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# Differential regulation of the expression of cytochrome P450 aromatase, estrogen and androgen receptor subtypes in the brain–pituitary–ovarian axis of the Japanese eel (*Anguilla japonica*) reveals steroid dependent and independent mechanisms

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ABSTRACT

This study aimed at investigating the role of sexual steroids in the regulation of the expression of the single aromatase gene and steroid receptor subtypes in the brain–pituitary–ovarian axis of the Japanese eel. Unlike other teleosts, which possess duplicated genes for aromatase, *cyp19a1a* and *cyp19a1b*, expressed in the gonads and in the brain, respectively, eel species possess a single *cyp19a1*. Phylogenetic analysis indicated that eel brain/gonadal *cyp19a1* branches at the basis of both teleost gonadal *cyp19a1a* and brain *cyp19a1b* clades. Female eels treated with catfish pituitary homogenate (CPH) to induce sexual maturation showed an increase in the expression of *cyp19a1* and aromatase enzymatic activity in the brain and in the ovaries. Treatments with sex steroids (estradiol-17$\beta$, $E_2$ or testosterone, T) revealed that the increase in *cyp19a1* expression in the brain may result from $E_2$-specific induction. In contrast, the increase in *cyp19a1* expression in the ovaries of CPH-treated eels is a result of steroid-independent control, probably from a direct effect of gonadotropins contained in the pituitary extract. Analysis of the expression of estrogen and androgen receptor subtypes, *esr-α*, *esr-β*, *ar-α* and *ar-β*, in eels treated with CPH or sex steroids revealed differential regulations. In CPH-treated eels, the expression of *esr-α* and *ar-α* was significantly increased in the brain, while the expression of ar-α and ar-β was increased in the ovaries. No change was observed in esr-β in any organ. Steroid treatments induced an upregulation by $E_2$ of esr-α, but not esr-β expression, in the brain, pituitary and ovaries, while no autoregulation by T of its own receptors could be observed. These results reveal both steroid-dependent and -independent mechanisms in the regulation of *cyp19a1* and steroid receptor subtype expression in the eel.



## 1. Introduction

Estrogens are involved in various aspects of sexual differentiation, vitellogenesis, gonadal development, reproduction and reproductive behaviors [29]. Cytochrome P450 aromatase is the key enzyme that catalyzes the conversion of androgens to estrogens [50]. It has been shown that aromatase is expressed in various tissues, especially in brain and gonads, in different vertebrate species [6,50]. In mammals, there is only a single copy of the aromatase gene, *CYP19A1*, except in pigs, which have multiple copies of the *CYP19A1* gene [20]. The tissue-specific regulation of *CYP19A1* is achieved through distinct promoters and alternative splicing of 5′-untranslated exons under the control of various factors, including gonadotropins, growth factors and steroids [49].

Early studies revealed that teleosts exhibit a much higher brain aromatase enzymatic activity (100- to 1000-fold) than mammals [3,6]. This high enzymatic activity is related to the specific expression of the duplicated aromatase gene *cyp19a1b* in the brain of teleosts. In most teleosts, two separate genes, named *cyp19a1a* and *cyp19a1b*, have been identified. As other duplicated teleost genes,

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they are supposed to be related to the whole genome duplication that occurred early in the teleost lineage, before the separation of the Elopomorpha superorder group [2,21]. *Cyp19a1a* and *cyp19a1b* are predominantly expressed in the gonads and brain, respectively, and encode the distinct aromatase isoforms aromatase A and aromatase B (goldfish, *Carassius auratus*: [17,54]; zebrafish, *Danio rerio*: [28,48]; European sea bass, *Dicentrarchus labrax*: [5,11]; rainbow trout, *Oncorhynchus mykiss*: [12,53]; rice field eel, *Monopterus albus*: [67]). In rainbow trout, due to additional salmonid gene duplication, two distinct *cyp19a1b* genes, *cyp19a1b1* and *cyp19a1b2*, were found; the *cyp19a1b2* gene has been identified as an unusual duplicate of *cyp19a1b1* lacking the first two exons of *cyp19a1b1* [12]. In addition, von Schalburg et al. [61] showed that there are at least five *cyp19a1b1* transcripts with different 5′-untranslated regions in the ovaries and testis of rainbow trout.

In contrast, our previous studies in the Japanese eel, *Anguilla japonica*, indicated that the enzymatic properties of brain aromatase in this species are similar to those of mammalian aromatase, with a lower affinity and a lower maximal reaction rate than brain aromatase from other teleots [25]. In addition, as suggested by Ijiri et al. [22] for the Japanese eel and Tzchori et al. [60] for the European eel, our data supports the hypothesis that eels (*Anguilla* species) may have retained a single *cyp19a1* gene [25], expressed both in the gonads and in the brain, similar to mammals.

Brain aromatase enzymatic activity is significantly increased during the spawning season in various teleost species (goldfish: [45]; black porgy, *Acanthopagrus schlegeli*: [32]; European sea bass: [19]). Both E2 and aromatizable androgens were shown to upregulate brain *cyp19a1b* expression and enzymatic activity (goldfish: [46]; black porgy: [9]; zebrafish: [40]). Gonadal *cyp19a1a* transcript levels and enzymatic activity in ovarian follicles are also significantly increased during vitellogenesis in teleosts (rainbow trout: [53]; tilapia, *Oreochromis niloticus*: [8]; red seabream, *Pagrus major*: [18]; Atlantic croaker, *Micropogonias undulatus*: [43]), but the role of sex steroids in this upregulation is less clear than for brain *cyp19a1b*. Several steroid nuclear receptor subtypes have been identified in teleosts. At least two major subtypes of estrogen receptors (*esr-α* and *esr-β*) are found in teleosts including the European silver eel and Japanese eel [44,56]. As first discovered in the Japanese eel [24,57], there are two androgen subtypes (*ar-α* and *ar-β*) in teleosts, likely resulting from the whole genome duplication in the teleost lineage.

Eels present a unique life cycle with a blockade of sexual maturation at a prepubertal stage (silver stage) as long as the oceanic reproductive migration is not achieved (for review: [15]). Deciphering special endocrine mechanisms of eel reproduction is a current challenge with both basic and applied perspectives.

To investigate the potential roles of sex steroids and their receptors in the differential regulation of the single aromatase gene along the brain–pituitary–ovarian axis, we analyzed the variations in aromatase transcript levels, enzymatic activity, and transcript levels of estrogen and androgen receptor subtypes during induced ovarian development and after sex steroid treatment in Japanese eels.

## 2. Materials and methods

### 2.1. Animals

Three-year-old female Japanese eels (at the prepubertal silver stage), *A. japonica*, were obtained from an aquaculture farm in Taiwan and transferred at the culture station of the National Kaohsiung Marine University, Taiwan. Experimental fish were placed in outdoor 2.5 ton-tanks (one tank/experimental group), with running freshwater, under natural light and temperature (water temperature range: 20–27 °C). All procedures and investigations were approved by the National Kaohsiung Marine University Institutional Animal Care and Use Committee and were performed in accordance with standard guiding principles.

### 2.2. Gonadotropic treatment

Chronic treatments with teleost (carp or salmon) pituitary homogenates are usually applied to induce ovarian development of female European and Japanese eels [14,26,64]. In the present study, we chose to use catfish (*Clarias fuscus*) pituitary homogenates (CPH) prepared in saline (0.75% NaCl) from acetone-dried pituitaries purchased from Kaohsiung local fishermen. CPH is commonly used for inducing final maturation and spawning in various teleost species in aquaculture farms in Taiwan.

Female eels ($n = 38$, BW = 752.4 ± 16.7 g, BL = 75.7 ± 0.6 cm) were divided into two groups: control eels (for aromatase enzymatic activity, $n = 8$; for *cyp19a1* and steroid receptor subtype transcript levels, $n = 12$) and treated eels (for aromatase enzymatic activity, $n = 8$; for *cyp19a1* and steroid receptor subtype transcript levels, $n = 10$). The treated fish were injected intraperitoneally (ip) weekly with the homogenate of one catfish pituitary (10 mg dry weight) in 0.5 ml saline/fish for 17 weeks. The control fish were injected with saline alone. Fish were sacrificed 3 days after the last injection.

### 2.3. Sex steroid treatments

Chronic treatments with sex steroids were performed according to the protocols previously described [26]. $E_2$ and T were purchased from Sigma and dissolved in coconut oil (Sigma–Aldrich Corp., St. Louis, MO).

Female eels (BW = 746.5 ± 79 g, BL = 85.6 ± 3.3 cm) were divided into five groups ($n = 8$ eels per group). Treated eels received ip injections of 0.75 mg or 3.75 mg of steroid (T or $E_2$)/kg BW. Control eels received injections of coconut oil alone. Fish were injected weekly for 6 weeks and were sacrificed 1 week after the last injection.

### 2.4. Sampling procedure

Control and treated eels were anesthetized with 800 ppm 2-phenoxyethanol before being sacrificed. The total body weight (BW), liver weight and ovarian weight were measured for the calculation of the hepatosomatic index (HSI = liver weight/BW × 100%) and gonadosomatic index (GSI = gonad weight/BW × 100%). The forebrain, midbrain, pituitary and ovaries were collected and stored at −80 °C for enzymatic and quantitative real-time PCR analysis. A piece of ovarian tissue was also stored in Bouin's solution for histological observation. Blood samples were allowed to clot at 4 °C, and serum was collected and stored at −20 °C for steroid EIAs.

### 2.5. Measurement of aromatase enzymatic activity

Aromatase enzymatic activity of brain, pituitary and ovaries was measured using the tritiated water assay as previously described [9,25]. The protein concentrations of the crude supernatant fraction were measured with a Bio-Rad protein assay kit (Bio-Rad Co., Hercules, CA). Aromatase enzymatic activity was expressed as fmol$^3$ $H_2O$/h mg protein.

### 2.6. Quantification of cyp19a1, esr-α, esr-β, ar-α and ar-β transcripts by real-time PCR analysis

Quantitative real-time PCR (qPCR) analysis for gene transcripts was conducted according to previously described methods [26]. Partial sequences of Japanese eel *cyp19a1* (700 bp), *esr-α* (318 bp), *esr-β* (687 bp), *ar-α* (995 bp), and *ar-β* (790 bp) cDNAs were cloned and

used as standards for qPCR. These cDNAs were cloned from the total RNA of Japanese eel brain (*cyp19a1*), liver (*esr-α* and *esr-β*) or testis (*ar-α* and *ar-β*) by RT-PCR using specific primers designed from the published sequences of Japanese eel neural aromatase (GenBank Accession No. AY616117.1; [25]), European eel *esr-α* (GenBank Accession No. EU073125.1; [44]), Japanese eel *esr-β* (GenBank Accession No. AB003356.1; [56]), *ar-α* (GenBank Accession No. AB023960.1; [57]), and *ar-β* (GenBank Accession No. AB025361.1; [24]). The sequences obtained were 100% identical to the published Japanese eel sequences (*cyp19a1*, *esr-β*, *ar-α*, and *ar-β*) and 97% identical to the published European eel esr-α (GenBank Accession No. HM545084 for Japanese eel *esr-α*).

Specific primers for *cyp19a1*, *esr-α*, *esr-β*, *ar-α*, *ar-β* and reference gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (GenBank Accession No. AB049458.1; [52]) were designed for qPCR (Table 1). Gene quantification of standards (plasmids with cDNA sequence) and samples were conducted simultaneously by qPCR (GeneAmp 5700 Sequence Detection System; Applied Biosystems, Foster City, CA) with SYBR green I as a dsDNA minor-groove binding dye. Melting curves indicated the amplification of a single amplicon for each gene. The slopes of the respective standard and sample curves of the log (cDNA concentrations) vs. Ct (the calculated fractional cycle number at which the PCR-fluorescence product is detectable above a threshold) were −3.3 to −3.5, indicating an amplification efficiency of 100–90%. The transcript values of each gene were calibrated with the internal reference gene (*gapdh*). No significant changes were observed in *gapdh* transcript levels after steroid treatments.

### 2.7. Immunoenzymatic assays of serum $E_2$ and T

The concentrations of $E_2$ and T in serum were measured by Cayman Chemical's ACE™ enzyme immunoassay kit (EIA) Kits (Estradiol EIA Kit and Testosterone EIA Kit, Cayman Chemical Company, Ann Arbor, MI).

### 2.8. Statistics

The data from the enzymatic assays, qPCR and EIAs were expressed as means ± SEM. All statistical analyses were performed with SPSS10.0 for Windows. The values were subjected to a one-way ANOVA to test significance, followed by a *t*-test or Duncan multiple-range test.

### 2.9. Phylogenetic analysis

The amino acid sequences of vertebrate *cyp19a1* were first aligned using ClustalW [55]. The protein substitution matrix of the resulting alignment was determined as JTT + I + G + F using the ProTest software [1]. The phylogenetic analysis of the *cyp19a1* sequence alignment was performed using a maximum likelihood method using the RaxML software [51] with 1000 bootstrap replicates.

## 3. Results

### 3.1. Analysis of aromatase sequences

Partial cloning (1032 bp) of *cyp19a1* from Japanese eel brain cDNA (GenBank Accession No. AY616117.1) resulting in a sequence that was 100% identical to that of Japanese eel ovarian *cyp19a1* [22]. No other *cyp19a1* sequence could be detected in the eel. Sequence alignment and comparisons were made using available *cyp19a1* sequences in vertebrates and chondrichtyan *cyp19a1* as a rooting group (Fig. 1). The phylogenetic tree shows two main clusters corresponding to tetrapod *cyp19a1* and teleost *cyp19a1*. The eel single gonadal/brain *cyp19a1* branches at the base of the teleost *cyp19a1* cluster. All the other teleost sequences are distributed into two clades corresponding to gonadal *cyp19a1* (*cyp19a1a*) and brain *cyp19a1* (*cyp19a1b*). These two clades reflect the duplication of *cyp19a1* in teleosts. The single eel *cyp19a1* does not appear to be more related to one teleost clade than to another.

### 3.2. Effect of gonadotropic treatment in female

#### 3.2.1. Biometric parameters and serum steroid levels

Chronic treatment with CPH induced a significant development of the ovaries, as shown by the large increase in GSI (13.6 ± 1.3% in the treated eels vs. 0.70 ± 0.06% in the control eels, $p < 0.001$). Histological observation showed that the oocytes of control eels were at the previtellogenic stage (no visible yolk globules), while the oocytes of CPH-treated eels were at the advanced vitellogenic stage (with numerous yolk globules) (data not shown). HSI was significantly increased in CPH-treated eels (1.50 ± 0.12% in treated eels vs. 1.13 ± 0.05% in the control eels, $p < 0.05$). The concentrations of sex steroids in serum were significantly increased in CPH-treated eels ($E_2$ levels: 2.50 ± 0.42 ng/ml in the treated eels vs. 0.35 ± 0.10 ng/ml in the control eels, $p < 0.001$; T levels: 2.13 ± 0.37 ng/ml in the treated eels vs. 0.54 ± 0.13 ng/ml in the control eels, $p < 0.05$).

#### 3.2.2. Aromatase enzymatic activity

Aromatase activity could be measured in the brain, pituitary and ovaries. The activity was the highest in the pituitary. Aromatase enzymatic activity was significantly increased in the CPH-treated eels in the forebrain (20-fold, $p < 0.001$), midbrain (17-fold, $p < 0.001$) and ovaries (12-fold, $p < 0.05$), while no significant change was observed in the pituitary as compared to the controls (Fig. 2a).

#### 3.2.3. Cyp19a1 transcripts

As for enzymatic activity, *cyp19a1* transcript levels were higher in the pituitary than in the brain and ovaries as indicated by comparison of Ct values (data not shown). *Cyp19a1* transcript levels

**Table 1**
Specific primers used for quantitative real-time PCR analyses (S, sense strand; AS, antisense strand).

| Gene | | Sequences | Amplicon size | GenBank Accession No. |
|---|---|---|---|---|
| *cyp19* | S | 5′-TCCTGCTCTACCTCTGGGTTCT-3′ | 84 bp | AY616117.1 |
| *cyp19* | AS | 5′-GATCATAGCCGCACCAGACA-3′ | | |
| *esr-α* | S | 5′-CGCTCCACGACCAGGTGCAG-3′ | 318 bp | HM545084.1 |
| *esr-α* | AS | 5′-GCGCCACACCAGCCCGATTA-3′ | | |
| *esr-β* | S | 5′-AGGATCCAAGGGCACAATG-3′ | 65 bp | AB003356.1 |
| *esr-β* | AS | 5′-CTTGCGCCGGTTCTTGTC-3′ | | |
| *ar-α* | S | 5′-CCCTACTCTGGTTCCTTGAAAAAC-3′ | 81 bp | AB023960.1 |
| *ar-α* | AS | 5′-CGCCCTCGTACCTGTAACTGA-3′ | | |
| *ar-β* | S | 5′-TGTGTTTCCCATGGAGTTCTTCTT-3′ | 63 bp | AB025361.1 |
| *ar-β* | AS | 5′-GCCTCGTCTGCGCAAATC-3′ | | |
| *gapdh* | S | 5′-GCCAGCCAGAACATCATC-3′ | 110 bp | AB049458.1 |
| *gapdh* | AS | 5′-GACACGGAAAGCCATACC-3′ | | |

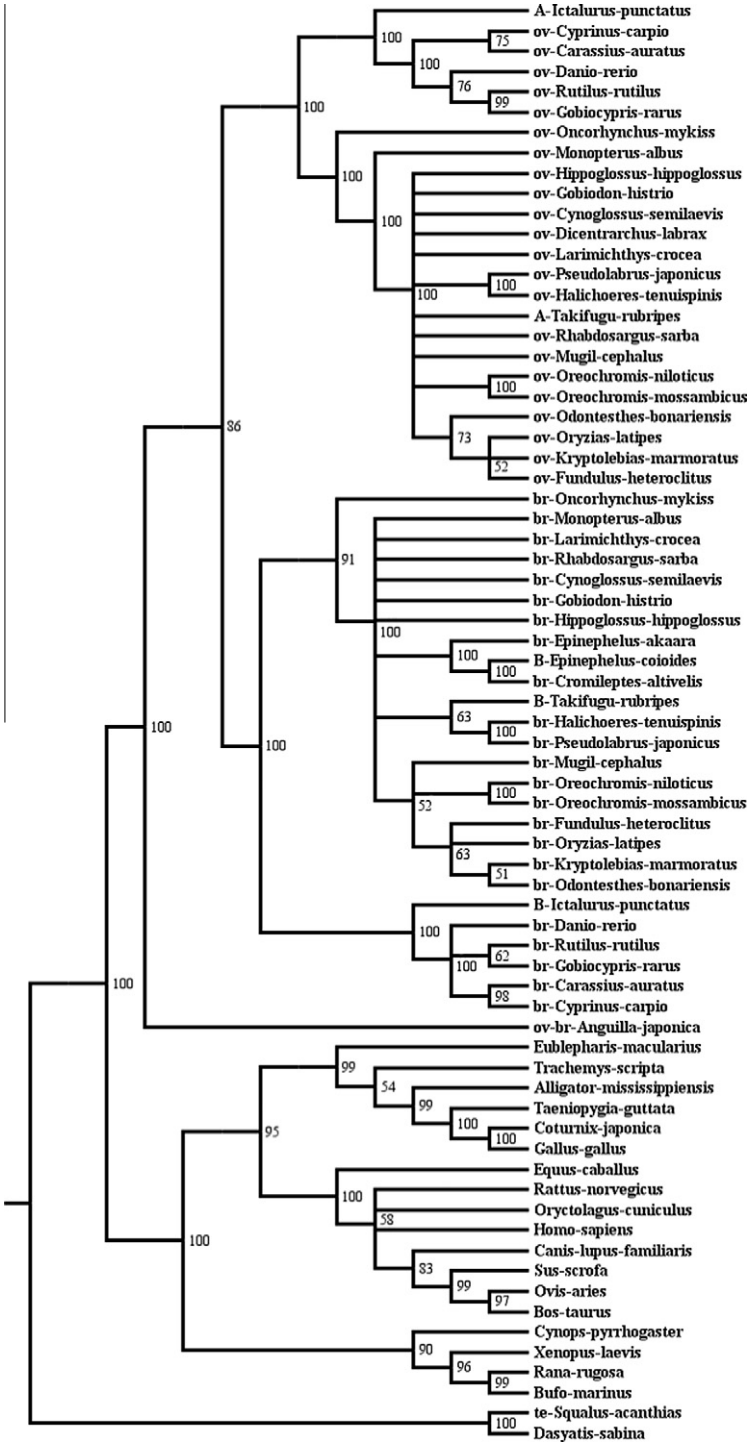


**Fig. 1.** Consensus phylogenetic tree of the vertebrate cyp19a1 constructed based on the amino-acid sequences of cyp19a1 using the maximum likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%); only values and branching above 50% are shown. The sequences used for this phylogenetic reconstruction were taken from NCBI data base (GenBank Accession No. between brackets): ov/br/*Cyprinus carpio* (ACB13197.1, ACB13198.1), ov/br/*Ictalurus punctatus* (AAB32613.1, NP_001187092.1), ov/br/*Kryptolebias marmoratus* (ABC68614.1, ABC68613.1), A/B/*Takifugu rubripes* (NP_001166967.1, BAF93506.1), ov/br/*Gobiocypris rarus* (ADB29065.1, ADB44882.1), ov/br/*Fundulus heteroclitus* (AAR97268.1, AAR97269.1), ov/br/*Oreochromis mossambicus* (AAD31031.1, AAD31030.1), ov/br/*Cromileptes altivelis* (AAV91178.1, AAV91181.1), ov/br/*Gobiodon histrio* (AAV91177.1, AAV91180.1), ov/br/*Larimichthys crocea* (ACO35041.1, ACO35042.1), ov/br/*Cynoglossus semilaevis* (ABL74474.1, ABM90641.1), A/B/*Epinephelus coioides* (AAR97601.1, AAR97602.1), ov/br/*Halichoeres tenuispinis* (AAR37048.1, AAR37047.1), ov/br/*Pseudolabrus japonicus* (ABB96485.1, ABB96486.1), ov/br/*Rhabdosargus sarba* (ABC70868.1, ABC70869.1), ov/br/*Anguilla japonica* (AAS47028.1, AAT36641.1), ov/*Salmo trutta* (AAR04775.1), ov/br-I/br-II/*Oncorhynchus mykiss* (1806325A, CAG32835.1, CAG32835.1). o, ov/br/*Danio rerio* (AAB65788.1, AAV41033.1), ov/br/*Dicentrarchus labrax* (CAC43178, AAM95455), ov/br/*Rutilus rutilus* (BAD91037.1, BAD91038), ov/br/*Epinephelus akaara* (AAS58448.1, AAS58447.1), ov/br/*Hippoglossus hippoglossus* (CAC36394.1, AAY26901.1), ov/br/*Mugil cephalus* (AAW72732.1, AAW72730.1), ov/br/*Monopterus albus* (ABX45101.1, ABX45102.1), ov/br/*Oreochromis niloticus* (AAB16814, AAG18458.1), ov/br/*Carassius auratus* (BAA23758.1, BAA23757.1), ov/br/*Oryzias latipes* (BAA11656.1, AAP83449.1), ov/br/*Larimichthys crocea* (ACO35041.1, ACO35042.1), ov/br/*Odontesthes bonariensis* (ABK30807.1, AAQ88434.2), *Homo sapiens* (NP_000094.2), *Equus caballus* (AAC04698.1), *Bos taurus* (CAA83651.1), *Xenopus laevis* (BAF48354.1), *Taeniopygia guttata* (NP_001070159.1), *Squalus acanthias* (ABB53418.1), *Dasyatis Sabina* (AAF04617.1), *Rattus norvegicus* (NP_058781.2), *Cynops pyrrhogaster* (BAD12119.1), *Trachemys scripta* (AAG09376.1), *Bufo marinus* (ACN54526.1), *Rana rugosa* (BAD21352.1), *Gallus gallus* (AAA48738.1), *Coturnix japonica* (AAN04475.2), *Ovis aries* (CAB40563.1), *Canis lupus familiaris* (CAH69600.1), *Sus scrofa* (NP_999594.1), *Oryctolagus cuniculus* (NP_001164392.1), *Eublepharis macularius* (BAE20061.1), *Alligator mississippiensis* (AAK31803.1). ov: ovarian form aromatase, br: brain form aromatase, te: testicular form aromatase. The tree was rooted using the sequences of *Squalus acanthias* and *Dasyatis sabina* cyp19a1.

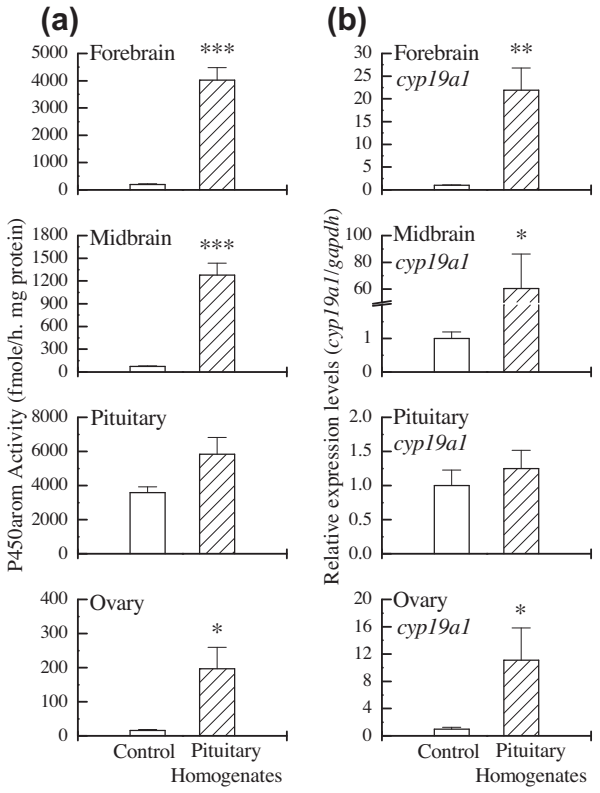


**Fig. 2.** Effect of catfish pituitary homogenates on forebrain, midbrain, pituitary and ovary (a) *cyp19* transcript levels and (b) aromatase enzymatic activity in female Japanese eels, as measured by real-time quantitative PCR. Bars represent mean ± SEM. Significant differences between the two groups are denoted by the following: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

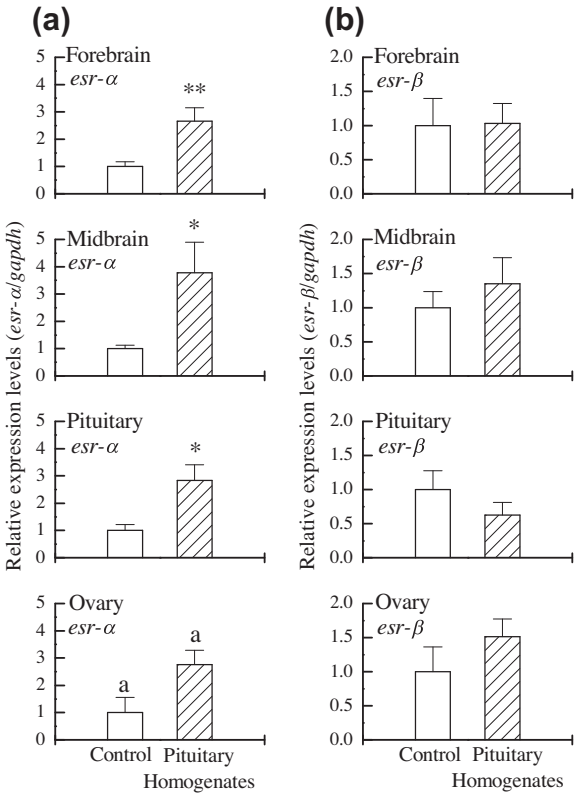


**Fig. 3.** Effect of catfish pituitary homogenates on forebrain, midbrain, pituitary and ovary (a) *esr-α* and (b) *esr-β* transcript levels in female Japanese eels, as measured by real-time quantitative PCR. Bars represent mean ± SEM. Significant differences between the two groups are denoted by the following: $^{*}p < 0.05$ and $^{**}p < 0.01$.

were significantly increased in CPH-treated eels in the forebrain (22-fold, $p < 0.01$), midbrain (60-fold increase, $p < 0.05$) and ovaries (11-fold increase, $p < 0.05$), whereas pituitary *cyp19a1* transcripts remained at a similar level in the CPH-treated eels as compared to the control eels (Fig. 2b).

### 3.2.4. Estrogen receptors (esr-α and esr-β) transcripts

*Esr-α* transcript levels were significantly increased in CPH-treated eels in the forebrain (3-fold, $p < 0.01$), midbrain (4-fold, $p < 0.05$) and pituitary (3-fold, $p < 0.05$) when compared with the control eels, whereas *esr-α* transcripts in the ovaries were not significantly different (Fig. 3a). *Esr-β* transcript levels were not significantly different in any tissue in the CPH-treated eels as compared with the control eels (Fig. 3b).

### 3.2.5. Androgen receptors (ar-α and ar-β) transcripts

*Ar-α* transcript levels were significantly increased in the CPH-treated eels in the midbrain (3-fold, $p < 0.05$) and ovaries (3-fold, $p < 0.05$), while a significant decrease was observed in the pituitary (3-fold decrease, $p < 0.01$) and no change was observed in the forebrain as compared to the control eels (Fig. 4a). *Ar-β* transcript levels were significantly increased in the ovaries of CPH-treated eels (7-fold, $p < 0.05$), while no significant changes were observed in the forebrain, midbrain and pituitary as compared to the control eels (Fig. 4b).

## 3.3. Effect of sex steroid treatments

### 3.3.1. Biometric parameters and serum steroid levels

No significant changes in GSI were observed after treatment with $E_2$ or T (0.77 ± 0.12% in the controls, 0.64 ± 0.06% in the low $E_2$-, 0.93 ± 0.09% in the low T-, 0.55 ± 0.17% in the high $E_2$-, 0.95 ± 0.11% in the high T-treated eels, *NS*). Histological observation showed that oocytes remained at the previtellogenic stage (no visible yolk globules) in steroid-treated eels as observed in the controls (data not shown). The HSI was not significantly changed after low $E_2$- (1.26 ± 0.06%, *NS*) or low T-treatment (1.30 ± 0.05%, *NS*), but significantly increased after high $E_2$- (1.57 ± 0.09%, $p < 0.05$) or high T-treatment (1.46 ± 0.09%, $p < 0.05$) as compared to the controls (1.08 ± 0.06%). $E_2$ serum levels were significantly increased after low $E_2$- (8.00 ± 0.49 ng/ml, $p < 0.001$) and high $E_2$-treatment (77.40 ± 6.17 ng/ml, $p < 0.001$) as compared to controls (0.30 ± 0.04 ng/ml). T serum levels were significantly increased after low T- (1.84 ± 0.26 ng/ml, $p < 0.01$) and high T-treatment (5.45 ± 1.21 ng/ml, $p < 0.01$) as compared to the controls (0.36 ± 0.04 ng/ml). $E_2$ serum levels remained low even after high T-treatment (0.64 ± 0.11 ng/ml, *NS* as compared to the controls).

### 3.3.2. Cyp19a1 transcripts

*Cyp19a1* transcript levels were significantly increased by $E_2$ treatment in a dose-dependent manner both in the forebrain and midbrain. In the forebrain, a 5-fold increase ($p < 0.001$) was induced by a low dose of $E_2$, and a 8-fold increase ($p < 0.001$) was induced by high dose of $E_2$ as compared to the controls. In the midbrain, a 4-fold increase ($p < 0.05$) was induced by low dose of $E_2$, and an 11-fold increase ($p < 0.01$) was induced by high dose of $E_2$ as compared to the controls. In contrast, pituitary and ovary *cyp19a1* transcript levels remained unchanged after $E_2$ treatment. Brain, pituitary and ovary *cyp19a1* transcripts were not significantly changed by any dose of T treatment (Fig. 5).

### 3.3.3. Estrogen receptor (esr-α and esr-β) transcripts

*Esr-α* transcript levels were significantly increased in the forebrain, pituitary and ovaries (2-, 7- and 3-fold, respectively;

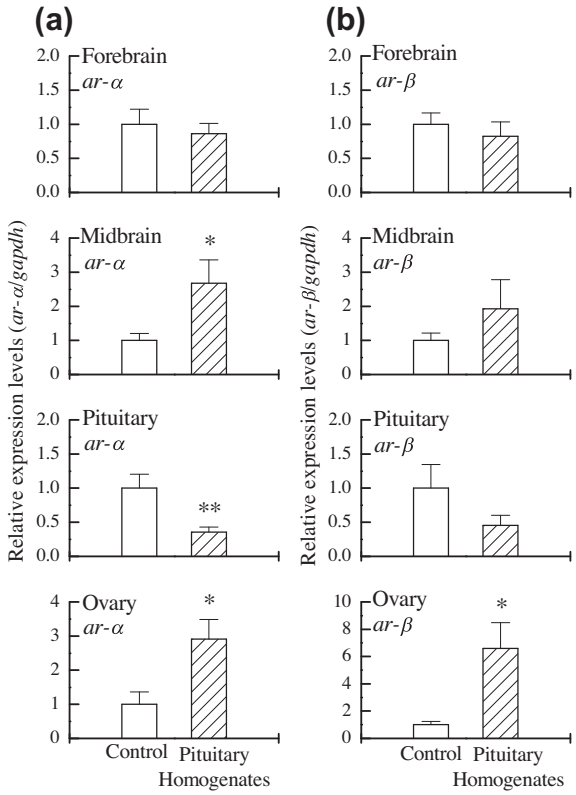


**Fig. 4.** Effect of catfish pituitary homogenates on forebrain, midbrain, pituitary and ovary (a) *ar-α* and (b) *ar-β* transcript levels in female Japanese eels, as measured by real-time quantitative PCR. Bars represent mean ± SEM. Significant differences between the two groups are denoted by the following: $^{*}p < 0.05$ and $^{**}p < 0.01$.

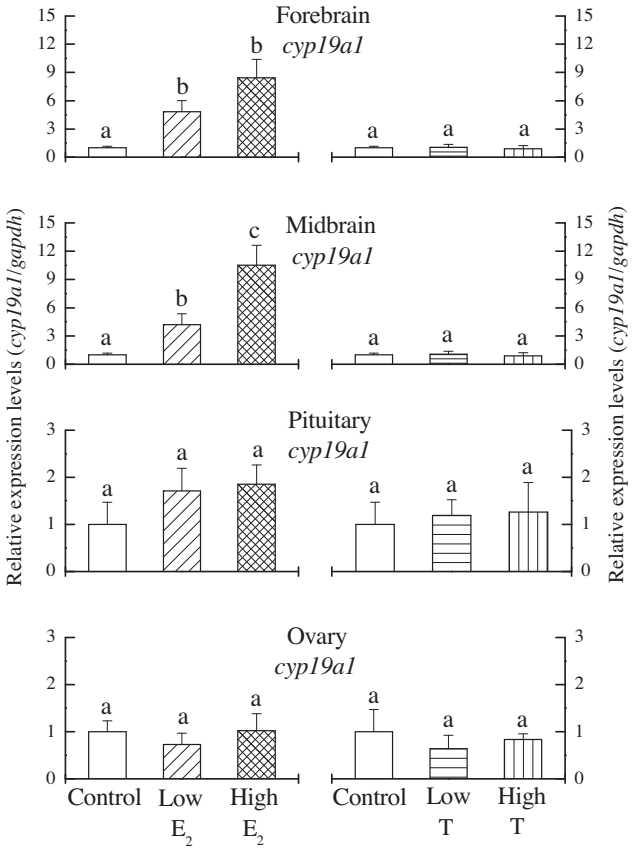


**Fig. 5.** Effect of sex steroid treatments on forebrain, midbrain, pituitary and ovary *cyp19* transcript levels in female Japanese eels, as measured by real-time quantitative PCR. Three-year-old cultivated female eels received weekly injections of estradiol-17-β ($E_2$) or testosterone (T) (low dose: 0.75 mg or high dose: 3.75 mg in coconut oil/kg BW) for 6 weeks. Control eels were injected with coconut oil alone. Bars represent mean ± SEM. Different letters represent significant differences among the groups. ($p < 0.05$ or $p < 0.001$).

$p < 0.05$) after a high dose of $E_2$ treatment, while a low dose of $E_2$ had no significant effect (Fig. 6). T at a low or high dose did not induce any changes in *esr-α* transcript levels in any tissue, except for a slight increase (2-fold increase, $p < 0.05$) in the forebrain in eels treated with a low dose of T (Fig. 6). Neither $E_2$ nor T induced any changes in *esr-β* transcript levels in the brain, pituitary and ovaries (Fig. 7).

### 3.3.4. Androgen receptors (ar-α and ar-β) transcripts

Treatments with $E_2$ or T did not induce any changes in *ar-α* (Fig. 8) or *ar-β* (Fig. 9) transcript levels in the brain, pituitary and ovaries.

## 4. Discussion

### 4.1. A single cyp19a1 gene is expressed in the brain and gonads in the eel

Duplicated genes for *cyp19a1* have been found in teleosts, likely resulting from the whole genome duplication event that occurred at the basis of their lineage [2]. Teleost *cyp19a1a* is expressed in the gonads, while *cyp19a1b* is expressed in the brain. In contrast, a single *cyp19a1* gene is found in eel species, and we showed that it is expressed in both the brain and the gonads, similar to tetrapods. To further understand the unique characteristics of eel *cyp19a1*, we performed a phylogenetic analysis of *cyp19a1* sequences. Eel *cyp19a1* branches at the base of the teleost *cyp19a1* cluster in agreement with the basal phylogenetic position of Elopomorphs among teleost species. Eel *cyp19a1* does not appear to be more related to teleost *cyp19a1a* than to teleost *cyp19a1b* clades. This suggests that single eel *cyp19a1* may have conserved ancestral properties of actinopterygian *cyp19a1*.

We may consider two alternative scenarios for the single *cyp19a1* gene in the eel. In the first scenario, whole genome duplication would have occurred early in the teleost lineage, before the separation of the Elopomorph group from the other teleosts, and eel single *cyp19a1* would reflect a specific loss of one duplicated *cyp19a1* gene in Elopomorh/Anguilla lineage. In the alternative scenario, whole genome duplication would have occurred after the separation of the Elopomorph lineage from the other teleosts groups and the single *cyp19a1* in the eel would simply reflect the ancestral situation of a unique gene. Increasing data are indicating the presence of various duplicated genes in the eel as well as in other teleosts, in favor of the first scenario. This is the case for instance for duplicated androgen receptors (*ar-α* and *ar-β*) first evidenced in the eel [57,24], or for duplicated dopamine D2 receptors as investigated by our group [47]. Furthermore, this has been recently highlighted by the study of hox genes in the Japanese eel revealing whole genome duplication [21]. This supports the hypothesis that the single *cyp19a1* gene in the eel would result from a specific *cyp19a1* gene loss in the Elopomorph/Anguilla lineage. The presence of a single *cyp19a1* gene in the eel may account for its low brain aromatase activity, similar to mammals, but differently from the other teleosts [25].

### 4.2. Upregulation of cyp19a1 expression in the brain and ovaries during induced maturation in the eel

The *cyp19a1* transcripts coding for the single brain/gonadal aromatase in the eel were significantly increased in the CPH-induced

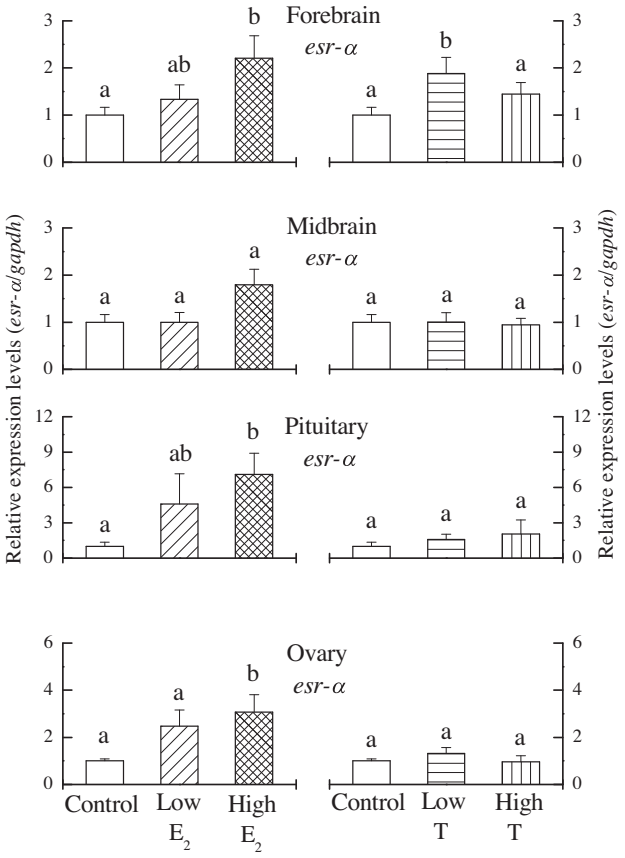


**Fig. 6.** Effect of sex steroid treatments on forebrain, midbrain, pituitary and ovary *esr-α* transcript levels in female Japanese eels, as measured by real-time quantitative PCR. Three-year-old cultivated female eels received weekly injections of estradiol-17-β ($E_2$) or testosterone (T) (low dose: 0.75 mg or high dose: 3.75 mg in coconut oil/kg BW) for 6 weeks. Control eels were injected with coconut oil alone. Bars represent mean ± SEM. Different letters represent significant differences among the groups. ($p < 0.05$).

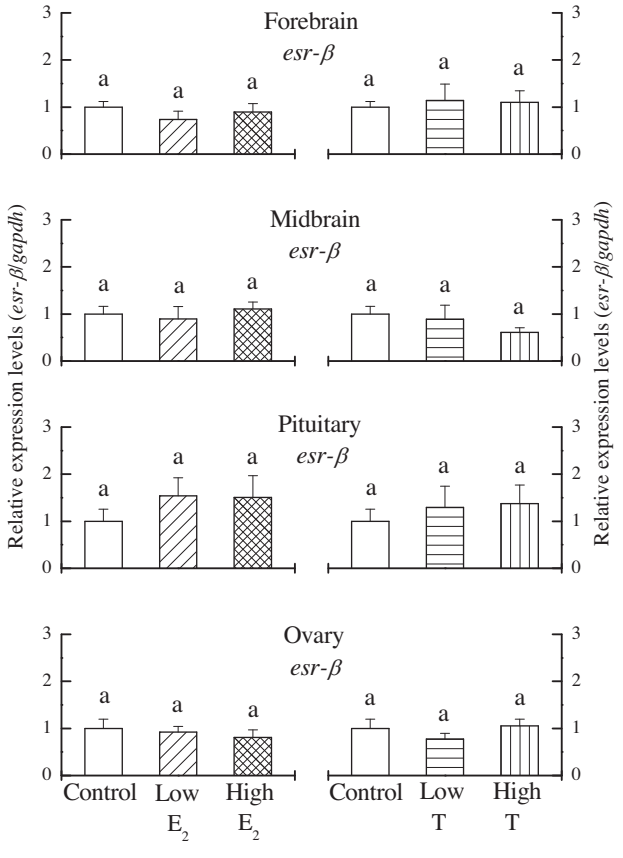


**Fig. 7.** Effect of sex steroid treatments on forebrain, midbrain, pituitary and ovary *esr-β* transcript levels in female Japanese eels, as measured by real-time quantitative PCR. Three-year-old cultivated female eels received weekly injections of estradiol-17-β ($E_2$) or testosterone (T) (low dose: 0.75 mg or high dose: 3.75 mg in coconut oil/kg BW) for 6 weeks. Control eels were injected with coconut oil alone. Bars represent mean ± SEM. Different letters represent significant differences among the groups. ($p < 0.05$).

matured eels in both the brain and ovaries. Similar increases were measured for aromatase enzymatic activity in the brain and ovaries of CPH-induced matured eels. These results are consistent with other studies in teleosts concerning both types of aromatase genes. For instance, the brain *cyp19a1b* transcripts levels and aromatase enzymatic activity were significantly increased during the spawning season in goldfish [17], black porgy [32], sea bass [19] and blue gourami (*Trichogaster trichopterus*) [16]. Similarly, the ovarian *cyp19a1a* transcript levels and aromatase enzymatic activity were significantly elevated during vitellogenesis in rainbow trout [53], Nile tilapia [8] and red seabream [18].

In contrast to the increases in *cyp19a1* transcripts levels in the brain and ovaries, no significant changes were observed in the pituitary in CPH-treated female eels as compared to control female eels. The expression of *cyp19a1b* transcripts also increased significantly in the brain, but not in the pituitary of grey mullet (*Mugil cephalus*) when the fish reached the puberty stage [42]. In contrast, Kazeto and Trant [27] indicated that brain and pituitary *cyp19a1b* expression levels increased dramatically prior to spawning in channel catfish (*Ictalurus punctatus*).

### 4.3. Estradiol-specific role in the upregulation of cyp19a1 expression in the brain

Circulating $E_2$ and T levels were elevated in the CPH-induced matured female eels, in agreement with previous studies in various eel species ( *A. japonica*: [23]; *Anguilla anguilla*: [10]; *Anguilla dieffenbachii*: [35]). Both $E_2$ and T could therefore be potentially involved in feedback regulations of gene expression during sexual maturation. For instance, our previous studies demonstrated an $E_2$-dependent up-regulation of mGnRH (mammalian type gonadotropin releasing hormone) and testosterone-dependent down-regulation of cGnRH-II (chicken 2 type GnRH) in the brain of matured female European eels [38]. We investigated the potential role of these sex steroids in the increase in *cyp19a1* expression by performing steroid treatments. We observed a dose-dependent stimulatory effect of $E_2$ on *cyp19a1* expression in the brain, but not in the ovaries. This result reveals a differential regulation by steroids of eel *cyp19a1* transcripts in the brain and in the ovaries, with an estradiol-specific upregulation in the brain. The effect of $E_2$ in the brain was specific, as no change was induced by androgen.

In other teleosts, both aromatizable androgens and estrogens are known to upregulate brain *cyp19a1b* transcripts (goldfish: [17]; zebrafish: [28,37]). Androgens are considered to act through estrogen receptors after local aromatization, but not via androgen receptors (for review: [13]). In zebrafish, Mouriec et al. [40] recently found that brain *cyp19a1b* was upregulated by T and also by dihydroxytestosterone (DHT), an androgen generally considered as non-aromatizable in mammals. They suggested that the effect of DHT on the brain *cyp19a1b* gene was due to the conversion of DHT into a metabolite with estrogenic activity in zebrafish. In our study, T had no significant effect on brain *cyp19a1* expression in contrast to the upregulatory effect of $E_2$. The low level of brain aromatase activity in eel, unlike in zebrafish and other teleosts, likely accounts for the

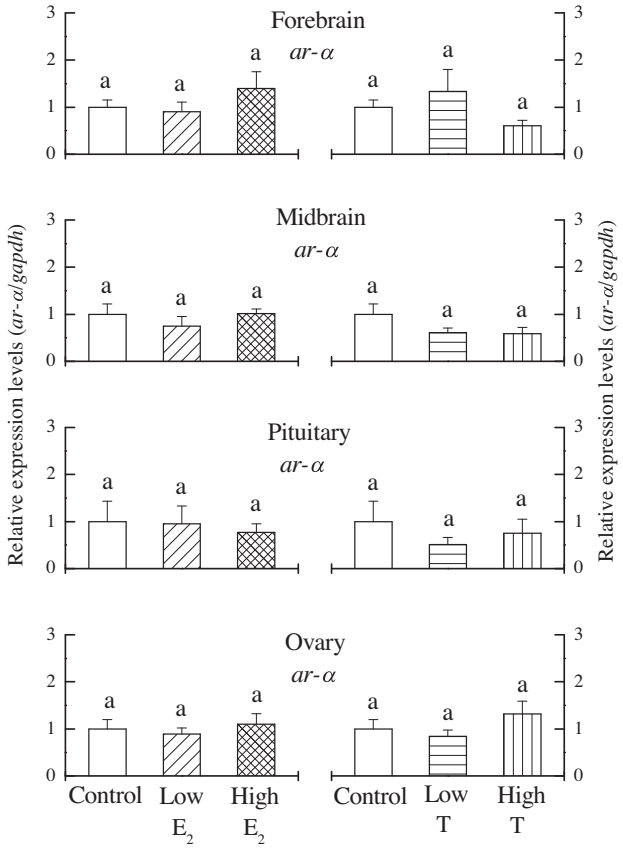


**Fig. 8.** Effect of sex steroid treatments on forebrain, midbrain, pituitary and ovary *ar-α* transcript levels in female Japanese eels, as measured by real-time quantitative PCR. Three-year-old cultivated female eels received weekly injections of estradiol-17-β ($E_2$) or testosterone (T) (low dose: 0.75 mg or high dose: 3.75 mg in coconut oil/kg BW) for 6 weeks. Control eels were injected with coconut oil alone. Bars represent mean ± SEM. Different letters represent significant differences among the groups. ($p < 0.05$).

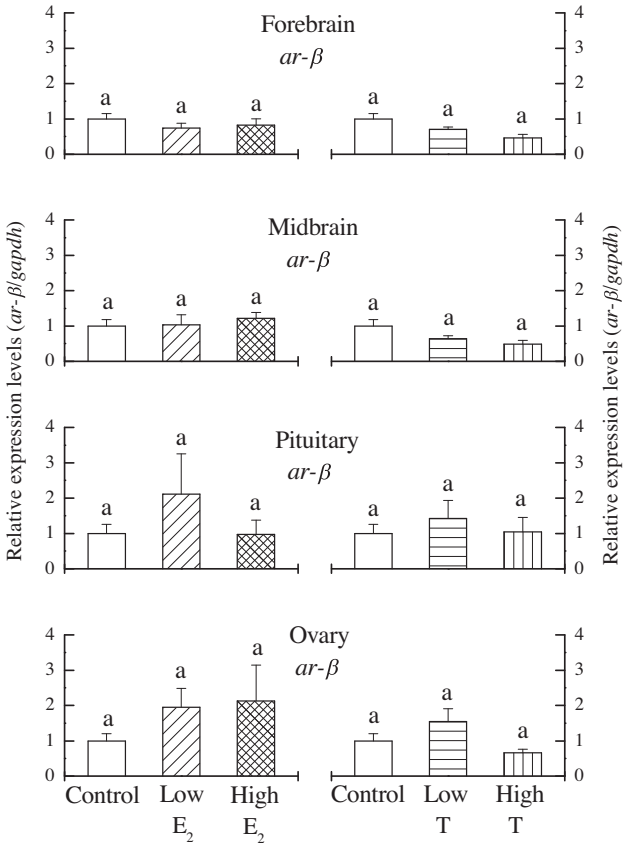


**Fig. 9.** Effect of sex steroid treatments on forebrain, midbrain, pituitary and ovary *ar-β* transcript levels in female Japanese eels, as measured by real-time quantitative PCR. Three-year-old cultivated female eels received weekly injections of estradiol-17-β ($E_2$) or testosterone (T) (low dose: 0.75 mg or high dose: 3.75 mg in coconut oil/kg BW) for 6 weeks. Control eels were injected with coconut oil alone. Bars represent mean ± SEM. Different letters represent significant differences among the groups. ($p < 0.05$).

lack of an effect of T on *cyp19a1* expression. The low central aromatase activity in the eel, a unique feature among teleosts, may have favored various estrogen-specific or inversely androgen-specific regulatory processes, as also highlighted by our previous neuroendocrine studies (i.e., [4,47,63]). Estrogen response elements have been found on the brain *cyp19a1b* promoter, but not on the gonadal *cyp19a1a* promoter in all investigated teleosts (goldfish: [7], zebrafish: [58], grey mullet: [42]). This ERE is supposed to mediate the upregulatory effect of $E_2$ on brain *cyp19a1b* in these teleosts. In mammals, no typical ERE was found in the promoter of the single *CYP19A1*. Yilmaz et al. [65] demonstrated that in the mouse, $E_2$ upregulates brain *CYP19A1* expression via the brain-specific promoter I.f by enhancing the binding of an estrogen receptor-α/JUN complex to distinct activator protein1 (AP-1) motifs in hypothalamic cells. Further studies in the eel will aim at deciphering if the $E_2$-dependent stimulation of the expression of the single aromatase gene *cyp19a1* in the brain is mediated via an ERE similar to teleost *cyp19a1b* or via a mammalian-like pathway.

### 4.4. Steroid-independent upregulation of cyp19a1 expression in the ovaries

In contrast to the $E_2$ upregulatory effect on *cyp19a1* expression in the brain, neither $E_2$ nor T had any effect on *cyp19a1* expression in eel ovaries. Although a previous study in black porgy had demonstrated higher gonadal aromatase enzymatic activity after long-term treatment with $E_2$ [9], most of the studies on teleosts showed that ovarian *cyp19a1a* expression was not estrogen-inducible (goldfish: [7]; zebrafish: [28]). As mentioned above, in teleosts, no ERE could be detected on the promoter of gonadal *cyp19a1a*.

These findings suggest a steroid-independent regulatory mechanism for the increase in *cyp19a1* expression in the ovaries of CPH-induced matured eels. In mammals, Yong et al. [66] indicated that FSH and LH strongly stimulated human *CYP19A1* mRNA expression in mature human granulosa cells. In salmonids, Montserrat et al. [39] also showed that coho salmon FSH directly stimulated ovarian *cyp19a1a* expression levels and aromatase enzymatic activity in vitellogenic ovarian follicles of brown trout (*Salmo trutta*) *in vitro*. We may therefore hypothesize that the increase in ovarian *cyp19a1* expression and enzymatic activity in CPH-treated eels may result from a direct stimulation by gonadotropins, without excluding possible other indirect effects.

### 4.5. Differential regulation of steroid receptor subtype expression during induced maturation and under the effects of sex steroids

We observed a differential regulation of the expression of eel estradiol receptor subtypes during induced maturation. The expression levels of *esr-α* increased in the brain and pituitary in CPH-treated eels, with no significant change in the ovaries. No change in the expression of *esr-β* was observed in any organ. Treatment with a high dose of $E_2$ was also able to increase *esr-α*, but not *esr-β* expression. This upregulation was $E_2$-specific, as no significant effect was observed with T treatment on *esr-α* or *esr-β* expression. This

suggests that the selective increase in *esr-α* expression in CPH-treated matured eels may be mediated by endogenous $E_2$.

These results indicate a differential autoregulation of $E_2$ receptor subtypes by $E_2$ in the eel, with an up-regulation of *esr-α*, but not *esr-β* expression. In contrast, in goldfish, Nelson et al. [41] observed that the ovarian transcripts of *esr-α*, *esr-β1* and *esr-β2* were all significantly upregulated by $E_2$. The presence of an ERE in the promoter of *esr-α* has been first demonstrated in the rainbow trout [31]. More recently, in zebrafish, an imperfect ERE has been found in the promoter of *esr-α* along with an ERE half site with no ERE consensus in the promoter of *esr-β* [30,36].

Concerning androgen receptor subtypes, we found in CPH treated eels, an increase in *ar-α* expression levels in the midbrain, no change in the forebrain and a decrease in the pituitary. However, neither $E_2$ nor T treatment induced any significant up- or down-regulation of *ar-α* and *ar-β* in the eel brain and pituitary. This suggests that up and down regulation of *ar-α* in CPH-treated eels may not result from auto-regulation by androgens nor from cross regulation by estrogens, but from other indirect maturation-related mechanisms. Recently, a single *ar* was identified in a cyprinid fish, *Spinibarbus denticulatus*, and no significant variation of its mRNA levels was found throughout the ovarian recrudescence, in brain, pituitary and ovaries [33].

The ovarian *ar-α* and *ar-β* transcript levels were remarkably increased in CPH-treated eels. The importance of ARs in eel maturation has been suggested because exogenous androgen, but not estrogen, in combination with pituitary extracts can enhance eel maturation [62]. In *in vitro* studies, 11-ketotestosterone (11-KT), but not $E_2$, induced a significant increase in the diameters of previtellogenic oocytes and oocyte nuclei in a dose-dependent manner in New Zealand shortfinned eel [34]. More recently, data showed that *ar-α* transcript levels were high from the late oil droplet stage to the late vitellogenic stage, while *ar-β* transcript levels were high from the late oil droplet stage to the midvitellogenic stage in the Japanese eel [59]. In contrast to the clear-cut effect of CPH treatment on ovarian *ar-α* and *ar-β* expression levels, $E_2$ or T treatment did not have any significant effect; this suggests that the stimulatory effect of CPH would not be mediated by endogenous $E_2$ and T in female eels. Further studies are required to decipher whether this effect on ovarian AR may be the result from a direct action of pituitary hormones, as hypothesized above, for the increase in ovarian aromatase.

In conclusion, the present study showed that the expression of the single aromatase gene *cyp19a1* present in the eel is upregulated in both the brain and the ovaries during maturation induced by CPH treatment. However, different regulatory mechanisms may be involved, with an estradiol-specific induction of *cyp19a1* expression in the brain, but not in the ovaries, where *cyp19a1* would be submitted to a steroid-independent control. The differential regulation of the expression of sex steroid subtypes esr-α, ar-α and ar-β was evidenced during induced maturation, while no changes were observed for esr-β. An upregulation of esr-α, but not esr-β expression, by $E_2$ in the brain and ovaries was evidenced. In contrast, no apparent autoregulation by T of the ar-α and ar-β receptors was found. These results reveal both steroid-dependent and -independent mechanisms in the regulation of aromatase and steroid receptor expression in the eel.

## Acknowledgment

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