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A MECHANISTIC MODEL FOR STUDYING THE INITIATION OF ANGUILLID VITELLOGENESIS BY COMPARING THE EUROPEAN EEL (*Anguilla anguilla*) AND THE SHORTFINNED EEL (*A. australis*)

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Abstract

An inverse relation exists between the maturation stage at the start of the oceanic reproductive migration and the migration distance to the spawning grounds for the various eel species. The European eel *Anguilla anguilla* migrates up to 5-6,000 km and leaves in a previtellogenic state. The shortfinned eel *A. australis* migrates 2-4,000 km and leaves in an early vitellogenic state. In this study, we compared the early pubertal events in European silver eels with those in silver shortfinned eels to gain insights into the initiation of vitellogenesis. Immediately after being caught, yellow and silver eels of both species were measured and sampled for blood and tissues. Eye index (EI), gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated. Plasma 11-ketotestosterone (11-KT) and 17β-estradiol (E2) levels were measured by radioimmunoassay. Pituitary, liver and ovaries were dissected for quantitative real-time PCR analyses (pituitary dopamine 2b receptor *d2br*, gonadotropin-releasing hormone receptors 1 and 2 *gnrhr1* and *gnrhr2*, growth hormone *gh* and follicle-stimulating hormone-β *fshb*; liver estrogen receptor 1 *esr1*; gonad follicle-stimulating hormone receptor *fshr*, androgen receptors α and β *ara* and *arb*, vitellogenin receptor *vtgr* and P450 aromatase *cyp19*). Silver eels of both species showed a drop in pituitary *gh* expression, progressing gonadal development (GSI of ~1.5 in European eels and ~3.0 in shortfinned eels) and steroid level increases. In shortfinned eels, but not European eels, expression of *fshb*, *gnrhr1* and *gnrhr2*, and *d2br* in the pituitary was up-regulated in the silver-stage as compared to yellow-stage females, as was expression of *fshr*, *ara* and *arb* in the ovaries. Expression of *esr1* in European eels remained low while *esr1* expression was up-regulated over 100-fold in silver shortfinned eels. The mechanistic model for anguillid vitellogenesis that we present suggests a first step that involves a drop in Gh and a second step that involves Fsh increase when switching in the life history trade-off from growth to reproduction. The drop in Gh is associated with gonadal development and plasma steroid increase but precedes brain-pituitary-gonad axis (BPG) activation. The Fsh increase marks BPG activation and increased sensitivity of the liver to estrogenic stimulation, but also an increase in D2br-mediated dopaminergic signaling to the pituitary.

Keywords

Eel reproduction; silvering; growth hormone; sex steroids; brain-pituitary-gonad axis; dopaminergic inhibition

Abbreviations: BW, body weight; BL, body length; EI, eye index; GSI, gonadosomatic index; HSI, hepatosomatic index; T, testosterone; 11-KT, 11-ketotestosterone; E2, 17β-estradiol;

BPG, brain-pituitary-gonad axis; DA, dopamine; D2br, dopamine 2b receptor; Gnrh, gonadotropin-releasing hormone; Gnrhr1, gonadotropin-releasing hormone receptor 1; Gnrhr2, gonadotropin-releasing hormone receptor 2; Gh, growth hormone; Lh, luteinizing hormone; Fsh, follicle-stimulating-hormone; Fshb, follicle-stimulating hormone-β; Fshr, follicle-stimulating hormone receptor; Esr1, estrogen receptor 1; Ara, androgen receptor α; Arb, androgen receptor β; Vtg, vitellogenin; Vtgr, vitellogenin receptor; Cyp19, P450 aromatase; Igf-1, insulin growth factor 1; Elf, elongation factor 1; L36, 60s ribosomal protein; PE, pituitary extract.

1. Introduction

Eels spend their growth stage as immature yellow eels in brackish estuaries and inland fresh waters (reviewed by Tesch, 2003). After a long growth stage (4-30^{+} years), in a still largely undefined physiological state, yellow eels cease feeding and change into migratory silver eels that swim for thousands of kilometres to their spawning areas. During silvering, eels undergo numerous morphological, physiological and behavioural changes that prepare them for their oceanic migration (reviewed by Durif et al., 2009); one of the most distinctive morphological changes is the enlargement of the eyes (Pankhurst, 1982). Parallel to these changes, eels advance their reproductive stage, initiating vitellogenesis during silvering (Aroua et al., 2005).

Vitellogenesis is essential for accumulation of yolk in the oocytes to accrue the nutritional reserves that will be called upon during the early stages of ontogeny. Although the spawning grounds of several eel species (e.g. *A. anguilla*, *A. rostrata*, *A. japonica*) have been discovered, only few fully matured eels (*A. japonica*) have been captured in the open ocean (Chow et al., 2009; Tsukamoto et al., 2011) which has prevented researchers from studying vitellogenesis in these fish in nature. Propagation efforts of eels in captivity by pituitary extract (PE) injections have enabled researchers to study vitellogenesis during artificial maturation (Okumura et al., 2001, 2002; Palstra et al., 2010a; Tosaka et al., 2010; Pérez et al., 2011). However, the long-term treatment of weekly PE injections to induce full sexual maturation is often leading to abnormal oogenesis and consequently poor quality eggs (Adachi et al., 2003). Alternative approaches have centred on shortening the duration of PE treatment by conditioning the broodstock through feminization (Kagawa et al., 1997; Ohta et al., 1997; Ijiri et al., 1998), by simulating migration (Mes et al., 2016) and/or by administering androgens (Lokman et al.,

2015). The first key step is to activate the brain-pituitary-gonad (BPG) axis, to sensitize the liver and trigger the initiation of vitellogenesis, or to initiate puberty. Basic information on the initiation of vitellogenesis in eels from the wild is, however, still largely lacking.

Vitellogenesis in teleost fish is regulated by the BPG axis (reviewed by Babin et al., 2007; Planas and Swanson, 2008; Hara et al., 2016). Preoptic hypothalamic neurons induce the secretion of gonadotropin-releasing hormone (Gnrh) that in turn activates pituitary gonadotrophs *via* its receptors (Gnrhr) to synthetize and release follicle-stimulating hormone (Fsh). The action of Fsh, which is mediated by its ovarian receptor (Fshr), promotes E2 synthesis by stimulating the activity of ovarian aromatase (Cyp19), an enzyme that converts testosterone (T) into E2 (Montserrat et al., 2004). Once released into the circulation, E2 induces the production of vitellogenin (Vtg) by binding to hepatic nuclear estrogen receptors (Esrs). Esr1 was found to be highly inducible by E2 in zebrafish, *Danio rerio* (Menuet et al., 2004), and largemouth bass, *Micropterus salmoides* (Sabo-Attwood et al., 2004), unlike Esr2. Also in eels, the Esr1 showed high sensitivity to hormone treatment by a strong response to a single injection of carp pituitary extract (Palstra et al., 2010ab). Although the exact mechanism still needs to be clarified, 11-KT, acting *via* its ovarian androgen receptors (Ara and Arb), and Gh may potentiate the effect of E2 on Vtg production (Kwon and Mugiya 1994; Peyon et al., 1996; Asanuma et al., 2003). Ultimately, Vtg is incorporated in the oocytes by receptor-mediated endocytosis after binding to its receptor (Vtgr) and is cleaved into small units of yolk that are stored as nutrients for developing embryos in the future larval yolk sac (Sire et al., 1994).

In many teleosts, including eels, the central dopaminergic system exerts an inhibitory action on reproduction by counteracting the stimulatory effect of Gnrh on gonadotropin release (reviewed by Dufour et al., 2005, 2010). Dopamine (DA) acts on pituitary gonadotropes through its main receptor, D2br (Jolly et al., 2016). Vidal et al. (2004) showed that the removal of dopaminergic inhibition is required to induce a dramatic increase in gonadotropin synthesis and release, that in turn stimulated hepatic Vtg release and uptake in the oocytes. These authors observed that oocytes of silver eels under DA inhibition had large nuclei with numerous lipid vesicles. In contrast, oocytes of silver eels treated to remove the dopaminergic inhibition had oocytes with yolk granules which are characteristic of entry into vitellogenesis. As for most studies on dopaminergic inhibition in eels, Vidal et al. (2004) focused on luteinizing hormone (Lh) synthesis and release rather than on Fsh due to the lack of tools (reviewed by Dufour et al., 2005, 2010). Fsh and Lh play a differential role in reproductive physiology (Suetake et al.,

2002; Kazeto et al., 2008); in eels, Fsh is involved in the initiation of vitellogenesis while Lh mediates the late vitellogenic and final maturational stages (Kajimura et al., 2001; Suetake et al., 2002). Recently, Jolly et al. (2016) found that DA inhibits *fshb* expression in eels. Therefore, the role of dopaminergic signalling during the initiation of vitellogenesis in eels needs to be further clarified.

When embarking on their oceanic migration, shortfinned silver eels *A. australis* have yolky oocytes which are still absent in the gonads of migratory European silver eels *A. anguilla* (in The Netherlands). Indeed, the early vitellogenic oocytes with peripheral yolk granules in shortfinned silver eels (Lokman et al., 1998) resembled the oocytes in dopamine antagonist-treated European eel (see above), described by Vidal and co-workers (2004). Colombo et al. (1984) observed that European silver eels had previtellogenic oocytes with large nuclei and numerous lipid vesicles in the cytoplasm. This difference in oocyte development probably relates to the migration distance (Todd, 1981): while previtellogenic *A. anguilla* swim approximately 5,000 to 6,000 km to reach their spawning site in the Sargasso Sea (Schmidt 1923), vitellogenic *A. australis* swim 2,000 to 4,000 km to reach their spawning grounds in the South Pacific, somewhere in the vicinity of Fiji (Kuroki et al., 2008; Miller and Tsukamoto 2017). We propose that a cross-specific comparison between previtellogenic European eel and early vitellogenic shortfinned eel will be helpful to comprehend the initiation of vitellogenesis in anguillid eels.

In the present study, we investigated the expression of a number of candidate genes along the BPG axis (*gnrhr1*, *gnrhr2*, *fshb*, *gh*, *fshr*, *ara, arb*, *vtgr*, *cyp19*) and in the liver (*esr1*) in wild yellow and silver eels of *A. anguilla* and *A. australis*. Furthermore, we investigated the expression of the main dopamine receptor (*d2br*) to further comprehend the role of the dopaminergic system during the initiation of vitellogenesis. Comparing the changes during silvering in both species might elucidate the mechanistic changes during the initiation of vitellogenesis in eels.

2. Materials and methods

2.1 Ethics

The measurements and sampling procedure in European eels complied with the current law of the Netherlands and was approved by the Dutch central committee for animal experimentation

(CCD nr. AVD401002017817) and the animal experimental committee of Wageningen University (IvD nr. 2017.D.0007.001). Experimental protocols on shortfinned eels were approved by the University of Otago Animal Ethics Committee in accordance with the guidelines of the Australian & New Zealand Council for the Care of Animals in Research and Teaching.

2.2 Experimental fish sampling

Shortfinned eels and European eels were captured during their seaward migration with fyke nets by local fishermen in Lake Ellesmere on March 24th 2017 (Christchurch, New Zealand) and in the Harinxma Canal on October 4th 2017 (Harlingen, The Netherlands), respectively. Immediately after being caught, the female eels were classified as 'yellow' and 'silver' by the fishermen on basis of several characteristics: body color, the shape of the snout (more acute in silver eels than yellow eels) and the pectoral fin color (dark in silver eels). Twelve similar sized eels were selected for each species (N=6 yellow eels and N=6 silver eels), measurements were performed and eels were sampled for blood and tissues. Eels were euthanized with an overdose anaesthetic (0.3 g l^{-1} benzocaine or 1 ml l^{-1} clove oil) and measured for body length (BL), and body weight (BW). Eye diameters (horizontal and vertical) were measured to calculate the EI (Pankhurst, 1982). Blood was retrieved after tail transection (shortfinned eels) or by using heparin-flushed syringes (European eels) which were placed on ice immediately after use. The blood was then centrifuged (4 °C, 5 min, 10,000 rpm) and plasma was stored at -80 °C until later measurements by radioimmunoassay (Section 3.3). Liver and gonads were dissected and weighed to calculate HSI and GSI, respectively. Gonad and liver tissues (<100 mg) and whole pituitaries were frozen on dry ice and stored at -80°C until use in quantitative real-time PCR (Section 3.6).

2.3 Plasma analysis

11-KT and E2 were assayed by radioimmunoassay. Plasma was dispensed into 12x75 borosilicate glass tubes and topped up with phosphate-buffered saline (pH 7.5) to a total volume of 100 µl. Samples were subjected to 95°C for 5 mins to denature plasma proteins. Steroids were subsequently extracted by vortexing for 15 secs after addition of 1 ml of diethyl ether. The aqueous phase was frozen on dry ice and the organic solvent phase decanted into a clean tube. A further two rounds of extraction with diethyl ether were done and the extracts added to that of the first extract. Solvent was largely evaporated overnight and any remainder removed in a vacuum oven for a further 1-2 hours.

Dry residues were reconstituted in PBS-BSA and subsequently assayed as reported previously (Lokman et al., 1998). All analyses were done in a single run on two replicate aliquots for each sample. The minimum level of detection was estimated at 0.07 ng ml^{-1} for E2 and at 0.18 ng ml^{-1} for 11-KT. The within-assay coefficient of variation equated to 13% for the E2 assay and to 18% for the 11-KT assay, whilst extraction recoveries averaged 74% for E2 and 94% for 11-KT. The antisera for both assays were previously used for estimation of plasma steroids in shortfinned eel. To validate the method for European eel, serial dilutions of pooled plasma were run and this was found to parallel the standard curve.

2.4 Sequence alignments and primers design

Target and reference sequences (Table 1) were obtained from the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the genomes of *A. anguilla* (Henkel et al., 2012; Jansen et al., 2017) and an unpublished multi-tissue transcriptome of *A. australis* (e.g. Thomson-Laing et al., 2018). From NCBI, the following sequences were obtained: Elongation factor 1 (*elf*) (*A. anguilla*: EU407825; *A. australis*: HM367094), 60s ribosomal protein l36 (*l36*) (*A. australis*: HM357467), *gnrhr1* (*A. anguilla*: JX567770), *gnrhr2* (*A. anguilla*: JX567771), *gh* (*A. anguilla*: AY148493; *A. australis*: HQ436341), *fshb* (*A. anguilla*: AY169722; *A. australis*: HQ436344), *d2br* (*A. anguilla*: DQ789977), *esr1* (*A. anguilla*: LN879034), *vtgr* (*A. australis*: HQ454301), *cyp19* (*A. anguilla*: KF990052; *A. australis*: HQ436343), *fshr* (*A. anguilla*: LN831181; *A. australis*: AB605267), *ara* (*A. anguilla*: FR668031; *A. australis*: AB710174) and *arb* (*A. anguilla*: FR668032; *A. australis*: AB710175). Since the reference sequence *l36* and the target sequence *vtgr* were not described for *A. anguilla* in NCBI, these sequences were obtained from the genome of the European eel (Henkel et al., 2012; Jansen et al., 2017). Similarly, missing sequences in NCBI of *A. australis* (*gnrhr1*, *gnrhr2* and *esr1*) were obtained from the unpublished multi-tissue transcriptome of the shortfinned eels (e.g. Thomson-Laing et al., 2018). Target and reference sequences were aligned with CLC Sequence Viewer 7 (Qiagen, Hilden, Germany) between *A. anguilla* and *A. australis* to search for 100% sequence identity regions between both species. Primers previously developed for *A. australis* (Table 1) were aligned with the *A. anguilla* sequence to check whether the primers shared 100% sequence identity between species. Primers that were not 100% identical nor previously described, were newly designed (Table 1) with Primer3 v.0.4.0 (Koressaar and Remm 2007; Untergasser et al., 2012). The *d2br* sequence was not described for *A. australis* in NCBI nor present in the

transcriptome sequence. Therefore, *A. anguilla* and *A. japonica* sequences (GenBank: JX305467) were aligned and primers designed in the regions with 100% sequence identity.

2.5 RNA isolation

Total RNA was isolated from pituitary, liver and ovaries with Trizol Reagent as described by the manufacturer (Invitrogen, California, USA). Possible contaminant traces of DNA were digested with recombinant DNAse I (Ambion, California, USA). Complementary DNA was generated from RNA using oligo-dT and random hexamers with PrimeScript RT Reagent kit (Takara, Kusatsu, Japan).

2.6 Quantitative RT-PCR

Quantitative real-time PCR was performed with SYBR Green Master Mix (Takara, Kusatsu, Japan) on a QuantStudio™-5 Real-Time PCR system (ThermoFisher, Waltham, Massachusetts, USA). Reactions were heated for 2 min at 95 °C and run for 40 cycles of denaturation (95 °C, 5 min), annealing (60-64 °C, 10 s) and extension (72 °C, 5 s). Melting curve analysis was performed to check for primer-dimers artefacts and reaction specificity. RT-PCR products of *A. australis* were electrophoresed on agarose gel, excised, extracted with NucleoSpin Gel PCR Clean-up (Macherey-Nagel, Düren, Germany) and sequenced. Sequence identity was confirmed using CLC Sequence Viewer and the Basic Local Sequence Alignment Search Tool (BLAST) in NCBI database. Primer efficiencies were determined by generating standard curves for each of the housekeeping and target genes. R^2 values and efficiency for all standard curves were > 0.98 and 90-110%, respectively (c.f., MIQE guidelines in Bustin et al., 2009).

Samples and standard curves were run in duplicate on the same well-plate. A pooled cDNA from *A. australis* was generated and run with the shortfin samples for each of the reference and target genes. This pooled cDNA was later run with *A. anguilla* samples to validate the cross-specific comparison. For the reference and target genes, Ct values of the pooled cDNA were highly similar between the quantitative real-time PCR runs of *A. australis* and *A. anguilla*. Data were expressed as fold change of yellow *vs*. silver eels by using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001) for *A. australis* and *A. anguilla*.

The reference genes *elf* and *l36* were evaluated for relative transcript copy number between yellow and silver eels in the pituitary, liver and gonads. Relative copy numbers of these reference genes were not significantly different between yellow and silver eels. Liver *esr1* was

normalized over *elf* and pituitary genes *d2br*, *gnrhr1*, *gnrhr2*, *gh* and *fshb*, as well as gonad genes *fshr*, *ara*, *arb*, *vtgr* and *cyp19*, were normalized over *l36*.

2.7 Statistical analysis

BW and BL, and log-transformed plasma 11-KT and E2 values, were pair-wise compared between silver and yellow eels using Student's t-tests. Means of biometric indices and of normalized copy numbers of target genes were compared between silver and yellow eels for each species using the non-parametric Wilcoxon test. One outlier value with over 4 times SD more *d2br* expression than the average was removed. Statistical analysis was performed in R (version 3.2.4) and differences were considered significant at $P < 0.05$.

3. Results

3.1 Morphometrics

Silver European eels were not different from the yellow European eels in BL ($P>0.05$, Table 2) and BW ($P>0.05$, Table 2). Similarly, shortfinned eels were not different in BW and BL between yellow and silver eels (BL: $P>0.05$; BW: $P>0.05$, Table 2). The EI was 7.8 ± 0.7 in European yellow eels and 6.1 ± 0.2 in shortfinned yellow eels, respectively (Table 2). In silver eels, EI was significantly higher for both species up to values of 10.3 ± 0.2 in European silver eels ($P<0.01$) and 8.0 ± 9.3 in shortfinned silver eels ($P<0.001$; Table 2).

3.2 Gonadosomatic and hepatosomatic indices

The GSI values of yellow eels were below 1% for both species: 0.7 ± 0.2% in European eels and 0.4 ± 0.2% in shortfinned eels (Table 2). In silver eels, the GSI was significantly higher with values of 1.5 ± 0.1% in European eels ($P<0.01$) and 3.0 ± 0.2% in shortfinned eels ($P<0.001$; Table 2). The HSI did not differ in European eel (Table 2); silver and yellow eels had HSI values of 1.2 ± 0.1% and 1.0 ± 0.1 %, respectively. For the shortfinned eels, the HSI was significantly higher in silver than in yellow eels (1.0 ± 0.0% *vs*. 0.6 ± 0.1%, respectively; $P<0.05$; Table 2).

3.3 Plasma 11-KT and E2

Plasma levels of 11-KT in yellow eels were low for both species: 0.5 ± 0.1 $ng.ml^{-1}$ in European eels and 1.3 ± 0.5 $ng.ml^{-1}$ in shortfinned eels (Table 2). In silver eels, the 11-KT concentration

was significantly higher than in yellow eels (European eel: P<0.0001; Shortfinned eel: P<0.0001; Table 2). 11-KT plasma levels were much higher in shortfinned silver eels than in European silver eels (82.3 ± 11.3 ng.ml^{-1} *vs*. 1.2 ± 0.3 ng ml^{-1}; Table 2).

Plasma levels of E2 in yellow eels averaged 1.9 ± 0.3 ng.ml^{-1} in European eels and 0.3 ± 0.1 ng.mL^{-1} in shortfinned eels (Table 2). Similar to 11-KT, E2 concentrations were significantly higher in silver eels when compared to yellow eels (European eel: P<0.01; Shortfinned eel: P<0.001) (Table 2). Plasma levels of E2 were about two times higher in European silver eels than in shortfinned silver eels (3.1 ± 0.5 *vs*. 1.5 ± 0.1 ng.ml^{-1}; Table 2).

3.4 Gene expression

Pituitary

In the pituitary, *d2br*, *gnrhr1*, *gnrhr2* and *fshb* expression did not change in European silver eels *vs*. yellow eels (Fig. 1A-D). In contrast, *d2br* (P<0.05), *gnrhr1* (P<0.001), *gnrhr2* (P<0.05) and *fshb* (P<0.05) expression was up-regulated in shortfinned silver eels *vs*. yellow eels (Fig. 1A-D). Expression of *gh* was down-regulated in silver eels *vs*. yellow eels for both species (European eel: P<0.05; Shortfinned eel: P<0.001; Fig. 1E).

Liver

In the liver, *esr1* expression was low and did not change between European yellow and silver eels (Fig. 2). For the shortfinned eels, *esr1* (P<0.001) was up-regulated over 110-fold in the silver compared to the yellow stage (Fig. 2).

Gonads

In the gonads, *fshr*, *ara*, *arb* and *cyp19* expression did not change in European eel (Fig. 3 A-C, E). *Vtgr* (P<0.001) was down-regulated in European silver eels (Fig. 3D). In shortfinned eel, on the other hand, ovarian *fshr* (P<0.01), *ara* (P<0.001) and *arb* (P<0.05) expression was up-regulated (Fig. 3A-C); up-regulation of both ar subtypes in shortfinned silver eel ovaries was comparable, reaching 3-fold for *ara* and 2-fold for *arb* (Fig. 3B-C). Expressions of *vtgr* and *cyp19* did not change between yellow and silver shortfinned eels (Fig. D-E).

4. Discussion

In European eels, spontaneous progression of vitellogenesis *only* occurs during and/or after the oceanic reproductive migration in their natural environment. Under conditions of captivity, vitellogenesis will not occur, except if induced by long-term hormonal treatment. However, this long-term treatment can lead to abnormal oogenesis and poor quality eggs. These abnormalities arguably result from inadequate initiation of vitellogenesis. In this study, we compared the previtellogenic European eel with the early vitellogenic shortfinned eel. Comparing the changes that occur during silvering between both species may provide a mechanistic model for studying the initiation of vitellogenesis in eel. This model may then reveal ways to trigger vitellogenesis other than by injecting hormones.

4.1 Ovarian development and sex steroid production during silvering

In this study, ovarian development on the basis of GSI for both species was more advanced in silver eels than in yellow eels (Table 3). Yellow eels of both species had previtellogenic oocytes (confirming earlier research for shortfinned eel: Todd, 1974, Lokman et al., 1998; European eel; Colombo et al., 1984) in small gonads relative to total body weight (< 1%). In silver eels, GSI was higher in *A. australis* than in *A. anguilla* (GSI > 3% *vs*. GSI < 2%). Silver eels of both species also displayed an increased eye index and increased plasma levels of sex steroids. This is in good agreement with previous studies since an increase in eye size, thought to be mediated by 11-KT (Rohr et al., 2001; Thomson-Laing et al., 2018), has been reported to correlate positively with gonadal development in European eels (Pankhurst 1982). Both 11-KT and E2 plasma levels significantly increased in silver eels for both species (Table 3), which is consistent with previous studies (shortfins: Lokman et al., 1998, European eel: Sbaihi et al., 2001; Aroua et al., 2005). Similar results were reported during silvering of the Japanese eel *A. japonica* (Han et al., 2003; Jeng et al., 2014) and of the American eel *A. rostrata* (Cottrill et al., 2001). 11-KT is increasingly associated with regulating several key life history events. For example, exposure to 11-KT *in vitro* increased oocyte diameters by 10-20% (Lokman et al., 2007) and correlated positively with lipid deposition in Japanese eel *in vivo* (Matsubara et al., 2003). More recently, Endo et al. (2011) and Damsteegt et al. (2015), using *in vitro* approaches and supplementation with isolated lipoproteins, provided compelling evidence for the relationship between 11-KT and lipid accumulation to be causative.

By stimulating the expression of the hepatic nuclear receptor *esr1* (Todo et al., 1996) and binding to it, E2 stimulates the liver in the production of vitellogenins. E2 levels were notably higher in silver than in yellow eels, but *cyp19* mRNA levels did not differ between both stages

363 in either species. Similar findings were reported by Setiawan et al. (2012), who deemed overall
364 *cyp19* transcript copy numbers to be higher in silver shortfinned eels when accounting for
365 increased ovarian size in the silver compared to the yellow stage. Increased steroid levels could
366 also be attributable to increased expression of genes higher up in the steroidogenic cascade,
367 such as steroidogenic acute regulatory protein, *star* (c.f. Reid et al., 2013).
368
369 **4.2 Liver sensitivity to estrogenic stimulation during the initiation of vitellogenesis**
370 Shortfinned silver eels with GSI > 3% have oocytes with peripheral yolk granules (also Todd,
371 1974, Lokman et al., 1998), whereas evidence for yolk in oocytes from European eels with GSI
372 < 2 has not been found (Sbaihi et al., 2001; Palstra et al., 2007, 2010ab; Mordenti et al., 2013).
373 The vitellogenic state of shortfinned eels and the previtellogenic state of European eels was
374 confirmed by the changes in HSI and *esr1* expression (Table 3). In shortfinned eels, the
375 increased HSI and the up-regulation over 100-fold of *esr1* expression in silver eels reflected
376 their vitellogenic state, which was in contrast with the unchanged HSI and *esr1* expression in
377 silver European eels. While E2 probably did not bind much in silver European eels due to a
378 lack of Esr1, E2 in the shortfinned eel likely bound to its receptor in the liver. From our result,
379 we can conclude that there is an increased hepatic sensitivity for E2 in silver shortfinned eels.
380
381 **4.3 Stimulation of the brain-pituitary-gonad axis during the initiation of vitellogenesis**
382 In both species, a significant decrease of *gh* expression occurred in the pituitaries of silver eels
383 (Table 3). Marchelidon et al. (1996) and Durif et al. (2005) similarly observed a Gh level
384 decrease in the pituitary of European silver eels when compared to yellow eels. The drop in Gh
385 concentrations is not specifically a fasting effect (Marchelidon et al., 1996) but probably
386 induced by thyroid hormone action (Rousseau et al., 2002). Gh is a potent secretagogue of
387 insulin-like growth factor 1 (Igf-1), which is synthetized and secreted by the liver (Cao et al.,
388 1989; Duan et al., 1993). Igf-1 can increase pituitary Lh content and inhibit Gh release and
389 production in a dose-dependent manner in European eel (Huang et al., 1998; Rousseau et al.,
390 1998). Conversely, Igf-I may exert negative feedback on *gh* expression which is only apparent
391 *in vivo*.
392
393 In the pituitary, expression of *gnrhr1* and *gnrhr2* genes were up-regulated in silver shortfins.
394 In contrast, *gnrhr* expression did not change between yellow and silver European eels (Table
395 3). Pituitary expression of *gnrhr1* and *gnrhr2* genes in European eels were only found to
396 increase during artificially induced sexual development (Peñaranda et al., 2013). The up-

regulated *gnrhr* expressions in silver shortfinned eels agrees with an overall molecular activation of the BPG axis (Table 3).

In the ovary, the expression of *fshr* was up-regulated in silver shortfinned eels in contrast to European eel (Table 3). Increased Fsh sensitivity coincided with increased expression of *fshb* in the pituitary, and with higher GSI and sex steroid levels in silver shortfinned eels (Table 3). Increased pituitary *fshb* and ovarian *fshr* expression was previously reported in early vitellogenic shortfinned eel (Setiawan et al., 2012). Setiawan and colleagues further provided compelling evidence that 11-KT addition could inducethe *fshr* increase *in vitro* and *in vivo*. T and E2 failed to increase *fshr* transcript levels in the Japanese eel (Jeng et al., 2007).

The relative expression of ovarian androgen receptors (*ara* and *arb*) increased between yellow and silver shortfinned eels, but not European eels. Fold change indicated slightly higher increases in mRNA levels for *ara* than *arb*. Similar results were previously reported for shortfinned eels (Setiawan et al., 2012) and the Japanese eel (Tosaka et al., 2010). Setiawan et al. (2012) showed that the increase in plasma levels of 11-KT was accompanied by increased ovarian and pituitary expression of androgen receptors. Therefore, it is likely, that sex steroid levels in silver European eels have been elevated for a shorter period of time than in silver shortfinned eels and that in turn, sensitivity to hormonal signals associated with reproduction has remained lower.

In the European eel, *vtgr* expression slightly decreased between yellow and silver eels. In the rainbow trout, Perazollo et al. (1999) found that *vtgr* expression was highest in previtellogenic and early vitellogenic oocytes through ovarian development. Peak values were then followed by a gradual decrease in *vtgr* expression during oocyte growth. Similar results were found in various other fish species, supporting the hypothesis that Vtgr is recycled to the oocyte surface during vitellogenic oocyte growth (white perch; Hiramatsu et al., 2004; cutthroat trout; Mizuta et al., 2013: largemouth bass; Dominguez et al., 2012). A stronger decrease in *vtgr* expression could thus be expected for shortfinned eels than for European eels. However, in shortfinned eels, no changes were observed and that was consistent with a previous study by Damsteegt et al. (2015). As the drop in *vtgr* expression in European eel is significant but also small, *vtgr* expression may peak at a different phase during the yellow eel life stage. Alternatively, peak *vtgr* expression occurs in a rather short time span, making the chance of missing it high.

4.4 Dopamine signaling is increased during the initiation of vitellogenesis

Dopamine has a negative effect on gametogenesis by inhibiting the synthesis and release of gonadotropins via its main receptor D2br (Vidal et al., 2004; Jolly et al., 2016). The increase in pituitary *d2br* expression in this study suggests an increased inhibitory tone of DA on Fsh and/or Lh production and release during the initiation of vitellogenesis. This is in good agreement with previous studies since plasma Fsh and Lh levels are still low in silver eels (Aroua et al., 2005; Mes et al., 2016).

While the stimulating effect of Gnrh on Fsh regulation is well documented, the potential role of dopamine has been less investigated. Recently, Jolly et al. (2016) showed that DA negatively regulates Fsh cells and that the *d2br* is mainly expressed by Fsh cells in silver European eels. This finding is not consistent with our results in silver shortfinned eels where an up-regulation of *d2br* coincided with an increase of *fshb* pituitary expression.

Although Lh release does not seem to occur during silvering, Lh production could increase dramatically in silver eels as compared to yellow eels (Aroua et al., 2005). Lh production may be stimulated in response to sex steroids (Huang et al., 1997; Vidal et al., 2004) and Igf-1 (Huang et al., 1998; Rousseau et al., 1998) but Lh may not be released into the circulation due to DA action. This dual control would allow the storage of Lh that is required for the plasma Lh surge during final maturation when the dopaminergic inhibition is finally lifted. We therefore hypothesize that DA binding does not necessarily inhibit the production but the release of Lh. The increased dopaminergic tone may allow long-term Lh production and storage during vitellogenesis until the initiation of final maturation at the spawning grounds.

4.5 Synthesis: A mechanistic model for studying the initiation of vitellogenesis in eel

Surprisingly, silver European eels show increases in EI, GSI (~1.5) and plasma steroid levels without up-regulated expression of any molecular indicators that hint at activation of the BPG axis. What *is* apparent, though, is a significant down-regulated expression of pituitary *gh*. Silver shortfins only doubled in GSI as compared to European eels but showed dramatic changes in expression of genes associated with BPG axis activation, including up-regulated expression of pituitary *fshb* and gonadal *fshr*. Our model proposes that a drop in Gh levels may represent a first step in switching from previtellogenesis towards the initiation of vitellogenesis, reflecting a life history change from the growth stage to the reproductive stage. This decrease in Gh concentrations could be permissive for metabolic factors (insulin, leptin, IGF-I) to stimulate

steroidogenesis (e.g. via Star), or Gh may even be directly responsible by inhibiting the initiation of puberty. Gh may thus serve as a master switch in the classical life history trade-off between growth and reproduction in the semelparous eel. A second step in the initiation of vitellogenesis may include a major role for Fsh, triggered by GnRH, in stimulating the production of estrogen receptors in the liver and vitellogenin receptors in the oocytes (Tyler et al., 1997). This second step, representing the actual activation of the BPG axis, may be the point of no return in the life history of eels. Activation of the BPG axis coincides with an increased dopaminergic tone that may inhibit Lh release and allow for the Lh surge during final maturation when dopaminergic inhibition is lifted. We acknowledge that these arguments are based on gene expression data and further insights should be gained from measurements of Gh, Fsh and Lh protein levels and manipulation of yellow eels with recombinant Fsh.

4.6 Conclusions

In conclusion, the mechanistic model that we propose suggests a first step that involves a drop in levels of Gh and a second step that involves an increase in circulating levels of Fsh when switching from previtellogenesis to vitellogenesis in anguillid eels. The drop in Gh concentrations is associated with gonadal development and an increase in plasma steroid levels but precedes activation of the BPG axis. Subsequent activation of this axis leads to initiation of vitellogenesis in anguillid eels, characterized by increased sensitivity of the liver to estrogenic stimulation, but also by an increase in dopaminergic signaling to the pituitary. The activation of the BPG axis is further characterized by increases in sensitivity to Gnrh (up-regulation of *gnrhr1* and *gnrhr2*), in gonadotropin synthesis (*fshb*) and in ovarian ligand (*fshr*, *ara*, *arb*) sensitivities. Increased sensitivity of the liver to estrogenic stimulation is reflected in a dramatic increase in *esr1* expression.

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Tables

Table 1: Primers for each of the target genes with Abv: abbreviation; T: sequence obtained from an unpublished multi-tissue transcriptome of *A. australis* (e.g. Thomson-Laing et al., 2018); G: sequence obtained from the *A. anguilla* genome (Henkel et al., 2012; Jansen et al., 2017). Note: The D2br sequence was aligned between *A. anguilla* and the Japanese eel *A. japonica* since this sequence was not described for *A. australis*.

Target genes	Abv.	Accession number for *A. anguilla*	Accession number for *A. australis*	Primer sequences	T°	Product size (bp)	References
Reference genes							
Elongation factor 1	Elf	EU407825	HM367094	FW: CCCCTGCAGGATGTCTACAA RV: AGGGACTCATGGTGCATTTC	64	152	Setiawan & Lokman 2010
60s ribosomal protein l36	L36	G	HM357467	FW: CCTGACCAAGCAGACCAAGT RV: TCTCTTTGCACGGATGTGAG	62	160	Setiawan & Lokman 2010
Pituitary							
Gonadotropin releasing hormone receptor-1	Gnrhr1	JX567770	T	FW: TGACCCACGGTAGCTTTCA RV: GGCAGGACTCTCCACCTTTAC	60	165	
Gonadotropin releasing hormone receptor-2	Gnrhr2	JX567771	T	FW: CGCATGACCAAAGGGAAG RV: AAGGACACGACGATGACGA	60	116	
Growth hormone	Gh	AY148493	HQ436341	FW: GTTAACCGAGCACAGCACCT RV: TTCTCCTGCGTTTCATCTTTG	59	167	
Follicle stimulating hormone subunit-beta	Fshb	AY169722	HQ436344	FW: CCGTGGAGAATGAAGAATGC RV: TGGTTTCAGGGAGCTCTTGT	64	104	Setiawan et al. 2012
Dopamine receptor-2B	D2br	DQ789977		FW: CACGCTACAGCTCCAAAAGAA RV: TGAAGGGGACATAGAAGGACAC	60	186	
Liver							
Estrogen receptor-1	Esr1	LN879034	T	FW: GGCATGGCCGAGATTTTC RV: GCACCGGAGTTGAGCAGTAT	62	116	
Gonads							
Vitellogenin receptor	Vtgr	G	HQ454301	FW: TCTGAACGAACCCCAGGA RV: TTTGGGGAGTGCTTGTTGA	59	140	
Aromatase cytochrome P450	Cyp19	KF990052	HQ436343	FW: CGCACCTACTTTGCTAAAGCTC RV: AGGTTGAGGATGTCCACCTG	62	137	
Follicle-stimulating hormone receptor	Fshr	LN831181	AB605267	FW: CCTGGTCGAGATAACAATCACC RV: CCTGAAGGTCAAACAGAAAGTCC	63	173	Zadmajid et al. 2015
Androgen receptor-α	Ara	FR668031	AB710174	FW: AGGAAGAACTGCCCCTCTTG RV: ATTTGCCCGATCTTCTTCAG	62	90	Setiawan et al. 2012
Androgen receptor-β	Arb	FR668032	AB710175	FW: GCTTGGAGCTCGAAAATTGA RV: TTGGAGAGATGCACTGGATG	62	98	Setiawan et al. 2012

763 **Table 2:** Differences between yellow and silver stages in the European eel *A. anguilla* and the
764 shortfinned eel *A. australis* (average ± SE): body weight (BW); body length (BL); eye index
765 (EI); gonadosomatic index (GSI); hepatosomatic index (HSI); plasma 11-ketotestosterone level
766 (11-KT) and plasma 17β -estradiol level (E2). Values in bold indicate significant difference
767 between stages (yellow *vs*. silver) within species.
768

	Yellow	**Silver**	**P-values**
A. anguilla	**(N= 6)**	**(N= 6)**	
BW (g)	492 ± 75	473 ± 63	>0.05
BL (cm)	63.5 ± 3.3	64.7 ± 3.5	>0.05
EI	7.8 ± 0.7	**10.3 ± 0.2**	<0.01
GSI	0.7 ± 0.2	**1.5 ± 0.1**	<0.01
HSI	1.0 ± 0.1	1.2 ± 0.1	>0.05
11-KT ($ng.mL^{-1}$)	0.5 ± 0.1	**1.2 ± 0.3**	<0.0001
E2 ($ng.mL^{-1}$)	1.9 ± 0.3	**3.1 ± 0.5**	<0.01
A. australis	**(N= 6)**	**(N= 6)**	
BW (g)	979 ± 118	1057 ± 61	>0.05
BL (cm)	75.9 ± 3.1	80.4 ± 1.5	>0.05
EI	6.1 ± 0.2	**8.0 ± 9.3**	<0.001
GSI	0.4 ± 0.2	**3.0 ± 0.2**	<0.001
HSI	0.6 ± 0.1	**1.0 ± 0.0**	<0.05
11-KT ($ng.mL^{-1}$)	1.3 ± 0.5	**82.3 ± 11.3**	<0.0001
E2 ($ng.mL^{-1}$)	0.3 ± 0.1	**1.5 ± 0.1**	<0.001

Table 3: Comparison of changes along the BPG-axis that occur during silvering between the European eel *A. anguilla* and the shortfinned eel *A. australis*. Silvering is indicated by eye index increase which coincides with gonadal development and plasma steroid increase in both species. Between-species differences in HSI and *esr1* expression reinforce the previtellogenic state of European silver eels and the vitellogenic state of shortfinned silver eels at the start of oceanic migration. The vitellogenic state is characterized by both increased dopaminergic and Gnrh signaling. Furthermore, vitellogenesis is characterized by up-regulated *fshb* and down-regulated *gh* expression in the pituitary, and up-regulated (or not down-regulated) fsh receptor, androgen and vitellogenin receptors in the gonads. Statistical analysis: 0, no significant difference, + significantly higher at $P<0.05$, ++ significantly higher at $P<0.01$, +++ significantly higher at $P<0.001$, significantly lower at $P<0.05$, -- significantly lower at $P<0.01$. Full names of abbreviated indices: EI: Eye index; HSI: hepatosomatic index; GSI; gonadosomatic index. Full names of abbreviated plasma steroids: 11-KT: 11-ketotestosterone; E2: 17β-estradiol. Full names of abbreviated genes: *d2br*: dopamine 2b receptor, gonadotropin-releasing hormone receptors 1 and 2 *gnrhr1* and *2*, growth hormone *gh* and follicle-stimulating-hormone-β *fshb*; estrogen receptor 1 *esr1*; gonad follicle-stimulating hormone receptor *fshr*, androgen receptors α and β *ara* and *b*, vitellogenin receptor *vtgr* and P450 aromatase *cyp19*.

		A. anguilla	*A. australis*
	EI	++	++
plasma	11-KT	+++	+++
	E2	++	+++
pituitary	*d2br*	0	+
	gnrhr1	0	++
	gnrhr2	0	+
	fshb	0	+
	gh	-	--
liver	HSI	0	+
	esr1	0	++
gonad	GSI	++	++
	fshr	0	++
	ara	0	++
	arb	0	+
	cyp19	0	0
	vtgr	--	0

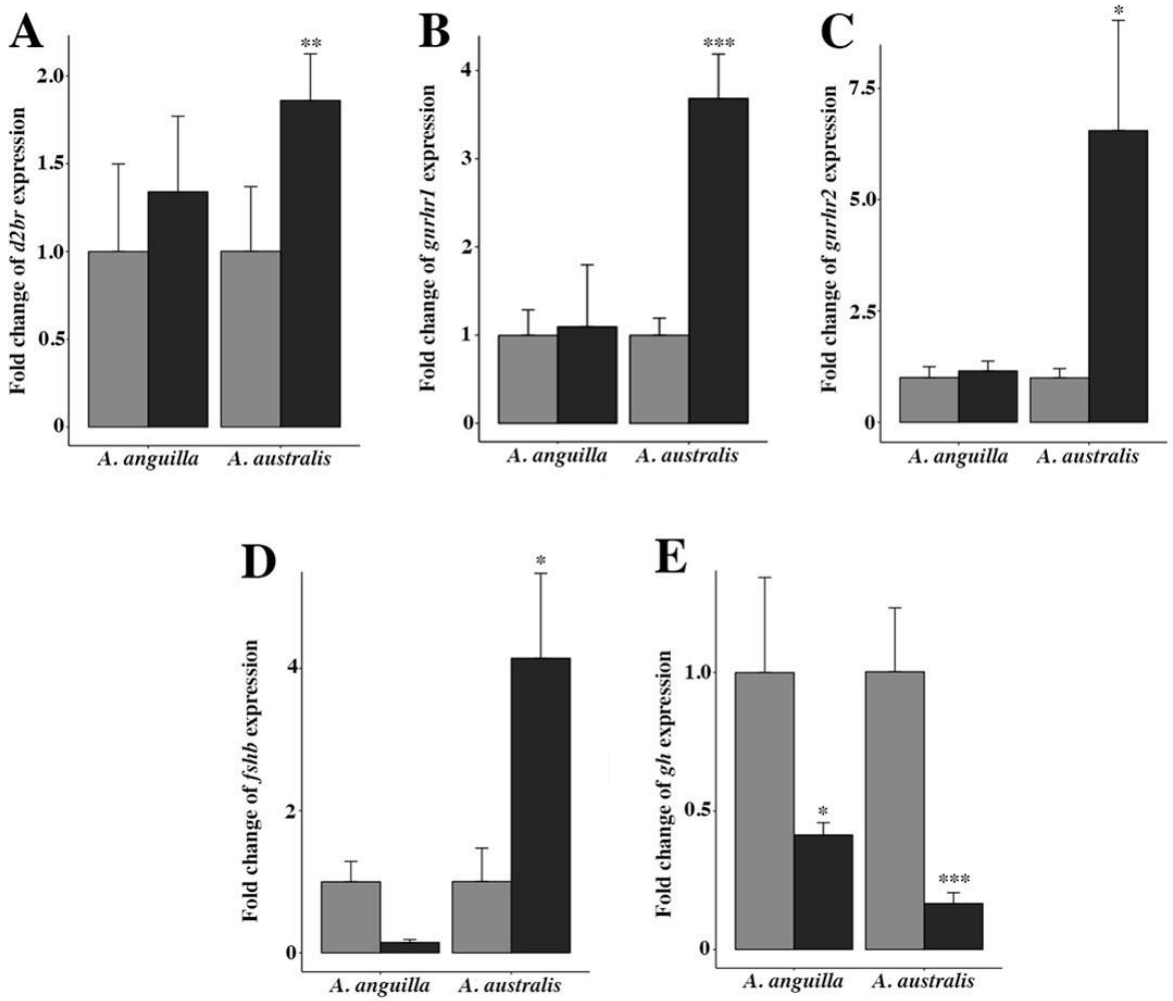
Figure Captions

Figure 1: Pituitary gene expression in the European eel *A. anguilla* (Yellow: N=5; Silver: N=5) and the shortfinned eel *A. australis* (Yellow: N=6; Silver: N=6), compared between yellow (grey bars) and silver eels (black bars). A: dopamine 2B receptor *d2br*; B: gonadotropin releasing hormone receptor 1 *gnrhr1*; C: gonadotropin releasing hormone receptor 2 *gnrhr2*; D: follicle stimulating hormone subunit beta *fshb* and E: growth hormone *gh*. Asterisks indicate statistical difference: * P<0.05, ** P<0.01 and ***P<0.001.

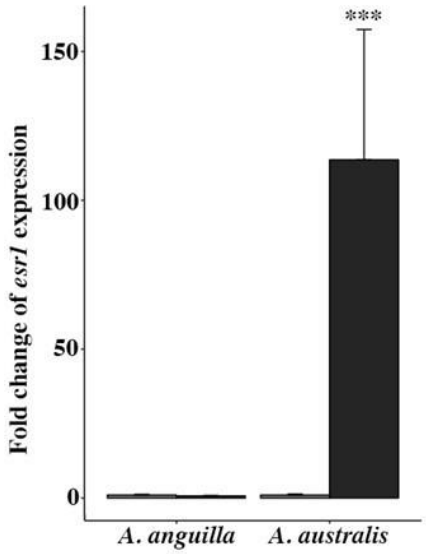
Figure 2: Liver gene expression of the estrogen receptor 1 *esr1* in the European eel *A. anguilla* (Yellow: N=6; Silver: N=6) and the shortfinned eel *A. australis* (Yellow: N=6; Silver: N=6), compared between yellow (grey bars) and silver eels (black bars). Asterisks indicate statistical difference: ***P<0.001.

Figure 3: Ovarian gene expression in the European eel *A. anguilla* (Yellow: N=6; Silver: N=6) and the shortfinned eel *A. australis* (Yellow: N=6; Silver: N=6), compared between yellow (grey bars) and silver eels (black bars). A: follicle stimulating hormone receptor *fshr*; B: androgen receptor alpha *ara*; C: androgen receptor beta *arb*; D: vitellogenin receptor *vtgr* and E: aromatase *cyp19*. Asterisks indicate statistical difference: * P<0.05, ** P<0.01 and ***P<0.001.

Figure 1

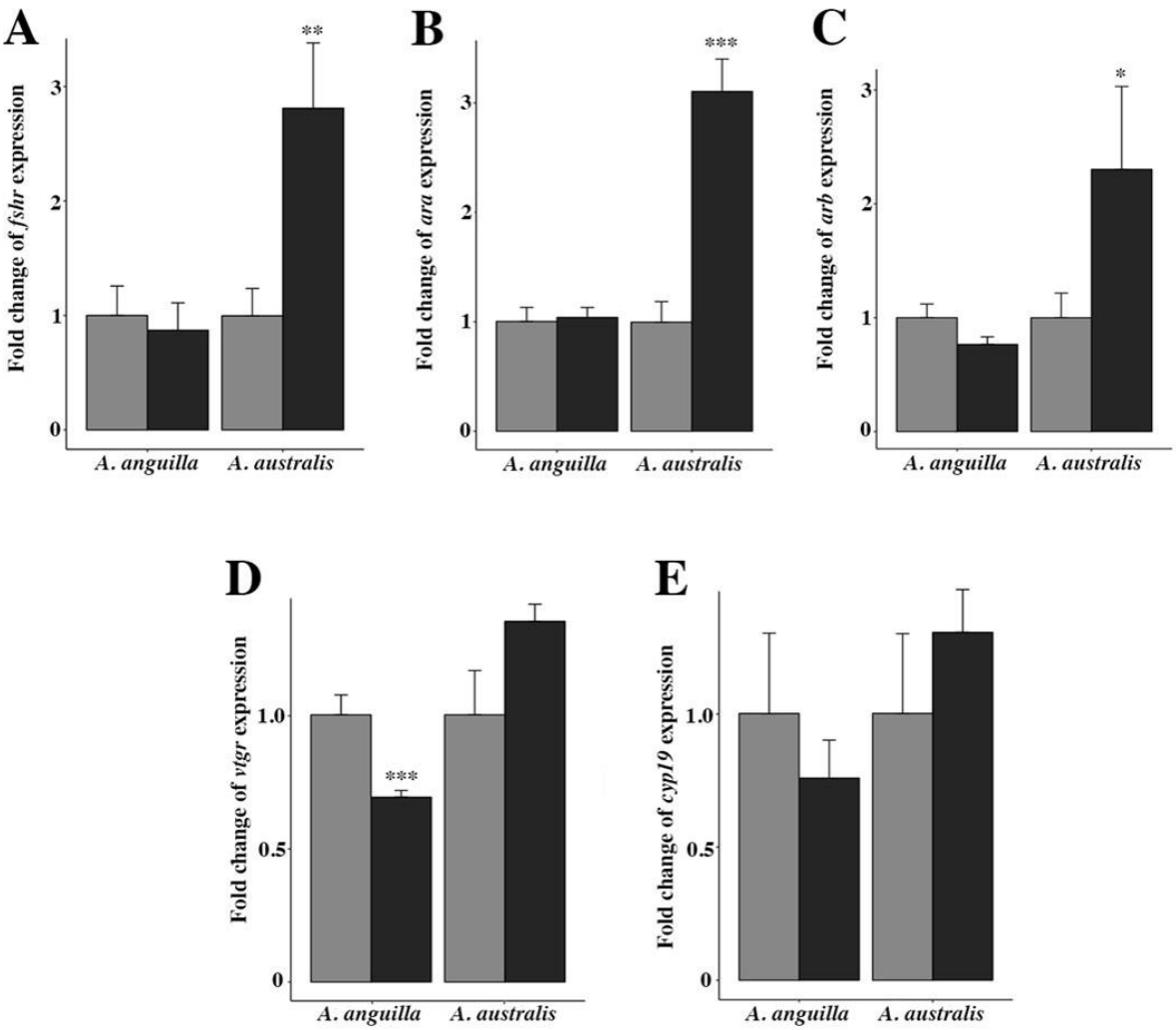

A
Fold change of d2br expression
2.0
1.5
1.0
0.5
0
**
A. anguilla
A. australis
B
Fold change of gnrhr1 expression
4
3
2
1
0
***
A. anguilla
A. australis
C
Fold change of gnrhr2 expression
7.5
5.0
2.5
0
*
A. anguilla
A. australis
D
Fold change of fshb expression
4
2
0
*
A. anguilla
A. australis
E
Fold change of gh expression
1.0
0.5
0
*
***
A. anguilla
A. australis

812 **Figure 2**



813

814 **Figure 3**



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