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Expressional regulation of gonadotropin receptor genes and androgen receptor genes in the eel testis

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ABSTRACT

Receptors for follicle-stimulating hormone (Fshr), luteinizing hormone (Lhcgr1 and Lhcgr2) and androgens (Ara and Arb) transduce the hormonal signals that coordinate spermatogenesis, but the factors that regulate the abundance of these transducers in fish testes remain little-understood. To mend this paucity of information, we first determined changes in transcript abundance for these receptors (*fshr, lhcgr1, ara* and *arb*) during spermatogenesis induced by human chorionic gonadotropin (hCG) injection in the eel, *Anguilla australis*. We related our findings to testicular production of the fish androgen, 11-ketotestosterone (11-KT), and to the levels of the transcripts encoding steroidogenic acute regulatory protein (*star*) and 11β-hydroxylase (*cyp11b*), and subsequently evaluated the effects of hCG or 11-KT on mRNA levels of these target genes *in vitro*. Testicular 11-KT production was greatly increased by hCG treatment, both *in vivo* and *in vitro*, and associated with up-regulation of *star* and *cyp11b* transcripts. *In situ* hybridization indicated that testicular *fshr* mRNA levels were higher in the early stages of hCG-induced spermatogenesis, while *lhcgr1* transcripts were most abundant later, once spermatids were observed. *In vitro* experiments further showed that hCG and its steroidal mediator 11-KT significantly increased *fshr* transcript abundance. These data provide new angles on the interactions between gonadotropin and androgen signaling during early spermatogenesis. Increases in levels of 11-KT following hCG injection elevated testicular *fshr* mRNA levels augmenting Fsh sensitivity in the testis. This evidence is suggestive of a positive feedback loop between gonadotropins and 11-KT that may be key to regulating early spermatogenesis in fish.

1. Introduction

Spermatogenesis is the process during which immature diploid spermatogonia develop into mature, fertile haploid spermatozoa. Although it is recognized that a wide range of factors are involved in regulating this event in vertebrates, it is evident that the reproductive axis, or brain-pituitary-gonad (BPG) axis, is its primary driver. As in other vertebrates, pituitary gonadotropins, i.e., follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), control fish spermatogenesis; this action is initiated by the binding of gonadotropin to specific G protein-coupled receptors (Fshr and Lhcgr; previously Lhr), respectively) on the cell membrane of testicular target cells. Fshr are usually located on Sertoli and Leydig cells whilst Lhcgr are more commonly positioned on the Leydig and germ cells (for details, see: Levavi-Sivan et al., 2010; Schulz et al., 2010). Whilst both Fsh and Lh are steroidogenic in fish, the Fshr seems to be promiscuous in most fish species (see: Levavi-Sivan et al., 2010). Transduction of the gonadotropin signal stimulates the production of 11-ketotestosterone (11-KT), a major fish androgen (Miura et al., 1991a) that affects target cell function through activation of nuclear androgen receptors (Ar). Fish have two nuclear Ars (Sperry and Thomas, 1999); Ara is usually associated with germ cells and Arb is generally situated in Sertoli cells (Chauvigné et al., 2014a,b). The resulting receptor-ligand complex can now act as a transcription factor, amongst others regulating spermatogonial proliferation (Ikeuchi et al., 2001; Ogino et al., 2009; Schulz et al., 2010). Gonadotropin and androgen receptors, thus, are key to conveying the hormonal signals released by the BPG axis. Yet, very little is known about the factors that regulate Fshr, Lhcgr and Ar abundance in the fish testes, and hence, about the factors that affect sensitivity to hormonal signals that are pivotal for reproductive development.

A substantial proportion of what has become known about

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regulation of spermatogenesis in fish was discovered using the Japanese eel, *Anguilla japonica*, as a model. Under aquaculture conditions, these fish have immature testes containing only non-proliferating spermatogonia, reflected in the presence of type A (single germ cells surrounded by Sertoli cells) and early type B spermatogonia (several germ cells in small clonal cysts; Miura et al., 1991a). A single injection of human chorionic gonadotropin (hCG) is sufficient to induce complete spermatogenesis from spermatogonial proliferation through to spermiogenesis (Miura et al., 1991a). Treatment of freshwater eels with hCG has been shown to activate only Lhcgr and not Fshr (Kazeto et al., 2012; Minegishi et al., 2012). However, recent research has identified two Lhcgr in eels, Lhcgr1 and Lhcgr2 (Maugars and Dufour, 2015). To date, eel spermatogenesis studies have focused largely on Lhcgr1 and until Lhcgr2 has been fully characterised its involvement in spermatogenesis remains unclear. Regardless, hCG treatment has proven to be an effective experimental tool to study the endogenous factors that orchestrate spermatogenesis (Miura and Miura, 2001).

Further attesting to the value of the eel as an excellent animal model for the study of spermatogenesis has been the establishment of a testicular organ culture system. Indeed, using immature testis, spermatogenesis can be successfully induced by gonadotropins or 11-KT treatment *in vitro* (Miura et al., 1991b,c; Miura and Miura, 2001; Ohta et al., 2007). The organ culture system enables direct effects of a multitude of factors on the testes to be readily examined, and its adoption has contributed significantly to the state of the art in the field of fish reproductive physiology (Schulz et al., 2010).

Yet, although the administration of even just a single injection of hCG is known to be very effective at inducing maturation in male eels (Khan et al., 1987; Miura et al., 1991a; Ohta and Tanaka, 1997) and indeed other fish species (Mylonas et al., 2010; Cejko et al., 2016; Mylonas et al., 2017), the underlying molecular sequence of events is only partly known. To provide new insights, we first determined how the transcript abundances of *fshr*, *lhcgr1*, *ara* and *arb* changed in the testes following a single *in vivo* hCG injection in early pubertal, silver (migrating) shortfinned eels, *Anguilla australis*. Testes in wild migrating shortfins are in the early spermatogonial proliferation phase (Type A and early Type B spermatogonia, see Lokman and Young, 1998), largely comparable to testes from cultured Japanese eels, and development is arrested at this stage upon capture and transfer of fish to captivity. Transcript abundances of *ars* and gonadotropin receptors were subsequently correlated to 11-KT production and expression of two steroidogenic proteins (Star, steroidogenic acute regulatory protein; Cyp11b, cytochrome P450 11β-hydroxylase) in the testis. We complemented these *in vivo* studies by a series of *in vitro* experiments to further tease out the effects of hCG and its steroidal mediator 11-KT on mRNA levels of these receptors.

2. Materials and methods

2.1. Animals

Male silver shortfinned eels were captured from Lake Ellesmere, New Zealand, in two consecutive autumns using fyke nets (c.f., Lokman et al., 1998) and used for *in vivo* and *in vitro* experiments. In keeping with the natural fast that occurs in silver eels, fish were starved for the duration of these experiments. Animal handling and manipulations were approved and conducted in accordance with the guidelines of the University of Otago Animal Ethics Committee.

2.2. Experiments and design

2.2.1. Experiment I – changes in levels of androgen and testicular target gene transcripts during hCG-induced spermatogenesis

Experiment I.1 Male silver eels (n = 65) were acclimated for one month to artificial seawater (30 ppt) in recirculating tanks at 18–20 °C. After acclimation, eels (120–220 g in body weight) received a single intramuscular injection of either phosphate-buffered saline (PBS; control group, n = 30) or of hCG (1000 IU/fish) dissolved in PBS (n = 30). Five eels were euthanized at the start of the experiment (Day 0) in 0.03% benzocaine and a further 5 eels from each group at 5, 10, 15, 20, 25 or 30 days after injection. Blood samples were collected and serum 11-KT levels measured by radioimmunoassay (RIA: Section 2.3.2.). Testes were retrieved from the eels and used for qPCR (Section 2.3.3.), *in situ* hybridization (Section 2.3.4.) and histological examination (Section 2.3.1).

*Experiment I.2*Eight wild male silver eels (180–230 g in body weight) were captured and sampled within 15 h ("wild eels") to provide baseline data for reference. Wild eels were euthanized in 0.03% benzocaine and blood was collected for the measurement of serum 11-KT levels (Section 2.3.2.), whereas testes were dissected for use in qPCR (Section 2.3.3.) and measurement of tissue 11-KT levels (Section 2.3.2.).

A second batch (n = 36) of male silver eels was used to repeat Experiment I.1 in order to ensure that findings were consistent between years. Eels (150–220 g body weight) were acclimated for two weeks to filtered natural seawater in recirculating tanks at 16–18 °C and treated with hCG or PBS as described for Experiment I.1. At 0, 5, 10, 20 or 30 days after injection, blood and testes were collected from 4 eels from each group, using the same procedure as outlined for wild eels.

2.2.2. Experiment II – steroidogenic enzyme activity in testis of wild silver eels in vitro

Testes were dissected from six wild-caught silver eels within 24 h of capture (c.f., Lokman et al., 1998) and 2–3 mm fragments were incubated in 1 ml eel Ringer (150 mM NaCl, 5 mM $CaCl_2$, 3.5 mM $MgCl_2$, 3 mM KCl, 10 mM HEPES, pH 7.4; Miura et al., 1991a) with or without pregnenolone (P5, 10 ng/ml) as substrate and/or additional supplementation with hCG (1 IU/ml). After incubation for 24 h at 20 °C, concentrations of 11-KT in the Ringer were measured by RIA (Section 2.3.2.).

2.2.3. Experiment III – effects of hCG and 11-KT on testicular target gene transcript levels in vitro

To accommodate for the longer incubation time required to demonstrate changes in baseline gene expression (versus changes in enzyme activity measured in Experiment II), further testicular organ culture was performed as described previously (Miura et al., 1991c, 2002a). Briefly, testicular fragments were placed on a nitrocellulose membrane supported by a cylinder of 500 µl of 1% (w/v) agarose placed in a 1 ml well that further contained 500 µl medium. The basal medium was Leibovitz L-15 supplemented with 0.5% (w/v) BSA, 0.1 mM glutamic acid, 0.1 mM aspartic acid, 1.7 mM proline, 1 mg/litre bovine insulin, 100 mg/litre streptomycin, 100,000 U/litre penicillin, and 10 mM HEPES adjusted to pH 7.4 (Miura et al., 1991c). Two *in vitro* experiments were subsequently run.

*Experiment III.1*To examine the effects of 11-KT on target gene transcript abundance, testicular fragments dissected from three eels (80–160 g in body weight) that were held captive for 8 months prior to use were cultured for 5 days at 20 °C in the presence of 11-KT (0, 1, 10, 100 ng/well) dissolved in ethanol. The cultured testicular fragments were processed for qPCR analysis (Section 2.3.3.).

Experiment III.2 To extend the results from Experiment III.1, testicular fragments from a second cohort of three eels (120–250 g in body weight; sampled 4 months after capture) were cultured for 5 days at 20 °C with 11-KT (1, 10, 100 ng/well), or hCG (0.1, 1 IU/well). Vehicle-only (ethanol for 11-KT, eel Ringer for hCG) was used as the zero-dose control. Cultured testicular fragments were used for qPCR (Section 2.3.3.) and the media were collected to measure 11-KT concentrations (Section 2.3.2.).

2.3. Analyses

2.3.1. Histological and immunohistochemical observations

Following preservation in Bouin's solution, testicular samples collected in Experiment I.1 were dehydrated through a series of ethanols and embedded in paraffin. They were subsequently sectioned at 5 μm, stained with Gill's hematoxylin and eosin and staged on the basis of morphology and cell numbers in each cyst, as described previously (Miura et al., 1991a; Ozaki et al., 2006). Sections were also stained immunohistochemically with an antibody against Japanese eel proliferating cell nuclear antigen (anti-Pcna) as a proxy for mitosis in proliferating spermatogonia (Miura et al., 2002b; Anand et al., 2016; Sarkar and Singh, 2018). An antibody against rabbit IgG conjugated to alkaline phosphatase (Vector Laboratories) was used as secondary antibody and the sections were stained with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) as substrates.

2.3.2. Radioimmunoassay

Tissue samples were homogenized in PBS (100 mg tissue/mL PBS). Steroids were extracted three times from serum, tissue homogenates or *in vitro* culture media in 3 × 1 ml diethyl ether. The eel Ringer from Experiment II was used directly, without steroid extraction. Levels of 11-KT in serum, tissue or incubation medium were determined by RIA, as described and validated in Lokman et al. (1998). Steroid recovery after extraction in diethyl ether was greater than 92.6%, the minimum level of detection was 0.08 ng/ml and estimates of variability were 7.6% (intra-assay coefficient of variation; n = 2) and 8.2% (inter-assay coefficient of variation; n = 2).

2.3.3. Quantitative real-time PCR (qPCR)

Total RNA was extracted from testicular tissue using TRIzol Reagent (Invitrogen). After DNase treatment (TURBO DNA-free Kit, Ambion), oligo-dT primed cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche). All samples were run in duplicate on a Mx3000P thermal cycler (Stratagene) as described in Setiawan and Lokman (2010) (See Table 1 for specific primer sequences). Where possible eel elongation factor 1 (*eef1*) was used as an internal reference gene (see: Setiawan and Lokman, 2010).

2.3.4. In situ hybridization

Complementary DNA fragments encoding shortfinned eel *ara*, *arb*, *fshr* and *lhcgr1* were amplified by RT-PCR (for primer sequences see: Table 2), subcloned into pGEM T-Easy vector (Promega Corp.) and confirmation sequenced. Purified cDNA fragments were used to synthesize digoxigenin (DIG)-labelled sense and anti-sense cRNA probes *in vitro* with the DIG RNA labelling Mix (Roche) following the manufacturers' instructions.

Testes fixed in 4% PFA in PBS were equilibrated in a graded series of sucrose in PBS and embedded in Cryomatrix (Thermo Shandon). Cryosections were cut at 10 μm thickness, re-fixed in 2% PFA in PBS and treated with 1 μg/ml proteinase K (Roche) in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Following further exposure to 2% PFA in PBS, sections were processed as described previously (Braissant and Wahli, 1998; Kusakabe et al., 2002) with minor modifications. Hybridized probes were detected immunologically with an alkaline phosphatase-conjugated DIG-antibody (Roche) and visualized with NBT/BCIP.

Table 2
Forward (FW) and reverse (RV) primer sequences and amplicon size in base pairs (bp) used for cloning in order to make probes for *in situ* hybridisation targeting androgen receptor alpha (*ara*), androgen receptor beta (*arb*), follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone receptor one (*lhcgr1*).

Gene	Primer sequence (5′ – 3′)	Amplicon size (bp)	Accession number
ara	FW: AGCGGCTGCAATTCGGGACAA RV: TCCTTCCCTTGAGACAACACC	882	AB710174
arb	FW: AACAGTTGCTCCGAGTCAGCC RV: CGACTCCGATGCAGATCTATC	840	AB710175
fshr	FW: AATCATGGTGTCGGAGAACGG RV: CACGCTGATGATCCAGATGAG	814	AB605267
lhcgr1	FW: GAAATTTCCCAGAGTGACTCCATC RV: TCGGGTTCCGGGGTGCACTT	827	AB605268

2.4. Statistical analysis

Treatment means (control vs hCG) were compared using *t*-tests with SPSS 18.0 (SPSS Inc.). In some cases, we opted to carry out the *t*-tests without the assumption of equal variances, instead using adjusted degrees of freedom. Data from qPCR analysis were normalized over those of *eef1*. In addition, one-way analyses of variance and Tukey's multiple comparisons were performed to detect differences between variable means at different end points within treatment groups (treated or control). In a few cases, *eef1* levels were not stable across compared groups, in which cases statistical analyses were not performed. When assumption of homogeneity of variance were not fulfilled based on Levene's test, data were analysed using the corresponding non-parametric test (Kruskal-Wallis or Mann-Whitney). In these cases, non-parametric tests gave the same conclusions as parametric tests, thus the

Table 1
Forward (FW) and reverse (RV) primer sequences used for qPCR, annealing temperatures (Ta) in degrees Celsius (°C) and amplicon size in base pairs (bp) for each qPCR product. Abbreviations: androgen receptor alpha (*ara*), androgen receptor beta (*arb*), cytochrome p450 11β-hydroxylase (*cyp11b*), elongation factor one alpha (*eef1*), follicle-stimulating hormone receptor (*fshr*), luteinizing hormone receptor one (*lhcgr1*) and steroidogenic acute regulatory protein (*star*). Unreferenced primer pairs were designed as part of this study.

Gene	qPCR primers (5′ – 3′)	T Ta (°C)	Amplicon size (bp)	Reference
ara	FW: AGGAAGAACTGCCCCTCTTG RV: ATTTGCCCGATCTTCTTCAG	62 °C	90	Setiawan et al. (2012)
arb	FW: GCTTGGAGCTCGAAAATTGA RV: TTGGAGAGATGCACTGGTG	62 °C	98	Setiawan et al. (2012)
cyp11b	FW: ATCACTGTCCAGCGATACC RV: CGCGTCGGCTTAAATATCTC	62 °C	132	Setiawan et al. (2012)
eef1	FW: CCCCTGCAGGATGTCTACAA RV: AGGGACTCATGGTGCATTTTC	64 °C	152	Setiawan and Lokman (2010)
fshr	FW: CCTGGTCGAGATAACAATCACC RV: CCTGAAGGTCAAACAGAAAGTCC	63 °C	173	Zadmajid et al. (2015)
lhcgr1	FW: GTACAGCGCTACGCATTCAAC RV: CGTAGAAGACACATCGAGCAGAC	62 °C	132	
star	FW: CAGTACCGAAGACCCCGATA RV: TGCCATCCTCTGTCTCAGGT	62 °C	142	

results presented are based on the parametric tests. As there were no suitable non-parametric multiple comparison tests, results from Tukey's posthoc comparisons are presented in the figures. Differences between treatment means are considered significant for all $p < 0.05$.

3. Results

3.1. Histological observations of testis from hCG-injected eels

In two replicate *in vivo* experiments (experiments I.1 and I.2), testicular development progressed comparably, developmental stage being highly dependent on time lapsed after hCG injection. Only single germ cells surrounded by Sertoli cells and small cysts containing few spermatogonia were observed in the testis of wild eels and time-zero controls ('initial controls') (Fig. 1A), and only a small number of germ cells displayed some immunoreactivity to anti-Pcna (Fig. 1B). By 5 and 10 days after hCG injection, seminiferous tubules had started to expand and both the number of cysts and the number of spermatogonia in these cysts had increased (Fig. 1C). Most germ cells were strongly and synchronously immunostained for Pcna (Fig. 1D). By 20 days, larger cysts containing many germ cells (Fig. 1E) that did not immunoreact with anti-Pcna had appeared; we identified these germ cells as putative

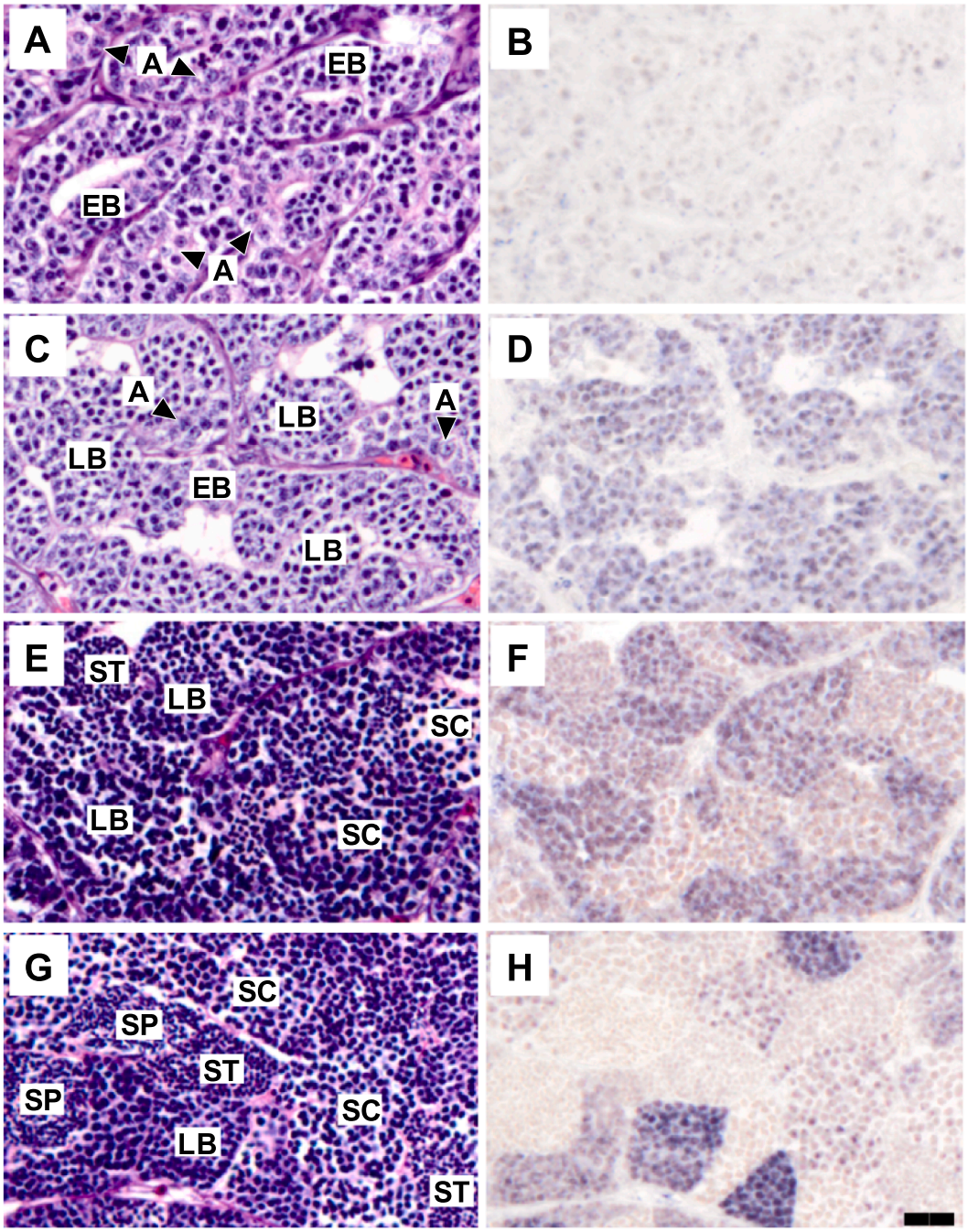

Fig. 1. Pairs of sequential sections from shortfinned eel (*Anguilla australis*) testes from Experiment I.1 stained with hematoxylin and eosin (A, C, E, G) or immunostained with anti-Pcna (proliferating cell nuclear antigen) (B, D, F, H). A and B, initial testis before human chorionic gonadotropin (hCG) injection. C and D, testis at 10 days after hCG injection. E and F, testis at 20 days after hCG injection. G and H, testis at 30 days after hCG injection. Abbreviations: A, type A spermatogonia; EB, early type B spermatogonia; LB, late type B spermatogonia; SC, spermatocytes; ST, spermatids; SP, sperm. Scale bar: 20 μm.

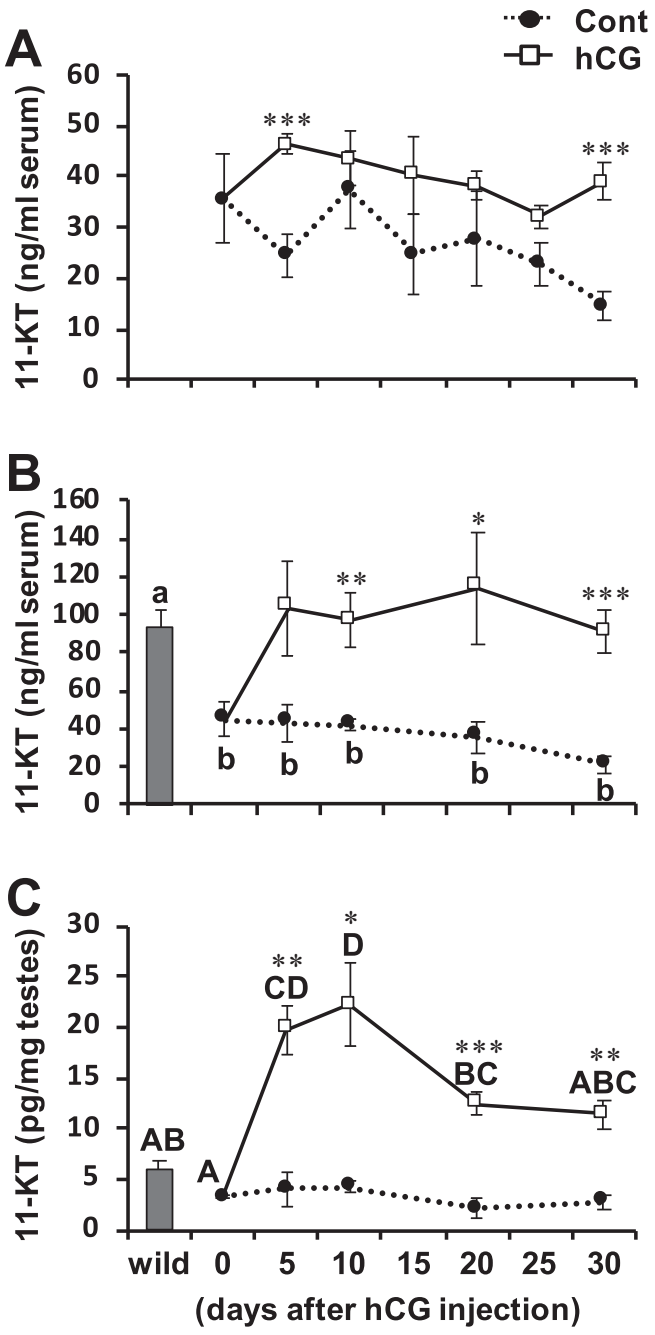

Fig. 2. Changes in 11-ketotestosterone (11-KT) levels in serum or testis homogenates in wild shortfinned male eels (*Anguilla australis*), sampled within one day of capture ("wild", n = 8), and in eels induced to undergo spermatogenesis artificially *in vivo* during Experiment I.1 (A: serum), and Experiment I.2 (B: serum; C: testis). Cont, control eels injected with PBS; hCG, eels injected with human chorionic gonadotropin (hCG) in PBS. Results are expressed as means ± SEM of five replicates (Experiment I.1) or four replicates (Experiment I.2). Means with different letters are significantly different between stages in each group; $p < 0.05$. Lower case letters indicate significant differences between control fish sampled at different time points. Upper case letters indicate differences between hCG treated fish sampled at different time points. Asterisks indicate values significantly different from control at each time point; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$.

spermatocytes (Fig. 1F). Spermatids, distinguishable by the smaller nuclei, and spermatozoa in the cyst lumen were first detected by Day 25, and the latter had greatly increased in number by Day 30 (Fig. 1G and H).

3.2. Changes in 11-KT levels during spermatogenesis

In shortfinned eels sampled immediately after capture from the wild, serum 11-KT levels averaged 94 ± 8.4 ng/ml (Fig. 2B). Levels decreased with time in captivity; average initial levels of 11-KT were 36 ± 8.7 ng/ml in Experiment I.1 (1 month of captivity prior to experimentation; Fig. 2A) and 45 ± 8.4 ng/ml in Experiment I.2 (2 weeks of captivity prior to experimentation; Fig. 2B). In both *in vivo* experiments, serum 11-KT levels increased after hCG injection and levels higher than those in the control group were maintained throughout the experimental period. In Experiment I.2, the 11-KT levels in hCG-injected eels were comparable to the levels seen in wild eels (Fig. 2B).

Concentrations of 11-KT within testicular tissue were remarkably increased after hCG injection ($F_{5,21} = 14.215$, $p < 0.001$) and much higher than in wild eels and the control group throughout the experimental period (Fig. 2C).

3.3. Steroidogenic enzyme activity in testis

Overnight incubation of testicular fragments from wild eels with hCG resulted in a large increase ($F_{3,20} = 32.035$, $p < 0.001$) in 11-KT production in both control and P5-supplemented incubates (*ca.* 16–18 fold that in controls, Fig. 3).

Similarly, following a five-day culture of testicular fragments in Experiment II, concentrations of 11-KT in the media were significantly, and dose-dependently, increased following addition of hCG compared to controls (data not shown).

3.4. Star and cyp11b transcripts during spermatogenesis

Star transcript abundance increased following hCG treatment (Experiment I.1: $F_{3,16} = 13.015$, $p < 0.001$; Experiment I.2: $F_{4,15} = 15.785$, $p < 0.001$), peaking at Day 5 after injection (Fig. 4A and B). Thereafter, *star* transcripts decreased in abundance and significant differences were no longer observed when comparing with the control group (Fig. 4A and B). *Cyp11b* transcript numbers were also increased significantly by hCG injection (Experiment I.1: $F_{3,16} = 19.936$, $p < 0.001$; Experiment I.2: $F_{4,15} = 22.734$, $p < 0.001$), showing an approximately-linear increase with time throughout the experimental period (Fig. 4C and D).

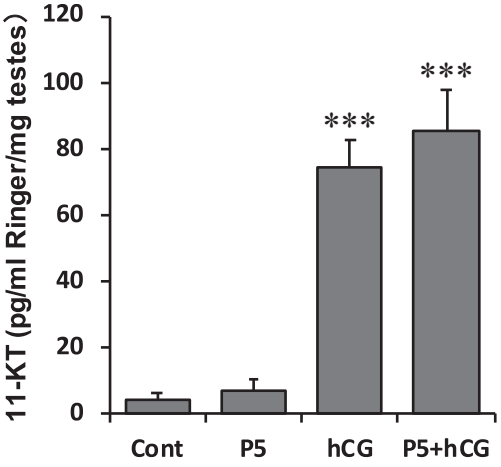

Fig. 3. 11-Ketotestosterone (11-KT) concentrations in incubation media. Cont, testicular fragments from shortfinned eel (*Anguilla australis*) incubated without pregnenolone (P5) or human chorionic gonadotropin (hCG); P5, testicular fragments incubated with P5 (10 ng/ml); hCG, testicular fragments incubated with hCG (1 IU/ml); P5 + hCG; testicular fragments incubated with P5 (10 ng/ml) and hCG (1 IU/ml). Results are expressed as means ± SEM of six replicates. Asterisks indicate values significantly different from control; *** = $p < 0.001$.

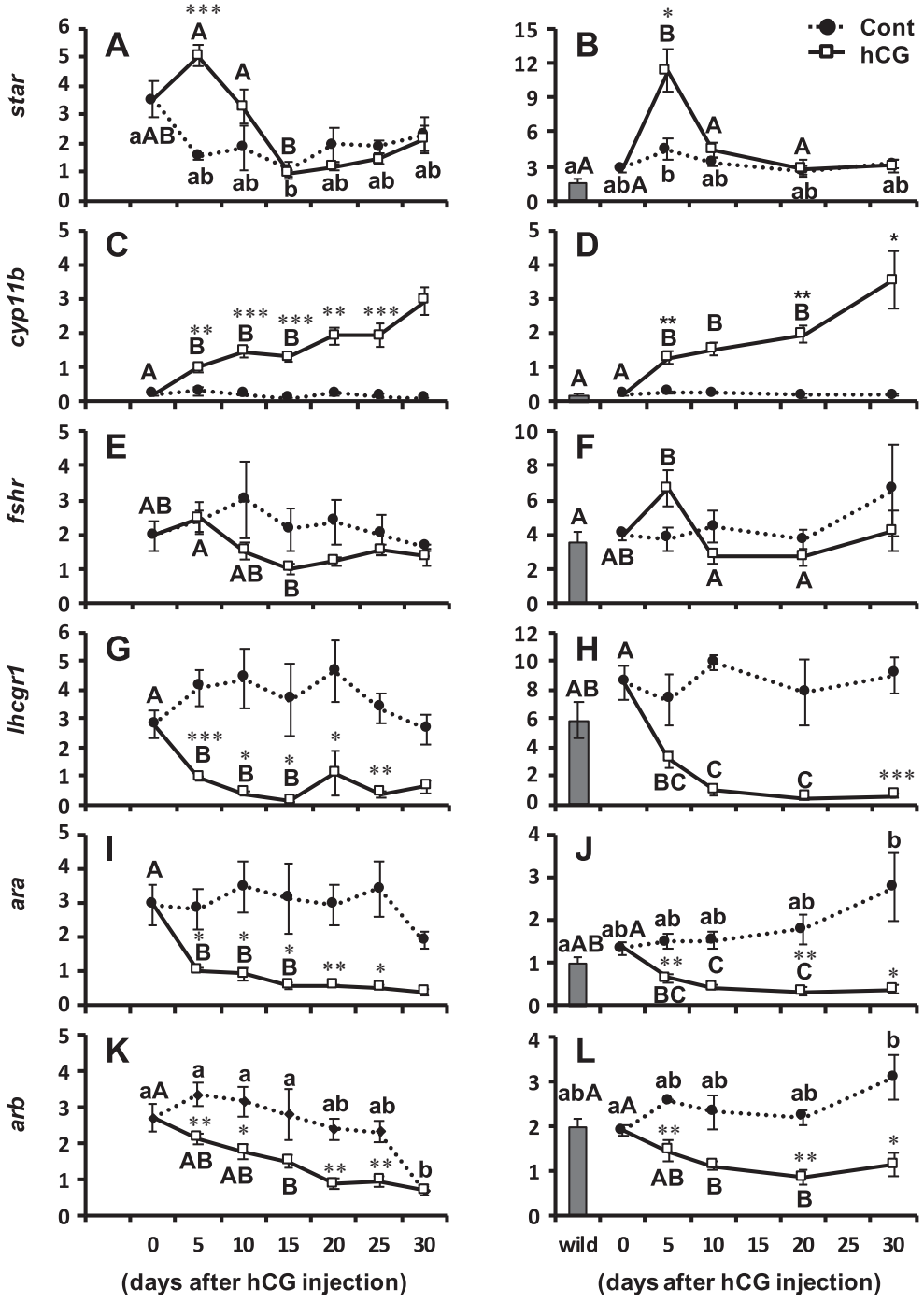

Fig. 4. Changes in relative transcript levels of *steroidogenic acute regulatory protein* (*star*) (A and B), *cytochrome P450 11* β *-hydroxylase* (*cyp11b*) (C and D), *follicle stimulating hormone receptor* (*fshr*) (E and F), *luteinizing hormone receptor one* (*lhcgr1*) (G and H), *androgen receptor alpha* (*ara*) (I and J) and *androgen receptor beta* (*arb*) (K and L) during spermatogenesis *in vivo* Experiment I.1 (A, C, E, G, I and K) and *in vivo* Experiment I.2 (B, D, F, H, J and L) in shortfinned eel (*Anguilla australis*) testis. Cont, control eels injected with PBS; hCG, eels injected with human chorionic gonadotropin in PBS. Results are expressed as means ± SEM of five replicates (Experiment I.1) or four replicates (Experiment I.2). Means with different letters are significantly different between stages in each group on the basis of Tukey's tests; $p < 0.05$. Lower case letters indicate significant differences between control fish sampled at different time points. Upper case letters indicate differences between hCG treated fish sampled at different time points. Asterisks indicate values significantly different from control at each time point on the basis of *t*-tests; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Not all groups were suitable for inclusion in the statistical analysis and therefore some groups do not have letters.

3.5. *fshr and lhcgr1 transcripts during spermatogenesis*

Results of *in situ* hybridization indicated that *fshr* mRNA was localized both inside and outside of seminiferous tubules (Fig. 5A–D). Its abundance within seminiferous tubules appeared higher in early stages of spermatogenesis (until Day 10) than in more mature stages, while in the interstitium, expression remained high throughout the experimental period (Fig. 5A–D). Significant differences in *fshr* transcript abundance were not observed in the testes between hCG-injected eels and control eels throughout the experimental period by qPCR analysis; however, the relative abundance of *fshr* transcripts in the testes of hCG-injected eels tended to decrease from 5 days post-hCG injection (Fig. 4E and F).

In situ hybridization analysis also revealed the presence of *lhcgr1* mRNA in both intra- and extra-tubular compartments of the testis of hCG-injected eels only at day 25 and 30 after hCG injection (Fig. 5E–H). Notwithstanding the "dilution" of signal by increasing numbers of germs cells with advancing spermatogenesis, these results contrast with those obtained from qPCR analysis; the latter approach saw transcript levels of *lhcgr1* in testes of hCG-injected eels decrease remarkably after hCG injection (Experiment I.1: $F_{316} = 29.983$, $p < 0.001$; Experiment I.2: $F_{4,15} = 16.880$, $p < 0.001$) and remained lower than those in the control group (Fig. 4G and H).

3.6. *ar transcripts during spermatogenesis*

Results of *in situ* hybridization showed that *ara* mRNA, which was localized both within and outside seminiferous tubules, was most abundant in the testes prior to, and decreased after, hCG injection

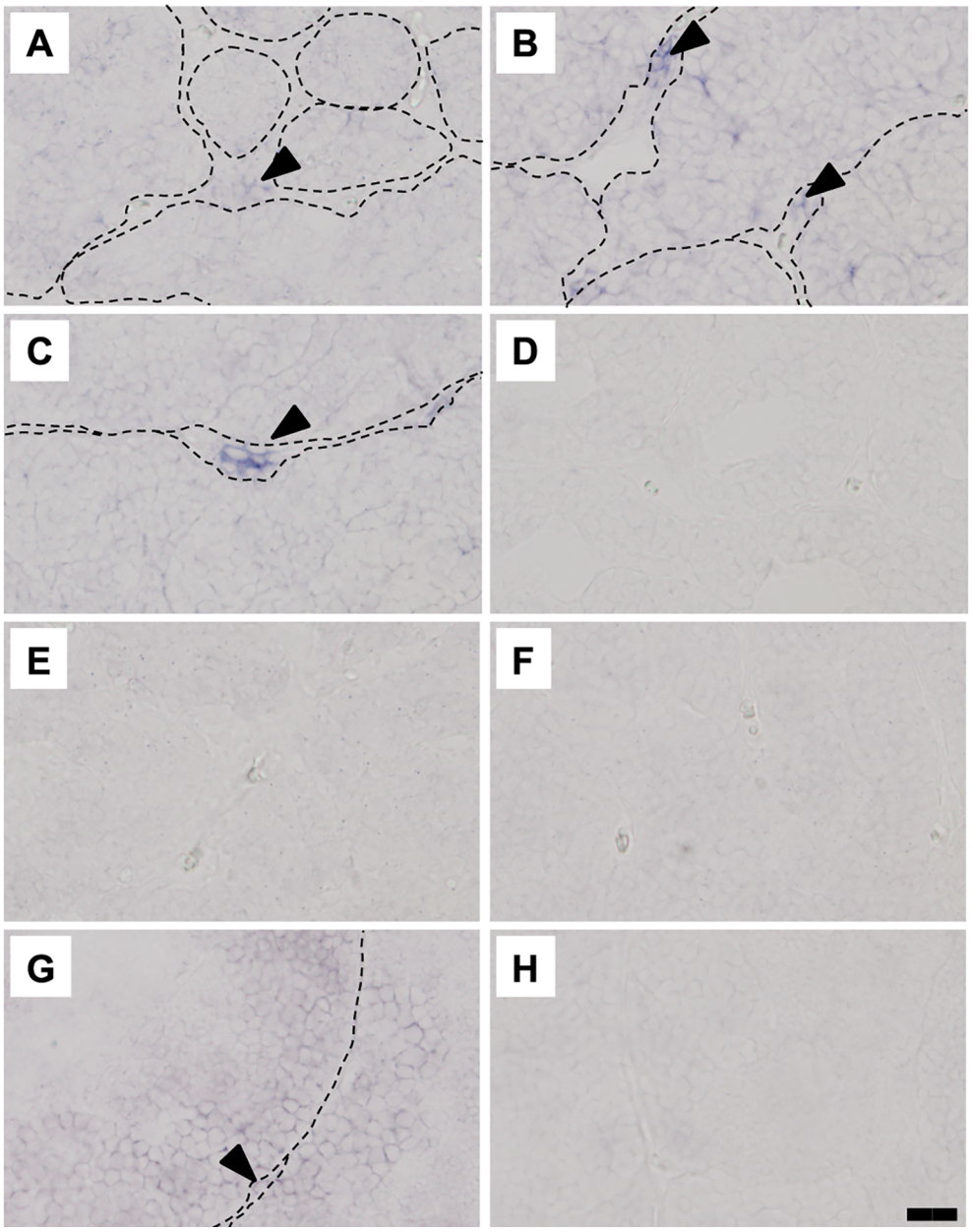

Fig. 5. Localization of *fshr* (A, B, C and D) and *lhcgr1* (E, F, G and H) mRNA in the testis by *in situ* hybridization. A and E, initial testis before hCG injection hybridized with anti-sense probe for *fshr* or *lhcgr1*. B and F, testis at day 10 after hCG injection hybridized with anti-sense probe for *fshr* or *lhcgr1*. C and G, testis at day 30 after hCG injection hybridized with anti-sense probe for *fshr* or *lhcgr1*. D testis at day 10 after hCG injection hybridized with sense probe for *fshr*. H, testis at day 30 after hCG injection hybridized with sense probe for *lhcgr1*. Dashed lines represent seminiferous tubule boundaries. Arrowheads indicate Leydig cells. Scale bar: 20 μm.

(Fig. 6), whereas *arb* mRNA remained undetectable in testes, regardless of developmental stage, by *in situ* hybridization (data not shown). Results of qPCR reinforced the *in situ* hybridization data, and showed that transcript levels of both *ara* and *arb* in testes of hCG-injected eels decreased after hCG injection (*ara* Experiment I.1: $F_{3,16} = 11.891$, $p < 0.001$; *ara* Experiment I.2: $F_{4,15} = 17.251$, $p < 0.001$) (*arb* Experiment I.1: $F_{3,16} = 4.614$, $p < 0.05$; *arb* Experiment I.2: $F_{4,15} = 8.418$, $p < 0.001$), tracking significantly lower than those of fish in the control group (Fig. 4I–L). At nearly all time points measured (with the exception of 30 days, Experiment I.1), transcript levels of both androgen receptors were reduced to less than half in hCG-injected eels when compared to control fish.

3.7. Effects of hCG and 11-KT on testicular target gene expression in vitro

Star transcript abundances were significantly increased in the testes cultured with hCG ($F_{2,6} = 11.387$, $p < 0.01$); 11-KT was without effect (Fig. 7A and B). *Cyp11b* transcript abundance similarly increased following exposure to hCG (Fig. 7C and D), but unlike *star*, 11-KT also induced increases in *cyp11b* mRNA levels ($F_{3,7} = 76.217$, $p < 0.001$) in a dose-dependent manner.

Fshr transcripts were increased by 11-KT treatment in a dose-dependent manner ($F_{3,7} = 24.276$, $p < 0.001$: Fig. 7F). The abundance of *fshr* transcripts in testicular fragments treated with hCG was also significantly higher than that in the control group (Fig. 7F). In contrast, *lhcgr1* transcripts were more abundant only in explants treated with

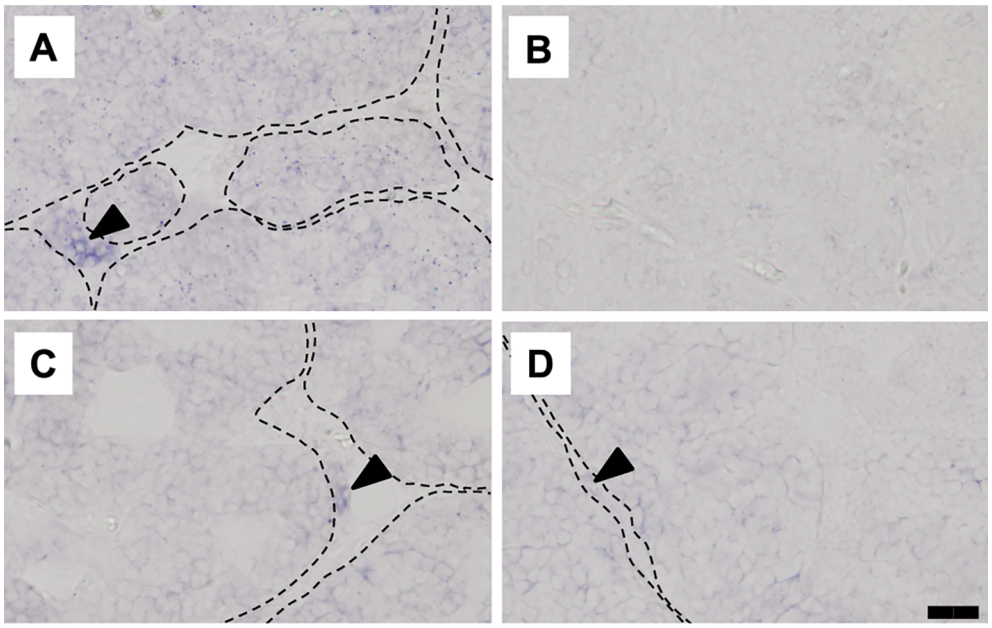

Fig. 6. Localization of *ara* mRNA in the testis by *in situ* hybridization. A, initial testis before hCG injection hybridized with anti-sense probe. B, initial testis before hCG injection hybridized with sense probe. C, testis at day 10 after hCG injection hybridized with anti-sense probe. D testis at day 30 after hCG injection hybridized with anti-sense probe. Dashed lines represent seminiferous tubule boundaries. Arrowheads indicate Leydig cells. Scale bar: 20 μm.

hCG at the lowest concentration (0.1 U/well; Fig. 5H). 11-KT and hCG at higher doses tended to decrease *lhcgr1* transcript mRNA levels (Fig. 7G and H).

Significant effects of 11-KT on *ara* and *arb* were not observed in *in vitro* Experiment III.1 (Fig. 7I and K). In *in vitro* Experiment III.2, transcript levels of *ars* in testes cultured with either 11-KT or hCG at the higher doses were lower than those of controls (Fig. 7J and L).

4. Discussion

A function for hormones, chiefly gonadotropins and androgens, in controlling spermatogenesis in fish is well-established, yet, surprisingly few reports have focused on the expressional regulation of genes encoding the receptors that mediate these hormonal signals. How do reproductive hormones and the expression of their receptor genes interact? Specifically, in eels, how do the interactions lead to the production of spermatozoa, even after just a single experimental administration of gonadotropin? And how does this scenario compare to what is likely to happen as eels come into spawning condition in the wild? We employed hCG to induce spermatogenesis in the shortfinned eel to address these questions.

Unlike Japanese eels, shortfinned males in the silver stage are easily captured from the wild - enabling us to generate baseline data from wild fish - and the testes are in a slightly more advanced, but still very early stage of spermatogenesis (Lokman and Young, 1998; this study). Transfer to captivity led to gametogenic arrest and this coincided with a decrease in 11-KT levels from around 90 ng/mL in eels sampled immediately after capture (c.f., Lokman and Young, 1998; Lokman et al., 2016), to 36 or 45 ng/mL in eels (Day 0) held captive for 4 (Experiment I.1) or 2 weeks (Experiment I.2). Thus, *despite* the notion that 11-KT was still circulating in the blood stream for up to 4 weeks post-capture, there was no evidence to suggest that spermatogenesis had progressed. These observations are in keeping with findings from our recent study in which experimental elevation of 11-KT to ~50 ng/mL barely seemed to affect the testes of captivity-acclimated shortfinned eels, whereas only limited development was evident after elevating 11-KT levels further to ~90 ng/mL (Lokman et al., 2016).

However, hCG injection successfully induced complete spermatogenesis, just as in European and Japanese eels (Khan et al., 1987; Miura et al., 1991a). Furthermore, in keeping with what has been reported for its Northern Hemisphere congeners, basal 11-KT production by testicular tissue from shortfinned eels *in vivo* and *in vitro* was enhanced by hCG stimulation. This response was more noticeable for fish in Experiment I.2 than for those in Experiment I.1, possibly reflecting higher *lhcgr1* transcript abundance in the former group after a shorter period spent in captivity at slightly lower temperatures (see: Peñaranda et al., 2016). Similarly, slight differences in responsiveness to 11-KT between *in vitro* experiments could reflect differences in time in captivity and temperature, possibly resulting in differences in developmental stage, prior to experimentation.

The initial increase in 11-KT production in response to hCG *in vivo* coincided with a notable increase in mRNA levels of *star*, a steroidogenic protein that facilitates cholesterol delivery across the mitochondrial membrane to Cyp11a (cytochrome P450 cholesterol side-chain cleavage enzyme) (Stocco, 2000). Our *in vivo* observations were reinforced by *in vitro* studies that saw *star* message increase in response to hCG, but not following exposure to 11-KT. The *star* gene in shortfinned eel testis thus appears to be under strong transcriptional control of a gonadotropic signal (at least an Lhcgr-mediated signal), similar to what was previously shown in testis from zebrafish, *Danio rerio* (García-Lópes et al., 2010), Senegalese sole, *Solea sengalensis* (Marin-Juez et al., 2011; Chauvigné et al., 2014a,b), rainbow trout, *Oncorhynchus mykiss* (Sambroni et al., 2013a,b) and Japanese sea bass, *Lateolabrax japonicas* (Chi et al., 2014).

The present study further revealed some valuable insights into the regulation of another steroidogenic protein, Cyp11b, in eel testes. Cyp11b is an enzyme that catalyzes the hydroxylation of testosterone to 11 β -hydroxytestosterone, a precursor of 11-KT (Kusakabe et al., 2002). *In vivo* increases in *cyp11b* mRNA levels during spermatogenesis, either during an annual cycle (e.g., salmonids: Kusakabe et al., 2006; Maugars and Schmitz, 2008 and sea bass: Rocha et al., 2009) or following hCG injection (i.e., *Anguilla* spp.: Jiang et al., 1996; present study) likely reflect the importance of Cyp11b for 11-KT production during early germ cell development. However, whilst changes in *cyp11b* mRNA levels during the sex determination period (e.g., *Danio rerio*, Wang and Orban, 2007; *Odontesthes bonariensis*, Blasco et al., 2010; *Lates calcarifer*, Banh et al., 2017), during a reproductive cycle (e.g., *Oncorhynchus mykiss*, Kusakabe et al., 2002; *Salmo salar*, Maugars and Schmitz, 2008; *Dicentrarchus labrax*, Rocha et al., 2009; Valero et al., 2015) or during natural or induced sex change of fish (e.g., *Amphiprion clarkii*, Miura et al., 2008; *Monopterus albus*, Liu et al., 2009b) have been reported, there appear to be no reports on the hormonal regulation of

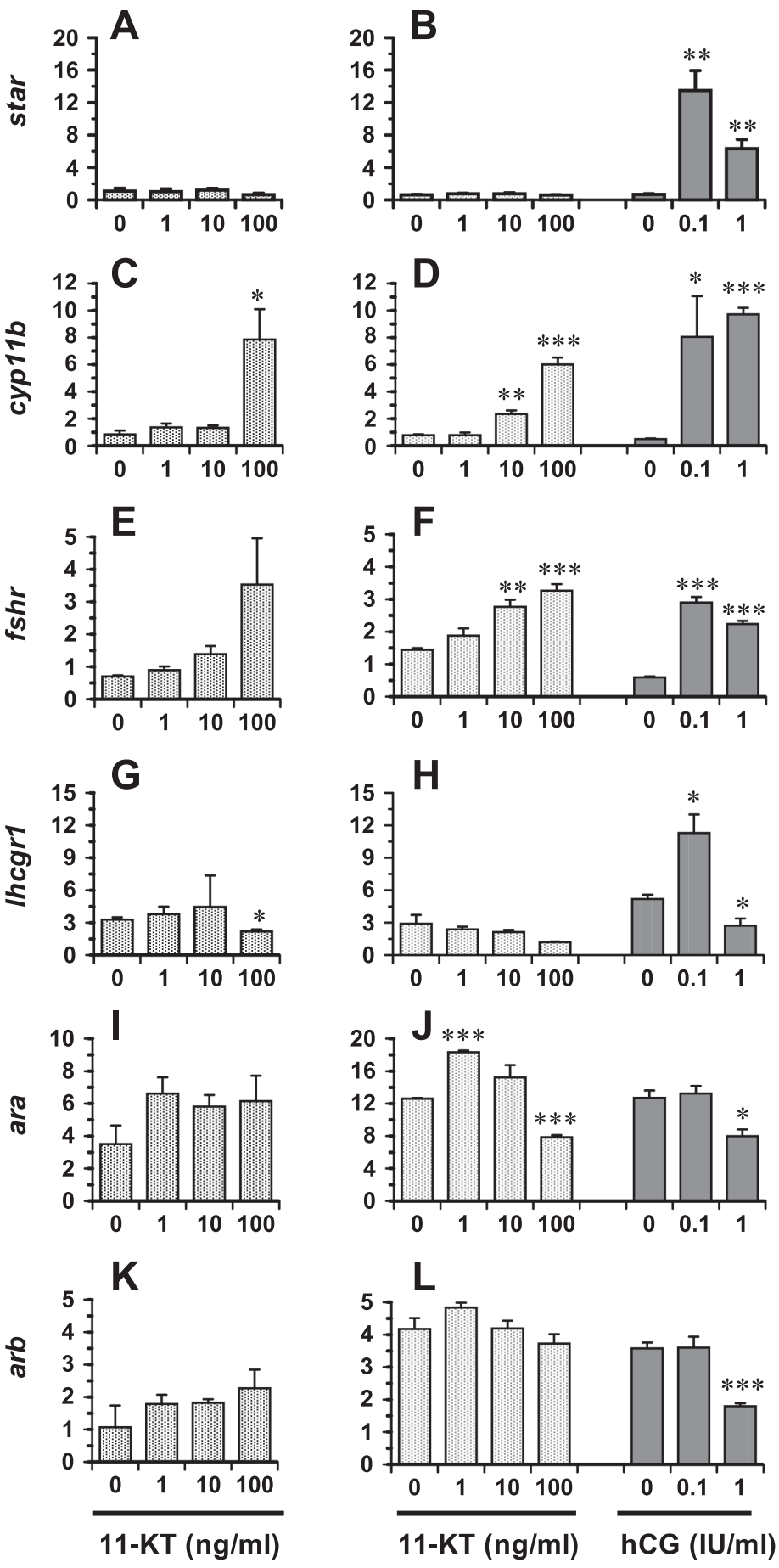

Fig. 7. Effects of 11-ketotestosterone (11-KT) and human chorionic gonadotropin (hCG) on relative transcript levels of *steroidogenic acute regulatory protein* (*star*) (A and B), *cytochrome P450 11* β *-hydroxylase* (*cyp11b*) (C and D), *follicle stimulating hormone receptor* (*fshr*) (E and F), *luteinizing hormone receptor one* (*lhcgr1*) (G and H), *androgen receptor alpha* (*ara*) (I and J) and *androgen receptor beta* (*arb*) (K and L) *in vitro* Experiment III.1 (A, C, E, G, I and K) and *in vitro* Experiment III.2 (B, D, F, H, J and L) in shortfinned eel (*Anguilla australis*) testis. Results are expressed as means ± SEM of triplicates. Asterisks indicate values significantly different (*t*-test) from each control without hormones; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$.

cyp11b gene expression in teleost testes. Our current findings from *in vitro* experiments showed unequivocally that hCG increased *cyp11b* transcript abundance in the testes. 11-KT similarly enhanced *cyp11b* expression in the testes, suggestive of a positive feedback loop that may drive the continuous increase in *cyp11b* mRNA levels observed throughout hCG-induced spermatogenesis *in vivo*.

In Japanese eel, Fshr was detected in both Sertoli and Leydig cells when employing immunolocalization studies (Ohta et al., 2007). These findings concur with our *in situ* hybridization localization data for *fshr* mRNA, which was detected both inside (putative Sertoli cells) and outside (putative Leydig cells) the seminiferous tubules. The localization of *fshr* mRNA to both Sertoli cells and Leydig cells has been reported (zebrafish: García-López et al., 2010; African catfish, *Clarias gariepinus*: García-López et al., 2009; Senegalese sole, *Solea senegalensis*: Chauvigné et al., 2012) or suspected (sea bass: Mazón et al., 2014) in several species of teleost fish, implying that aside from (probably) modulating Sertoli cell function (e.g., Sambroni et al., 2013a,b), Fsh may stimulate steroid production in Leydig cells. Indeed, it appears that a major, but not the only, function of Fsh in fish spermatogenesis is to increase androgen production in Leydig cells, through increasing steroidogenic protein gene expression.

Like *fshr*, *lhcgr1* mRNA appeared to be localized both inside and outside the seminiferous tubules. Expression of both the *fshr* and *lhcgr* in Leydig and Sertoli cells was recently reported in zebrafish (García-López et al., 2010), the first report of dual gonadotropin receptor expression in vertebrate Sertoli cells. A more recent study in zebrafish (Xie et al., 2017) produced fertile *lhb;lhr* and *fshb;fshr* mutant males, highlighting the promiscuity of Lh and Fsh signalling during spermatogenesis. Further support for the overlapping actions of gonadotropins comes from positively stained *lhcgr* mRNA in spermatids from several species of teleost displaying cystic germ cell development (Senegalese sole, zebrafish and seabream; Chauvigné et al., 2014a,b).

Both recombinant eel Fsh and Lh could induce 11-KT production and subsequent full spermatogenesis in Japanese eel (Kazeto et al., 2008). In contrast, recombinant Fsh but not recombinant Lh was shown to induce full spermatogenesis in European eel (Peñaranda et al., 2018). Our results indicated a greater expression of *fshr* than *lhcgr1* during early spermatogenesis. Furthermore, in the pituitary of wild male silver shortfinned eels, Fsh-producing cells are abundant (Y Ozaki, unpublished data). Collectively, these findings suggest that in wild eels, Fsh is the principal driver of testicular 11-KT production, resulting in the initiation of spermatogenesis in the eel. Testicular sensitivity to the Fsh signal subsequently increases, as reflected in increased *Fshr* gene expression following exposure to 11-KT *in vitro*, in accord with what was reported in female eels (Setiawan et al., 2012) – this further facilitates the early stages of spermatogenesis at least as long as Fsh levels remain elevated. Whether this is indeed the case is not easily answered, as getting access to wild eels in advanced stages of development has essentially remained impossible. Furthermore, relevant experimental studies are almost invariably performed on females because of their larger size, thus limiting the extent to which the findings from such studies can be extrapolated to anguillid males undergoing gonadal recrudescence. At present, observations on female silver European eels by Aroua and co-workers (2007) probably provide the best bench mark, steroid effects on gonadotropin β -subunit gene expression having been evaluated both *in vivo* and *in vitro*; accordingly, estrogens increased *lhb* expression *in vivo* (~8-fold) and *fshb* expression *in vitro* (~3-fold), whereas androgens (testosterone and 5α-dihydrotestosterone) increased *lhb* mRNA levels around 9-fold, but only *in vitro* (Aroua et al., 2007).

Interestingly, following the early increase in testicular 11-KT production which initiates spermatogonial proliferation, *ara* and *arb* transcript abundances dramatically decreased *in vivo*, as was also recently reported in European eel (Peñaranda et al., 2014). Higher expression of *ar* in the testis during early stages of spermatogenesis have similarly been observed in a cyprinid (*Spinibarbus denticulatus*: Liu et al., 2009a) and European sea bass (*D. labrax*: Viñas and Piferrer, 2008) supporting the notion that androgens are only needed during early spermatogenesis (Ohta et al., 2007; Peñaranda et al., 2016, 2018). In Senegalese sole, *in vitro* treatment with either recombinant Fsh or Lh increased expression of both *ars* in the early stages (stage I-II) of spermatogenesis but only Fsh had any effect (increased *arb* expression) in the later stages (stage III) (Chauvigné et al., 2014a,b). The mechanisms responsible for reduced *ar* mRNA levels remain to be elucidated, as neither 11-KT, nor hCG had clear effects on *ar* transcript abundance *in vitro*.

Our data have provided new insights into the mechanisms regulating fish spermatogenesis, particularly with regard to the expressional regulation of gonadotropin and androgen receptor genes and the genes encoding *star* and *cyp11b* by hCG or 11-KT; a positive feedback system appears to exist in which gonadotropin (endogenous Fsh or exogenous hCG) activates a Fsh-receptor which, in turn, drives increases of *star* and *cyp11b* expression. Resulting increases in 11-KT synthesis then augment the sensitivity to Fsh through up-regulation of *fshr* expression during early spermatogenesis, which is likely to be pivotal under natural conditions. *Ara* and *arb* mRNA levels decrease after the initiation of spermatogenesis, reinforcing the importance of androgen signaling for the initiation of spermatogenesis, but not necessarily its progression.

Declaration of interest

The authors declare that there is no conflict of interest that would impact on the impartiality of this work.

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References

Anand, S., Bhartiya, D., Sriraman, K., Mallick, A., 2016. Underlying mechanisms that restore spermatogenesis on transplanting healthy niche cells in busulphan treated mouse testis. Stem Cell Rev. 12, 682–697.

Aroua, S., Weltzien, F.-A., Le Belle, N., Dufour, S., 2007. Development of real-time RT-PCR assays for eel gonadotropins and their application to the comparison of *in vivo* and *in vitro* effects of sex steroids. Gen. Comp. Endocrinol. 153, 333–343.

Banh, Q.Q., Domingos, J.A., Zenger, K.R., Jerry, D.R., 2017. Morphological changes and regulation of the genes *dmrt1* and *cyp11b* during the sex differentiation of barramundi (*Lates calcarifer* Bloch). Aquaculture 479, 75–84.

Blasco, M., Fernandino, J.I., Guilgur, L.G., Strussman, C.A., Somoza, G.M., Vizziano-Cantonnet, D., 2010. Molecular characterization of cyp11a1 and cyp11b1 and their gene expression profile in pejerrey (*Odontesthes bonariensis*) during early gonadal development. Comp. Biochem. Physiol. 156A, 110–118.

Braissant, O., Wahli, W., 1998. A simplified *in situ* hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica 1, 10–16.

Cejko, B.I., Judycka, S., Juchno, D., Boroń, A., Leska, A., Jabłońska, O., Pecio, A., Kowalski, R.K., 2016. Hormonal treatment affects sperm motility in the spined loach (*Cobitis taenia*, Pisces, Cobitidae). Aquac. Res. 48, 5139–5145.

Chauvigné, F., Verdura, S., Mazón, M.J., Duncan, N., Zanuy, S., Gómez, A., Cerdà, J., 2012. Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells of an evolutionary advanced teleost. Biol. Reprod. 87, 1–11.

Chauvigné, F., Zapater, C., Gasol, J.M., Cerdà, J., 2014b. Germ-line activation of the luteinizing hormone receptor directly drives spermiogenesis in a nonmammalian

vertebrate. Proc. Natl. Acad. Sci. U.S.A 111, 1427–1432.
Chauvigné, F., Zapater, C., Crespo, D., Planas, J.V., Cerdà, J., 2014a. Fsh and Lh direct conserved and specific pathways during flatfish semicystic spermatogenesis. J. Mol. Endocrinol. 53, 175–190.
Chi, M.L., Wen, H.S., Ni, M., He, F., Li, J.F., Qian, K., Zhang, P., Chai, S.H., Ding, Y.X., Yin, H.X., 2014. Molecular identification of genes involved in testicular steroid synthesis and characterization of the responses to hormones stimulation in testis of Japanese sea bass (*Lateolabrax japonicas*). Steroids 84, 92–102.
García-Lópes, Á., Bogerd, J., Granneman, J.C.M., van Dijk, W., Trant, J.M., Taranger, G.L., Schulz, R.W., 2009. Leydig cells express follicle-stimulating hormone receptors in African catfish. Endocrinology 150, 357–365.
García-Lópes, Á., de Jonge, H., Nóbrega, R.H., de Waal, P.P., van Dijk, W., Hemrika, W., Taranger, G.L., Bogerd, J., Schulz, R.W., 2010. Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. Endocrinology 151, 2349–2360.
Ikeuchi, T., Todo, T., Kobayashi, T., Nagahama, Y., 2001. Two subtypes of androgen and progestogen receptors in fish testis. Comp. Biochem. Physiol. B 129, 449–455.
Jiang, J.Q., Kobayashi, T., Ge, W., Kobayashi, H., Tanaka, M., Okamoto, M., Nonaka, Y., Nagahama, Y., 1996. Fish testicular 11B-hydroxylase: cDNA cloning and mRNA expression during spermatogenesis. FEBS Lett. 397, 250–252.
Kazeto, Y., Kohara, M., Miura, T., Miura, C., Yamaguchi, S., Trant, J.M., Adachi, S., Yamauchi, K., 2008. Japanese eel follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): production of biologically active recombinant Fsh and Lh by *Drosophila* S2 cells and their differential actions on the reproductive biology. Biol. Reprod. 79, 938–946.
Kazeto, Y., Kohara, M., Tosaka, R., Gen, K., Yokoyama, M., Miura, C., Miura, T., Adachi, S., Yamauchi, K., 2012. Molecular characterization and gene expression of Japanese eel (*Anguilla japonica*) gonadotropin receptors. Zool. Sci. 29, 204–211.
Khan, I.A., Lopez, E., Leloup-Hâtey, J., 1987. Induction of spermatogenesis and spermiation by a single injection of human chorionic gonadotropin in intact and hypophysectomized immature European eel (*Anguilla anguilla* L.). Gen. Comp. Endocrinol. 68, 91–103.
Kusakabe, M., Kobayashi, T., Todo, T., Lokman, P.M., Nagahama, Y., Young, G., 2002. Molecular cloning and expression during spermatogenesis of a cDNA encoding testicular 11beta-hydroxylase (P45011beta) in rainbow trout (*Oncorhynchus mykiss*). Mol. Reprod. Dev. 62, 456–469.
Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., Young, G., 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. J. Endocrinol. 189, 541–554.
Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and receptors. Gen. Comp. Endocrinol. 165, 412–437.
Liu, J.-F., Guiguen, Y., Liu, S.-H., 2009b. Aromatase (P450arom) and 11 beta-hydroxylase (P45011 beta) genes are differentially expressed during the sex change process of the protogynous rice field eel *Monopterus albus*. Fish Physiol. Biochem. 35, 511–518.
Liu, X., Su, H., Zhu, P., Zhang, Y., Huang, J., Lin, H., 2009a. Molecular cloning, characterization and expression pattern of androgen receptor in *Spinibarbus denticulatus*. Gen. Comp. Endocrinol. 160, 93–101.
Lokman, P.M., Young, G., 1998. Gonad histology and plasma steroid profiles in wild New Zealand freshwater eels (*Anguilla dieffenbachii* and *A. australis*) before and at the onset of the natural spawning migration II. Males. Fish Physiol. Biochem. 19, 339–347.
Lokman, P.M., Vermeulen, G.J., Lambert, J.G.D., Young, G., 1998. Gonad histology and plasma steroid profiles in wild New Zealand freshwater eels (*Anguilla dieffenbachii* and *A. australis*) before and at the onset of the natural spawning migration I. Females. Fish Physiol. Biochem. 19, 325–338.
Lokman, P.M., Damsteegt, E.L., Wallace, J., Downes, M., Goodwin, S.L., Facoory, L.J., Wylie, M.J., 2016. Dose-responses of male silver eels, *Anguilla australis*, to human chorionic gonadotropin and 11-ketotestosterone *in vivo*. Aquaculture 463, 97–105.
Marin-Juez, R., Castellana, B., Manchado, M., Planas, J.V., 2011. Molecular identification of genes involved in testicular steroid synthesis and characterization of the response to gonadotropic stimulation in the Senegalese sole (*Solea senegalensis*) testis. Gen. Comp. Endocrinol. 172, 130–139.
Maugars, G., Dufour, S., 2015. Demonstration of the coexistence of duplicated LH receptors in teleosts, and their origin in ancestral Actinopterygians. PLOS One 10, e0135184.
Maugars, G., Schmitz, M., 2008. Gene expression profiling during spermatogenesis in early maturating male Atlantic salmon parr testes. Gen. Comp. Endocrinol. 159, 178–187.
Mazón, M.J., Gómez, A., Yilmaz, O., Carrillo, M., Zanuy, S., 2014. Administration of follicle-stimulating hormone *in vivo* triggers testicular recrudescence of juvenile European sea bass (*Dicentrarchus labrax*). Biol. Reprod. 90, 6.
Minegishi, Y., Dirks, R.P., de Wijze, D.L., Brittijn, S.A., Burgerhout, E., Spaink, H.P., van den Thillart, G.E.E.J.M., 2012. Quantitative bioassays for measuring biologically functional gonadotropins based on eel gonadotropic receptors. Gen. Comp. Endocrinol. 178, 145–152.
Miura, S., Horiguchi, R., Nakamura, M., 2008. Immunohistochemical evidence for 11β-hydroxylase (P45011β) and androgen production in the gonad during sex differentiation and in adults in the protandrous anemonefish *Amphiprion clarkii*. Zool. Sci. 25, 212–219.
Miura, T., Miura, C., 2001. Japanese eel: a model for analysis of spermatogenesis. Zool. Sci. 18, 1055–1063.
Miura, C., Miura, T., Yamashita, M., 2002b. PCNA protein expression during spermatogenesis of the Japanese eel (*Anguilla japonica*). Zool. Sci. 19, 87–91.
Miura, T., Yamauchi, K., Nagahama, Y., Takahashi, H., 1991a. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool. Sci. 8, 63–73.
Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991b. Human chorionic gonadotropin induces all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Dev. Biol. 146, 258–262.
Miura, T., Yamauchi, K., Takahashi, H., Nagahama, Y., 1991c. Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Proc. Natl. Acad. Sci. U.S.A 88, 5774–5778.
Miura, T., Miura, C., Konda, Y., Yamauchi, K., 2002a. Spermatogenesis-preventing substance in Japanese eel. Development 129, 2689–2697.
Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516–534.
Mylonas, C.C., Duncan, N.J., Asturiano, J.F., 2017. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture 472, 21–44.
Ogino, Y., Katoh, H., Kuraku, S., Yamada, G., 2009. Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. Endocrinology 150, 5415–5427.
Ohta, T., Miyake, H., Miura, C., Kamei, H., Aida, K., Miura, T., 2007. Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. Biol. Reprod. 77, 970–977.
Ohta, H., Tanaka, H., 1997. Relationship between serum levels of human chorionic gonadotropin (hCG) and 11-ketotestosterone after a single injection of hCG and induced maturity in the male Japanese eel, *Anguilla japonica*. Aquaculture 153, 123–134.
Ozaki, Y., Miura, C., Miura, T., 2006. Molecular cloning and gene expression of Spo11 during spermatogenesis in the Japanese eel, *Anguilla japonica*. Comp. Biochem. Physiol. B. 143, 309–314.
Peñaranda, D.S., Mazzeo, I., Gallego, V., Hildahl, J., Nourizadeh-Lillabadi, R., Pérez, L., Weltzien, F.-A., Asturiano, J.F., 2014. The regulation of aromatase and androgen receptor expression during gonad development in male and female European eel. Reprod. Domest. Anim. 49, 512–521.
Peñaranda, D.S., Morini, M., Tveiten, H., Vilchez, M.C., Gallego, V., Dirks, R.P., van den Thillart, G.E.E.J.M., Pérez, L., Asturiano, J.F., 2016. Temperature modulates testis steroidogenesis in European Eel. Comp. Biochem. Phsyiol A. 197, 58–67.
Peñaranda, D.S., Gallego, V., Rozenfeld, C., Herranz-Jusdado, J.G., Pérez, L., Gómez, A., Giménez, I., Asturiano, J.F., 2018. Using specific recombinant gonadotropins to induce spermatogenesis and spermiation in the European eel (*Anguilla anguilla*). Theriogenology 107, 6–20.
Rocha, A., Zanuy, S., Carrillo, M., Gómez, A., 2009. Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. Gen. Comp. Endocrinol. 162, 265–275.
Sambroni, E., Rolland, A., Lereyre, J.-J., le Gac, F., 2013b. Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis. J. Mol. Endocrinol. 50, 1–18.
Sambroni, E., Lereyre, J.-J., le Gac, F., 2013a. Fsh controls gene expression in fish both independently of and through steroid mediation. PLoS One 8, e76684.
Sarkar, D., Kumar Singh, S.K., 2018. Inhibition of testicular steroidogenesis and impaired differentiation of Sertoli cells in peripubertal mice offspring following maternal exposure to BDE-209 during lactation suppress germ cell proliferation. Toxicol. Lett. 290, 83–96.
Schulz, R.W., de França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.
Setiawan, A.N., Lokman, P.M., 2010. The use of reference gene selection programs to study the silvering transformation in a freshwater eel *Anguilla australis*: a cautionary tale. BMC Mol. Biol. 11, 75.
Setiawan, A.N., Ozaki, Y., Shoae, A., Kazeto, Y., Lokman, P.M., 2012. Androgen-specific regulation of FSH signalling in the previtellogenic ovary and pituitary of the New Zealand shortfinned eel, *Anguilla australis*. Gen. Comp. Endocrinol. 176, 132–143.
Sperry, T.S., Thomas, P., 1999. Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities. Endocrinology 140, 1602–1611.
Stocco, D.M., 2000. The role of StAR protein in steroidogenesis: challenges for the future. J. Endocrinol. 164, 247–253.
Valero, Y., Sanchez-Hernandez, M., Garcia-Alcazar, A., Garcia-Ayala, A., Cuesta, A., Chaves-Pozo, E., 2015. Characterisation of the annual regulation of reproductive and immune parameters on the testis of European sea bass. Cell Tissue Res. 362, 215–229.
Viñas, J., Piferrer, F., 2008. Stage-specific gene expression during fish spermatogenesis as determined by laser-capture microdissection and quantitative-PCR in sea bass (*Dicentrarchus labrax*) gonads. Biol. Reprod. 79, 738–747.
Wang, X., Orban, L., 2007. Anti-Mullerian hormone and 11 beta-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. Dev. Dyn. 236, 1329–1338.
Xie, Y., Chu, L., Liu, Y., Sham, K.W.Y., Li, J., Cheng, C.H.K., 2017. The highly overlapping actions of Lh signalling and Fsh signalling on zebrafish spermatogenesis. J. Endocrinol. 243, 233–246.
Zadmajid, V., Falahatimarvast, A., Damsteegt, E.L., Setiawan, A.N., Ozaki, Y., Shoae, A., Lokman, P.M., 2015. Effects of 11-ketotestosterone and temperature on inhibin subunit mRNA levels in the ovary of the shortfinned eel, *Anguilla australis*. Comp. Biochem. Physiol. B. 187, 14–21.