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Expression of gonadotropin subunit and gonadotropin receptor genes in wild female New Zealand shortfinned eel (*Anguilla australis*) during yellow and silver stages

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ABSTRACT

Despite tremendous importance of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) as primary controllers of reproductive development, information on the expression profiles of the genes encoding gonadotropin subunits and gonadotropin receptors (Fshr and Lhr) in wild eels are essentially non-existent. This study investigated pituitary *fshb* and *lhb* mRNA levels and ovarian *fshr* and *lhr* mRNA levels of wild shortfinned eels, *Anguilla australis* at different stages of oogenesis. Protein expression of Fsh in the pituitary was also quantified and visualized using slot blot and immunohistochemistry. Pituitary *fshb* and *lhb* mRNA levels showed a differential expression pattern, *fshb* mRNA levels increasing significantly from the perinucleolus (PN) to the oil droplet stage (OD) before slightly decreasing (not significantly) in the early vitellogenic stage (EV). A similar trend was observed in relative Fsh protein levels analyzed by slot blot and immunohistochemistry, but this trend was not reflected in the plasma levels of sex steroids. In contrast, pituitary *lhb* mRNA levels increased significantly from the PN to EV stage. A higher expression of Fsh at both mRNA and protein levels in the pituitary of eels at the OD stage compared to other investigated stages suggests that synthesis of Fsh production in the pituitary may reach a peak at the OD stage. In the ovary, transcript abundances of *fshr* and *lhr* gradually increased during previtellogenic follicle growth, but markedly and significantly increased thereafter. Taken together, our data suggest *i)* that Fsh release may be very limited, or absent, prior to onset of puberty in shortfinned eels and *ii)* that Lh is not functionally important in this fish during the EV stage.

1. Introduction

Gonadal development in female teleosts is dependent on the production of steroid hormones from the ovarian follicle in response to stimulation by pituitary gonadotropins (Nagahama, 1994). Gonadotropins (follicle-stimulating hormone, Fsh; luteinizing hormone, Lh) are members of the glycoprotein hormone family, each consisting of a common alpha and hormone-specific beta subunit (Pierce and Parsons, 1981). The effects of gonadotropins on gonadal physiology depend on their availability in the circulation and the abundance of their specific receptors in the gonad (Ascoli and Segaloff, 1989). Understanding the changes in abundance of gonadotropins and their receptors, both at the transcript and protein level, and evaluating some of their effects - associated changes in plasma sex steroid levels and oocyte cytology - during gonadal development are key to understanding the natural progression of oogenesis.

At present, acquired insights into gonadotropins and into regulation of their production in fish are mainly related to advanced stages of ovarian development, i.e., vitellogenesis and final oocyte maturation. Generally, Fsh is considered vitellogenesis-supporting, while Lh is related to final oocyte maturation (Nagahama and Yamashita, 2008). Recently, the use of transcription activator-like effector nuclease technology to disrupt the hormone-specific β-genes of both Fsh and Lh in the zebrafish indicated that the Fsh deficiency significantly delayed the development of both ovary and testis, whereas the disruption of *lhb* led to the failure of spawning in female (Zhang et al., 2015). Similarly, a gene knockout (KO) study on medaka showed that the follicular growth of Fsh (KO) was arrested at previtellogenic stage and that Lh knockouts were anovulatory (Takahashi et al., 2016).

In contrast, the role of pituitary gonadotropins in the previtellogenic stage, including primary and early secondary oocyte growth, is not clear and remains debatable. Following hypophysectomy,

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Table 1
Morphological and gonadal characteristics of the wild female shortfinned eels (*A.australis*) in different stages of ovarian development.

Stage					
Sampling date		PN	EOD	OD	EV
Oct 2015	Sample size	9	4	2	0
	TL (cm)	74.4 ± 2.6	78.8 ± 1.1	83.5 ± 5.5	–
	BW (g)	677.8 ± 64.6	1168 ± 99	1370 ± 92	–
Dec 2015	Sample size	9	5	2	2
	TL (cm)	65.8 ± 2.1	77.6 ± 1.1	84.1 ± 3.3	93.5 ± 2.3
	BW (g)	681.5 ± 66.9	992.6 ± 39.6	1413 ± 25	1671.5 ± 52.5
Feb 2016	Sample size	6	7	2	0
	TL (cm)	67.5 ± 3.4	73.9 ± 1.8	83.0 ± 6.0	–
	BW (g)	696.3 ± 125.7	926 ± 81.2	1370 ± 388	–
Mar 2016	Sample size	6	8	1	5
	TL (cm)	64.9 ± 1.2	77.1 ± 1.9	83	91.9 ± 1.6
	BW (g)	595.8 ± 36.1	1089.6 ± 92.6	1185	1555.3 ± 97.1
April 2016	Sample size	7	4	3	3
	TL (cm)	70.4 ± 1.7	76.6 ± 2.1	85.6 ± 3.4	79 ± 2.7
	BW (g)	712 ± 63.2	940.1 ± 113.1	1455.8 ± 141.2	1021.1 ± 90.1
Total number of fish		37	28	10	10

TL: total length, BW: body weight, PN: perinucleolus stage, EOD: early oil droplet stage, OD: oil droplet stage, EV: early vitellogenic stage.

pituitary–independence of primary oocyte growth was reported in goldfish, *Carassius auratus* (Khoo, 1979) and catfish, *Heteropneustes fossilis* (Sundararaj and Goswami, 1968). Conversely, there is compelling evidence for involvement of gonadotropic hormones during the previtellogenic stage in teleost fish. For instance, Fsh was deemed an important signal associated with appearance of cortical alveoli and accumulation of lipid droplets in oocytes of coho salmon, *Oncorhynchus kisutch* (Campbell et al., 2006). Likewise, Luckenbach et al. (2008) suggested that Fsh is a potential endocrine regulator during transition from primary to early secondary oocyte growth in coho salmon. A dramatic increase of ovarian *fshr* mRNA levels at the previtellogenic stage was also shown in zebrafish, *Danio rerio* (Kwok et al., 2005). Collectively, gonadotropins seem to be involved in ovarian development during early stages of oogenesis, depending on fish species.

Eels are catadromous fish with a striking life cycle that can be divided into six life history stages: egg, larva, glass eel, elver, yellow eel and silver eel (Tesch, 2003). Somatic growth principally occurs in the yellow eel phase, typically while inhabiting freshwater waterways. At this time, ovaries harbor oocytes that are in the previtellogenic stage and that may or may not contain oil droplets (Lokman et al., 1998). Extensive remodeling, comparable to the salmonid parr-smolt transformation (Folmar and Dickhoff, 1980), sees yellow eels transition to the silver eel phenotype, and this is temporally linked to activation of the reproductive axis.

What is the involvement of gonadotropins during early oogenesis in the yellow eel? Limited information on previtellogenic Japanese eels, *Anguilla japonica* indicated that both *fshb* and *lhb* mRNA were detectable and that ovarian mRNA levels of *fshr* were significantly higher than those of *lhr* (Jeng et al., 2007). Meanwhile, previtellogenic oocytes of *A. australis* responded to recombinant Fsh treatment *in vitro* with a significant increase in mRNA levels of *star* (steroidogenic acute regulatory protein) (Reid et al., 2013). Taken together, the involvement of gonadotropins in previtellogenic oocyte growth of eels is thus plausible.

However, data on profiles of gonadotropins and their receptor mRNA levels at different stages of ovarian development in wild eels are extremely limited, as eels in advanced stages of oogenesis are not accessible to science due to being in unknown offshore locations. Fortunately, wild eels are locally abundant in New Zealand; therefore, insights into gonadotropin biology during early oogenesis can be obtained. Similar to other freshwater eel species, the eggs of NewZealand shortfinned eels are spawned in the ocean and develop into transparent leaf-like larvae (leptocephalus). The leptocephali are transported to continental waters by oceanic currents and they undergo metamorphosis into glass eels (non-pigmented). Following this stage, the glass eels migrate toward estuaries and freshwater bodies and develop into pigmented elvers (Bruijs and Durif, 2009). These elvers enter the feeding phase and continuously grow in fresh water to become “yellow eels”. After residing in fresh water for a long time, the yellow eels develop to become “silver eels” and they undergo significant physiological, morphological and behavioural changes (see more details in Aoyama and Miller, 2003; Lokman et al., 2003). The silver eels migrate out towards the ocean for breeding and it is believed that they will die after spawning (Jellyman and Lambert, 2003). The spawning grounds of New Zealand shortfinned eels are proposed to be near Fiji (Aoyama et al., 1999).

The present study aimed to investigate mRNA levels of gonadotropin subunits (*fshb* and *lhb*), and their receptors (*fshr* and *lhr*) in wild New Zealand shortfinned eels at different stages of ovarian development from yellow to silver eels. Transcript abundance data were complemented by measurement of protein levels of Fsh in the pituitary using slot blot and immunohistochemistry. Additionally, due to the dependence of gonadal development on steroid hormones, the plasma levels of estradiol-17β (E2) and 11 ketotestosterone (11-KT) were measured using radioimmunoassay. Lastly, changes in mRNA levels of genes encoding steroidogenic enzymes (cytochrome P450 aromatase (*cyp19*); cytochrome P450 11β-hydroxylase (*cyp11b*)) were determined throughout the early stages of ovarian development.

2. Materials and methods

2.1. Animals

Female non-migrant (yellow eels) and/or migrant eels (silver eels) were captured approximately monthly at Lake Ellesmere (South Island, New Zealand) from October 2015 to April 2016, using fyke nets (Table 1). The eels were trapped during the night and specimens were selected for sampling in the morning. The non-migrant and migrant eels were distinguished depending on the color of the pectoral fins (black in migrants). During selection, the non-migrant eels were first divided between three groups based on body weight (< 700 g, 700 – 1000 g, and > 1000 g), anticipating that different developmental stages could thus be collected. The reproductive stage of sampled eels was later fine-tuned on the basis of ovarian histological observations, ovaries from non-migrants being assigned to perinucleolus (PN), early oil droplet (EOD), or oil droplet (OD) stages while those from migrants were assigned the early vitellogenic (EV) stage.

Table 2
QPCR primers, their annealing temperatures, amplicon size (bp) for each qPCR product, the efficiency of amplification of the standard curves (%) and the inter-assay variation shown as the coefficient of variation (%) for each target gene in qPCR assay (n = 3 plates per assay). Abbreviations: follicle-stimulating hormone β-subunit (*fshb*), luteinizing hormone β-subunit (*lhb*), follicle-stimulating hormone receptor (*fshr*), luteinizing hormone receptor (*lhr*), cytochrome p450 aromatase (*cyp19*), cytochrome p450 11β-hydroxylase (*cyp11b*), 60S ribosomal protein (*l36*) and elongation factor-1α (*eef1*). Lengths of the amplicons are in base pairs (bp), and annealing temperatures (Ta) in degrees Celsius (°C).

Gene	qPCR primers (5′– 3′)	Ta (°C)	Amplicon size (bp)	Tissue	Efficiency %	Coefficient of variation (%)	**Reference**
fshb	FW: CCGTGGAGAATGAAGAATGC RV: TGGTTTCAGGGAGCTCTTGT	64 °C	104	pituitary	96.5 to 102.7	8.3	Setiawan et al. (2012)
lhb	FW: TCACCAAGGACCCAAGCTAC RV: CCATGGTGCACAGGTTACAG	62 °C	171	pituitary	96.7 to 99.2	11.5	Setiawan et al. (2012)
fshr	FW: CCTGGTCGAGATAACAATCACC RV: CCTGAAGGTCAAACAGAAAGTCC	63 °C	173	ovary	97.8 to 101.1	5.4	Zadmajid et al. (2015)
lhr	FW: GTACAGCGCTACGCATTCAAC RV: CGTAGAAGACACATCGAGCAGAC	62 °C	132	ovary	96.3 to 97.9	5.8	Ozaki et al. (unpublished data)
cyp19	FW: AAAAAGCCCGCACCTACTTT RV: AGGTTGAGGATGTCCACCTG	62 °C	145	ovary	95.9 to 96.8	4.6	Setiawan et al. (2012)
cyp11b	FW: ATCACTGTCCAGCGATACC RV: CGCGTCGGCTTAAATATCTC	62 °C	132	ovary	95.1 to 100.2	12.5	Setiawan et al. (2012)
l36	FW: CCTGACCAAGCAGACCAAGT RV: TCTCTTTGCACGGATGTGAG	62 °C	160	ovary pituitary	97.1 to 97.8 96.9 to 101.1	4.4 5.6	Setiawan & Lokman (2010)
eef1	FW: CCCCTGCAGGATGTCTACAA RV: AGGGACTCATGGTGCATTTC	64 °C	152	ovary pituitary	95.0 to 102.0 99.5 to 101.3	7.7 5.2	Setiawan & Lokman (2010)

2.2. Sampling protocol

Within 8 h of retrieval from the nets and temporary housing in a flow-through tank with water at 12 °C, fish were euthanized in 0.3 g/l benzocaine and total body length and weight were measured before removal of the tail for blood collection. Blood was stored in polypropylene tubes containing 50 µl of 200 mg/ml ethylenediaminetetracetic acid on ice. Blood was then centrifuged at 4 °C and 1000*g* for 10 min for plasma collection. Plasma was aspirated and stored at −70 °C until assay for sex steroid levels (Section 2.4). Ovarian and pituitary tissues were collected immediately after decapitation. Portions of the right ovary and whole pituitaries were rapidly frozen on dry ice and stored at −70 °C until RNA extraction and subsequent molecular and slot blot analyses (Sections 2.5, 2.6 & 2.9), except for the pituitaries (April 2016) used for immunohistochemistry (fixed in 4% paraformaldehyde in 100 mM phosphate-buffered saline (PBS), pH 7.4; Section 2.10) and those lost during processing (1 from October, 2 from December, 1 from February and 1 from March). A few small ovarian tissue fragments were fixed in 10% neutral-buffered formalin for histological procedures. The left ovary was weighed to calculate gonadosomatic index (GSI) ([(single ovary weight × 2)/BW] × 100).

2.3. Histology

All fixed ovarian tissues were processed for embedding in Technovit 7100 (Heraeus Kulzer GmbH and Co., Hanau, Germany) following the manufacturer's instructions. Embedded samples were sectioned on a Reichert Jung microtome at 2 µm and were stained in 1.3% methylene blue and 0.2% azure II prior to being counterstained in 2% basic fuchsin. Images were captured by a compound microscope (Olympus BX51) equipped with an Olympus SC100 camera. The captured images were then used to identify the reproductive stage of eels.

2.4. Radioimmunoassay

The concentrations of 11-KT and E2 in plasma samples were measured by radioimmunoassay as described previously (Lokman et al. 1998). Due to the lack of space to analyze all samples in a single assay, duplicate samples were run in several assays. The inter-assay coefficients of variation were 13.5% for E2 and 8.7% for 11-KT. The minimum detectable concentrations of E2 between the first and second assays were comparable (0.051 ng/ml vs. 0.047 ng/ml). For the 11-KT assay, the minimum detectable levels were 0.19 ng/ml and 0.12 ng/ml for the first and second assay, respectively.

2.5. RNA extraction, DNase treatment and cDNA synthesis

RNA from frozen tissues was extracted using TRIzol® Reagent (Invitrogen) following the manufacturer's protocol. The concentration and relative purity of extracted RNA were checked via absorbance at 260/280 using a ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA). To minimize potential genomic DNA contamination, the isolated RNA (5 µg) was treated with TURBO™ DNase (Applied Biosystems, Invitrogen) according to the manufacturer's instructions. Subsequently, complementary DNA (cDNA) was synthesized from 1 µg of DNase-treated RNA using the High-Capacity cDNA Reverse Transcription Kit, using random hexamer pimers as outlined in the kit manual (Applied Biosystems, Invitrogen). The reverse-transcription reactions were carried out using an Eppendorf EpGradient S PCR machine under the following conditions: an initial step of 25 °C for 10 min, followed by incubation at 37 °C for 120 min and 85 °C for 5 min. The cDNA product was diluted to 10 ng RNA-equivalent/µl using milli-Q water prior to storage at −70 °C.

2.6. Real-time quantitative PCR (qPCR)

2.6.1. Primers for qPCR

All primers used for qPCR analysis in this study were designed and validated from previous studies (Table 2). These primers were re-validated prior to each qPCR assay in the present study.

2.6.2. qPCR assays

All qPCR assays were performed using the QuantStudio 5 Real-Time thermal cycler (Applied Biosystems) with a thermal profile of 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing for 10 s (see Table 2 for specific annealing temperature), and extension at 72 °C for 5 s. Thereafter, a final dissociation curve analysis was done. Duplicate samples were run on a single 96-well plate along with standards and a no-template control (distilled water). Each 10 µl reaction consisted of 1 µl of cDNA, 0.5 µl of each of the forward and reverse primers (10 µM), 5 µl of SYBR® Premix Ex Taq ™II (Takara Bio, Kyoto, Japan) and 3 µl of DNAse-free water. Due to the lack of space to fit all samples on a single plate, duplicate samples were run on replicate plates as quality control in order to evaluate inter-assay variation (Table 2). The concentrations of standards, generated from serial dilutions of qPCR product, varied between target genes (10 pg/µl to 1 fg/

μl for *l36* and *eef1*, and 0.1 pg/μl to 0.1 ag/μl for the others). Amplification efficiencies ranged from 95.1% to 102.7% for the different target genes (Table 2). Based on a selection of genes recommended in a previous study (Setiawan and Lokman, 2010), two candidate reference genes (*l36* and *eef1*) were evaluated for use with ovary and pituitary tissues. In the ovary, *l36* gene expression was not significantly different between reproductive stages, while *eef1* gene expression was significantly lower in ovaries in the PN stage than that in EOD and OD stages. Therefore, all qPCR data of the target genes from the ovary were normalized over *l36*. In the pituitary, the geometric means of *l36* and *eef1* showed no significant difference ($p > 0.05$) and proved more consistent between pituitaries from eels in the different developmental stages compared to normalizing over a single reference gene. Hence, the geometric means were used to normalize the transcript abundances of the target genes in the pituitary.

2.7. Method development for RNA and protein extraction

In order to quantify gene and protein expression from the *same* pituitary, a novel method for RNA and protein extraction was developed and validated. Briefly, pituitary tissue was well homogenized with 120 μl phosphate-buffered saline (PBS) containing 10 μl protease inhibitor (Roche, New Zealand) for one minute at 4 °C. Immediately thereafter, 60 μl of homogenate was transferred into TRIzol® reagent for RNA extraction and the remaining homogenate was centrifuged for 1 h at 10,000*g* at 4 °C. Subsequently, the supernatant was stored at 4 °C for measurement of Fsh protein levels using slot blot (Section 2.9).

To assess whether homogenization in plain buffer affected RNA quality, the following validation steps were conducted: three pituitaries from yellow eels were homogenized and centrifuged as mentioned above. Following these steps, 40 μl of supernatant was aliquoted and transferred into TRIzol® reagent at different times post-homogenization (30, 90 and 270 s). Subsequently, RNA extraction, cDNA synthesis and qPCR assay for *fshb* and *l36* were carried out as described above (Section 2.5). Accordingly, normalized *fshb* gene expression did not change between the three time points (Fig. 1). Therefore, this method was considered suitable to extract both protein and RNA from the same pituitary tissues, at least when employed under stringent time management.

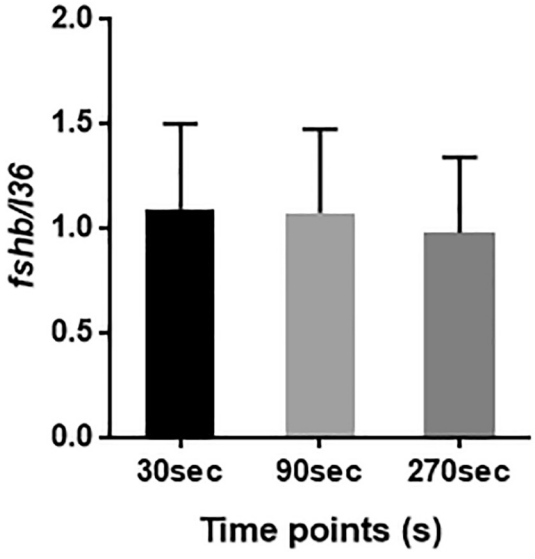

Fig. 1. Mean (± SE) relative transcript copy number of *fshb* at different time points (30, 90 and 270 s) after pituitary tissues were homogenized in plain buffer for protein extraction.

2.8. Slot blot and data analysis

The specificity of an Fsh antiserum was assessed using Western blot. The antiserum was raised against Japanese eel recombinant Fsh, expressed in *Escherichia coli* (Kazeto et al., 2008) To validate the antiserum for use with shortfinned eel, the methodology described by Kamei and co-workers was adopted. Briefly, homogenates of yellow and silver eel pituitaries were separated on 15% SDS-PAGE gels (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under reducing conditions before being subjected to Western blotting. Only one main band around 27 kDA was immunostained on the PVDF membrane (Suppl. Fig. 1). This result is somewhat different to that of *A. japonica* (around 15 to 20 kDA, see Kazeto et al. 2008) in spite of a large number of different biochemical conditions trialed. This inconsistency could be due to either the slight difference in glycosylation, or protein sequence between these species. Importantly, Western blotting recognized only one band in the present study. Therefore, the Fsh antiserum was deemed specific for the detection of Fsh in the pituitary of New Zealand shortfinned eels, and hence, suitable for use in slot blotting applications.

The extracted proteins from pituitaries (50 μl/sample) and standards were mixed with 2.5 μl of 5% Ponceau Red to visually aid protein loading. Immobilon™-P PVDF membrane with 0.45 μm pore size (Millipore) was soaked in 100% methanol for 15 s prior to use. The membrane and filter paper (blotting paper) were then briefly soaked in TBS (Tris-buffered saline; high salt, 500 mM of NaCl) before being assembled into the slot blot chamber (Hoefer Scientific Instruments). Each slot was washed well with 500 μl TBS (high salt) before 50 μl of sample was loaded onto the membrane. The membrane was then removed from the slot blot system and incubated with blocking buffer (skim milk 5%) for 2 h. The membrane-immobilized Fsh protein was detected using Fsh antiserum (1:1000 dilution; see Kazeto et al. (2008) and Setiawan et al. (2012) for details) which was in turn detected using anti-rabbit IgG (1 : 1000 dilution) conjugated to alkaline phosphatase (Invitrogen). Due to space limitations, all samples from October, December, February and March could not fit in a single slot blot run. Instead, the samples were assigned randomly and loaded onto four different membranes.

The staining intensity of each slot was measured using ImageJ software. Subsequently, the membranes were photographed, and ImageJ was used to obtain an estimate of the relative Fsh protein levels in the samples based on the intensity of colour of each slot instead. Due to the small amount of extracted protein, the intra and inter assay coefficients of variation could not be determined in this study. For the same reason, pituitary Lh levels in pituitary homogenates were not measured in the present study.

2.9. Immunohistochemistry of pituitary fshb

To localize Fsh protein in the pituitary between eels in different stages of ovarian development, fixed tissues (Section 2.2) were dehydrated through a series of ethanol baths, and embedded in paraffin. The embedded samples were sectioned (4 μm) and mounted onto poly-L-lysine-coated microscope slides (LabServ). Immunohistochemistry was performed following the methodology in Damsteegt et al. (2014) with slight modifications. In order to inactivate any endogenous peroxidase activity, the de-paraffinised and hydrated sections were incubated in boiled TE buffer for 15 min. Subsequently, sections were incubated with 10% skim milk for 2 h to block non-specific binding of antibodies. The sections were immunohistochemically stained with Fsh antiserum at a dilution of 1:1000 used alongside HISTOFINE SAB_AP(R) Kit (Nichirei, Tokyo, Japan). Anti-rabbit IgG conjugated with biotin was used as a secondary antibody. After 30 min of incubation with streptavidin-conjugated alkaline phosphatase, sections were stained with 0.5% 5-bromo − 4-chloro − 3-indolyl phosphate 0.4% 4-nitro blue tetrazolium chloride solution (Sigma-Aldrich) to visualize immunoreactive Fsh

proteins. Micrographs were taken using an Olympus BX51 microscope coupled to an Olympus SC 100 camera.

2.10. Data analysis

Data were tested for normality and equal variances using Shapiro-Wilk and Bartlett's test, respectively. Data which did not meet these assumptions were log-transformed to obtain homogeneity of variance. Although data were collected over the course of half a year, month effects were excluded in data analyses because the sample sizes did not meet the requirements for two-way ANOVA [developmental stage, with four levels (PN, EOD, OD and EV) and month, with 5 levels]; indeed, some developmental stages were either absent or in low numbers during several months (Table 1). Therefore, data from different months were pooled by stage and compared using one-way ANOVA, followed by Tukey's post-hoc test. Independent samples T-Test was used to compare 11-KT data between OD and EV stage due to the undetectable plasma 11-KT levels of PN and EOD eel groups. Regression analysis was carried out to examine the relationship between pituitary *fshb* mRNA levels and pituitary Fsh protein levels. All analyses were performed using SPSS 17.0 and all numerical data are presented as means ± SE using GraphPad Prism version 6.0 for Windows (GraphPad Software, California, USA).

3. Results

3.1. Morphometry

Basic morphometric data from female shortfinned eels captured during 5 sampling trips are summarized in Table 1. Out of a total of 85 eels sampled, there were 75 yellow eels and 10 silver eels. Based on histological observations of ovarian development, 37, 28 and 10 yellow eels were assigned to the PN, EOD and OD groups, respectively, whereas the 10 silver eels were considered as the EV group.

3.2. Gonadosomatic index

The GSI increased gradually but significantly as eels progressed through oogenesis ($F_{3,84}$ = 467.2, $p < 0.0001$). The GSI of non-migrant eels was < 2, while the GSI of migrant eels, which had oocytes at the EV stage, was approximately 3 (Fig. 2).

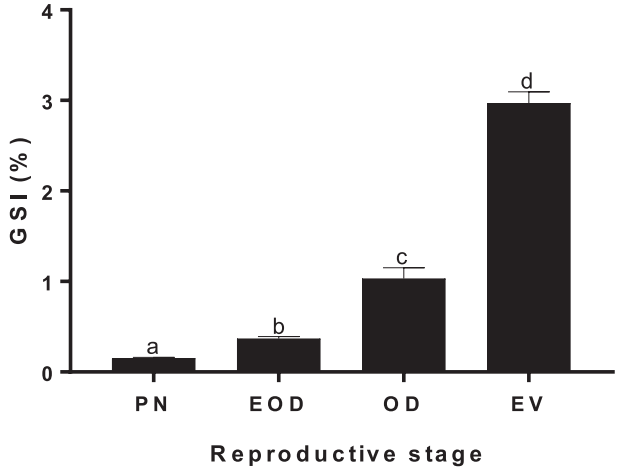

Fig. 2. Mean (± SE) GSI of wild female *A. australis* in either perinucleolus, early oil droplet, oil droplet or early vitellogenic oocyte stage. Different letters above bars denote significant differences between ovarian developmental stages. Abbreviations: PN = perinucleolus, (n = 37); EOD = early oil droplet (n = 28); OD = oil droplet (n = 10); EV = early vitellogenic (n = 10).

3.3. Histology

The four eel groups (PN, EOD, OD and EV) showed different degrees of oocyte development (c.f., Lokman et al. 1998). In the PN group, ovaries contained mainly chromatin nucleolus and perinucleolus oocytes, while oil droplets initially appeared at the periphery of oocytes from eels in the EOD group. In the OD group, the oocytes continued to develop, being larger and displaying ongoing lipid accumulation. In the EV group, the whole ooplasm of the oocytes was fully occupied by oil droplets and yolk granules were observed at the periphery of the oocytes (Fig. 3).

3.4. Plasma E2 and 11-KT

The plasma E2 levels increased gradually and significantly during ovarian development ($F_{3,84}$ = 32.768, $p < 0.001$) and, at about 0.9 ± 0.06 ng/ml, proved significantly higher in silver than in yellow eels ($p < 0.05$). In non-migrants, the highest E2 plasma levels (0.6 ± 0.12 ng/ml) were found in the OD group. There was no significant difference in the plasma E2 levels between the PN and EOD groups, averaging 0.2 ± 0.03 ng/ml and 0.3 ± 0.04 ng/ml, respectively (Fig. 4A). Plasma 11-KT levels in eels from the PN and EOD groups were undetectable. Low levels of 11-KT (0.3 ± 0.04 ng/ml) were found among fish in the OD group. In contrast, the plasma 11-KT levels were very high in EV fish (43.5 ± 7.95 ng/ml), some two orders of magnitude higher than in the OD group ($t = 5.435$, $df = 18$, $p < 0.0001$; Fig. 4B).

3.5. Target gene transcript abundance in the pituitary

3.5.1. Follicle-stimulating hormone β subunit (fshb)

Pituitary *fshb* mRNA levels varied significantly between different stages of ovarian development ($F_{3,62}$ = 10.076, $p < 0.0001$), being significantly lower in the PN stage than in other stages ($p < 0.05$). Relative transcript copy numbers of ovarian *fshb* increased steadily from the PN to OD stage before decreasing by over 50% in the EV stage. However, differences in the mean *fshb* mRNA levels between EOD, OD and EV stages were not significant (Fig. 5A).

3.5.2. Luteinizing hormone β subunit (lhb)

Relative transcript copy numbers of *lhb* in the pituitary increased with stage of oocyte development ($F_{3,60}$ = 14.733, $p < 0.0001$) (Fig. 5B). ANOVA confirmed that *lhb* relative transcript abundance in the PN stage was significantly lower than that in the EOD, OD and EV stages ($p < 0.05$). The mRNA level of *lhb* in the EV stage was significantly higher, by approximately 25-fold, than in the EOD stage ($p < 0.05$). Two samples (from PN and OD groups) were not amplified during the qPCR reactions and were therefore eliminated from data analyses.

3.6. Relative follicle-stimulating hormone protein level

Relative pituitary Fsh protein levels were significantly different between eels in different stages of ovarian development ($F_{3,62}$ = 4.552, $p < 0.05$). Relative Fsh protein levels significantly increased from approximately 3856 ± 303.7 colour intensity units (CIU) at the PN stage to 4512 ± 348.9 and 6503 ± 607.8 CIU at the EOD and OD stage respectively, before decreasing to 4064 ± 828.6 CIU at the EV stage (Fig. 5C). Changes in relative pituitary Fsh levels with developmental stage closely mirrored those in *fshb* expression, reflected in a positive relationship, R^2 = 0.52, $F_{1,61}$ = 65.55, $p < 0.0001$ (Fig. 5D).

3.7. Immunohistochemistry

Fsh protein was localized in the proximal pars distalis of the pituitary. Cells immunoreactive to anti-fshβ were visible by blue/dark

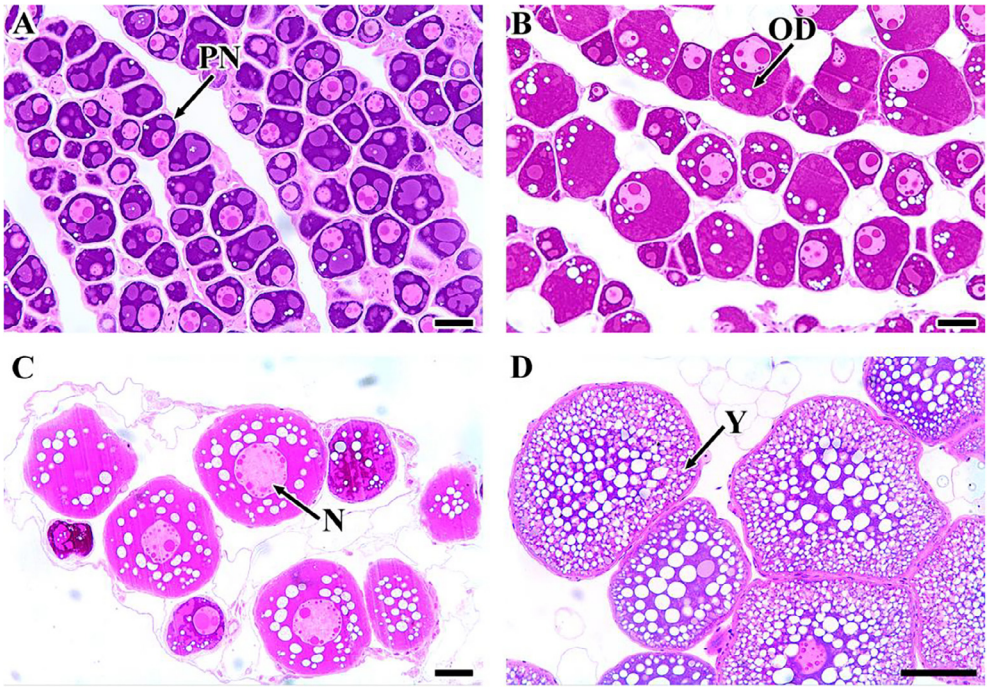

Fig. 3. Light micrographs of ovarian tissue from female eels (*A. australis*) at different stages of oogenesis. A) Perinucleolus stage; B) Early oil droplet stage; C) Oil droplet stage; D) Early vitellogenic stage. Abbreviations: CN = chromatin nucleolus; PN = perinucleolus; OD = oil droplet; N = nucleolus; Y = yolk globule. (A-C: scale bar = 100 µm; D: scale bar = 50 µm).

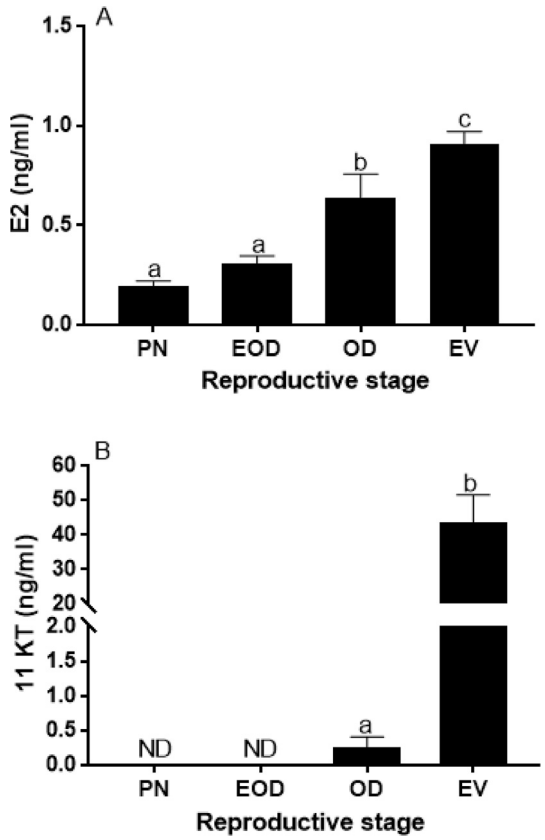

Fig. 4. Mean (± SE) plasma E2 (A) and 11-KT (B) levels of wild female eels (*A. australis*) at different stages of oogenesis. Different letters above bars denote significant differences in plasma E2 levels between oocyte developmental stages. Abbreviations: PN = perinucleolus, (n = 37); EOD = early oil droplet (n = 28); OD = oil droplet (n = 10); EV = early vitellogenic (n = 10).

staining (Fig. 6). A total of 17 pituitaries from fish (April 2016) in different stages of ovarian development were processed for immunohistochemistry. Variation in immunostaining between fish at the same stage of ovarian development was evident, but more pronounced differences in staining were observed between the reproductive stages. Notably, there was no evidence for staining of Fsh in eels at the PN stage (Fig. 6A). Cells immunoreactive to anti-fshβ were only found at the peripheral area of the pituitary in eels at the EOD and OD stages (Fig. 6B-C), although much darker staining was detected in the eels with oocytes at the OD stage in comparison with other stages (Fig. 6C). Immunostaining of Fsh in eels at the EV stage was evident as typical chords of blue-staining cells in the proximal pars distalis (Fig. 6D).

3.8. Target genes transcript abundance in the ovary

3.8.1. Follicle-stimulating hormone receptors (fshr)

Relative transcript copy numbers of *fshr* in the ovaries increased sharply and significantly between yellow (oocytes at the PN, EOD and OD stage) and silver eels (oocytes at the EV stage) ($F_{3,83} = 9.918$, $p < 0.0001$). There were no significant differences in the expression of *fshr* between the PN, EOD and OD groups (Fig. 7A). Complementary DNA from one sample in the PN group was not amplified during the qPCR reaction and was therefore excluded from data analyses.

3.8.2. Luteinizing hormone receptor (lhr)

Similar to ovarian *fshr* gene expression, relative transcript copy numbers of *lhr* were significantly higher in migrants (EV group) than in non-migrants (PN, EOD and OD group) ($F_{3,84} = 15.977$, $p < 0.0001$). Among non-migrants, the difference in the *lhr* expression between different stages of oogenesis was not statistically significant (Fig. 7B).

3.9. Steroidogenesis-related genes (cyp19 and cyp11b)

Relative transcript abundance of ovarian *cyp19* was not significantly different between stages of oogenesis (Suppl. Fig. 2A). Similarly, no significant changes or clear trends were detected in *cyp11b* gene expression throughout ovarian development (Suppl. Fig. 2B).

4. Discussion

Although gonadotropins (Fsh and Lh) are the major hormones to induce puberty in fish (Nagahama and Yamashita, 2008), there is little

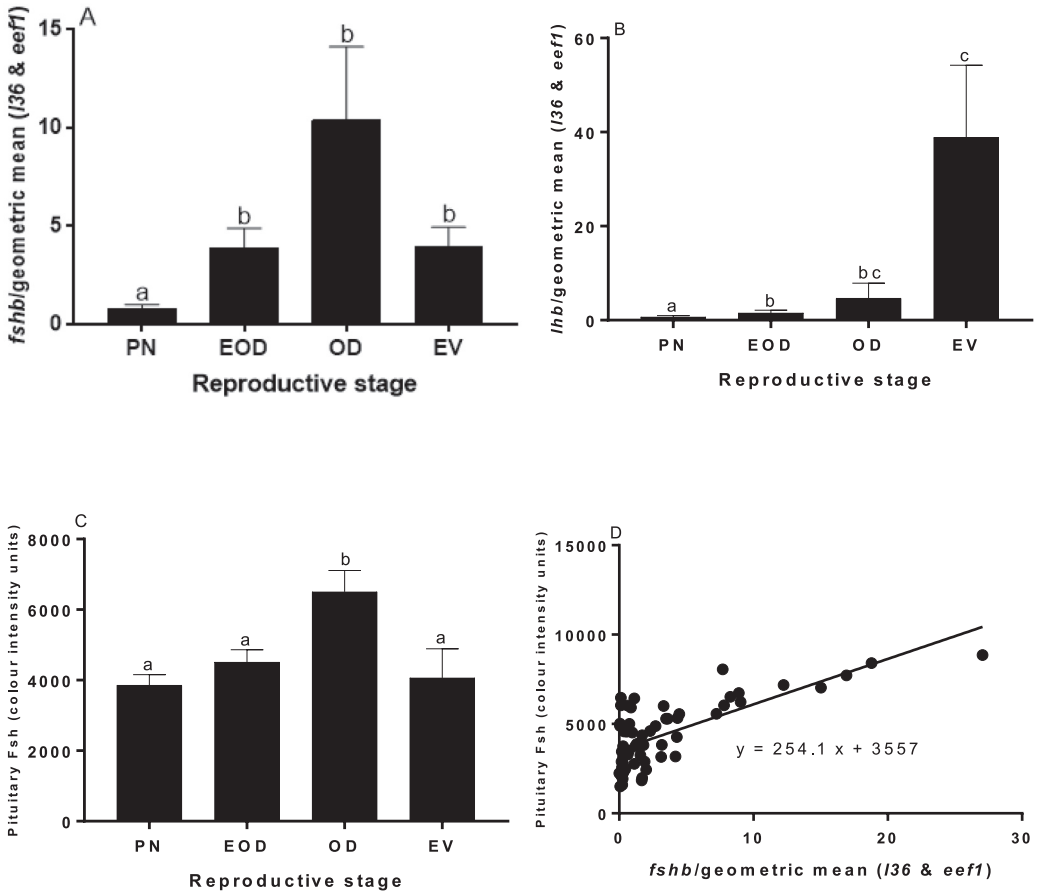

Fig. 5. Mean (± SE) relative transcript copy numbers of *fshb* (A), *lhb* (B), Fsh protein (C) and correlation between *fshb* mRNA and Fsh protein levels (D) in the pituitary of wild female eels (*A. australis*) at different stages of oogenesis. Different letters above bars denote significant differences in *fshb* gene expression between oocyte developmental stages. Abbreviations: PN = perinucleolus, (n = 27); EOD = early oil droplet (n = 22); OD = oil droplet (n = 7); EV = early vitellogenic (n = 7); follicle-stimulating hormone β-subunit (*fshb*); 60S ribosomal protein (*l36*), and elongation factor-1α (*eef1*).

information in fish relating to the role of these hormones during the early stages of oogenesis. At present, there is growing support for the notion that gonadotropic hormones are involved in regulating early stages of ovarian development in fish, for example through the data archived from previous studies on salmonids and yellowtail kingfish (Campbell et al., 2006; Luckenbach et al., 2011; Sanchis-Benlloch et al., 2017). The present study used the New Zealand shortfinned eel to elucidate the involvement of gonadotropic hormones (Fsh and Lh) during early oogenesis. Therefore, this study provides a phylogenetic contrast and different experimental approach to published work to date.

Results from the present study indicate that *fshb* mRNA levels increased significantly from the PN to OD stage (yellow eels) before showing a tendency to decrease at the EV stage. These data are consistent with the expression profile of pituitary gonadotropin subunit genes associated with the transition from yellow to silver female European eels. According to Aroua et al. (2005), an early increase of *fshb* mRNA levels was observed in the "intermediate" European eels which are equivalent to the EOD and OD eels in the present study and a decreasing trend was recorded between "intermediate" eels to silver eels. However, the transcript abundance pattern of pituitary *fshb* differed slightly between New Zealand shortfinned eels and Japanese eels. Pituitary *fshb* mRNA levels of New Zealand shortfinned eels decreased, albeit not significantly, from yellow OD to the silver eel EV stage. In contrast, although there were no significant differences, pituitary *fshb* mRNA levels of wild Japanese eels increased from yellow to silver eels (Han et al., 2003). Significantly, the trend in *fshb* mRNA levels in the present study is reinforced by that of protein, evident from the positive correlation between *fshb* mRNA levels and Fsh protein levels and by immunohistochemical observations. Similarly, a strong correlation between changes in *fshb* mRNA levels and Fsh pituitary content was reported in rainbow trout (Gomez et al. 1999). It is noticed that pituitary *fshb* mRNA levels in OD stage were approximately 12.5 times higher than those in PN stage whereas pituitary Fsh protein levels in OD stage were only about 1.5-fold those in PN stage. The discrepancy between the protein and mRNA levels in the present study is reasonable because the changes in gene expression are not always *per se* reflected at the protein level; examples of poor correlations between mRNA and protein levels abound (de Sousa Abreu et al., 2009; Vogel and Marcotte, 2012).

The presence of Fsh in equal quantities in prepubertal and early pubertal stages could suggest that Fsh initially just accumulates in the pituitary. Previous studies on the mRNA profiles of pituitary gonadotropin subunits in immature and artificially matured Japanese eels also suggested that Fsh would *accumulate* in the immature stage rather than in maturing and matured stages (Nagae et al. 1996; Yoshiura et al. 1999; Suetake et al. 2002).

Absence of yolk proteins in oocytes of yellow eels at all stages of ovarian development, together with low steroid levels imply that Fsh either is not released and/or not recognized by the ovary. In the present study, *fshr* mRNA levels increased in the early vitellogenic stage, but Reid et al. (2013) indicated that even in early stages of oogenesis (PN and EOD) the ovary can respond to recombinant Fsh *in vitro*, reflected by increases of *star* mRNA levels. Therefore, sensitivity of the ovary to recombinant Fsh *in vitro* suggests that the signal, if present, should be

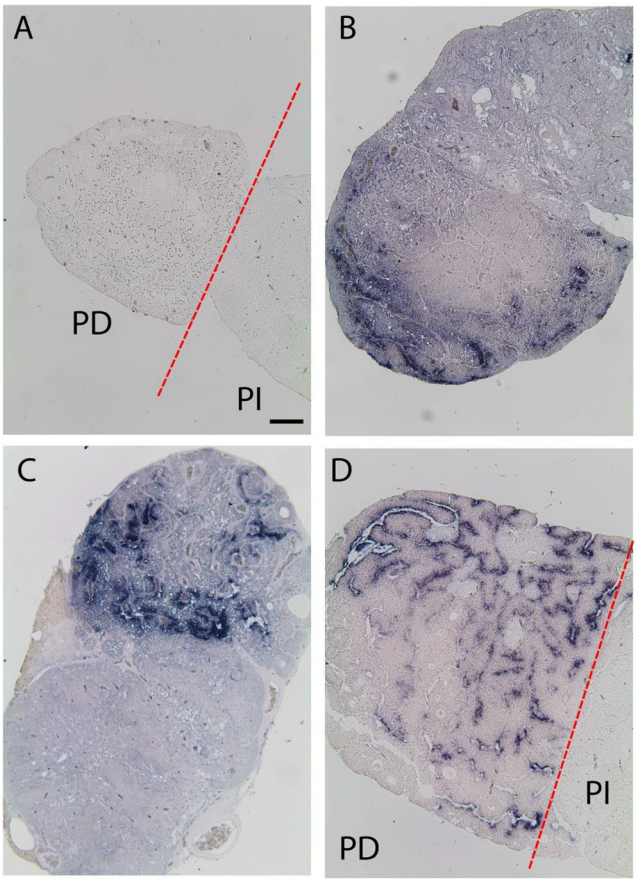

Fig. 6. Micrographs of pituitary sections, immunostained with anti-fshβ, from wild-caught female eels (*A. australis*) at different stages of ovarian development. A) Perinucleolus stage; B) Early oil droplet stage; C) Oil droplet stage; D) Early vitellogenic stage. All images at same magnification. Scale bar = 100 µm. Abbreviation: PD: pars distalis; PI: pars intermedia.

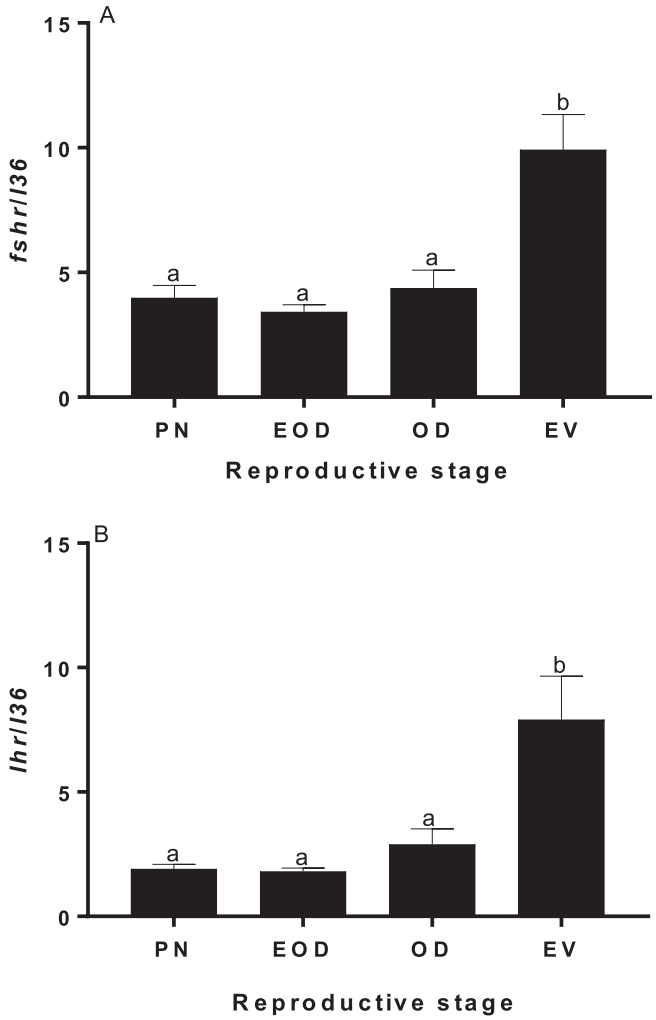

Fig. 7. Mean (± SE) relative transcript copy numbers of *fshr* (A) and *lhr* (B) in the ovary of wild female eels (*A. australis*) at different stages of oogenesis. Different letters above bars denote significant differences in *fshr* gene expression between oocyte development stages. Abbreviations: PN = perinucleolus, (n = 36); EOD = early oil droplet (n = 28); OD = oil droplet (n = 10); EV = early vitellogenic (n = 10); follicle-stimulating hormone receptor (*fshr*); 60S ribosomal protein (*l36*).

detectable *in vivo*. Accordingly, failure to release Fsh, rather than it not being recognized, is the most likely explanation for low steroid levels in yellow eels. This contradicts the generally held belief that Fsh is released upon being produced, in keeping with a strong correlation between pituitary and plasma Fsh in salmonids (Gomez et al. 1999; Campbell et al. 2006). It appears that in prepubertal eels this may not be the case, though unfortunately, we do not have an assay to measure Fsh levels in blood. It is possible that there is an unknown factor inhibiting the *release* of Fsh into the circulation at the yellow stage. A recent study on Nile tilapia (*Oreochromis niloticus*) by Golan et al. (2016) showed that stellate cells which produce follistatin (a potent inhibitor of Fsh release in fish) were distributed abundantly in close proximity to Fsh-producing cells in the proximal pars distalis. This may explain the higher levels of Fsh protein in the pituitary of yellow eels compared to silver eels as seen in the present study. The lack of an Fsh secretagogue, such as gonadotropin-releasing hormone (GnRH), could also be a reason for the higher Fsh protein level in pituitaries from fish in the OD than in the EV stage. GnRH is well-known secretagogue of Fsh and Lh and it stimulates the expression of the glycoprotein hormone alpha subunit, and that of *fshb*, and *lhb* genes in fish (Yaron et al., 2003).

Interestingly, *fshr* mRNA levels increased with puberty, increasing significantly at the EV stage. The dramatic increase in the transcript abundance of ovarian *fshr* between the OD and EV stage is consistent with a previous study on New Zealand shortfinned eels (Setiawan et al. 2012). Indirect, androgen-mediated increases in *fshr* mRNA levels seem to be the likely mechanism for the increase in *fshr* mRNA levels, as previously suggested by Setiawan et al. (2012). Increased sensitivity to the Fsh signal therefore, is possibly the driver for rapid changes to the reproductive physiology of the eel, reflected by significant increases in plasma E2 levels and yolk appearance in EV stage occurring subsequently.

Unlike *fshb* gene expression, the *lhb* mRNA level increased during gonadal development. *Lhb* mRNA profiles in the present study are also in agreement with results from wild Japanese eels in which *lhb* mRNA levels increased from pre-silver to silver stages (Han et al. 2003), equivalent to the PN and EV stages scored for *A. australis*. The natural dynamics in *lhb* mRNA levels are still unknown, as advanced stages of oogenesis in wild eels have not been observed; however, in samples from female longfins (*Anguilla dieffenbachii*; GSI 5–10%), stained with a traditional Herlant tetrachrome, two tinctorially different populations of PAS-positive cells were identifiable in the proximal pars distalis, whereas in shortfins (GSI 2–4%), only one such population was seen (PM Lokman & G Young; unpublished observations). These observations were reinforced by Ikeuchi et al. (1999), who demonstrated the presence of Lhβ^{+} cells in the pituitary from a silver longfin eel (GSI = 7.6%) using specific antisera. Together, these findings suggest that Lh may well become involved in regulation of oogenesis from midvitellogenesis onwards. Traditionally, increases in Lh content have

been associated with the *peri*-ovulatory period, but there are indications that in some fish, such as tilapia (Aizen et al. 2007), plasma Lh may increase well before ovarian follicles are fully grown.

In teleosts, the many of the principal actions of gonadotropins are mediated by downstream-produced steroids (Nagahama et al. 1995). In the present study, plasma 11-KT and E2 significantly increased from the OD to EV stage. These results are consistent with findings from previous studies on New Zealand shortfinned eels. Lokman et al. (1998) reported plasma levels of both 11-KT and E2 in migrant eels to be significantly higher than those of non-migrants. There were no significant changes in the expression of *cyp19* and *cyp11b* genes during ovarian development in wild *A. australis*, despite the remarkable increase in the levels of E2 and 11-KT. The mismatch between steroidogenic enzyme gene expression and sex steroid levels was also reported and discussed at length in previous studies on New Zealand shortfinned eels (Setiawan et al. 2012), Japanese eels (Matsubara, 2003; Ijiri et al., 2003; Sudo et al., 2011) and rainbow trout (Kusakabe et al. 2002).

In conclusion, this study is the first to measure pituitary *fshb* mRNA levels and pituitary Fsh protein levels in the *same* sample. A significant correlation was detected between these two variables. The pituitary *fshb* and *lhb* mRNA levels showed a differential expression pattern. Pituitary *fshb* mRNA and Fsh protein were detectable at the OD stage, and hence, Fsh may have been accumulating in eels at the OD pre-pubertal stage. However, the release of Fsh in this stage, which could not be confirmed due to the lack of assays that can measure plasma Fsh levels, seems unlikely, as steroid levels remained low. Despite the significant increase of *lhb* and *lhr* mRNA levels in the EV stage, an involvement of Lhr in the early stages of vitellogenesis is unlikely, given the anecdotal absence of pituitary Lh protein at that developmental stage.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.001.

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