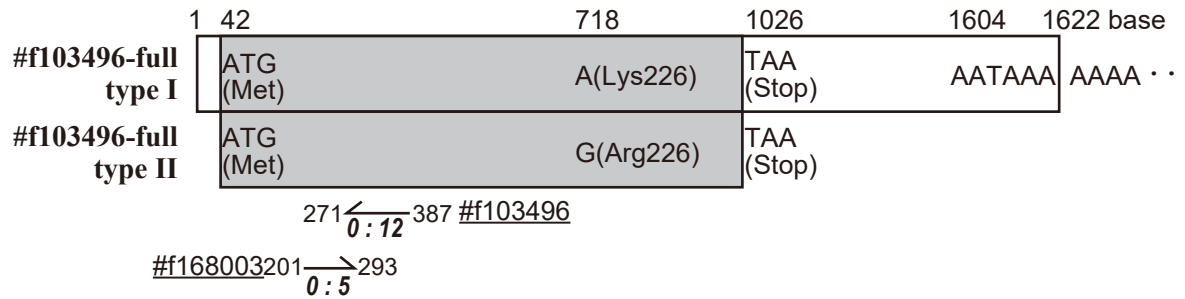
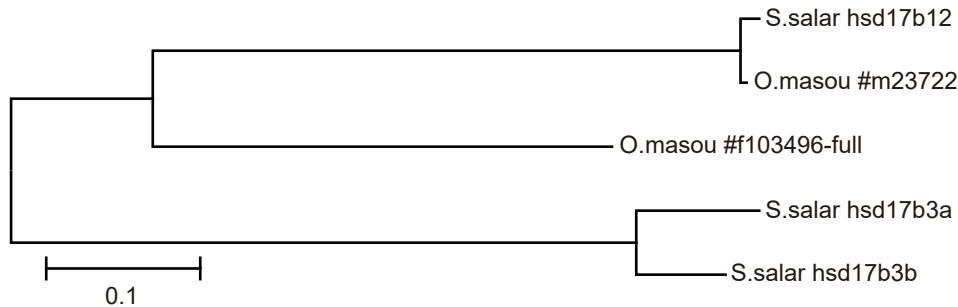


Figure 2. Sequence and expression of contig #f103496. Nucleotide and predicted amino acid sequences of contig #f103496, which encodes an amino acid sequence similar to hsd17 β type 12 (A). The number of reads mapped to contig #f103496 from control (Cont) and forskolin-treated (Fors) groups. ND, not detected (B). The raw levels of #f103496 and ef1 α mRNA in 45 ng of total RNA. Open and closed bars represent levels in the control or forskolin-treated groups, respectively (C). ef1 α -Normalized #f103496 mRNA levels (D). Each vertical bar represents the mean $\pm$ SE of five experiments using different females (B and C). The triple asterisk indicates a significant difference of $P < .0005$.

(A)



(B)



(C)

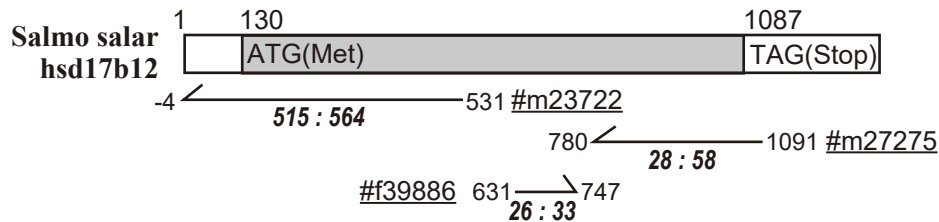


Figure 3. Schematic representation of the two coding sequence forms of full-length #103496-encoding cDNA (typeI and typeII). The closed bars represent the translated coding region and the open bars represent the 5'- and 3'-untranslated regions (A). Phylogenetic relationships were determined using predicted amino acid sequences from the masu salmon (*O. masou*) #f103496-full sequence; contig #m23722, which matched *Salmo salar* hsd17b12; *S. salar* hsd17b3a; and *S. salar* hsd17b3b (B). Schematic representation of the coding sequence of *S. salar* hsd17b12-encoding cDNA and matching contigs from *O. masou* (C). The numbers above the bars represent nucleotide positions, and the arrows underneath the bars represent the matching contigs. Right or left arrows indicate forward or reverse matching contigs, respectively. The numbers attached to arrows indicate the nucleotide position corresponding to the *S. salar* sequence, and underlined numbers indicate contig names. The bold italic numbers under the arrows indicate the number of mapped reads from the control and forskolin-treated groups.

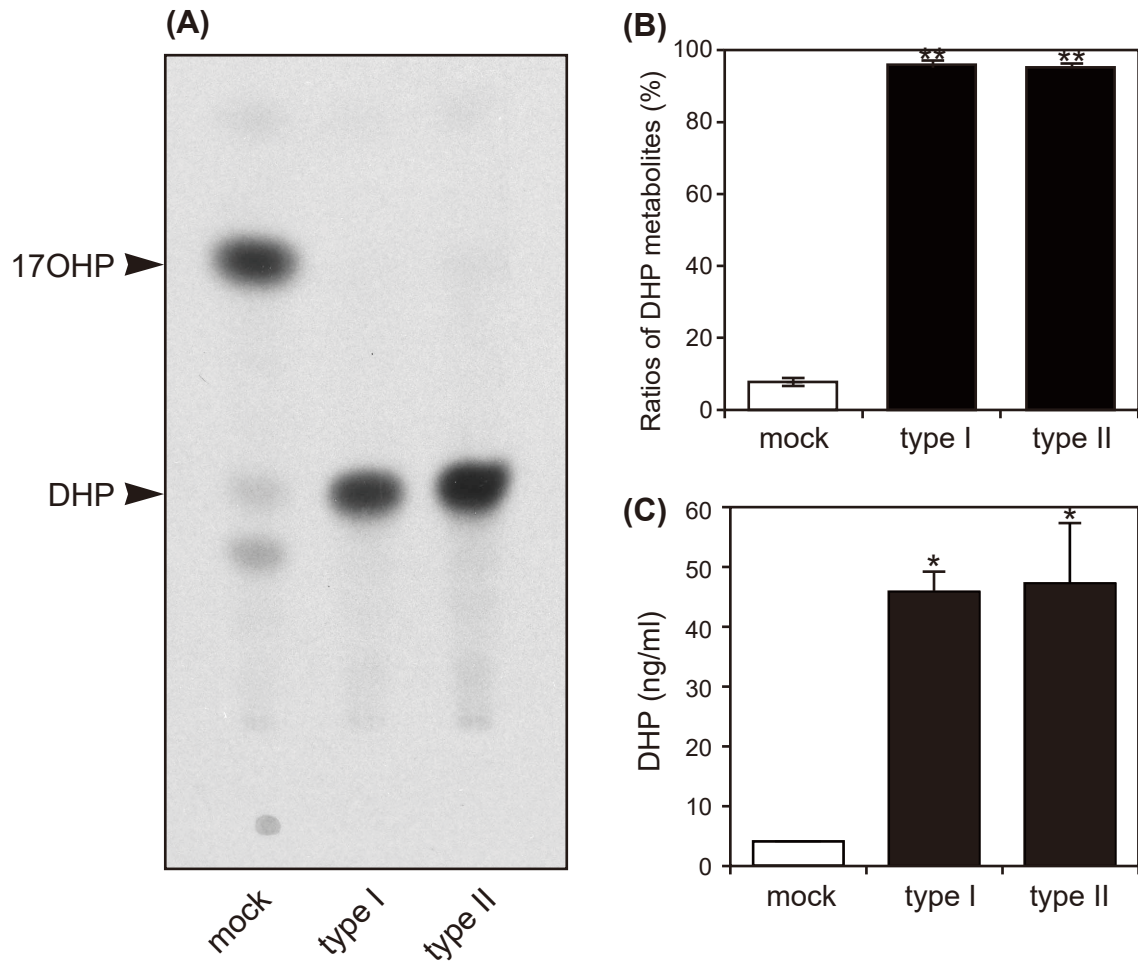


Figure 4. Enzyme activity of omhsd17 β 12l. Autoradiograph of thin-layer chromatography, illustrating the conversion of 3H-labeled 17 α -hydroxyprogesterone (17OHP) to 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) by HEK293T cells transfected with pSI containing either empty vector (mock) or omhsd17 β 12l typeI or typeII (A). The percent conversion of 17OHP to DHP was calculated from the autoradiograph using ImageJ (B). DHP production by HEK293T cells transfected with pSI constructs in the presence of 100 ng/mL cold 17OHP (C). In both experiments, three replicate independent transfections were conducted for each treatment. Each vertical bar represents the mean $\pm$ SE. Double and single asterisks indicate statistical differences at $P < .0001$ and $< .05$, respectively.

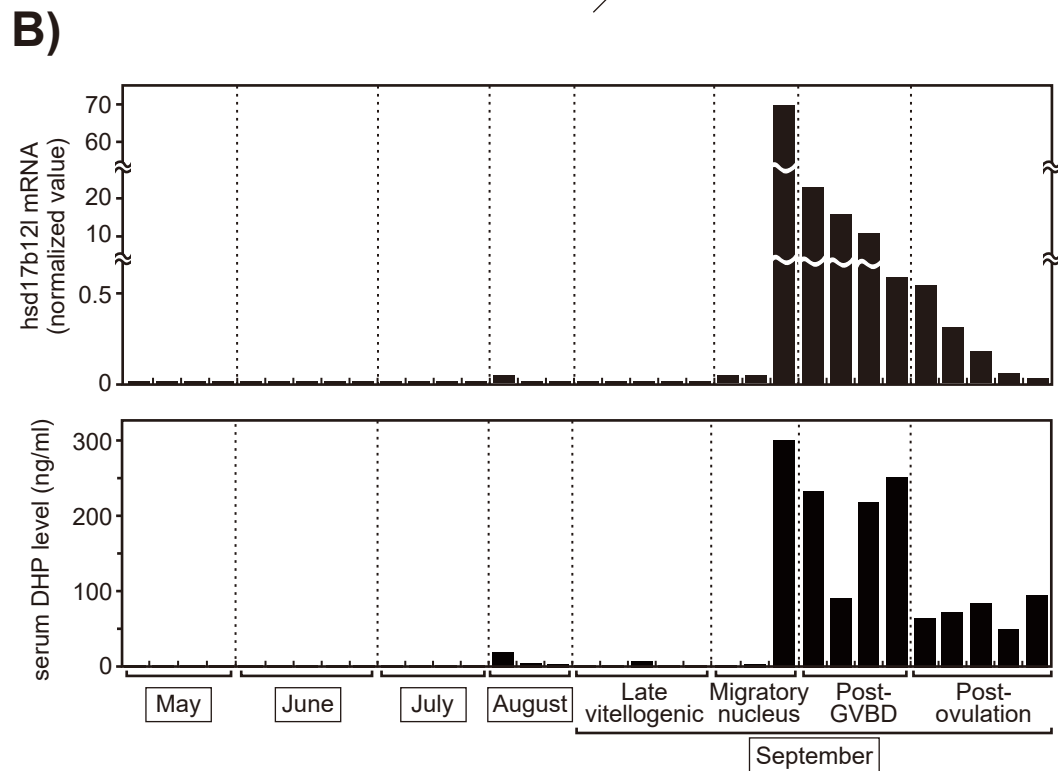
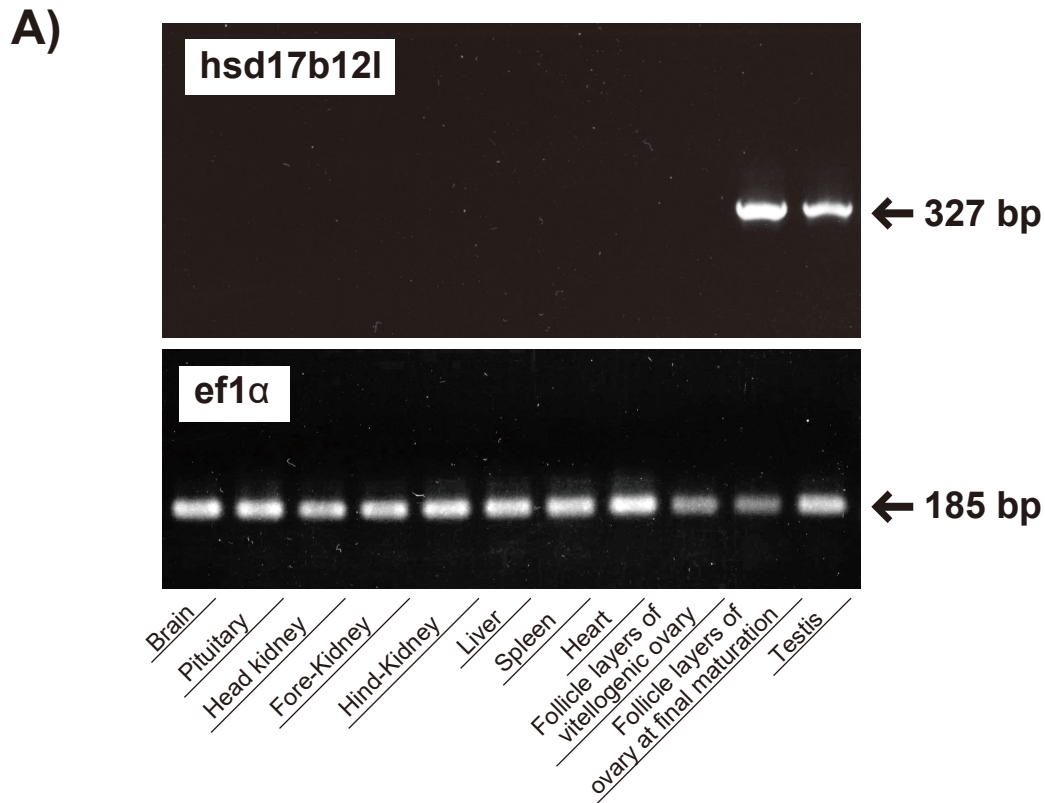


Figure 5. Tissue-specific omhsd17 β 12l expression and its relationship with serum DHP concentrations in ovarian follicles. Tissue-specific expression of the masu salmon (*O. masou*) omhsd17 β 12l transcript, as determined by RT-PCR. Amplification of ef1 α was used as a positive control (A). Changes in serum 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) concentration and normalized values of omhsd17 β 12l in follicle layers during sexual maturation in masu salmon (B). The corresponding upper and lower column values are derived from the same fish. Migratory nucleus, follicle layers from ovaries with oocytes in which germinal vesicles were visibly close to the cell periphery. Post-GVBD, follicle layers from ovaries with oocytes after germinal vesicle breakdown. Post-ovulation, follicle layers from ovaries after ovulation.

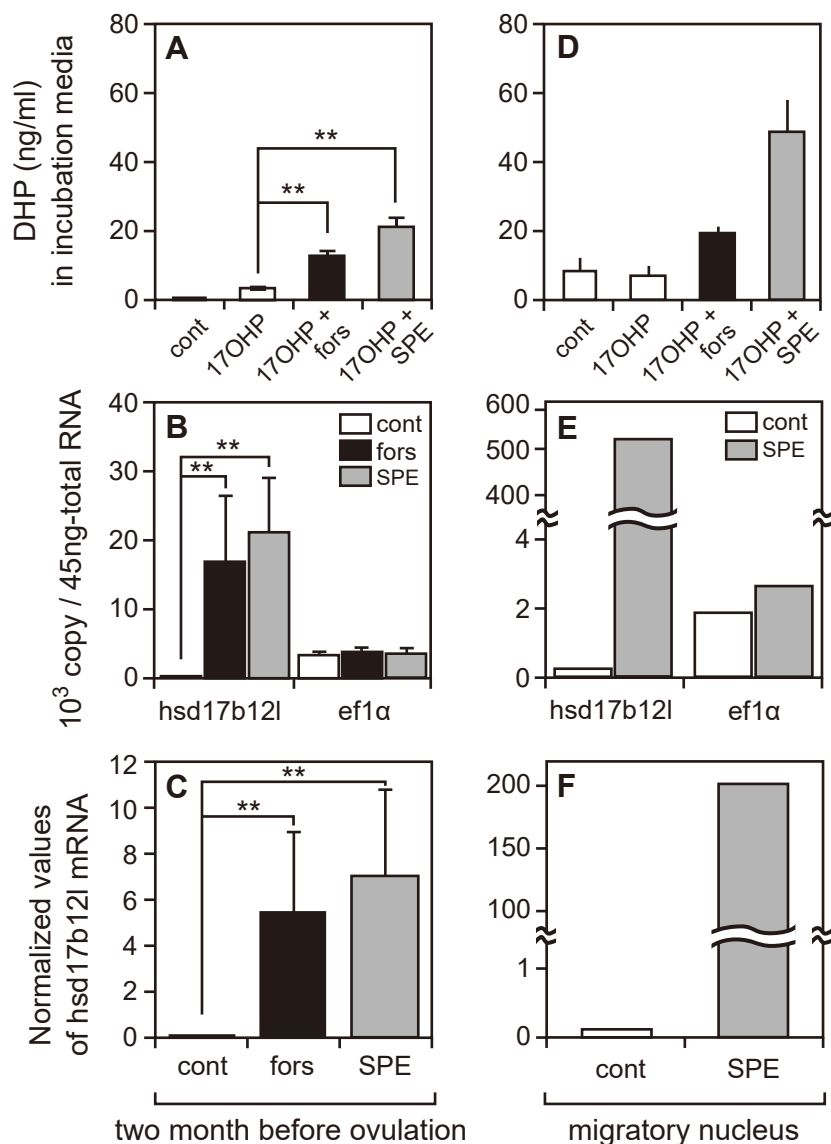


Figure 6. 20β -Hydroxysteroid dehydrogenase activity and omhds17 β 12l mRNA levels in incubated granulosa layers in female masu salmon. Measurements were taken at two months before ovulation (A, B, C) and at the migratory nucleus stage (D, E, F). The concentration of $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) in media after incubations, that were conducted in Ringer medium alone (cont) or in Ringer medium with 100 ng/mL 17α -hydroxyprogesterone (17OHP), 17OHP and 10 μ M forskolin (17OHP + fors), or 17OHP and 100 μ g/mL salmon pituitary extract (17OHP + SPE) (A, D). Levels of omhds17 β 12l and ef1 α mRNA in 45 ng of total RNA from granulosa layers incubated in Ringer medium alone (open column), Ringer medium with forskolin (closed column), or Ringer medium with SPE (gray column) (B, E). ef1 α -normalized omhds17 β 12l mRNA levels (C, F). Each vertical bar represents the mean $\pm$ SE from four experiments using different females (A, B and C), or mean $\pm$ SD from single female (D). The double asterisk indicates a significant difference of $P < .01$.

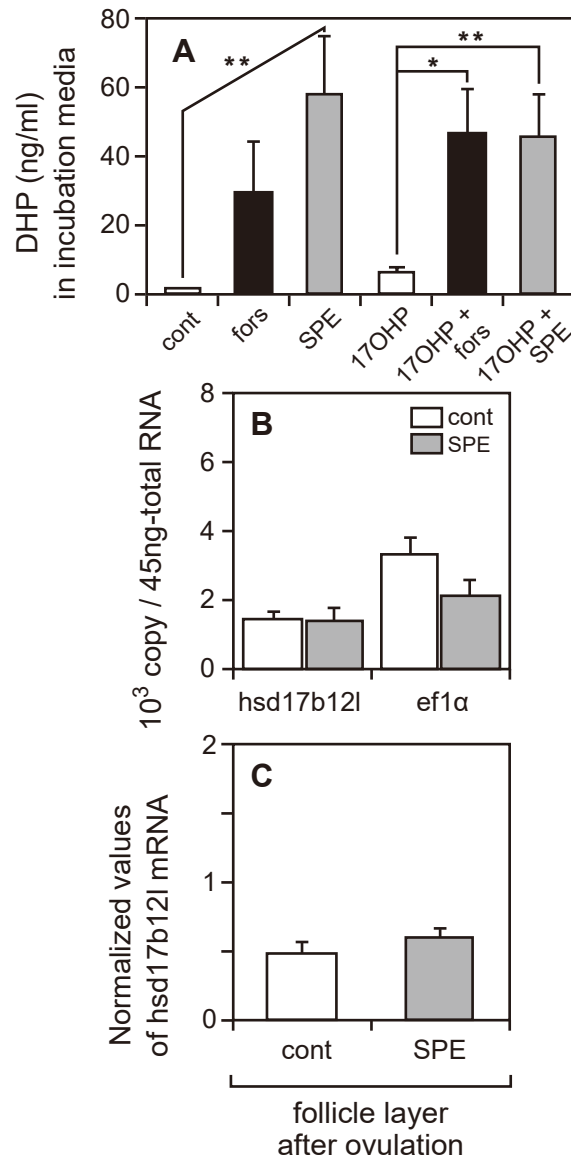


Figure 7. $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) production and omhsd17 β 12l mRNA levels in the incubated follicle layers. Incubations were conducted in Ringer medium with no additives (cont) or with 100 ng/mL 17α -hydroxyprogesterone (17OHP), 10 μ M forskolin (fors), 100 μ g/mL salmon pituitary extract (SPE), or a combination of 17OHP and either forskolin or SPE (A). Levels of omhsd17 β 12l and ef1 α mRNA in 45 ng of total RNA from follicle layers incubated in Ringer medium (cont, open columns) or with SPE (gray column) (B). ef1 α -Normalized omhsd17 β 12l mRNA levels (C). Each vertical bar represents the mean $\pm$ SE from five experiments using different females. The asterisk and double asterisk indicate significant differences of $P < .05$ and $P < .01$, respectively.

Masu salmon, *Oncorhynchus masou* ef1a partial cDNA sequence (GenBank Accession No. LC149908)

TCTGTGGAGATGCACCACGAGACCCTGGAAGCGGCTCTACCCGGTGACAATGTTGGCTTCAACGTCAAGAACGTGTCCGTCAA
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 ATCCTGAACCACCCGGGCCAGATCTCCCAGGGCTATGCCCCGTAAGTGGATTGCCACACCGCTCACATCGCCTGCAAGTTCAG
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 TCATTGTCATGGTGCCAGGAAAGCCCATGTGTGTGGAGAGCTTCGCCGCCTACCCTCCCTTG/GTCGTTTTGCCGTGCGCGA
 CATGAGACAGACCGTTGCCGTTGGTGTTCATCAAGGCCGTCGACAAGAAGGCTGCCAGCACTGGCAAGGTGACCAAGTCCGCCA
 TTAAGGCCACCAAAGCCAAA

"/" represents predicted exon/intron boundary

ef1a 1-forward: AAATCTGGAGACGCCGCCA

ef1a 2-reverse (complementary): CCTTG/GTCGTTTTGCCGT

ef1a 3-reverse (complementary): AAGGTGACCAAGTCCGCCAT

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TCTGTGGAGATGCACCACGAGACCCTGGAAGCGGCTCTACCCGGTGACAATGTTGGCTTC					
S V E M H H E T L E A A L P G D N V G F					
70	80	90	100	110	120
AACGTCAAGAACGTGTCCGTCAAGGATATCCGTCGTGGCAACGTGGCTGGAGACAGCAAG					
N V K N V S V K D I R R G N V A G D S K					
130	140	150	160	170	180
AATGACCCCCCAATGGAGGCCGGCAACTTCACAGCTCAGGTTCATCATCCTGAACCACCCC					
N D P P M E A G N F T A Q V I I L N H P					
190	200	210	220	230	240
GGCCAGATCTCCAGGGCTATGCCCCGTAAGTGGATTGCCACACCGCTCACATCGCCTGC					
G Q I S Q G Y A P V L D C H T A H I A C					
250	260	270	280	290	300
AAGTTCAGCGAGCTCAAGGAGAAGATCGACCGTCGGTCCGGCAAGAAGCTTGAGGACAAC					
K F S E L K E K I D R R S G K K L E D N					
310	320	330	340	350	360
CCCAAAGCCCTGAAATCTGGAGACGCCGCCATCATTGTCATGGTGCCAGGAAAGCCCATG					
P K A L K S G D A A I I V M V P G K P M					
370	380	390	400	410	420
TGTGTGGAGAGCTTCGCCGCCTACCCTCCCCTTGGTCGTTTTGCCGTGCGCGACATGAGA					
C V E S F A A Y P P L G R F A V R D M R					
430	440	450	460	470	480
CAGACCGTTGCCGTTGGTGTTCATCAAGGCCGTCGACAAGAAGGCTGCCAGCACTGGCAAG					
Q T V A V G V I K A V D K K A A S T G K					
490	500	510	520		
GTGACCAAGTCCGCCATTAAGGCCACCAAAGCCAAA					
V T K S A I K A T K A K					

Comparison with medaka genomic sequence: ENSORLT00000002805

exon5 GTATTGGAACAGTCCCTGTAGGTCGCGTGGAAGTGGGGTACTAAACCTGGCATGGTTG 60
 masu -----

exon5 TGACCTTTGCCCCAGTCAACGTGACCACTGAAGTGAAGTCTGTGGAGATGCACCATGAGG 120
 masu -----TCTGTGGAGATGCACCACGAGA 22

exon5 masu	CTCTAACCGAGGCTCTTCCTGGAGACAATGTGGGCTTTAATGTCAAGAATGTGTCCAGTCA CCCTGGAAGCGGCTCTACCCGGTGACAATGTTGGCTTCAACGTCAAGAACGTGTCCGTCA * * * *	180 82
exon5 masu	AAGATATACGCCGTGGGAATGTGGCTGGCGACAGCAAGAATGACCCCCCGCAGGAAGCTG AGGATATCCGTCGTGGCAACGTGGCTGGAGACAGCAAGAATGACCCCCCAATGGAGGCCG *	240 142
exon5 masu	CCA ACTTCACTGCTCAG----- GCAACTTCACAGCTCAG----- *	257 159
exon6 masu	-----GTGATCATTTCTCAACCACCCGGGCCAGATTAGTGCAGGCTACGCTCCTGT -----GTCATCATCCTGAACCACCCGGGCCAGATCTCCCAGGGCTATGCCCCCGT *	50 209
exon6 masu	GCTAGACTGTCACACTGCTCACATTGCCTGCAAGTTTGCAGAGCTGAAGGAAAAAATTGA ACTGGATTGCCACACCGCTCACATCGCCTGCAAGTTTCAGCGAGCTCAAGGAGAAGATCGA *	110 269
exon6 masu	TCGTCGCTCAGGGAAGAAGCTAGAGGACAACCCCAAGTCTCTCAAGTCAGGAGATGCCGC CCGTCGGTCCGGCAAGAAGCTTGAGGACAACCCCAAAGCCCTGAAATCTGGAGACGCCGC *	170 329
exon6 masu	CATCGTAGACATGATTCTGCGGAAGCCCATGTGTGTTGAGAGCTTCTCCGAGTACCCTCC CATCATTGTGATGGTGCCAGGAAAGCCCATGTGTGTTGAGAGCTTCCGCCCTACCCTCC *	230 389
exon6 masu	ACTGG----- CCTTG----- * * *	235 394
exon7 masu	GTCGTTTTGCGGTTTCGCGACATGCGCCAGACCGTTGCCGTTGGTGTGATAAAGGGGGGTGG GTCGTTTTGCCGTGCGCGACATGAGACAGACCGTTGCCGTTGGTGTGATCAAGGCCGCTCG *	60 454
exon7 masu	AGAAGAAAGTCTCCACCACTGGTAAGGTCACCAAGTCTGCTCAGAAGGCCCCAGAGGAACA ACAAGAAGGCTGCCAGCACTGGCAAGGTGACCAAGTCCGCCATTAAAGGCCACCAAGCCA *	120 514
exon7 masu	AATGA AA-- * *	125 516

Masu salmon, *Oncorhynchus masou* omhsd17β12l complete cDNA sequence (GenBank Accession No. LC149904 (typeI), LC149905 (typeII))

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GCAAATGAG/TTGGCCAGAAAGAGGTCTGGACATTGTACTGGTCAGCCGGTCAAAAGATAAACTCCACATTGTCGCCAAGGAG**A**
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"/" represents predicted exon/intron boundary

omhsd17β12l 1-forward: **GAGTGAAATTTGCCAGACATGG**

omhsd17β12l 2-reverse (complementary): **GATTG/AGAGCCAACATGGACG**

omhsd17β12l 3-forward: **ATGGACAATGGGCAG/TTGTCA**

omhsd17β12l 4-reverse (complementary): **TACGCAAATGAG/TTGGCCAGAA**

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M D T V S D S					
70	80	90	100	110	120
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M L V R G L V F I G G F T V L Y Y M L K					
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W S W I C W C G F R V Y V L S K V W Q T					
190	200	210	220	230	240
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D L K A Y G Q W A V V T G A T A G I G K					
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310	320	330	340	350	360
AAGATAAACTCCACATTGTCGCCAAGGAGATTGAGAGCCAACATGGACGCCGGACCCAGA					
D K L H I V A K E I E S Q H G R R T Q I					
370	380	390	400	410	420
TCATCCAGACAGACTTCACAAAGGGCCATGACATCTACCCTGCTATAGCTGAGGCACTAC					
I Q T D F T K G H D I Y P A I A E A L R					
430	440	450	460	470	480
GGGACCTGGACATAGGCATCCTGGTTAACAATGTAGGCATGAAGTATTCTGACAAGTTGG					
D L D I G I L V N N V G M N Y S D K L V					

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  S  A  T  K  I  F  V  T  Y  F  S  R  S  L  N  S  E  Y  #  S

      730      740      750      760      770      780
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  Q  G  I  T  V  Q  C  V  A  P  F  M  V  S  T  N  M  T  H  N

      790      800      810      820      830      840
ACTTGCCCCCAACCTGTTGTTGAAGAGTGCCAGTGCTTTTGCCCGTGAAGCTCTGAACA
  L  P  P  N  L  L  L  K  S  A  S  A  F  A  R  E  A  L  N  T

      850      860      870      880      890      900
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  V  G  H  S  S  Y  T  S  G  C  V  S  H  A  L  Q  N  I  A  L

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      970      980      990      1000      1010      1020
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  D  *

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      1390      1400      1410      1420      1430      1440
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      1570      1580      1590      1600      1610      1620
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#: K or R

Comparison with medaka genomic sequence: ENSORLT00000009884

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masu              151 CTGTCAAAGTTTGGCAAACCTGATTTAAAGGCATATGGACAATGGGCAG 210
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                   ***** ** * * * * * * * * * * * * * * * * *
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medaka_exon3      GTTGCCAAGGAGATCG 76
masu              76 GTCGCCAAGGAGATTG 333
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medaka_exon4      AAAAGGAGTTTGGACAGAAGACTCGCACCATCCGAGTGGACTTCACAGACGGCTGCAGCA 60
masu              334 AGAGCCAACATGGACGCCAGACCCAGATCATCCAGACAGACTTCACAAAGGGCCATGACA 393
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Masu salmon, *Oncorhynchus masou* CR/20 β -HSD typeA partial cDNA sequence (GenBank Accession No. LC149906)

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GCTGCCCTGGCTGGGT

CR/20 β -HSD typeA forward: CCGGTGCCAATAAAGGCAT

CR/20 β -HSD typeA reverse (complementary): CTTGCGATTGTGAGGGAGCT

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K V A V V T G A N K G I G L A I V R E L					
70	80	90	100	110	120
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C K A K F T G D V I L T A R N E K L G N					
130	140	150	160	170	180
GAGGCAGTGAAGATGCTGAAGTCTGAAGGATTTGAAGTTGCTTTCCACCACCTTGATATC					
E A V K M L K S E G F E V A F H H L D I					
190	200	210	220	230	240
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250	260	270	280	290	300
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D V L I N N A G M A F K N D A T E T F G					
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E Q A E V T M R T N F W G T L W V C H A					
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CTCCTACCCCTCCTCAGACCAAATGCCAGAGTGGTGAATGTCTCCAGCTTTGTTAGCAAG					
L L P L L R P N A R V V N V S S F V S K					
430	440	450	460	470	480
AAGGCTCTTGATACATGCAGCCCTCAACTACAAGCCAAGTTCCGTGATACTGAGCTCTCT					
K A L D T C S P Q L Q A K F R D T E L S					
490	500	510	520	530	540
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E E E L C L L M G Q F V I A A Q Q G N H					
550	560	570	580	590	600
CAGGCCAGGGGTGGCCAAACACAGCCTATGGCACAACAAAGATCGGAGTGACTGTGCTG					
Q A Q G W P N T A Y G T T K I G V T V L					
610	620	630	640	650	660
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A C C P G W					

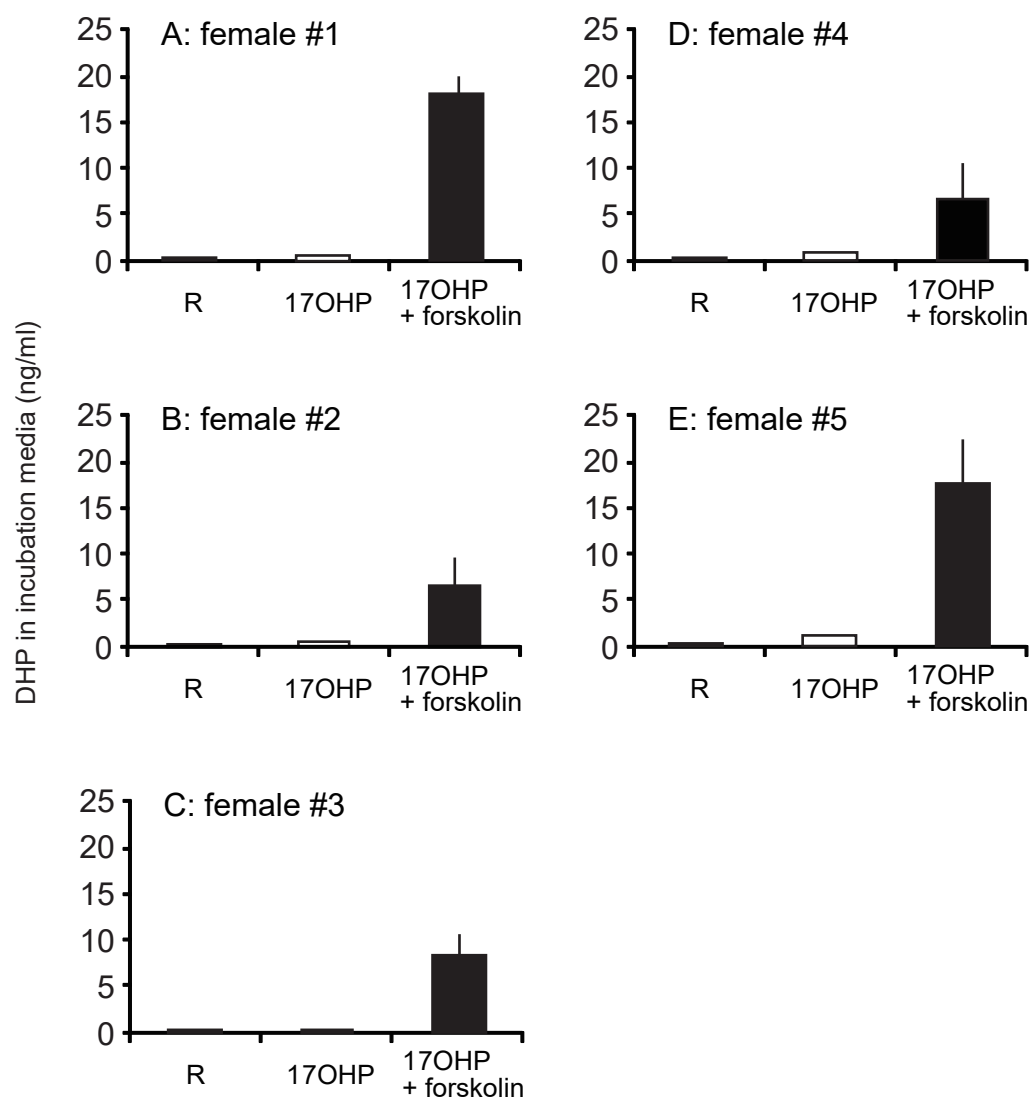
Masu salmon, *Oncorhynchus masou* CR/20 β -HSD typeB partial cDNA sequence (GenBank Accession No. LC149907)

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ATTAACAACGCTGGAATGTCCTTTA**AAAATGATGCGACTGAGACTTTTGG**GGAACAGGCTGAGGTGACCATGCGCACCAACTT
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GATCGGAGTGACTGTGTTGTCCAGGATTCAGGCTCATTTTCTGACTAAGACCCGGGCAGCTGATGGAATCCTGCTCAACGCCT
GCTGCCCTGGCTGGGT

CR/20 β -HSD typeB forward: **TCATTAACAACGCTGGAATGTCC**

CR/20 β -HSD typeB reverse (complementary): **AAAATGATGCGACTGAGACTTTTGG**

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70	80	90	100	110	120
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C K A K F T G D V I L T A R N E K L G N					
130	140	150	160	170	180
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E A V K M L K S E G F E V A F H H L D I					
190	200	210	220	230	240
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D V L I N N A G M S F K N D A T E T F G					
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E Q A E V T M R T N F W G T L W V C H A					
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L L P L L R P N A R V V N V S S F V S K					
430	440	450	460	470	480
AAAGCTCTTGATACATGCAGCCCTCAACTACAAGCCAAGTTCCGGGATACTGAGCTCTCT					
K A L D T C S P Q L Q A K F R D T E L S					
490	500	510	520	530	540
GAGGAGGAGCTGTGCTTGCTGATGGGGCAGTTTGTATTGCCGCTCAGCAGGGAAACCAT					
E E E L C L L M G Q F V I A A Q Q G N H					
550	560	570	580	590	600
CAGGCCAGGGGTGGCCAAACACAGCCTATGGCACAAACAAAGATCGGAGTGACTGTGTTG					
Q A Q G W P N T A Y G T T K I G V T V L					
610	620	630	640	650	660
TCCAGGATTCAGGCTCATTTTCTGACTAAGACCCGGGCAGCTGATGGAATCCTGCTCAAC					
S R I Q A H F L T K T R A A D G I L L N					
670	680				
GCCTGCTGCCCTGGCTGGGT					
A C C P G W					



Supplemental fig. 2

cDNA#: 0503I

Predicted mevalonate (diphospho) decarboxylase:

ATAGTAGACAGTCTCGCGTTTCTAGTAATTTTTGTTATTTTATTGATACGGGTTTGACTTTTTATCAAAATGCCCGAGGACAG
CGTCAAAGAGCTACCATGGTAACATGCAGTGCGCCTGTGAATATTGCTGTTATCAAATACTGGGGGAAGAGGGATGAGGAGT
TGATTCTACCTATAAATTCATCTTTGAGTGCCACATTACATCAAGACCAGCTCAAGACGACCACAACAGTGGCATGCAGCAGG
TCGTTCCAGGAGGATCGTATCTGGCTCAACGGCAAAGAGGAGGACATAACCCAGCCCAGACTACAGTCCTGCCTTAGGGAGAG
TCAGTTACTATATGCAATGATATGATGGAGGGAGAGGGGGAAGAGTAAGAGGGCAAAGGACAAGCCATGATGTGTTGTTGTTG
GTTTCCTTACTTAGTTTCGGCGCCTGGCCCGTAAGCGACGTAGTGATGGGGTGGCTGTACATAAGAGGGACTTTACTGCGTTTCG
CTGAGCTGACCATGAAGGATAGCAATCAGTTCCATGCCACTTGCCTGGACACCTACCCACCCATCTTCTACCTCAACGACGTG
TCGCGCCGTGTTATCAACCTTGTGCACCGCTATAACCGTCACTACAGGGAAACAAAGGTGGCATAACATTTGATGCACGTCC
CAATGCAGTGATCTACACTCTTCAGCAGAATGTGGAAGAGTTTGTCCAGGTGGTCAAACACTTCTTCCCACCAGAGACCAATG
GAGGACAGTTTCTAAAGGGTCTTCCGGTTGCCCAACA

qPCR primer set:

0503I_1F-forward: CGTATCTGGCTCAACGGCA

0503I_2R-reverse: CCCCTCTCCCTCCATCAT

cDNA#: 0414B

Unknown

GATGTATCTTTAGATAGATTTGGCACTTACAACTGTAGAGTTTGTGCTTATTTTGAGAACAATAGGATGATAACCCACAGAA
GGGTAAGTAGAGATTTACGTGACAGAGATACCAAACCAAAAGGCCCCCGGACAGTGTAATTCAGTGTATCTTTGGGTCAGCAT
TAAAACCATCCATGCCTGAAAATCTGCTAGGTAGATCTTCCCTTATAGGTCTCCATGACTGGTTTTTCAGATATCACCATCCT
CCCTCCATTGTTCCCTTAGGTTTACTGACATACTCAAAGGTACTTCAACTGCCTTATTGACTATACCCTCACTCCAGCAGACT
TACATGGCAATCGTGTGTGTGTCAGTGAGTGTGTGTGTGCGTGTGCGTGTACGAGTATCAGAAAGTGTGTTTGTGAGAGTGAGTG
TGTGTGCGTCCGTGTAGCCTTTGCCACCTGGCCTGACCGGACTCCACACTCCTATTCTGTTTGTCACTAGAGCTCAAGTTGG
TCCTTTGCTGTTTATAACAGTGTATTTATTACTTTTCAGCTGTACATAAGAATACTGTTTCAGTCAGTGTAATCTATTTAAAT
GTATATGCACTTGCATTACCTGGAAGTTGGTGGTGTCTAGCGTGTTCAGGCTCTGCGTCGGAGTCTTTTTTTAGATTTCGTTA
TTATGATATTGTTTCTTTTTTTTTTAAATATATTATATCCTAAGTAAATATATTCACACAAATGTTTGAGTGCCCTGAGCAA
CAGCTAACGAGACATATACACAACCTGTAGGTTGACAA

qPCR primer set:

0414B_1F-forward: ATCCTCCCTCCATTGTTCCCT

0414B_2R-reverse: CTGACACACACGATTGCCATG

cDNA#: 0509L

Predicted cAMP-dependent protein kinase catalytic subunit beta:

GGTGGTGACAGGATTTGGACTCCTTCTTTTCATATTTGGCTGTAACGTTATCAATTGAATTTCTCTATATTGTTCCAAATTAAC
GATTTTGCCTACAAGGGGAACGTCACTCAACAACAGCTGTAGCTAGCTACCGCCATCTCCCGCAGTCTGAGCGATCGCCGTGT
CTTCATAAGATGGGAAACGCTGCTACCGCCAAGAAAGGCAACGAGCTGGAGAGCGTGAAAGAGTTCCTAGCCAAAGCCAAAG
AAGACTTCTTACGGAAATGGGAATGTCCACCACAGACCACGACGGGCCTAGATGACTTTGACGGGCTAAAGACCCTGGGTACA
GGCTCATTTGGAAGAGTCATGCCGGTCAAACATAAAGGAACCGAGCAGTTCTTCGCCATGAAAATACTGGACAAGCAAAAGGT

GGTGAAACTCGAGCAAATAGAACACACGTAAATGAGAAGAGAATATTGCAGGCCGTGTCTTTTCCCTTCCTCGTCAGACTTG
ATTATGCCTTCAAGGACAACCTCCAATTTGTACATGGTGATGGAGTATGTTCCAGGAGGAGAGATGTTTTCGCATCTAAGACGA
ATCGGAAGGTTTCAGTGAACCCACGCACGGTTTTACGCGGCCAGATAGTGCTGACATTTGAGTACCTCCGTTCACTAGACCT
TATCTACAGAGATCTGAAGCCTGAGAACCTTCTAATCGATCAGCATGGCTACATCCAGGTGACAGACTTTGGATTTGCCAAGA
GGGTAAAAGGCAGAACCTGGACGTTGTGTGAACTCCAGAGTACCTGGCGCCCGAGAT

qPCR primer set:

0509L_1F-forward: GAAAGGCAACGAGCTGGAGAG

0509L_2R-reverse: TCTAGGCCCGTCGTGGTCT

cDNA#: 0408O

Predicted CREB cAMP responsive element modulator:

TTTCTGTTAGAGAGCTGAGATAAAGGGTTTCTATTGGACCTCTTAAGAACCGATATGGCTGTTGCTGGGGATGAGACTGAGTC
AGCTTCCACTGGTGACATGTCAGGCTACCAGATCTGCACTCCACCTCCAGCCTGCCCCAGGGCGTTGTGATGGCTGGCTCCC
CAGGCTCCCTGCACAGCCCCCCCCCACGTGGCTGACGAGACCACACGCAAGAGACAACCTCCGCCTCATGAAGAACAGGCAGGC
AGCCAAAGAGTGCCGGCGGAGGAAGAGAGAGTATATTGCTGTCTGGAGACTCGCCTCACCATGCTGGAGGCCAGAACAGA
AGCTCATGGATGAGCTAACTACTTTAAGGAAATGTATGGAATTAAGTCGAGTCAGTAACAGTACAGCATACTGTACATACAC
ACCCTGTAGAGACATGTTATTTATGTCTATCATAACCAGCTACTGTACATAACATTGCACAGTAATGTGTTTTATTTCAATAA
AATATGCCATATGGTTGTATGGCATTTCGCTTTTAAATTGTTTTAAACATTCTCATCGAAGATCTGTTATTTGAAGATTTATCA
GACAAAATGCTCCAGAGGTTTATCTTGAATGACATGTGGAGTCTCAGGCCTGCACATATAGCAATGTATAGGCATTATTTTG
TAGAGACTAGGGCTTGCATAGAGACCTCTGTCTTAAATGACATTTTACAAAGAAACGAACCAGCAT

qPCR primer set:

0408O_1F-forward: AAGAACAGGCAGGCAGCCA

0408O_2R-reverse: TGAGCTTCTTGTCTGGGCC

cDNA#: 0510B

Predicted phosphatase, orphan 1:

CAGATGCGGTAGCCGGGCACCAACCACTCAGGACGAGGGGCACAGAGCCGACCATTTGAAAACACATGTTACCCGGTGTG
GGCGAGCCACCGCTCCACCGCTCCTCAAACATGGCTGCCTCATCTGTCCCTTCCACGGCCCCAGTCCCGTCCCTCCCAAACG
CTTCCTGGTTCTCTTTGACTTTGACGAGACTATCGTCAACGAGAGCAGCGACGATGCAGTGGTGCGCGCCGCCCGGGCCAGC
AGCTCCCCGATTGGCTGAGACACTCTATATCTGGGTTGCTATCCACCCCCAAAAAGGCAGAGAATCAACTAATGTCCTCCAG
AAATGGTTATTGGAGACTAGTATCGAATTATAGGGGATGCCTGGAGATGGTGGTGGGTTCTACCATAAGACACCAGGGCATT
CCGTAAGCATTCAAATATGAACATCTCCTCCAGTGATAGAAATACTACCTTGGTCCAGCACACTAGTTCAGTGCAACCTCACA
TACTGAGTCTATGTGCATGTCTATGTAATTTGAACCCTATATAAGACAAGTGTGTGCTCAAAGTCGTAAATCACTGTGAACAA
ATGGCCATGGGTTGGAGTGTGTTGTAACCTTGACCCTGATGCAACAAACAAACAAAAATCCAAAAGGCTTCCTTGAAAATGAAC
AAACCTGCATGTTGATGTGTTAATGTCATCAAAGAAGTACCTTTGACAAATTATGAATCAGTGTTACA

qPCR primer set:

0510B_1F-forward: TATCCACCCCCAAAAAGGC

0510B_2R-reverse: ACCCACCACCATCTCCAGG

cDNA#: 0422E

Predicted C-type lectin domain family 4 member E:

CAGTTGGAGAGGGAGAGAGACCAGTTACAGATCAGTTATAAACGCTTGACTAAAGAAAGAGACCAGTCACAAACTCATTATAA
TACCATAACTAGAGAGAGAGACCAGTTACAAAGGAAAATTTTCAGAGCAGGAGAAAAAGATATCAGAAGAACTGGGATGCTGCC
CTGGAGGTTGGAGGAGATTCGGATCTAGCTGCTATTACCTTTCAACAGAGAGTAAACTTTGGACTGAGAGCAGAGCAGACTGT
ATAAGGGGAGGGGAGACCTGGTGATCATAAACAGCAGAGAGGAACAGGTGTTTCTAAACAAAATGGAAAAAGATGTGCGCTT
CTGGATTGGTCTGACTGACTCAGAGATGGAAGGGTTCTGGAAATGGGTGGATGGAACAACATCACCAACAACGTTCTGGAGAA
GAGGAGAGCCAAATAATGCTGATGGGGAGGAGGATTGTGTGGTATTCAACAGTTTTCATTAATCTGTCTTGGGCCTGGGAAATG
ATTCAGTCATGGAATGACCAGCCTTGCTCAGTGAGTACTCAATGGGTATGTGAGAGCAGTGTCTATAACAAATCCATCCACAAC
AGGAGAATAATATCTGAATTCCTTCTGAGTTAGCAATTGTATTGTTAGTTAGCAATTGTATTATCACAGATTATTGTTGAA

qPCR primer set:

0422E_1F-forward: TAATCTGTCTTGGGCCTGGG

0422E_2R-reverse: GAGCAAGGCTGGTCATTCCA

cDNA#: 0519L

Uncharacterized predicted protein:

GAGTTTTGAGAGGTAAAGTGCGGATGAGGAGAGAAAAGTTGGTATATTAAGGAAAAACAAGACGAAACAAATACTAAAAAA
TTAAGGGAAGAGAAAAAAATCAAGGAAGGTATAGAGCAGCCAGAAGCAGGGTGGTGGCAACTGGCTGCTGGTGGGCCTCCTG
GTGCCCCCTCTGCATCTTCATCATTATTATTGTGGCACTGGGTATCGTCTACTGCACCCGCTGCGCCGTCATGCCGCGCAACAC
GCCGGCCACCAACTGCTACCATTGGATCTCCGGAGCGCACGACAAGCAGGGCGCGCCCAATCCCAGCAAGGGGATACAGTCAC
ATGTTTTAAACAGATGAATGAGGAAAGACTTGTACATAAATAAACTGTTAAATAAATAAGAGACAGGAAGAAGGTAAGAGAGGG
GTGGTTGTTGTACTGTATCTGCCTCCTCCCGTCCCATTGCCACTGTGTTGCCGTACTGGGACTCTTTGAACACTTGAATGTGT
CTGTGGAATATCGGTTGAAAGAGAAGAACAGGGAAGCAATACTAAAAAACGCTAATCCCTTTTCATGGGGTCCTTTAGATGTG
GTAGAAAAAACGGCATAATTGTTGCTTCCACTGATCTCAAAGCACAATATATAGGCCTATGATTGCACTATAGGTACAA

qPCR primer set:

0519L_1F-forward: GCCTCCTCCCGTCCCAT

0519L_2R-reverse: GTTCAAAGAGTCCCAGTACGGC

cDNA#: 0510F

No hit:

CGTCATGCCGCGCACACGCCGGCCACCAACTGCTACCATTGGATCTCCGGAGCGCACGACAAGCAGGGCGCGCCCAATCCCAG
CAAGGGGATACAGTCACATGTTTTAAACAGATGAATGAGGAAAGACTTGTACATAAATAAACTGTTAAATAAATAAGAGACAGG
AAGAAGGTAAGAGAGGGGTGGTTGTTGTACCGTATCTGCCTCCTCCCGTCCCATTGCCACTGTGTTGCCGTACTGGGACTCTT
TGAACACTTGAATGTGTCTGTGGAATATCGGTTGAAAGAGAAGAACAGGGAAGCAATACTAAAAAACACTAATCCCTTTTCAT
GGGGTCCTTTAGATGTGGTAGAAAAAACGGCATAATTGTTGCTTCCACTGATCTCAAAGCACAATATATAGGCCTATGATTGC
ACTATAGGTACAATAGAAACCGTCCATTGGGATTGTTGTTGAATACATCTGGTCATATTTACGGATGTTGTGGGAATATACTG
TCAATATATCACCAGTGCCATCTGAAACCAAGCCAAAGAACTGTGCTGTAGCTTTTAGCATTTTTGTAGTCCATGAAATCTG
GGTGTGGGTCTTTCAAAAAATGCTGAGGTTTTTAATGCTCAATTGAGCTCATTTCCAAGTATGTGTTGCAATGACCATTTA
TGTCAACTTTGTTAAAGAAGGATCACATGCAGCTTCATATTTAGCTATGCAAGCTATGCAAGCATGACTGTCATGACGCTGTT

GAGGAAAAGCA

qPCR primer set:

0510F_1F-forward: TCACCAAGTGCCATCTGAAACC

0510F_2R-reverse: CTAAGAGCTACAGCACAGTTCTTTGG

cDNA#: 0412C

Predicted interferon-inducible protein Gig1:

GCCACACACCGTAATAAGCTATCTCTCCATCCCATCTTTTATCATCCCTACAGTTCAAGATGGTGCGGTCTGTCAGAGAAGAA
TGTAAGTCAGCTGAGCACCATAACAAGACCTGAAGGACTCTGGCTTCGGCCGTCTCCCCCGACACGGCCTCAAGCTCCTCTT
CTGGTTCGCCAACGAGTGCGTGGCGTTCAATCACACGGCAACATGCTGGTAAAGTGCCACCCTGAGAGAGGCGACTTCGGCT
TCCACTACTTTGGCAACTTCGAGGAGATTCTCCAGTTCTATCGCGAGACCGCAGGGAAAGCTACTTCGAGGTGGGCAACCTG
AACACAGAGACCTATTCCAAAGCCGAGGACCTACCGGACTACGTGAGACAGGACTACGGGCTTTCCCTGGGCTACCGCCTATG
CAACAAGGACCGCATCATCATCAGGCTGAAGCAGGGGGATGTGAAGGCCACCTATGTCACCGAGCACAAGGAGAACGGCGGCC
GGGGGGAGTTTGACTCCGAACGTACCCACCTTGTAATCCGGATTTGATCCGCATCATCTGGGATCCTGAGCTGGAGCTAGCG
ACATTCTGGACAGACGGGCTACATGGGACTGTCTCCGAGAGAGCGCCTCCTCAGAGCTTTCAAGACAAACTCGGATGTCAT
CTCGTGGGAGTATGA

qPCR primer set:

0412C_1F-forward: GCATCATCATCAGGCTGAAGC

0412C_2R-reverse: TCGGTGACATAGGTGGCCTT

cDNA#: 0507O

Uncharacterized predicted protein:

ATCTGCAGTCTACTGCTGCCTCAGGACAGACACAGTCAGAGACAGTTAGAGCCACAGAGCGCGCTCAGAGACCAGCATCGCTG
CTTCTCTTTTTTTAAAGCTACACAGCCAGATCTTATCTGCACAGACGTTTATTACAGATTCTCCTGAAGAGTTAACGAGAGA
GGCCAAACTACAGGAGCAGAGAAAAGACTTGGATATACACTATACCTTTTAATACAGCAGCCAAGAGTCCCATGTCCAAATCG
GTGTCCCAGAAGAGATTACAGCTGCCCACTATCAGCGAGGGCTACGAGGACCTGGTCAGAGACATGAACCAGACGAACCACTC
CAACACCCTTCACTCTGCCCCGGCCTACCACCCACCCAGGGCCCTGTCCACACACCCTCTCCTCAGAGGACTACCTGCTGTCCA
TCTGCCACCTGGCTACCCACCTCCCTTACACCAATGGCATCATCAACCCTCATCAGCAGGTATCGCTGCACCAGAGGCCA
CGGCTGCCGCGGCTTGCTGTGCAAGATGAAAACCTCTCCAAAGACCACTGCACTTTGTCCCAGGGAAAAGTCCCATGGGAAGGA
CAGGCCTGGGATGTTCTCTGACTTCATCACTGCATGCGGCAGACAGGGGAAAGATTCCAAGTGCATGAGCCTGAGTTTGTT
GCCTTGGTGCCTCAGACCCCTTAGAGT

qPCR primer set:

0507O_1F-forward: CTGTCCATCTGCCACCTGG

0507O_2R-reverse: GATGCCATTGGTGTGAAGGG

cDNA#: 0404O

No hit:

CGCTTGACTAAAGAAAGAGACCAGTCACAACTCATTATAATACCATAACTAGAGAGAGAGACCAGTTACAAAGGAAAATTTTC

AGAGCAGTGGTATTCAACAGTTTCATTAATCTGTCTTGGGCCTGGGAAATGATTCAGTCATGGAATGACCAGCCTTGCTCAGT
 GAGTACTCAATGGGTATGTGAGAGCAGTGTCTATAACAAATCCATCCACAACAGGAGAATAATATCTGAATTCCTTCTGAGTT
 AGCAATTGTATTGTTAGTTAGCAATTGTATTATCACAGATTATTGTTGAAGTGTAATTTAAATCAATTATAACATTAGTTATG
 ATTAGTAATGATTGTTCTTAGGATTAGTAATGATTGTTCTTAGGATTAGTAATGATTGTTCTTATGATTAGTAATGATTGTAG
 TTATGATTTGGAATAATTTAATTTATATAATATAGAATTAATTATAAAGAATCATAGTATTATGAATGTGAAACTTTAACCTG
 AAAGTTAATGTAGTGAATTGACATGCAGGGTCAAATCTAATCTAATCATCTAACAAGGCAAGGCAAGTGCTATGCCTCAATTT
 GTGTTTATTTCTTTGTCAGATGGGATGATAATTATAGGCTACTTACTAATTCATTTTAGCTCCTAGTGGATTATATTAAATAC
 ATATTTTGAAAG

qPCR primer set:

0404O_1F-forward: GTGAATTGACATGCAGGGTCA

0404O_2R-reverse: TTGAGGCATAGCACTTGCCTT

cDNA: Predicted aquaporin4

GGCATGGATCATTTTCATCGGACAGACCCACTACTACTTTCTCCTCCCGTCTCGCACTGTGTCATTACCAGCCAACCTGCTGCC
 TGTCTGAGCTGCCTGTGCGGTGTAACCAGCATCCCATGATGGTGGCATTCAAAGGAATCTGGACCAAGAACTTCTGGAGGGC
 TGTCTCAGCTGAGTACCTAGCAACCACGATCTTCGTCTCCTCAGCCTGGGCTCCACCATCAATTGGGCGGCCGAGTCAGACA
 ACCCTCCCCCTGCAGACCTGGTCCTCATCTCATTTTGTCTCGGGCTGTCCATTGCCACAATGGTGCAGTGCTTTGGTCACATC
 AGCGGTGGCCACATCAACCCGGCAGTCACTGCAGCCATGGTGGTGACCCGGAAGTTGAGCCTGGCCAAGGGTGTGTTCTACCT
 AGCTGCCCAGTGCCTGGGGGCGATCACTGGGGCAGGGCTCCTCTACCTGGTGACGCCAGTGTCTGTGAGAGGGGGCCTGGGCT
 GCGGCCTGTTGGTGGAGCTCCTGATCACCTTCGAGCTGGTCTTCACGGTGTTCGCCACCTGTGACCCCAAACGCTCTGTTAAC
 GGCTCGGCTGGCCTCGCCATCGGATTCGCTGTAGCCATCGGTCATCTGTTTCGAATTCCATACACAGGAGCCAGTATGAACCC
 CGCTCGCTCCTTTGGACCTGCAGTCGTCACCATGAACTTTGAGAACCACTGGGTGTACTGGGTGGCCCAATCCTGGGT

qPCR primer set:

aquaporin4-1F-forward: TGGATCATTTTCATCGGACAGA

aquaporin4-2R-reverse: GCGAGACGGGAGGAGAAAGTA

cDNA: Predicted lectin

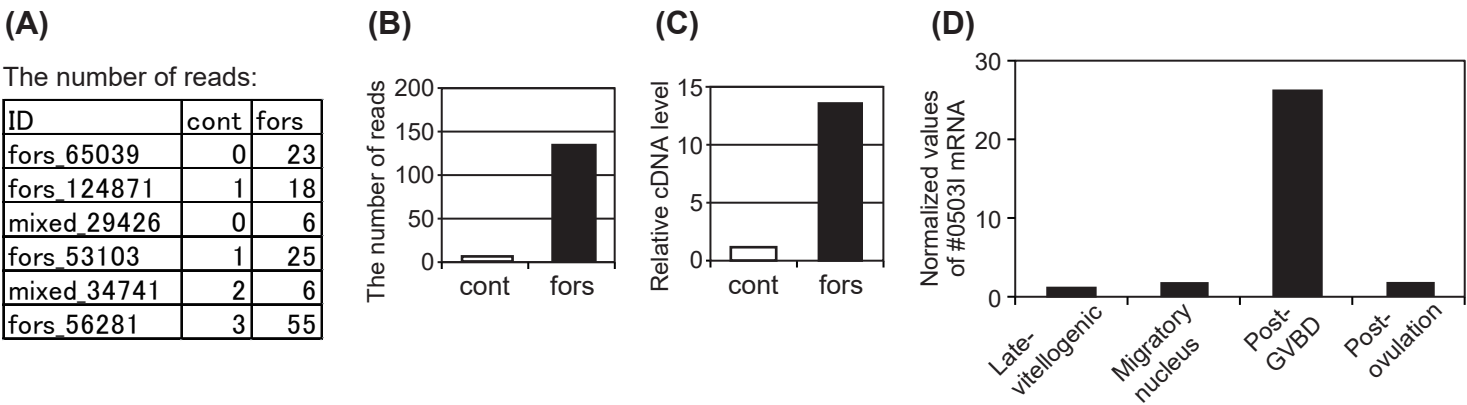
GATCAGTTATAAACGCTTGACTAAAGAAGAGACCAGTCACAACTCATTATAATACCA~~t~~AACTAGAGAGAGAGACCAGTTACA
 AAGGAAAATTTTCAGAGCAGGAGAGAAAAAGATATCAGAAGAACTGGGATGCTGCCCTGGAGGTTGGAGGAGATTCGGATCTAGCT
 GCTATTACCTTTCAACAGAGAGTAAACTTTGGACTGAGAGCAGAGCAGACTGTATAAGGGGAGGGGCAGACCTGGTGATCATA
 AACAGCAGAGAGGAACAGGTGTTTCTAAACAAAATGGAAAAAGATGTGCACTTCTGGATTGGTCTGACTGACTCAGAGATGGA
 AGGGTTCTGGAAATGGGTGGATGGAGCGACATCACCAACAACGTTCTGGAGAAGAGGAGAGCCAAATAATGCTGATGGGGAGG
 AGGATTGTGTGGTATTCAACAGTTTCATTAATCTGTCTTGGGCCTGGGAAATGATTCAGTCATGGAATGACCAGCCTTGCTCA
 GTGAGTACTCAATGGGTATGTGAGAGCAGTGTCTATAACAAATCCATCCACAACAGGAGAATAATATCTGAATTCCTTCTGAG
 TTAGCAATTGTATTGTTAGTTAGCAATTGTATTATCACAGATTATTGTTGAAGTGTAATTTAAATCAACTATAACATTAGTTA
 TGATTAGTAATGATTGTTCTTAGGATTAGTAATGATTGTTCTTAGGATTAGTAATGATTGTTCTTATGATTAGTAATGATTGT
 AGTTATGA

qPCR primer set:

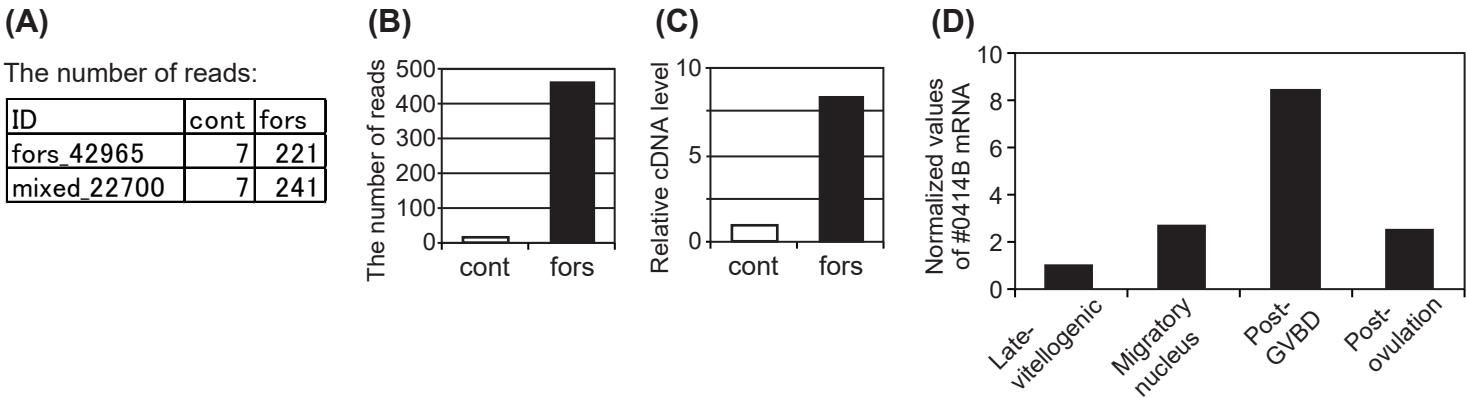
lectin-1F-forward: TTCTGGAAATGGGTGGATGG

lectin-2R-reverse: GAATACCACACAATCCTCCTCCC

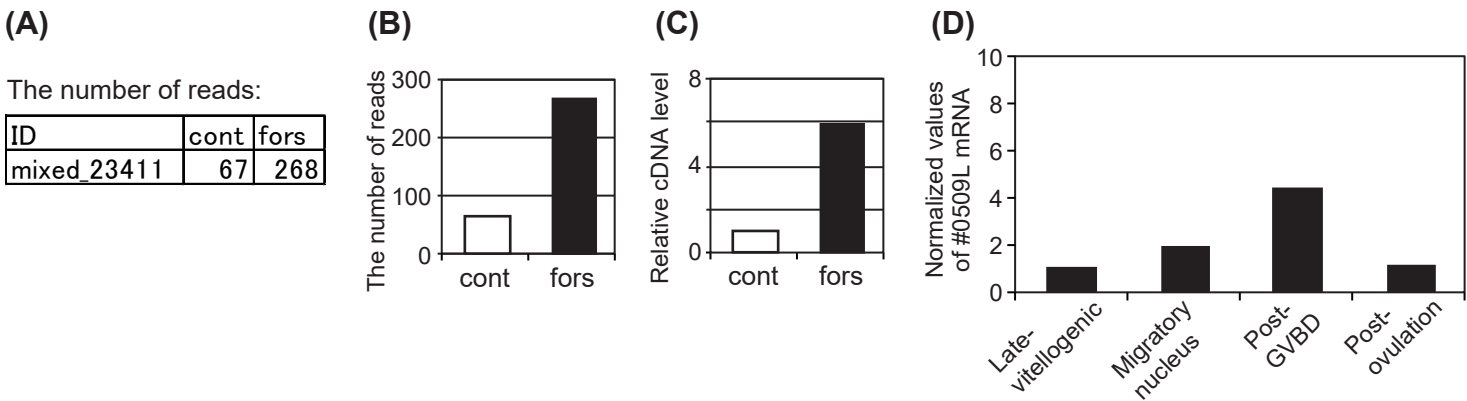
cDNA#: 0503I, Predicted mevalonate (diphospho) decarboxylase



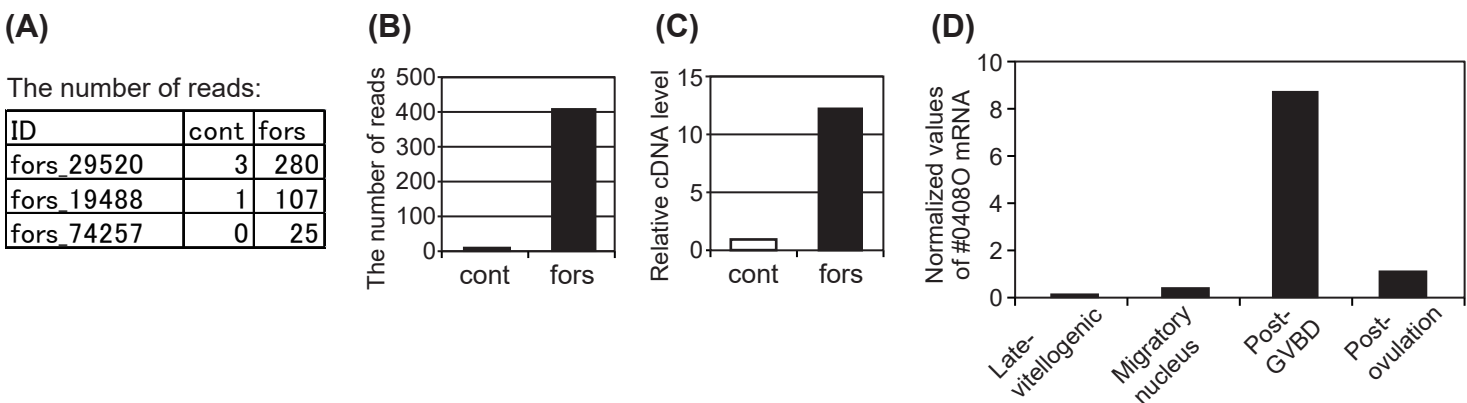
cDNA#: 0414B, Unknown



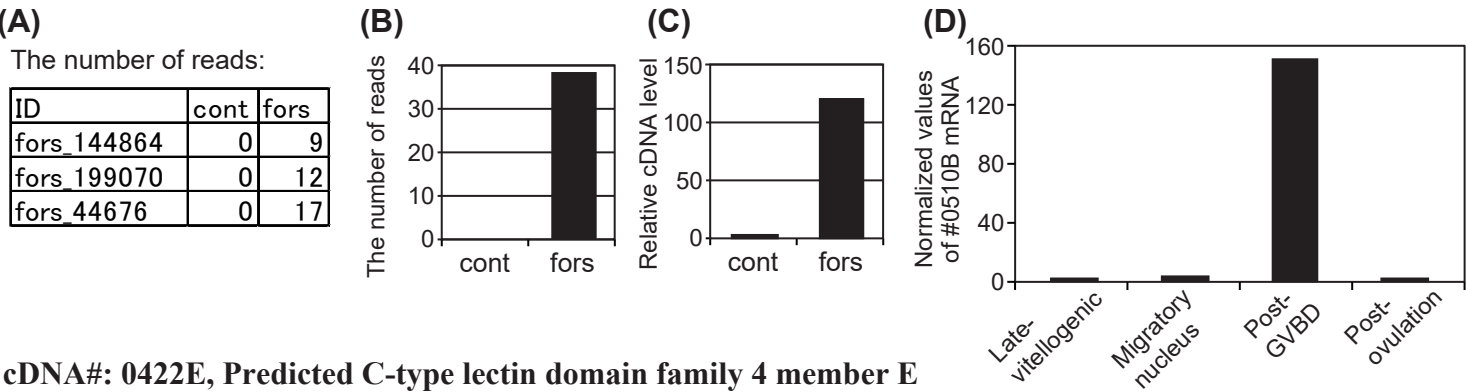
cDNA#: 0509L, Predicted cAMP-dependent protein kinase catalytic subunit beta



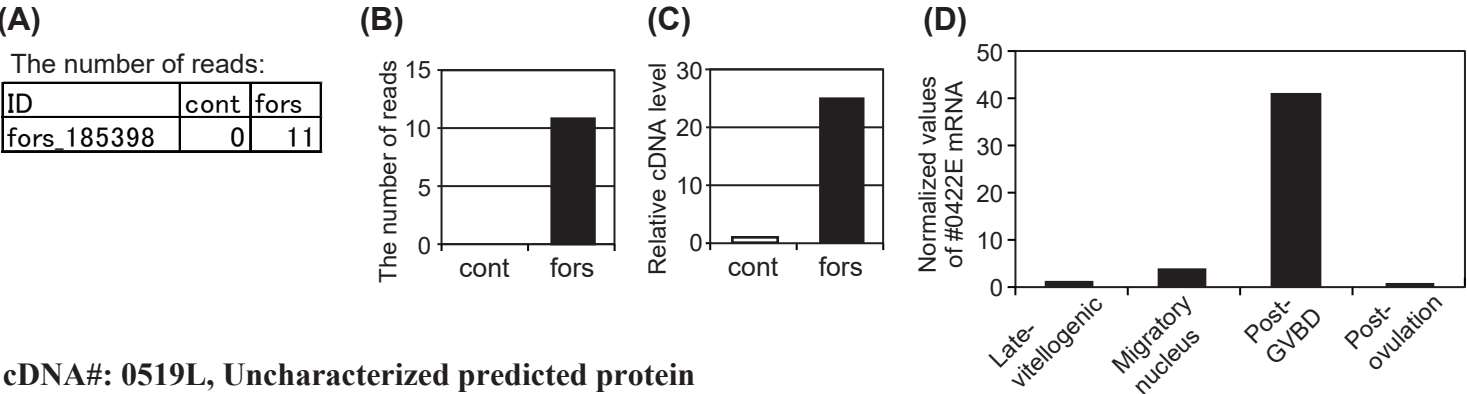
cDNA#: 0408O, Predicted CREB cAMP responsive element modulator



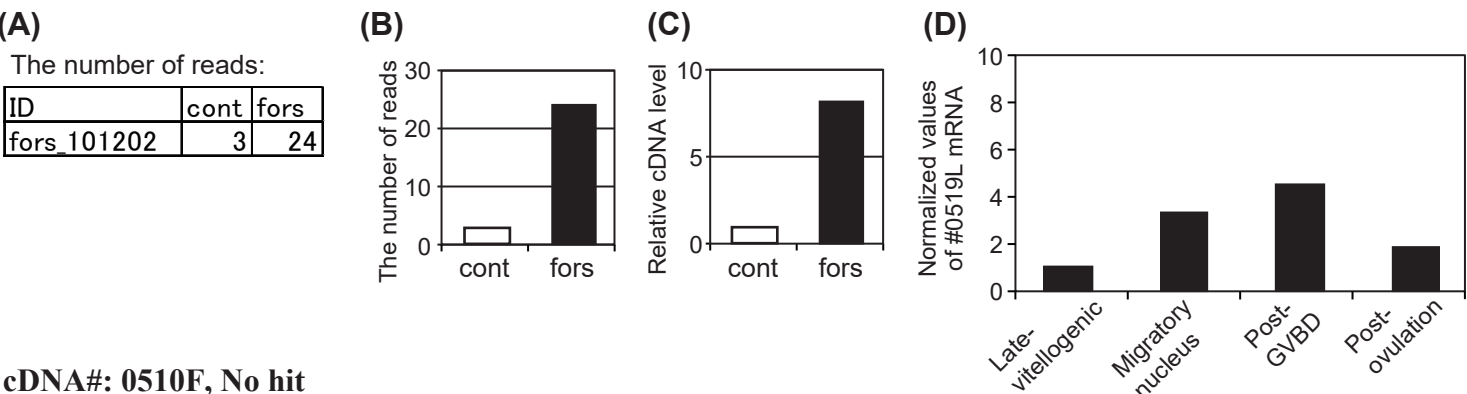
cDNA#: 0510B, Predicted phosphatase, orphan 1



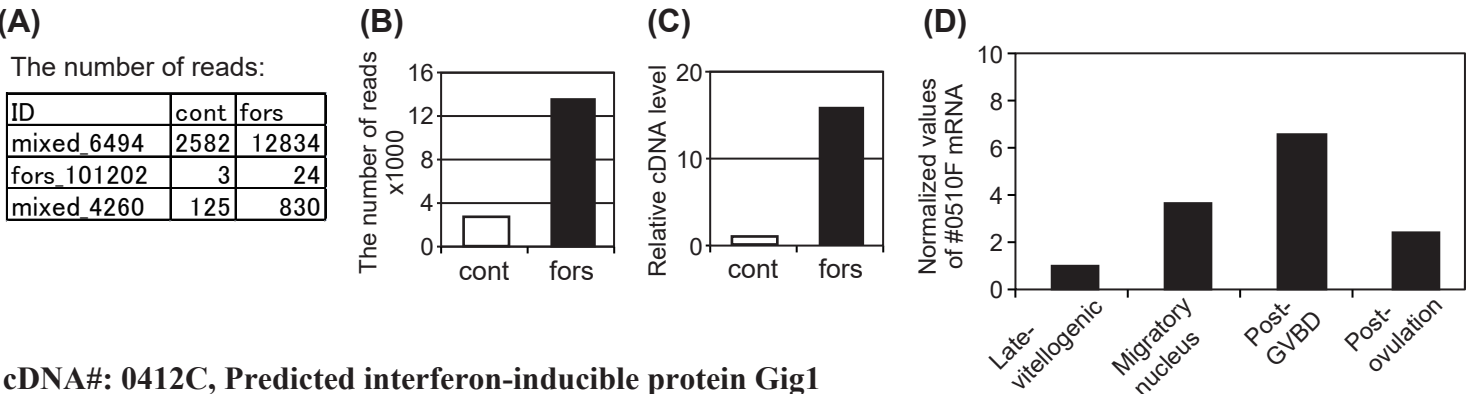
cDNA#: 0422E, Predicted C-type lectin domain family 4 member E



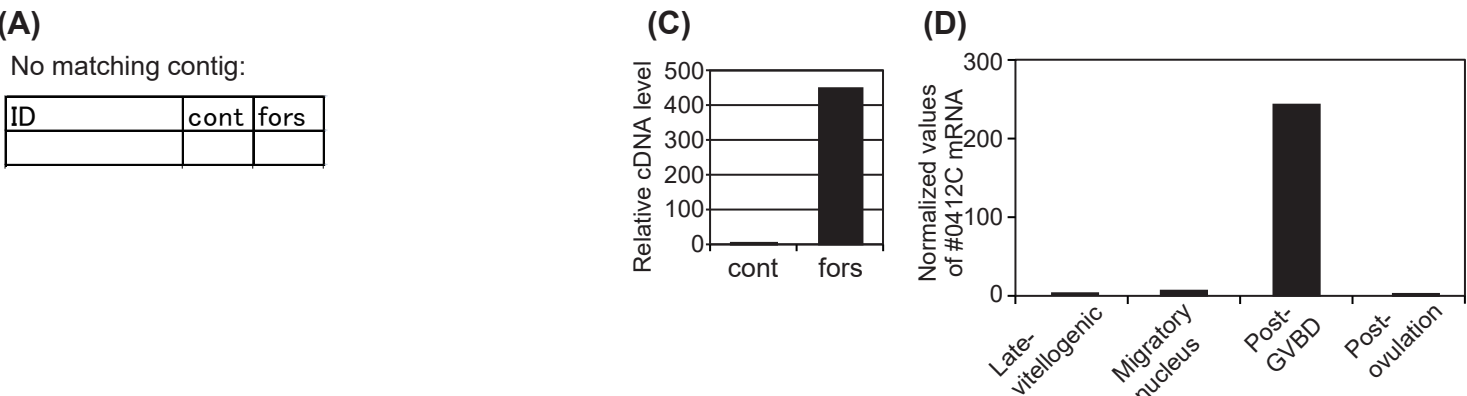
cDNA#: 0519L, Uncharacterized predicted protein



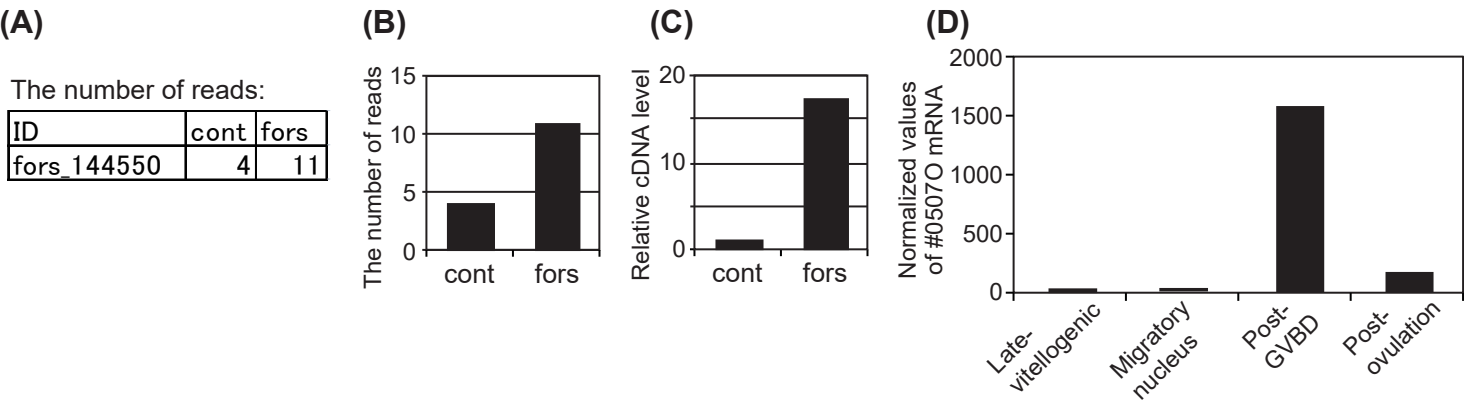
cDNA#: 0510F, No hit



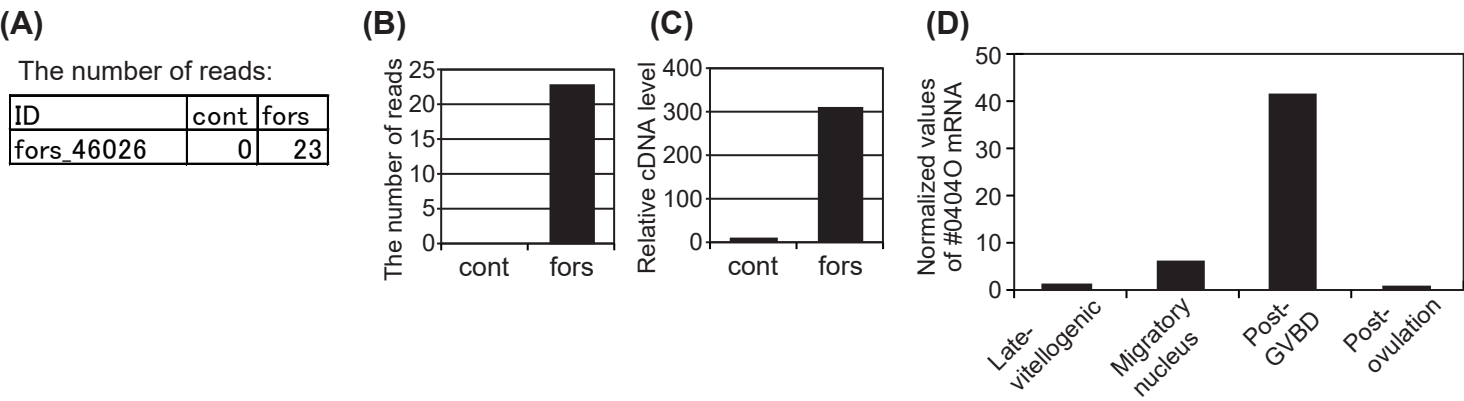
cDNA#: 0412C, Predicted interferon-inducible protein Gig1



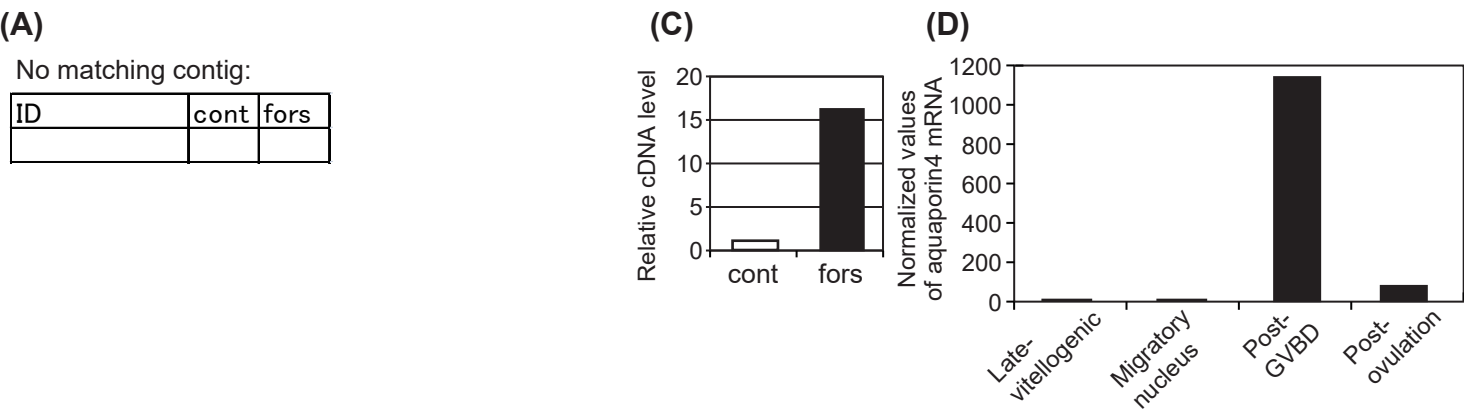
cDNA#: 0507O, Uncharacterized predicted protein



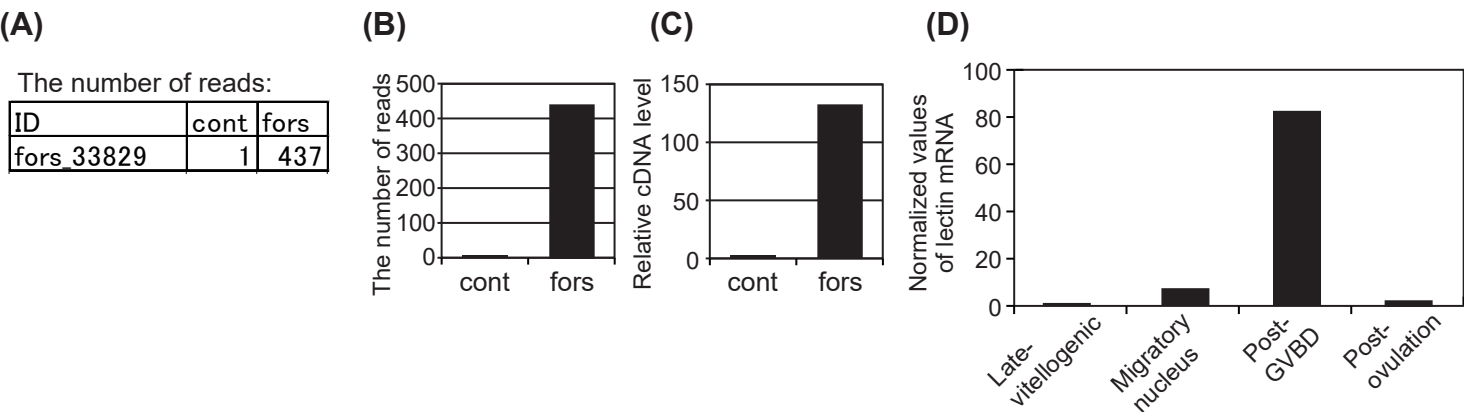
cDNA#: 0404O, No hit

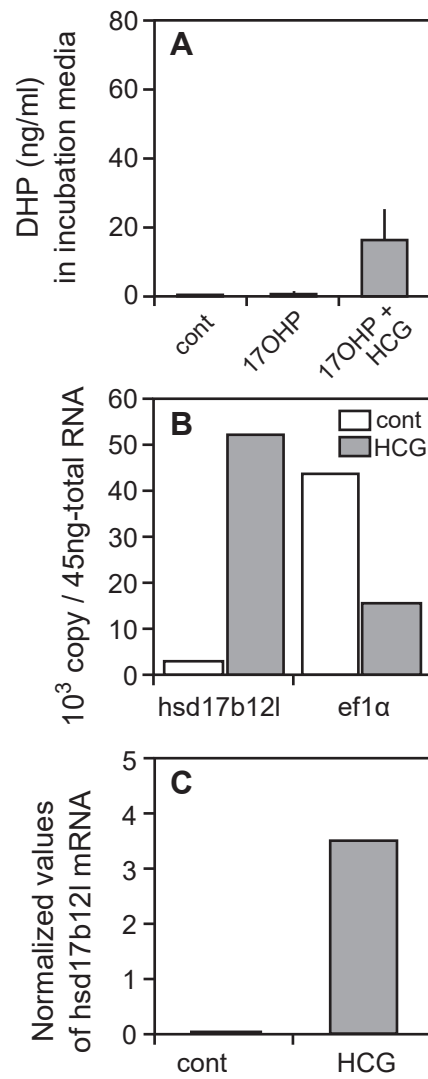


cDNA: Predicted aquaporin4



cDNA: Predicted lectin





Supplemental Fig. 5