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The regulation of aromatase and androgen receptor expression during gonad development in male and female European eel

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Abstract

This research investigated the regulation of aromatase and androgen receptor gene expression in the brain-pituitary-gonad (BPG) axis of male and female European eels (*Anguilla anguilla*) during induced sexual maturation. Complete *Anguilla anguilla* aromatase (*aa-cyp19a1*) and partial androgen receptor α and β (*aa-ara* and *aa-arb*) sequences were isolated, and qPCR assays were validated and used for quantification of transcript levels for these three genes. Expression levels of the genes varied with sex, tissue, and stage of maturation. *aa-arb* was expressed at higher levels than *aa-ara* in the pituitary and gonad in both sexes, suggesting *aa-arb* is the physiologically most important androgen receptor in these tissues. In the female brain, a decrease in *aa-ara* and an increase in *aa-cyp19a1* were observed at the vitellogenic stage. In contrast, a progressive increase in all three genes was observed in the pituitary and ovaries throughout gonadal development, with *aa-arb* and *aa-cyp19a1* reaching significantly higher levels at the vitellogenic stage. In the male pituitary, a decrease in *aa-arb* and an increase in *aa-cyp19a1* were observed at the beginning of spermatogenesis, and thereafter remained low and high, respectively. In the testis, the transcript levels of androgen receptors and *aa-cyp19a1* were higher during the early stages of spermatogenesis and decreased thereafter. These sex-dependent differences in the regulation of the expression of *aa-ara*, *aa-arb*, and *cyp19a1* are discussed in relation to the role of androgens and their potential aromatization in the European eel during gonadal maturation.

1. Introduction

The lifecycle of the European eel, *Anguilla anguilla* (L.) includes both oceanic and continental phases. European eels reproduce in the Atlantic Ocean, supposedly in the Sargasso Sea, after which the leptocephalus larvae drift towards the European coast, where they metamorphose into glass eels before entering the long growth phase as yellow eels in fresh or coastal water. At the start of their reproductive migration towards the Sargasso Sea, yellow eels transform into silver eels, which are still immature and remain blocked in the pre-pubertal stage as long as migration is prevented. This makes the eel an interesting model organism in the investigation of the regulatory mechanisms of reproductive development.

The pre-pubertal stagnation is due to insufficient release of pituitary gonadotropins, resulting from both a lack of stimulation by the gonadotropin-releasing hormone (GnRH) and a strong dopaminergic inhibition (Dufour et al. 1988, Vidal et al. 2004). Therefore, in order for eels to mature in captivity, gonadotropin treatment is necessary (e.g. Miura et al. 1991, Pérez et al. 2000, 2011). Upon gonadotropin stimulation, synthesis of gonadal androgens (testosterone (T) and 11-ketotestosterone (11-KT)) and estrogens (17β-estradiol (E_2)) increases. These androgens play crucial roles during gonad development, both by stimulating gamete development, and through feedback mechanisms to the brain and pituitary.

Unlike in mammals, plasma levels of androgens are relatively high in both female and male fish (Borg 1994). The effects of the androgens on their target cells may be direct, through binding to androgen receptors, or indirect, through local aromatization into estrogens by the complex enzyme aromatase. Aromatase is a member of the P450 cytochrome superfamily of enzymes and acts as a catalyst in the creation of estrogens from androgens. In contrast to the single form found in mammals (Harada, 1988), two paralogous genes of P450 aromatase have been found in teleosts, one of which is expressed mainly in the brain (*cyp19a1b*) and the second mainly in the gonad (*cyp19a1a*). This phenomenon of fish possessing two aromatase genes was first reported in goldfish (*Carassius auratus*, Gelinas et al. 1998), zebrafish (*Danio rerio*, Kishida and Callard 2001), and tilapia

(*Oreochromis niloticus*, Chang et al. 1997, Kwon et al. 2001), and has since been confirmed in many other teleost species (e.g. Blázquez and Piferrer 2004, Choi et al. 2005). In eels, however, only one aromatase cDNA has been identified (termed *cyp19a1*), and is expressed in the ovary, brain and pituitary (Ijiri et al. 2003, Tzchori et al. 2004).

In teleosts, two androgen receptor paralogs (ARα and ARβ, encoded by the genes *ara* and *arb*, respectively) have been identified through phylogenetic analyses, and evaluation of tissue distribution and ligand affinity (Sperry and Thomas 1999a, Olsson et al. 2005, Harbott et al. 2007). The expression of both androgen receptors (AR) has been demonstrated in a wide range of tissues in different fish species (in the brain, pituitary, gonad and peripheral tissues) (Todo et al. 1999; Blazquez and Piferrer, 2005). Although the biochemical characteristics of ARβ are similar to those of mammalian AR (Wilson and French 1976), the binding characteristics of ARα make this receptor unique to teleosts. While ARα binds only T with high affinity, ARβ is able to bind a wide spectrum of synthetic and natural androgens with approximately the same steroid-binding specificity. In addition, the tissue distribution differs, with ARα found exclusively in the brain, and ARβ in both the central nervous system and the peripheral tissues (Sperry and Thomas 1999a,b). Both AR genes are expressed in Japanese eel (*A. japonica*; Ikeuchi et al. 1999, Todo et al. 1999), but no significant differences in their androgen binding capacity have been detected. Both eel ARs are activated by 11-KT, and also by 5α-dihydrotestosterone which, along with T, is the most important androgen in mammals (Ikeuchi et al., 2001).

The purpose of this study was to measure the levels of *aa-cyp19a1*, *aa-ara*, and *aa-arb* in the brain, pituitary, and gonads during the artificial maturation of European eels of both sexes in order to improve our understanding of their regulation during eel reproductive development.

2. Materials and Methods

2.1. Fish maintenance, hormonal treatment and sampling

2.1.1 Maturation and sampling of female European eel

Forty-four wild female eels at the silver stage (mean body weight 847±28 g; mean body length 71.4±0.8 cm) caught by local fishermen in the Albufera Lagoon (Valencia, Spain) in October and November during their reproductive migration to the sea, were transported to the Aquaculture Laboratory at the Universitat Politècnica de València. They were kept in two 500 L fiberglass tanks equipped with separate recirculation systems. Over the course of two weeks the fish were acclimatized from fresh water to seawater (salinity 37.0±0.3%). Water temperature was maintained at 18 ºC throughout the experiment. The eels were treated weekly with intraperitoneal injections of carp pituitary extract (CPE, Catvis Ltd, Netherlands) for 12 weeks, at a dose of 20 mg/kg body weight (Pérez et al. 2011). The CPE was diluted in NaCl solution (0.9 g/l), centrifuged (1260 *g*, 10 min) and the supernatant was stored at -20 ºC until its use between 1 and 4 weeks later. Groups of 6 eels were anaesthetized and sacrificed by decapitation at weeks 4, 8 and 12. Additionally, before starting the hormonal treatment, two groups of female eels also from Albufera Lagoon (n=6) were sacrificed in fresh (F) and seawater (S) conditions with the aim to evaluate the possible influence of salinity.

Samples from the ovary, pituitary, and brain were collected from the eels at sacrifice and stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 ºC until extraction of total RNA (Peñaranda et al. 2010). The brain samples were divided into three sections: the olfactory bulb (OB), telencephalon (Tel), and di- and mesencephalon (DM), by the method described by Weltzien et al. (2005). In addition, ovarian tissue samples were fixed in 10 % formalin buffered at pH 7.4 for histological processing and measurement of oocyte diameter in order to determine the maturational status (Pérez et al. 2011).

2.1.2 Maturation and sampling of male European eel

One hundred and fifty male eels at the silver stage (mean body weight 124.1±12.6 g; mean body length 39.9±0.21 cm) were purchased from the Valenciana de Acuicultura, S.A fish farm (Puzol, Valencia) and transported to the facilities at the Universitat Politècnica de València. The fish were gradually acclimatized to seawater (salinity 37±0.3%; 20 ºC) over the course of one week and equally

distributed into three tanks of 500 L equipped with separate recirculation systems.

In order to induce maturation and spermiation, the eels were treated with weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU g/fish; Angelini Farma-Lepori, Barcelona, Spain) over the course of 13 weeks, as previously described by Pérez et al. (2000). Groups of 10 eels were anaesthetized and sacrificed by decapitation each week along the hormonal treatment. Additionally, before starting the hormonal treatment, 6 male eels also from Valenciana de Acuicultura, S.A fish farm were sacrificed in seawater (S) conditions, being considered as the control group.

Samples from the testis and pituitary were collected from the eels at sacrifice and then stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 ºC until the extraction of total RNA (Peñaranda et al. 2010). As the different sections of the brain were not collected during the first few weeks of treatment, the brains from male fish were not included in this study. In addition, testicular tissue samples were fixed in 10 % formalin buffered at pH 7.4 for histological processing and subsequent determination of maturational status.

2.2 Histology processing

The ovarian and testicular samples were processed and analyzed as described by Peñaranda et al. (2013). The diameters of the largest oocytes in the ovarian samples ($n \geq 100$/female) were measured using Camera Control Unit software (Nikon, Japan).

Three stages of the ovarian development were distinguished: 1) previtellogenic (PV; mean ovarian diameter 155±6.7 µm) - perinucleolar stage with no or few lipid droplets, or in lipid droplet stage but without yolk vesicles. 2) Early vitelogenic (EV; 211±10.7 µm) - small yolk vesicles restricted to the periphery of the oocyte. Vitellogenic (V; 402±22.6 µm) - mid- and late vitellogenesis are included in this stage; and 3) Vitellogenic (V; 402±22.6 µm) - mid- and late vitellogenesis are included in this stage. Mid-vitellogenesis is characterized by numerous yolk vesicles in the cytoplasm from the membrane to the nucleus, and a lower proportion of yolk vesicles compared to lipid droplets.

Late vitellogenesis is characterized by fewer, enlarged yolk vesicles and a higher proportion of lipid droplets compared with yolk vesicles (Supplementary Figure 1A) (Perez et al, 2011; Mazzeo et al, 2012).

Spermatogenesis stages were determined by identifying the most advanced germ cell type present and relative abundance (Peñaranda et al. 2010, 2013; Supplementary Figure 2A). Stage 1 was characterized by the presence of spermatogonia type A and/or B; Stage 2 by the presence of spermatogonia and spermatocytes; Stage 3 by spermatids appearing in the testis tissue, and Stages 4, 5 and 6 by spermatozoa appearing inside the lumen of the lobules. Distinguishing between stages 4-6 was based on the percentage of spermatozoa present in relation to the lobule lumen surface.

2.3 Measurement of gene expression by real-time quantitative RT-PCR (qPCR)

2.3.1 Primer design

Eel acidic ribosomal phosphoprotein P0, *aa-arp* (Table 1, Weltzien et al. 2005, Aroua et al. 2007, Peñaranda et al. 2010) was used as a reference gene in the qPCR analysis because its mRNA expression is stable throughout hormonal treatment (Weltzien et al. 2005). The expression stability of the reference gene was determined using the BestKeeper program (Pfaffl et al. 2004), reporting a standard deviation (SD[±Cq]) lower than 1 (testis: 0.59; ovary: 0.21; brain and pituitary: 0.53, $p<0.05$) and a Cq arithmetic mean of 19.51±1.11 in the testis, 10.1±0.72 in the ovary, and 19.32±1.67 in the brain. The BestKeeper calculated variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. The Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used to design specific primers for *aa-cyp19a1*, *aa-ara*, and *aa-arb* (Table 1). To avoid detection of genomic DNA (gDNA), at least one primer per pair was designed to span an exon-exon boundary. All the primers were tested on gDNA and RNA to confirm that potentially contaminant gDNA was not amplified. The specificity was confirmed by melting curve analysis, gel electrophoresis, and sequencing of the qPCR products.

184 *2.3.2 SYBR Green assay (qPCR)*

185 Total RNA was isolated from the RNAlater preserved tissues as described by Hildahl et al. (2011),
186 and then treated with DNase I (Turbo DNA-free; Ambion) at 37 °C for 30 min. First-strand cDNA
187 was synthesized from either 1 µg (brain and testis) or 2 µg (ovary) total RNA, using random hexamer
188 primers and superscript III reverse transcriptase (Invitrogen).

189 qPCR assays were performed as described by Weltzien et al. (2005) and Peñaranda et al.
190 (2013), using a Light Cycler 480 system with SYBR Green I detection (Roche, Meylan, France).
191 After an initial activation of *Taq* polymerase at 95 ºC for 10 min, 42 PCR cycles were performed at
192 the following cycling conditions: 95 ºC for 10 s, 60 ºC for 10 s, and 72 ºC for 7 s. Each PCR reaction
193 contained a total of 10 µl, comprising 1:10 diluted cDNA template (3 µl), forward and reverse primers
194 (250 nM each), and SYBR Green Master Mix (5 µl). Transcript levels were determined using an
195 efficiency-adjusted relative quantification method as described by Weltzien et al. (2005). Target and
196 reference genes in unknown samples were run in duplicate PCR reactions, and a cDNA pool from
197 various samples was included in each run as a calibrator. Non-template control (cDNA was replaced
198 by water) for each primer pair was run in duplicate on all plates.

199

200 *2.3.3 Tissue-Specific Expression of aa-cyp19a1*

201 Three silver males from the Valenciana de Acuicultura S.A fish farm, and three silver females from
202 the Albufera lagoon, with average body weights of 118±14.7 g and 632±46.5 g, respectively, were
203 sacrificed. RNA was extracted as described above from nine different tissues: the gonad, liver,
204 pectoral fin, anterior and posterior kidney, heart, gill, pituitary, and brain. The brain was divided into
205 five parts: olfactory bulbs, telencephalon, di- and mesencephalon, cerebellum, and medulla oblongata,
206 as previously reported by Weltzien et al. (2005). Total RNA was treated with DNase I (Turbo DNA-
207 free; Ambion) at 37 °C for 30 min. cDNA was prepared from 0.5 µg total RNA using superscript III
208 reverse transcriptase (Invitrogen) and random hexamer primers according to the standard protocol.
209 The existence of *aa-cyp19a1* transcripts in the various tissues was analyzed by qPCR.

2.4 Molecular cloning of *aa-cyp19a1*, *aa-ara* and *aa-arb* cDNAs

First-strand cDNA was synthesized from 2 µg of total RNA (extracted from the ovary and brain and DNase treated as described in section *2.3.3*), using random hexamer primers and superscript III reverse transcriptase (Invitrogen). Primer3 shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3) was used for the design and theoretical evaluation of PCR primers. Primers for initial fragment cloning were designed from the corresponding Japanese eel sequences (GenBank accession numbers: *ara* AB023960, *arb* AB025361, and *cyp19a1* AY540622).

PCR amplification was performed in an ABI GeneAmpTM system 2700 thermo cycler. The reaction mixture of 25 µl contained 1x PCR buffer (Invitrogen), 200 µM dNTPs (Invitrogen), 0.1 IU of Taq DNA polymerase (Invitrogen), 500 nM of each primer and 1 µl of cDNA template. The first PCR amplification was run as follows: denaturation at 94 ºC for 3 min, followed by 40 cycles at 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, and finally an extension step at 72 ºC for 10 min. The PCR products were visualized in 2 % agarose gel stained with ethidium bromide and bands of expected size were purified using a Qiaquick Gel Extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent *E. coli* JM109 cells (Promega). Positive colonies were isolated and plasmids extracted by a Qiagen Plasmid Mini Kit (Qiagen). Plasmids with inserts were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, University of Oslo sequencing service, Oslo, Norway).

The partial AR sequences were confirmed by comparing the results with those obtained by Lafont et al. (Unpublished results) (GenBank accession no. FR668031 and FR668032). The *aa-cyp19a1* full-length sequence was confirmed by comparison with the recently sequenced eel genome (Henkel et al. 2012) and the published sequence from Tzchori et al. (2004). The eel genome (Henkel et al. 2012) was also used to search for potential additional paralogous AR or aromatase genes.

2.5 Statistical analysis

Differences in the data were analyzed by analysis of variance (One-way ANOVA). A Student-Newman-Keuls test was used to compare means and the differences were considered significant when $P<0.05$. The results are presented as means ± standard error of means (SEM). All statistical procedures were run using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

3. Results

The gene expression data in the case of both male and female eels are presented according to the stage of gonad development.

3.1 Female eels

3.1.1 Ovarian development

Four hormonal injections were sufficient to stimulate ovarian development to the EV stage in most female eels (83 %; Supplementary Figure 1B), while eight weeks were necessary to reach the V stage (34 %). After 12 weeks of treatment, 83 % of females had reached the V stage.

3.1.2 aa-cyp19a1 and aa-ARs expression levels in the brain

In OB and DM, *aa-ara* expression levels were significantly lower in V stage than the previous stage (EV; Fig. 1A). Similar results were also seen in Tel, although this was not significant. *aa-arb* expression levels increased from the PV to the EV stage in OB, while non-significant differences were found in Tel and DM (Fig. 1B). For *aa-cyp19a1,* transcript levels in OB increased continuously from the PV to the EV stage and continued to the V stage (Fig. 3A). In DM, the increase was visible at the V stage (Fig. 3C). No differences in *aa-cyp19a1* expression levels between the developmental stages were found in Tel (Fig. 3B).

3.1.3 aa-cyp19a1 and aa-ARs expression levels in the pituitary

Expression of *aa-ara* and *aa-arb* in the female pituitary differed from each other. While transcript

levels of *aa-arb* increased from the PV to the EV stage and further to the V stage, similar but non-significant results were observed for *aa-ara* transcript levels (Fig. 2B). Additionally, the *aa-arb* expression levels were approximately eight times higher than *aa-ara*. *aa-cyp19a1* expression levels increased markedly (about eight-fold) from the PV and EV stages to the V stage (Fig. 3D). The *aa-cyp19a1* expression levels in the pituitary in the V stage were higher than those found at any stage in the different brain regions.

3.1.4 aa-cyp19a1 and aa-ARs expression levels in the ovary

Ovarian *aa-ara* and *aa-arb* expression levels increased during oogenesis (Fig. 4C and 4D). *aa-arb* gene expression levels increased significantly from the PV to the EV stage, and continued into the V stage, whereas a significant increase in *aa-ara* gene expression levels was only seen from the PV to the V stage. Based on Cq values, *aa-arb* expression levels were about 27-times higher than *aa-ara* levels. Ovarian *aa-cyp19a1* levels varied markedly between samples from the EV and V groups. The average levels were higher than those in the brain, and increased in line with the gonad development, with a significant increase at the V stage (Fig. 5B).

3.2 Male eels

3.2.1 Testicular development

After having been treated with hCG for two weeks, all the males reached spermatogonial stage 2 (Supplementary Figure 2B). Stages 3 and 4 were seen after three weeks, whereas stages 5 and 6 were first observed after four and seven weeks, respectively. From seven weeks until the end of the experiments most males were in stage 5 or 6, with some, possibly non-responders, remaining in stage 4.

3.2.2 aa-cyp19a1 and aa-ARs expression levels in the pituitary

Both *aa-ara* and *aa-arb* differed between male and female pituitaries (Fig. 2). While *aa-ara* expression levels increased in the female pituitaries during the gonad development, expression levels in the male pituitaries remained relatively stable with no significant changes between the stages. However, expression levels of *aa-arb* decreased between stages 2 and 3/4, and thereafter remained stable. Although comparisons between genes/primer pairs should be treated cautiously, the *aa-arb* expression levels were again higher than the *aa-ara* levels (about 7-fold). A significant increase in the *aa-cyp19a1* expression levels in the pituitary coincided with the appearance of spermatocytes in the testis (stage 2), with expression levels remaining elevated in the subsequent developmental stages (Fig. 3E)

3.2.3 aa-cyp19a1 and aa-ARs expression levels in the testis

Both *aa-ara* and *aa-arb* expression levels followed similar results, decreasing at the beginning of spermatogenesis and thereafter remaining at low levels (stage 2; Fig. 4A and 4B). Similar to results from the pituitary, *aa-arb* gene expression levels were higher than *aa-ara* levels (>100 fold). The highest mean expression level of testicular *aa-cyp19a1* gene expression (Fig. 5A) occurred in untreated males (stage 1), and this was followed by a progressive decrease until stages 3 and 4. Low expression levels continued during the more advanced stages of development.

3.3 Detection of aa-cyp19a1mRNA transcript in eel tissues

The tissue-specific expression pattern demonstrated different *aa-cyp19a1* transcript levels in the different parts of the brain, although similar levels were observed in both sexes (supplementary Figure 4). Higher *aa-cyp19a1* gene expression levels were observed in Tel and the pituitary than in the cerebellum and medulla oblongata. Lower expression levels were also found in the gonads than Tel and the pituitary, with levels in the testes being no higher than those in the ovaries. A variation in expression levels between the sexes was also observed in the gills; the females did not express *aa-cyp19a1*, whereas relatively low levels were found in male gills. No transcripts were observed in

either sex in the other tissues investigated from prepubertal silver eels.

3.4 Identification and characterization of the *aa-cyp19a1* gene

One form of aromatase (*aa-cyp19a1*), identical to the one published by Tzchori et al. (2004), was identified from the European eel genome (Henkel et al. 2012), and its sequence confirmed by molecular cloning. A single open reading frame of 1536 bp was identified as sharing a high identity (98%) with Japanese eel *cyp19a1* (supplementary Table 1). Additionally, multiple conserved domains were identified, including a transmembrane-spanning domain, an I-helix region, an Ozol's peptide region, an aromatic region, and a heme-binding region (supplementary Figure 3). Alignment of genomic DNA and cDNA sequences revealed nine exons and eight introns, with the following sizes: exon 1 (186 bp, 62 aa), intron 1 (1054 bp), exon 2 (153 bp, 51 aa), intron 2 (313 bp), exon 3(153 bp, 51aa), intron 3(397 bp), exon 4 (177 bp, 59 aa), intron 4 (839 bp), exon 5 (114 bp, 38 aa), intron 5 (281 bp), exon 6 (114 bp, 38 aa), intron 6 (239 bp), exon 7 (165 bp, 55 aa), intron 7 (620 bp), exon 8 (240 pb, 80 aa), intron 8 (411), exon 9 (234 bp, 78 aa). Sequence alignment confirmed the intermediate nature of the eel *cyp19a1* gene, sharing some sequence features unique to the other teleost *cyp19a1a* gene and other features unique to teleost *cyp19a1b*.

4. Discussion

4.1 aa-cyp19a1 cDNA characterization

The expression levels of *aa-ara*, *aa-arb*, and *aa-cyp19a1* in the brain, pituitary, and gonads of male and female European eels during hormonally induced maturation are measured and described, along with a complete description of the *aa-cyp19a1* sequence. The sequence analysis has demonstrated that classification of the *aa-cyp19a1* gene as a typical teleost ovarian variant or as a brain aromatase variant is not possible, as it appears to contain sequence domains that are specific to both the brain and ovarian variants found in other teleost species (supplementary Table 1 and supplementary Figure

3). It has been suggested that the two fish variants arose following the teleost specific round of whole genome duplication (WGD), which was followed by the loss of tissue specific regulatory elements resulting in two independent genes (Diotel et al. 2010). According to this hypothesis, we would expect the eel also to have two variants, as it is assumed that the teleost-specific WGD predates the divergence of the Elopomorphs order, which includes the European eel, as well as other teleosts (Henkel et al. 2012). Elopomorphs, however, diverged early in the evolution of teleost fish and one of the duplicate *cyp19a1* genes was probably lost at an early point in the diversification process. This theory is supported by the observation that, as with tetrapods, *aa-cyp19a1* lacks the additional intron in the 5'UTR.

4.2 Gene expression during female gonadal development

4.2.1 aa-cyp19a1 and aa-ARs expression levels in the brain

The significantly higher levels of *aa-ara* and *aa-arb* observed in the EV stage could indicate increased sensitivity of the brain to androgens during this stage of development. The decrease in *aa-ara* gene expression levels in the OB and DM (with similar results in Tel) of stage V females may be related to the positive effect of T on the expression of tyrosine hydroxylase (Weltzien et al. 2006) and dopamine receptors (D2A and D2B, Pasqualini et al. 2009) in the forebrain; with the lower *aa-ara* levels in stage V indicating less dopaminergic inhibition of gonadotrope activity and thus stimulation of gonad development.

In contrast to the brain *aa-ara* gene expression levels, which decreased from EV to V, the brain *aa-cyp19a1* expression levels increased in V stage females. The opposing nature of this regulation again supports the hypothesis that there is a reduction in inhibition of gonad development by androgens in the later stages, due both to a decrease in AR and to an up-regulation of aromatase expression (Pasqualini et al. 2009, Weltzien et al. 2006). In addition to decreased inhibition by androgens, the increase in expression levels of *aa-cyp19a1* in the forebrain as maturation progresses indicates an increase in the local synthesis of estrogens. Previous studies have shown a decrease in

tyrosine hydroxylase and *d2rb* gene expression in the eel forebrain following E_2 treatment (Weltzien et al. 2006, Pasqualini et al. 2009, Dufour et al. 2010). Additionally, Montero et al. (1995) observed an up-regulation of GnRH1 (mGnRH) in the forebrain following E_2 treatment. Consequently, *aa-cyp19a1* activity may have a dual effect in the eel forebrain during maturation, reducing androgenic stimulation of dopamine inhibition and also stimulating maturation through increased stimulation of GnRH synthesis by E_2.

4.2.2 aa-cyp19a1 and aa-ARs expression levels in the pituitary

Coinciding with higher expression levels of *aa-ara* and, in particular, *aa-arb* in the pituitary at the V stage, a significant increase was found in *aa-cyp19a1* levels. These significant differences could be related to the higher T levels found in the plasma of the same fish at the V stage (supplementary figure 5A).

In previous studies, E_2 did not stimulate gonadotropin synthesis (mRNA or peptide) in the European eel pituitary *in vitro*, but did so *in vivo* (Montero et al. 1996, Vidal et al. 2004). T on the other hand, has been shown to strongly induce pituitary *lhb* mRNA levels *in vitro*, but no effect was observed *in vivo* (Aroua et al. 2007). The lack of *in vivo* effect from T can be explained by T having a positive effect on the *d2rb* receptor and tyrosine hydroxylase gene expression in the eel forebrain (Weltzien et al. 2006, Pasqualini et al. 2009). In addition, it should be noted that the female eels used in the *in vivo* experiment by Aroua et al. (2007) were juvenile silver eels, and therefore *aa-ara*, *aa-arb*, and *aa-cyp19a1* transcript levels had probably not yet increased in the pituitary. The stimulation of gonadotropin synthesis *in vivo* by E_2 could be explained by E_2 having a positive effect on GnRH receptors in the pituitary (Levavi-Sivan et al. 2006, Lin et al. 2010). These data support our results, as both *aa-cyp19a1* and GnRH receptor transcripts increased in the pituitary during the same stage of development (V stage; See Fig. 3D and Peñaranda et al. 2013).

4.2.3 aa-cyp19a1 and aa-ARs expression levels in the ovary

As observed in the pituitary, the T increase (supplementary Fig. 5A) coincided with increased ovarian *aa-ara*, *aa-arb*, and *aa-cyp19a1* transcript levels at the V stage. This coincidence could indicate that T has a positive feedback on these genes in the pituitary and gonad, but not in the brain. In Japanese eel, *aj-ara* (*Anguilla japonica* ARα) mRNA transcripts were stable during maturation until the migratory nucleus stage, whereas *aj-arb* increased in mid-vitellogenesis (Tosaka et al. 2010). Although comparison of transcript levels between different genes in relative qPCR experiments should be treated with caution, the Japanese results are consistent with our data, since *aa-arb* was expressed in higher levels than *aa-ara* in the pituitary and gonad during vitellogenesis. In fact, Ikeuchi et al. (1999) reported that *aa-arb* has a higher androgen-binding affinity than *aa-ara* in Japanese eel, supporting the predominant presence of the *aa-arb* gene during maturation. No differences were found in 11KT plasma levels during the treatment (supplementary figure 5A), but it has been demonstrated that 11-KT plays an important role in controlling pre-vitellogenic oocyte growth in *A. japonica* and *A. australis* (Lokman et al. 2003, Matsubara et al. 2003b, Kazeto et al. 2011), promoting the growth and lipid transfer and/or accumulation of previtellogenic oocytes (Lokman et al. 2007, Endo et al. 2008). No apparent correlation was found between E_2 plasma levels and *aa-cyp19a1* expression, possibly because E_2 in the ovary has only a local effect. Our gene expression results are consistent with Japanese eel data. Matsubara et al. (2003a) reported increased levels of ovarian *cyp19a1* during vitellogenesis, and a decrease in the subsequent stages. The aromatase activity in hormonally treated Japanese eels remained low when the Gonadosomatic Index (GSI) was below 8 % (in the PV and EV stages), but increased in late vitellogenesis (GSI 12-15%) (Jeng et al. 2005).

4.3 Gene expression during male gonadal development

4.3.1 aa-cyp19a1 and aa-ARs expression levels in the pituitary

The different expression profiles and transcript levels observed for *aa-ara* and *aa-arb* in the male pituitaries could indicate that they have roles in spermatogenesis, but published information on this is

417 limited to a few tissues and/or a single season/reproductive stage (Harbott et al. 2007, Diotel et al.
418 2010). In *Spinibarbus denticulatus* (Cyprinidae), significantly higher AR expression was observed in
419 the pituitaries of fully recrudesced fish when compared with early and late recrudesced fish (Liu et al.
420 2009). Further studies are necessary in order to clarify the roles of these genes during reproductive
421 maturation in male teleosts, but the elevated expression levels of *aa-arb* in stage 1 and 2 male eels
422 could indicate that androgens in the pituitary play a role at the start of spermatogenesis.

423 As a consequence of the hormonal induction in eels, androgen production is greatly stimulated
424 at the beginning of treatment (Khan et al. 1987, Miura et al. 1991), thus allowing the possibility of T
425 being transformed to E_2 by the pituitary aromatase (Miura et al. 1999). This is consistent with the
426 increase in *aa-cyp19a1* observed in our experiment. In fact, it has been observed that locally
427 synthesized E_2 can increase the pituitary responsiveness to exogenous GnRH analogs in male goldfish
428 (Trudeau et al. 1991). In addition, directly following the increase in *aa-cyp19a1* (stage 3/4), an
429 increase in *lhb* levels was observed in the male pituitaries of the same fish, with high levels remaining
430 throughout the subsequent stages of development (Peñaranda et al. 2010).

431

432 *4.3.2 aa-cyp19a1 and aa-ARs expression levels in the testis*

433 11-KT is the natural androgen with the highest affinity to both AR in Japanese eel (Ikeuchi et al.
434 2001). It is possible that the increase in 11-KT could have a negative effect on the expression of *aa-*
435 *ara* and *aa-arb* in testis. In fact, the quantity of 11-KT in the blood of the same fish increased 16 fold
436 from stage 1 to stage 3/4 (Supplementary data 5B; Peñaranda et al. 2010). As in the pituitary, *aa-arb*
437 expression levels were much higher (~100 fold) than *aa-ara* levels, thus demonstrating that *aa-arb* is
438 also the physiologically most relevant AR in the testis.

439 It has been shown that E_2 stimulates synthesis of platelet-derived endothelial growth factor in
440 Japanese eel testis, which is a spermatogonial stem cell renewal factor during spermatogenesis (Miura
441 and Miura 2011). Therefore, high *aa-cyp19a1* expression levels at the start of spermatogenesis are

consistent with the role of E_2 during male maturation, decreasing over the course of gonad development.

4.4 Conclusion

In summary, *aa-arb* was expressed at higher levels than *aa-ara* in the pituitary and gonad of both sexes of European eels, suggesting that *aa-arb* could be considered the physiologically most relevant AR in these tissues. In the brain, however, it seems that a decrease in *aa-ara* and an increase in *aa-cyp19a1* levels coincides with gonad development, suggesting decreased androgen and increased estrogen levels are necessary for advancement of the maturation process. In the female pituitary and ovary, *aa-ar*, *aa-arb*, and *aa-cyp19a1* expression levels increased in line with gonad maturation. However, in the males the results were different; in the male pituitary, opposing patterns were observed in the expression of *aa-cyp19a1* and *aa-ara*/*aa-arb*, with *aa-cyp19a1* increasing with gonad development, while *aa-ara*/*aa-arb* levels decreased. In the testis, both *aa-cyp19a1* and *aa-ara* gene expression levels decreased during spermatogenesis. A clear sex differentiation related to *aa-cyp19a1* and *aa-ara* and *aa-arb* gene expression along the BPG axis was observed, but it seems that, irrespective of the sex, the balance between androgens and estrogens could be one of the mechanisms by which gonad maturation is controlled.

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used in the experiments. The English revision was done by Professor Lucy Robertson (Lucy Robertson Writing Services, Norway).

6. Conflict of interest

None of the authors have any conflict of interest to declare.

7. Author contributions

DSP, JFA, FAW and LP participated in the design of the study. VG and IM carried out the experimental work with animal facilities. DSP, JH and RNL performed the molecular biology analyses. DSP analyzed the data and wrote the manuscript, and all co-authors commented on it.

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Figure Legends

Figure 1. Gene expression, analyzed by qPCR, of *Anguilla anguilla androgen receptor a* (*aa-ara*) (A) and *Anguilla anguilla androgen receptor b* (*aa-arb*) (B) in female eel brains during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-15). See main text for definition of gonad developmental stages. PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage. OB= Obfactory bubls, Tel= Telencephalon and DM= Di- and mesencephalon.

Figure 2. Gene expression of *Anguilla anguilla androgen receptor a* (*aa-ara*) and *Anguilla anguilla androgen receptor b* (*aa-arb*) analyzed by qPCR in male (A) and female (B) pituitary during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-13). See main text for definition of gonad developmental stages. PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage.

Figure 3. Gene expression of *Anguilla anguilla aromatase P450 a1 (aa-cyp19a1)* analyzed by qPCR in eel brains (females) and pituitaries (males and females) during artificially induced sexual maturation. A) Female OB, B) Female Tel, C) Female DM, D) Female pituitary, E) Male pituitary. Different superscript letters mean significant differences ($p<0.05$; n=6-15). OB= Olfactory bulbs, Tel= Telencephalon, DM= Di- and mesencephalon, Pit= Pituitary. See main text for definition of gonad developmental stages.

Figure 4. Gene expression, analyzed by qPCR, of *Anguilla anguilla androgen receptor a* (*aa-ara*) (A), *Anguilla anguilla androgen receptor b* (*aa-arb*) (B) in male and *aa-ara* (C), *aa-arb (D) in female* eel gonads in during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-15). See main text for definition of gonad developmental stages. PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage.

Figure 5. Gene expression of *Anguilla anguilla aromatase P450 a1 (aa-cyp19a1)* analyzed by qPCR in male (A) and female (B) eel gonads during artificially induced sexual maturation. Different superscript letters mean significant differences ($p<0.05$; n=6-15). See main text for definition of gonad developmental stages. PV= Previtellogenic stage, EV= Early vitellogenic stage, V= Vitellogenic stage.

Tables

Table 1. Primer sequences used in quantitative PCR and in reverse transcriptase-PCR cloning experiments.

Name	Sequence (5'- 3')	Orientation	Usage	GenBank Accession number.	Reference
aa-cyp19a1	CTC ACA CCA TGA AGC ACC TGG AG	Forward	PCR		Tzchori et al. 2004,
	GAT GGA AGC TGC CGC TTT ACT GTC	Reverse	PCR[1] (1560bp)		confirmed in this work
	TTC AAG GGA ACG AAC ATC ATC	Forward	qPCR[2] (115 bp)		
	AGA AAC GGT TGG GCA CAG T	Reverse	qPCR[3] (BE=2.044)/(GE=2.067)		
aa-ara	CTG TGA AAT GCG TCA GGA GA	Forward	PCR	FR668031	confirmed in this work
	CCG CCA TTT TGT TTA GCA TT	Reverse	PCR[1] (2487 bp)		
	CGG AAG GGA AAC AGA AGT ACC	Forward	qPCR[2] (104 bp)		
	AGC GAA GCA CCT TTT GAG AC	Reverse	qPCR[3] (BE=2.058)/(GE=2.006)		
aa-arb	CCC GTA ACA GAC GGA AGA TA	Forward	PCR	FR668032	confirmed in this work
	GTG CTC GTA CAT GCT GGA GA	Reverse	PCR[1] (1658 bp)		
	CGC TGA AGG AAA ACA GAG GT	Forward	qPCR[2] (115 bp)		
	CAT TCC AGC CTC AAA GCA CT	Reverse	qPCR[3] (BE=2.173)/(GE=2.033)		
aa-arp	GTG CCA GCT CAG AAC ACG	Forward	qPCR[2] (107 bp)	AY763793	Weltzien et al. 2005
	ACA TCG CTC AAG ACT TCA ATG G	Reverse	qPCR[3] (EB=2.142)/(GE=2.181)		

[1] PCR amplicon length is given in parenthesis following the reverse PCR primers.

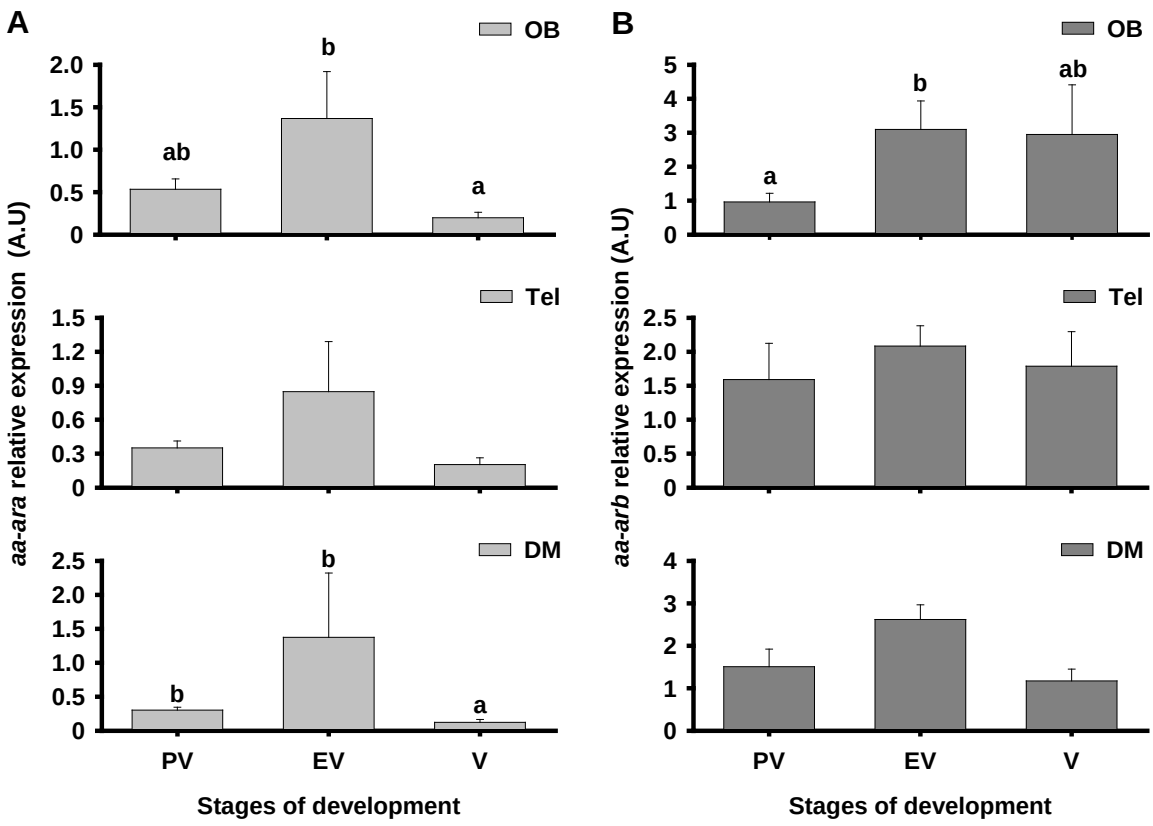
[2]qPCR amplicon length is given in parenthesis following the forward qPCR primers.

[3] qPCR efficiency for each primer pair in brain (BE) and gonad (GE) is given in parenthesis following the reverse qPCR primer

aa-cyp19a1= Anguilla anguilla aromatase P450 a1; aa-ara= Anguilla anguilla androgen receptor a; aa-arb= Anguilla anguilla androgen receptor b; aa-arp: Anguilla anguilla acidic ribosomal phosphoprotein P0.

676 **Figures**

677



678

679 Figure 1

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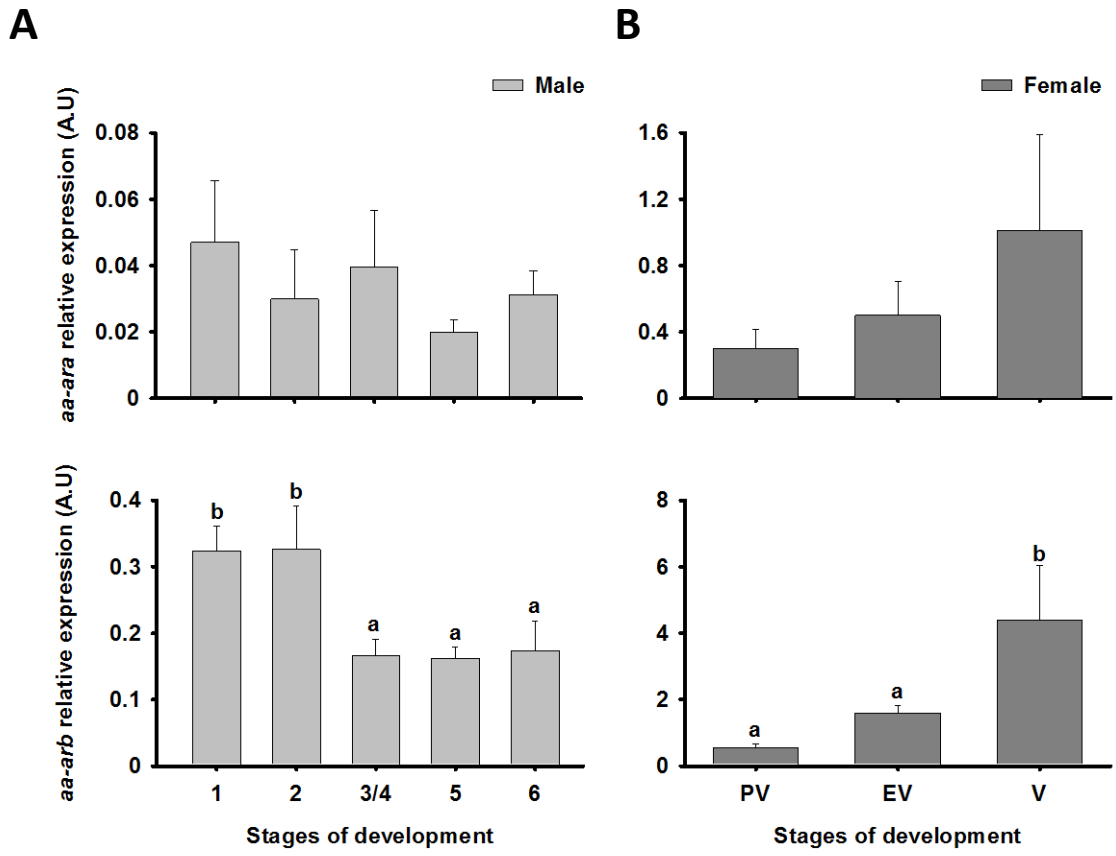

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683 Figure 2

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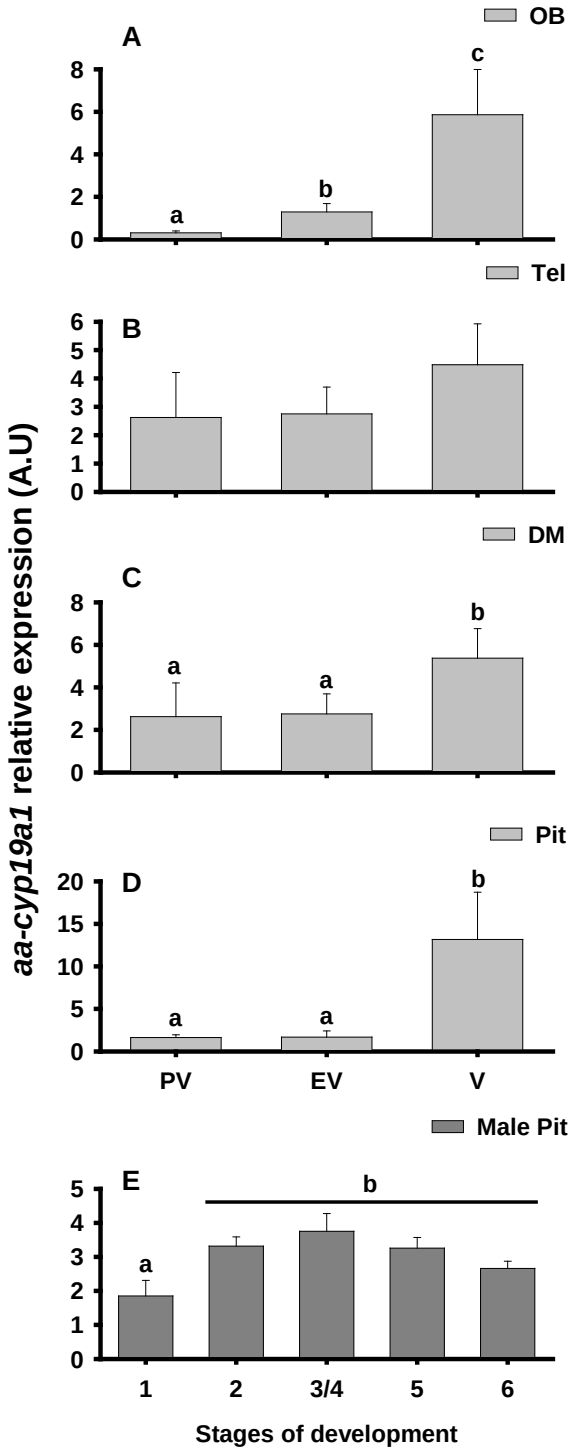
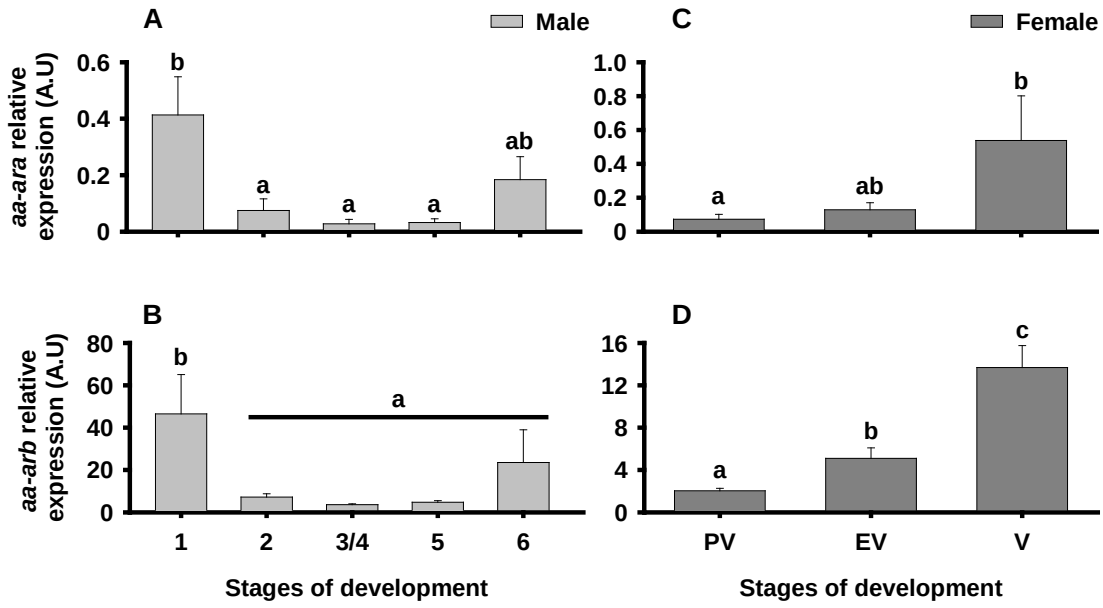
685



686

687 Figure 3

688



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690 Figure 4

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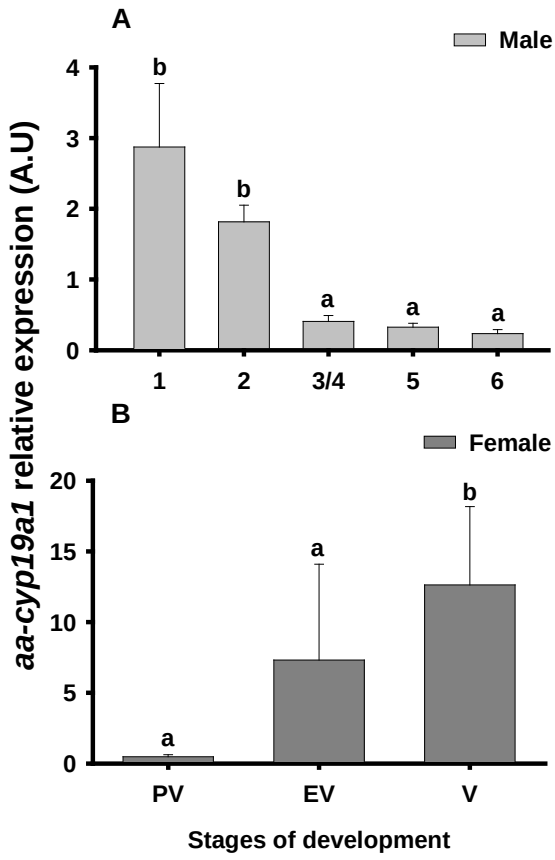

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693 Figure 5

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