

Fsh and Lh direct conserved and specific pathways during flatfish semicystic spermatogenesis

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Abstract

The current view of the control of spermatogenesis by Fsh and Lh in non-mammalian vertebrates is largely based on studies carried out in teleosts with cystic and cyclic spermatogenesis. Much less is known concerning the specific actions of gonadotropins during semicystic germ cell development, a type of spermatogenesis in which germ cells are released into the tubular lumen where they transform into spermatozoa. In this study, using homologous gonadotropins and a candidate gene approach, for which the genes' testicular cell-type-specific expression was established, we investigated the regulatory effects of Fsh and Lh on gene expression during spermatogenesis in Senegalese sole (*Solea senegalensis*), a flatfish with asynchronous and semicystic germ cell development. During early spermatogenesis, Fsh and Lh upregulated steroidogenesis-related genes and nuclear steroid receptors, expressed in both somatic and germ cells, through steroid-dependent pathways, although Lh preferentially stimulated the expression of downstream genes involved in androgen and progesterone syntheses. In addition, Lh specifically promoted the expression of spermatid-specific genes encoding spermatozoan flagellar proteins through direct interaction with the Lh receptor in these cells. Interestingly, at this spermatogenic stage, Fsh primarily regulated genes encoding Sertoli cell growth factors with potentially antagonistic effects on germ cell proliferation and differentiation through steroid mediation. During late spermatogenesis, fewer genes were regulated by Fsh or Lh, which was associated with a translational and posttranslational downregulation of the Fsh receptor in different testicular compartments. These results reveal that conserved and specialized gonadotropic pathways regulate semicystic spermatogenesis in flatfish, which may spatially adjust cell germ development to maintain a continuous reservoir of spermatids in the testis.

Key Words

- ▶ teleost
- ▶ follicle-stimulating hormone
- ▶ luteinizing hormone
- ▶ transcription
- ▶ semicystic spermatogenesis

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Introduction

The pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the master regulators of spermatogenesis through the specific binding to their respective cognate receptors in the gonad, the FSH receptor (FSHR) and the LH/chorionic gonadotropin receptor (LHCGR) (Holdcraft & Braun 2004). In mammals, expression of FSHR and LHCGR is segregated respectively in Sertoli and Leydig cells. In Sertoli cells, FSHR mediates the functions of FSH to enhance spermatogonial proliferation and the entry of spermatogonia into meiosis. In Leydig cells, LH acting via LHCGR induces the production of androgens for the completion of meiosis and spermiogenesis. FSH acts directly or indirectly to modulate gene expression in somatic cells, while affecting few genes in germ cells, whereas less is known concerning the subset of genes controlled by LH during spermatogenesis (Maclean & Wilkinson 2005, Abel *et al.* 2009). In non-mammalian vertebrates, such as teleosts, Fsh and Lh are also preferentially involved in early spermatogenesis and spermiogenesis respectively (Schulz *et al.* 2010). However, both gonadotropins are potent steroidogenic hormones (Planas & Swanson 1995) due to direct interaction with their cognate receptors in Leydig cells (Ohta *et al.* 2007, García-López *et al.* 2009, 2010, Alam *et al.* 2010, Chauvigné *et al.* 2012), while estrogens, androgens, and progestins play important roles during spermatogonial renewal and proliferation, meiosis initiation, and spermiogenesis (Miura *et al.* 1991a,b, 1999, 2006, Miura & Miura 2003, Scott *et al.* 2010).

To gather a more complete picture of the specific actions of gonadotropins on spermatogenesis in non-mammalian vertebrates, recent studies of rainbow trout (*Oncorhynchus mykiss*), and to a lesser extent of zebrafish (*Danio rerio*), have begun to identify Fsh- and Lh-dependent genes expressed in the testis and their regulation by steroids (García-López *et al.* 2010, Rolland *et al.* 2013, Sambroni *et al.* 2013a,b). Results from these studies indicate that in contrast to mammals, Fsh and Lh similarly regulate a considerable number of genes in teleosts, where Fsh exerts both steroid-independent and -dependent regulatory functions on many genes relevant for the onset of spermatogenesis and sperm maturation. However, the extrapolation of these findings to Teleostei as a group is limited since these model species have cystic germ cell development, and in some cases, highly cyclic testicular development, whereas some ancient and more evolutionary advanced teleosts, such as

Pleuronectiformes (flatfishes) show semicystic spermatogenesis (Mattei *et al.* 1993, Weltzien *et al.* 2004, Schulz *et al.* 2005, Almeida *et al.* 2008). In this type of spermatogenesis, germ cells (spermatocytes or spermatids) are released into the seminiferous lumen where they complete meiosis and/or spermiogenesis without direct contact with the Sertoli cells (Mattei *et al.* 1993, Schulz *et al.* 2010), but very little is known concerning its regulation by gonadotropins.

The flatfish Senegalese sole (*Solea senegalensis*) represents a teleost model for studying semicystic spermatogenesis, in which spermatids are released into the lumen of the seminiferous lobules close to the collecting duct where they transform into spermatozoa (García-López *et al.* 2005, 2006a,b). This species also shows highly asynchronous spermatogenesis since once males reach sexual maturity spermatogenic activity and spermiogenesis are maintained almost all year-round, although spermiation is slightly enhanced during the annual fluctuation of female ovulation (García-López *et al.* 2006a,b). Recent studies of sole have revealed a complex cell-type-specific and stage-specific pattern of gene expression during spermatogenesis, and that human chorionic gonadotropin (hCG) can regulate the expression of genes potentially involved in steroidogenesis, progression of spermatogenesis and germ cell maturation (Cerdà *et al.* 2008, Forné *et al.* 2009, 2011, Marín-Juez *et al.* 2011, 2013, Mechaly *et al.* 2012). However, the specific actions of Fsh and Lh on gene expression during spermatogenesis in Senegalese sole remain unknown. In addition, a non-steroidal Lh/Lhcgr form ba (Lhcgrba) pathway in released spermatids directing spermiogenesis has recently been uncovered in this species (Chauvigné *et al.* 2014), although the endocrine pathways regulating this process remain to be fully understood.

To gain insights into the gonadotropic regulation of spermatogenesis in Senegalese sole, we performed a comprehensive study to establish the cell-type-specific expression in the testis of candidate genes known to be involved in germ cell proliferation and differentiation in other teleosts. We further investigated their regulation *in vitro* by Fsh and Lh using homologous recombinant hormones. Our results reveal previously unidentified conserved as well as specialized Fsh and Lh-mediated pathways regulating semicystic spermatogenesis in the testis of sole with respect to other teleosts with cystic spermatogenesis.

Materials and methods

Animals and gonad staging

Adult Senegalese sole males raised in captivity (1032 ± 70 g in weight; mean \pm S.E.M.) were maintained under natural photoperiod and temperature as described previously (Agulleiro *et al.* 2006). Fish were anesthetized with 500 ppm of phenoxyethanol, and after a blood sample was taken, fish were weighed and killed by decapitation. The testes were removed and weighed in order to determine the gonadosomatic index (GSI; testis weight/body weight \times 100). Different biopsies were deep-frozen in liquid nitrogen and stored at -80°C , used for *in vitro* culture, or processed for histology, immunohistochemistry, and *in situ* hybridization (ISH) as described previously (Chauvigné *et al.* 2010). The testes were classified as stages I–II (early and mid spermatogenesis respectively) or stage III (late spermatogenesis) (García-López *et al.* 2006a) after histological analysis carried out as described previously (Agulleiro *et al.* 2007). The procedures for the care and use of animals were approved by the Ethics Committee from the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) in accordance with the European Union Council Guidelines (86/609/EU).

Culture of testis explants

In vitro incubation of testis explants (10–30 mg) and production of Senegalese sole recombinant Fsh and Lh (rFsh and rLh respectively) were carried out as described previously (Chauvigné *et al.* 2012). For each experiment *in vitro*, the testis from one male was dissected transversally into small fragments to assure the presence of the cortical and medullar regions. The explants were incubated in triplicate (one explant per well) with 100 ng/ml rFsh or rLh at 18°C in a temperature-controlled incubator. The replicate explants were treated with $5\ \mu\text{M}$ of MDL-12330A (MDL; Sigma–Aldrich) or H-89 (Cayman Chemical Company, Ann Arbor, MI, USA), inhibitors of adenylyl cyclase and of cAMP-dependent protein kinase A (PKA), respectively, or trilostane (TRIL; Selleck Chemicals, Houston, TX, USA), an inhibitor of 3β -hydroxysteroid dehydrogenase (Hsd3b), 1 h before the hormone treatments. The control explants were incubated with the same concentration of hormone and drug vehicle. After 30 h of culture, the medium from each replicate was collected, and the testis explants were weighted, deep-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Three independent experiments on three different males were carried out for each testis stage.

Steroid determination

The plasma levels of testosterone and 17β -estradiol (E_2) were determined in duplicate by RIA (Schering-CIS) as described previously (Palstra *et al.* 2010), whereas those of 11-ketotestosterone (11-KT) were measured by enzyme immunoassay (EIA; Cayman Chemical Company) as reported (Chauvigné *et al.* 2012). The levels of testosterone and 11-KT in culture media were determined by EIA and normalized with respect to the weight of the testis explants.

cDNA cloning

Total RNA extraction from the testis, cDNA synthesis, and RT-PCR were carried out as described previously (Chauvigné *et al.* 2010). To isolate partial cDNAs encoding Senegalese sole structural maintenance of chromosomes protein 1b (*smc1b*), cytochrome P450 19a1 (*cyp19a1*, aromatase), nuclear androgen receptor isoform alpha (*ara*), nuclear androgen receptor isoform beta (*arb*), gonadal soma-derived factor variant (*gsdf*), and estrogen receptor beta (*erb*), oligonucleotide primers were designed from the expressed sequence tags (ESTs) available in GenBank or in the Solea-DB database v2.0 (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/) (Supplementary Table 1, see section on supplementary data given at the end of this article). Alternatively, degenerate primers were used to isolate cDNAs for cytochrome P450scc (*cyp11a1*), cytochrome P450c17-I (*cyp17a1*), cytochrome P450c17-II (*cyp17a2*), 11β -hydroxysteroid dehydrogenase type 2 (*hsd11b2*), and Piwi-like protein 2 (*piwi*). In some cases, 3'-end UTR regions were amplified using 3'RACE (Life Technologies Corp.). Nucleotide sequences for *smc1b*, *cyp19a1*, *ara*, *arb*, *gsdf*, *amh*, *erb*, *cyp11a1*, *cyp17a1*, *cyp17a2*, *hsd11b2*, and *piwi* were deposited in GenBank with the accession numbers listed in Supplementary Table 1.

Immunohistochemistry, immunofluorescence, and ISH

Immunostaining for sole gonadotropin receptors, Fshr form a (Fshra) and Lhcgr form ba (Lhcgrba) (Chauvigné *et al.* 2010), were carried out using characterized receptor-specific affinity-purified antibodies as described previously (Chauvigné *et al.* 2012). For immunofluorescence, sections were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:5000; Sigma–Aldrich) for 3 min before mounting. ISH was carried out as described previously (Cerdà *et al.* 2008)

using gene-specific riboprobes (Supplementary Table 1). The first post-hybridization washing (50% formamide in $2\times$ SSC at 50 °C) was followed by two washes with 0.1– $2\times$ SSC at 42–55 °C depending on the probes.

Real-time quantitative RT-PCR

Transcript levels in each replicate testicular explant before (time zero) and after hormone treatment *in vitro* were determined by real-time quantitative RT-PCR (qRT-PCR) as described previously (Chauvigné *et al.* 2010, Zapater *et al.* 2012). The specific primers used are listed in Supplementary Table 1. The reference gene was elongation factor 1A1 (*ef1a1*). Real-time qPCR amplifications were carried out in duplicate using 2 μ l of cDNA diluted 1:10–1:200 depending on the transcript. The expression levels of *fshra* and *lhcrba* at time zero were normalized only to relative gonad size (receptor mRNA levels = qRT-PCR value/*ef1a1* value \times GSI) (Kusakabe *et al.* 2006) since testis biopsies of similar weight and total RNA content were employed. Changes in gene expression in testicular explants cultured *in vitro* were determined as fold-changes with respect to untreated explants at time zero using the $2^{-\Delta\Delta Ct}$ method. The clustering analysis and heatmap generation for genes differentially regulated by rFsh and rLh were carried out using the Euclidean method in R software (<http://cran.r-project.org>).

Immunoblotting

Protein extraction from testes, treatment with *N*-glycosidase F (PGNAseF, New England Biolabs, Inc., Ipswich, MA, USA), and immunoblotting using 10% SDS–PAGE, were performed as previously described (Chauvigné *et al.* 2012). Triplicate membranes were incubated overnight at 4 °C with anti-Senegalese sole *Fshra* or *Lhcrba* antisera (1:500), or with anti- α -tubulin antibody (Tuba, 1:2000, Sigma–Aldrich T9026). Relative protein abundance was determined from the blots of three different males using the Quantity one software (Bio–Rad) and normalized to relative gonad size (receptor protein levels = receptor band intensity/Tuba band intensity \times GSI).

Statistical analyses

Data are the mean \pm S.E.M. and were statistically analyzed by the Student's *t*-test, or one- or two-way ANOVA, after log-transformation of the data when needed, followed by the Duncan's multiple range test. A *P* value <0.05 was considered statistically significant.

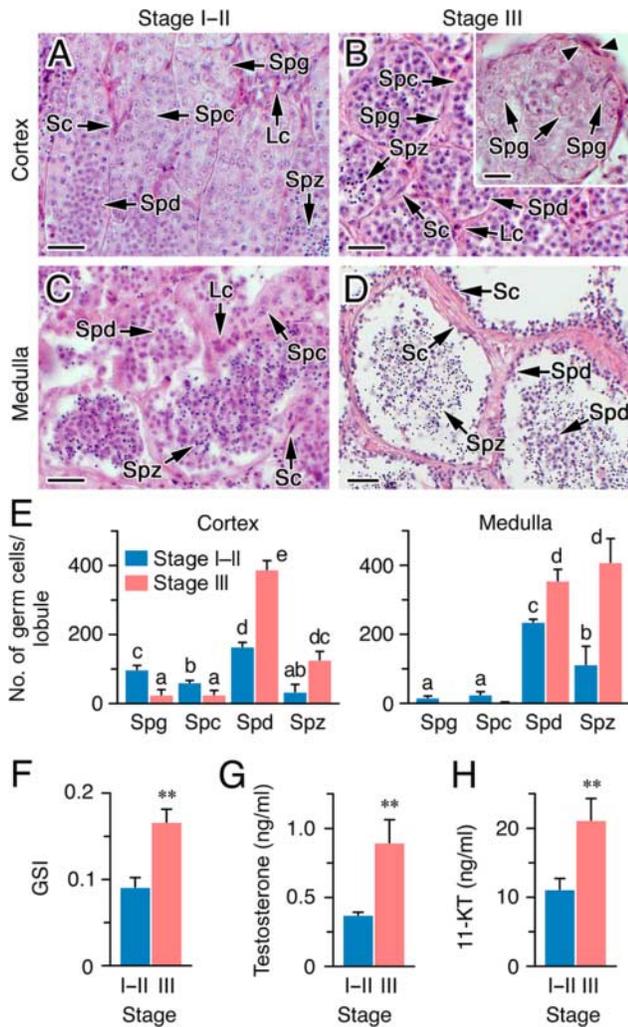
Results

Germ cell development and androgen plasma levels during final spermatogenesis

To identify the Senegalese sole spermatogenic stages investigated in this study, the progression of germ cell development in the cortex and medulla of the testis was examined histologically. At stages I–II, the cortical seminiferous lobules, in which for the majority the central lumen is not yet formed, contained germ cells at all developmental stages (spermatogonia, spermatocytes, and spermatids) and very few spermatozoa (Fig. 1A and E), indicating initiation of meiosis. In the testis medulla, the spermatogenic cysts opened showing mainly free spermatids within the central lumen, which was lined by a thin layer of Sertoli cells, where they start the transformation into spermatozoa (Fig. 1C and E). At stage III, most of the cortical lobules showed an open lumen filled almost exclusively with spermatids and some spermatozoa, although some residual spermatogonia and spermatocytes were also noted in the periphery of the lobules (Fig. 1B and E). However, in the most cortical region of the cortex, beneath the tunica albuginea, spermatogonia and associated Sertoli cells proliferate (Fig. 1B, inset). In the medulla of the testis at stage III, the diameter of the lumen of the lobules was considerably increased and contained large quantities of spermatids and spermatozoa, whereas Sertoli cells were still covering the tubules (Fig. 1D and E). The progression of spermatogenesis from stages I–II to III was associated with a 1.8-fold increase in GSI (Fig. 1F), and with a 2.4- and 1.9-fold increase in plasma levels of testosterone (Fig. 1G) and 11-KT (Fig. 1H), respectively, while circulating levels of E_2 were not detected (data not shown).

Fshra is differentially expressed during final spermatogenesis

The cellular localization of *Fshra* and *Lhcrba* in the testis was investigated by immunohistochemistry, employing specific affinity-purified antibodies (Chauvigné *et al.* 2012). In the cortex of the testis at stages I–II, Sertoli cells enclosing spermatogonia, spermatocytes, and spermatids strongly expressed the *Fshra* (Fig. 2A and C), whereas at stage III *Fshra* expression was no longer detected in the cortical Sertoli cells (Fig. 2B and D). In the medulla, the *Fshra* was detected in the Sertoli cells forming a thin epithelial-like layer covering the lumen of the seminiferous lobules in the testis at stages I–II and III (Fig. 2E and F). In contrast, *Fshra* expression in Leydig cells was homogeneous regardless of

**Figure 1**

The final stage of spermatogenesis in Senegalese sole is associated with increased plasma androgen levels. (A, B, C, and D) Representative histological sections of the cortex and medulla of testes at stages I–II and III stained with hematoxylin and eosin. Scale bars, 25 μ m. The inset in B shows the most distal region of the cortex beneath the tunica albuginea of stage III testis. Arrowheads point to proliferating Sertoli cells. Scale bar, 10 μ m. (E) Number of germ cells per seminiferous lobule in the cortex and medulla of testes at stages I–II and III. Bars with different superscript letters are significantly different ($P < 0.05$). In A, B, C, D, and E, Lc, Leydig cells; Sc, Sertoli cells; Spg, spermatogonia; Spc, spermatocytes; Spd, spermatids; Spz, spermatozoa. (F) GSI of males showing testes at stage I–II or III. (G and H) Plasma levels of testosterone (G) and 11-KT (H) in males showing testes at stage I–II or III. In E, F, G, and H, values (mean \pm s.e.m.; $n = 3$ fish) with asterisks (**) are significantly different ($P < 0.01$). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0087>.

their location or the spermatogenic stage of the testis (Fig. 2A, B, C, D, E, and F). The Leydig cells from the cortical and medullar regions of the testis also expressed the Lhcgrba in a similar fashion at stages I–II and III (Fig. 2G, H, I, J, K, and L). However, while the Lhcgrba was not detected in any of the germ cells of the cortex at either stage I–II or III

(Fig. 2I and J), in the medulla the receptor was present in free spermatids released into the tubular lumen at both stages (Fig. 2K and L, insets), thus confirming previous observations (Chauvigné *et al.* 2012, 2014).

Both the mRNA and protein levels of Fshra and Lhcgrba, as determined by qRT-PCR and western blotting, respectively, were not significantly different between stages I–II and stage III when normalized to the relative gonad size (Supplementary Figure 1, see section on supplementary data given at the end of this article). Immunoblotting of the receptors on the whole-testis extracts revealed, however, a different pattern of glycosylation of Fshra, but not of Lhcgrba, in stages I–II and III (Fig. 2M). At stages I–II, one major Fshra-reactive band of approximately 120 kDa was detected, which disappeared after PNGase F treatment, while a approximately 90-kDa band was induced *de novo* (Fig. 2M). In contrast, two reactive polypeptide bands of approximately 100 and approximately 110 kDa, respectively, were observed in testes at stage III, which were apparently not N-linked glycosylated (Fig. 2M). In both stages, however, the non-modified Fshra monomer of approximately 78 kDa, based on *in silico* determination of its molecular mass, was barely detectable (Fig. 2M, arrows). In stages I–II and III, testicular Lhcgrba was detected in immunoblotting as major reactive bands with molecular masses of approximately 78, 85, 110, and 150 kDa, while PNGase F treatment only reduced the size of the two higher-molecular-mass bands to approximately 95 kDa, probably indicating complex post-translational modifications of Lhcgrba (Fig. 2N).

Lh is more potent than Fsh at stimulating androgen production during final spermatogenesis

To investigate the steroidogenic action of Fsh and Lh during sole spermatogenesis, explants at stages I–II or III were incubated *in vitro* in the presence of rFsh and rLh for up to 30 h and the production of androgens was determined in the culture medium (Fig. 3). At stages I–II, both rFsh and rLh were equally able to stimulate the secretion of testosterone and 11-KT by approximately three- and fivefold above basal levels respectively. At stage III, however, rLh was much more potent than rFsh at stimulating testosterone (approximately seven- and threefold above basal levels, respectively) and 11-KT (approximately 16-fold and sixfold above basal levels, respectively) production. As expected, in both spermatogenic stages the steroidogenic action of rFsh and rLh involved the cAMP/PKA pathway and β 3-Hsd activation since MDL, H-89, and TRIL completely blocked androgen production *in vitro* (Fig. 3).

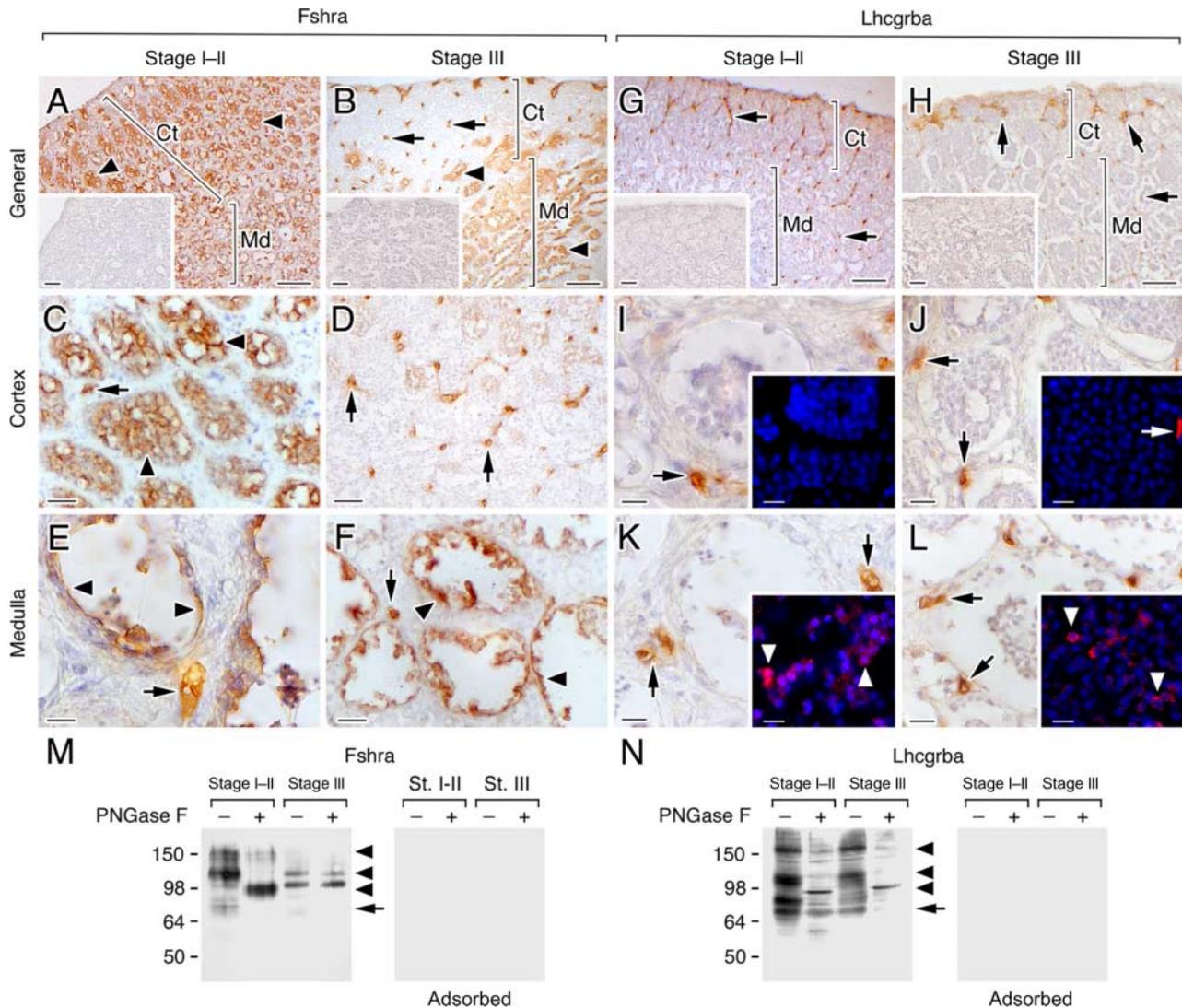


Figure 2

The expression pattern of *Fshra* and *Lhcgrba* in Senegalese sole testes at stages I–II and III of spermatogenesis. (A, B, C, D, E, F, G, H, I, J, K, and L) Immunolocalization of the *Fshra* (A, B, C, D, E, and F) and *Lhcgrba* (G, H, I, J, K, and L) in the cortex (Ct) and medulla (Md) regions of testes at both stages. The insets in A, B, G, and H show control sections incubated with the corresponding antisera preadsorbed with the immunizing peptides. The insets in I, J, K, and L show immunofluorescence staining for *Lhcgrba* (red) in spermatids (white arrowheads) and Leydig cells (white arrow) superimposed on DAPI (blue) nuclear staining. Arrowheads indicate Sertoli cells, whereas arrows point to Leydig cells. Scale bars, 100 μm (A, B, G, and H),

20 μm (C, D, E, F, I, J, K, and L), 10 μm (insets in D, I, J, K, and L). (M and N) Representative western blotting of *Fshra* (M) and *Lhcgrba* (N) in testes at stages I–II and III. Plus or minus signs indicate preincubation of protein extracts with PNGase F before SDS–PAGE and western blotting. The arrow indicates *Fshra* or *Lhcgrba* monomers, whereas the arrowheads indicate the products of PNGase F-sensitive and insensitive posttranslational modifications of the proteins. Duplicated blots (right) were incubated with the primary antibodies preadsorbed with the antigenic peptides to test for specificity. Molecular mass scales (kDa) are shown on the left. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0087>.

Cell type-specific expression of spermatogenic genes

The cellular localization in the testis of candidate genes involved in spermatogenesis was determined by ISH using specific riboprobes (Table 1). Most of the transcripts investigated showed a similar cell-type-specific expression in cortex and medulla. The mRNAs encoding steroidogenic enzymes, such as StAR protein (*star*), *hsd3b*,

17 β -hydroxysteroid dehydrogenase (*hsd17b*), *cyp17a1*, *cyp17a2*, *hsd11b2*, 20 β -hydroxysteroid dehydrogenase/carbonyl reductase-like (*cbr1*) and *cyp11a1*, were detected in Leydig cells (Fig. 4A, B, D, E, F, and G), whereas *cbr1* and *cyp11a1* were also found in spermatids (Fig. 4H and I). In contrast, *cyp19a1* was clearly detected only in spermatids (Fig. 4C). The members of the transforming growth factor

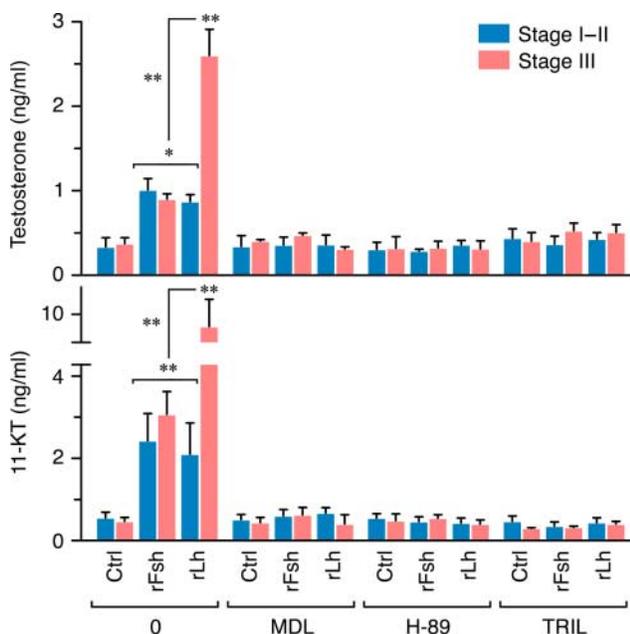


Figure 3

In Senegalese sole both recombinant gonadotropins stimulate testosterone and 11-KT production *in vitro*. The amounts of testosterone and 11-KT were measured in incubation media after 24 h of exposure of testis explants at stage I–II or III to 100 ng/ml of rFsh and rLh, in the presence or absence of 5 μ M of inhibitors of the cAMP/PKA pathway (MDL and H-89 respectively) and Hsd-3 β (TRIL). Values (mean \pm s.e.m.) are from three independent experiments on three different males, each with three replicates per condition. Asterisks denote significant differences ($P < 0.01$) with respect to the groups treated with the inhibitors, or as indicated. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0087>.

beta (Tgfb) superfamily, such as *gsdf* and anti-Müllerian hormone (*amh*), were expressed in Sertoli cells only (Fig. 5A and B), and in the case of *gsdf* exclusively in the cortical region (Fig. 5A, inset). The Sertoli cells also expressed insulin-like growth factor 1a (*igf1a*) (Fig. 5G). Interestingly, late germ cells (spermatocytes and spermatids) expressed insulin-like growth factor 1 receptor-like (*igf1rl*) (Fig. 5H). The nuclear androgen receptors *ara* and *arb* showed specific localization in germ cells (spermatogonia, spermatocytes, and spermatids) (Fig. 5D) or in Sertoli cells (Fig. 5E) respectively. The *erb* and nuclear progesterin receptor (*pgr*) transcripts were also observed in all germ cells (Fig. 5C and F), as well as in Leydig and Sertoli cells respectively. Finally, the meiosis-related genes *piwil2* and *smc1b* were expressed in spermatogonia, spermatocytes, and spermatids (Fig. 5I and J), whereas transcripts encoding spermatozoa differentiation genes such as septin 7a (*sept7a*) were only found in spermatids (Fig. 5K).

Fsh and Lh regulation of spermatogenic genes *in vitro*

The gonadotropic regulation of the expression of the 20 selected transcripts, plus *fshra* and *lhcrba*, as well as radial spoke head 1 homolog (*rsph1*) and StAR protein-like (*starl*) previously characterized (Chauvigné *et al.* 2010, 2014, Marín-Juez *et al.* 2011), was investigated in testes explants at stages I–II and III using rFsh and rLh. The results of the qRT-PCR experiments showed that the levels of all transcripts changed in response to rFsh and/or rLh except for *fshra* and *piwil2* (Supplementary Figure 2, see section on supplementary data given at the end of this article). Clustering analysis of significantly differentially expressed transcripts indicated that most of the genes were under gonadotropic regulation at stages I–II while very few were regulated at stage III (Fig. 6). In stages I–II, three main clusters of genes were defined based on the relative potency of rFsh and rLh for regulating gene expression (Fig. 6A). The first cluster contained genes preferentially upregulated by rFsh and included the Sertoli cell growth factors *gsdf* and *amh*, together with some steroidogenesis-related genes such as *hsd3b*, *cyp17a1*, and *cyp17a2* in Leydig cells; *gsdf* and *amh* being the most stimulated (approximately fivefold) genes. The second group was formed by genes primarily activated by rLh and included other steroidogenesis-related genes such as *cbr1*, *hsd11b2*, and *cyp11a1*; the nuclear receptors *pgr* and *arb*; the *lhcrba*; and the spermatozoa differentiation genes *sept7a* and *rshp1*. The transcript most highly stimulated by rLh was *hsd11b2* (approximately fourfold). Finally, the third cluster included those genes equally upregulated by both gonadotropins such as the rest of the steroidogenic genes, *star*, *starl*, *hsd17b*, and *cyp19a1* and *erb*, *ara*, and *scm1b*. At stage III, rFsh stimulated predominantly the expression of *arb*, *star*, and *pgr*, rLh specifically increased *igf1r* and *hsd17b*, and both gonadotropins slightly promoted the expression of *igf1*, *cyp17a1*, and *cbr1* (Fig. 6B).

Steroid-dependent and -independent actions of gonadotropins on testicular gene expression

To investigate whether the effect of gonadotropins on gene expression was dependent on steroid production, the expression levels of gonadotropin-regulated genes in explants at stages I–II treated with rFsh and rLh in the presence of MDL, H-89, or TRIL were determined by qRT-PCR (Fig. 7). The gonadotropic regulation of most of the genes analyzed, including Sertoli cell factors (*amh* and *gsdf*) (Fig. 7A and M), steroidogenic genes (*hsd3b*, *cyp17a1*, *cyp17a2*, *cyp11a1*, *hsd17b*, *cbr1*, *hsd11b2*, and *starl*)

Table 1 Cell type-specific gene expression in the Senegalese sole testis determined by ISH

Gene	Protein product	Cell type
<i>fshra</i>	Follicle-stimulating hormone receptor form a	Sc, Lc ^a
<i>lhcrba</i>	Luteinizing hormone receptor form ba	Lc, Spd ^{a,b}
<i>gsdf</i>	Gonadal soma-derived factor	Sc (only cortical)
<i>amh</i>	Anti-Müllerian hormone	Sc
<i>igf1a</i>	Insulin-like growth factor 1 form a	Sc
<i>igf1rl</i>	Insulin-like growth factor 1 receptor-like	Spc, Spd
<i>ara</i>	Nuclear androgen receptor isoform alpha	Spg, Spc, Spd
<i>arb</i>	Nuclear androgen receptor isoform beta	Sc
<i>pgr</i>	Nuclear progesterin receptor	Sc, Spg, Spc, Spd
<i>erb</i>	Estrogen receptor beta	Lc, Spg, Spc, Spd
<i>piwil2</i>	Piwi-like protein 2	Spg, Spc, Spd
<i>smc1b</i>	Structural maintenance of chromosomes protein form b	Spg, Spc, Spd
<i>rsph1</i>	Radial spoke head 1 homolog	Spd ^b
<i>sept7a</i>	Septin 7a	Spd
<i>star</i>	StAR protein	Lc
<i>starl</i>	StAR protein-like	Lc ^a
<i>cyp11a1</i>	Cytochrome P450scc	Lc, Spd
<i>hsd3b</i>	3 β -hydroxysteroid dehydrogenase	Lc
<i>cyp17a1</i>	Cytochrome P450c17-I	Lc
<i>cyp17a2</i>	Cytochrome P450c17-II	Lc
<i>hsd17b</i>	17 β -hydroxysteroid dehydrogenase	Lc
<i>cyp19a1</i>	Cytochrome P450 19a1 (aromatase)	Spd
<i>cbr1</i>	20 β -hydroxysteroid dehydrogenase/carbonyl reductase-like	Lc, Spd
<i>hsd11b2</i>	11 β -hydroxysteroid dehydrogenase type 2	Lc

Lc, Leydig cell; Sc, Sertoli cell; Spg, spermatogonia; Spc, spermatocyte; Spd, spermatid.

^aReported by Chauvigné *et al.* (2010, 2012).

^bReported by Chauvigné *et al.* (2014).

(Fig. 7B, C, D, H, I, J, K, O, and P), nuclear receptors *arb* and *pgr* (Fig. 7L and F), and *smc1b* (Fig. 7N), was completely abolished by MDL, H-89, and TRIL, indicating that the effect of both rFsh and rLh on these genes was dependent on steroids produced via the cAMP/PKA pathway. However, the stimulation of *star* by rFsh (Fig. 7O), and of *lhcrba* and *sept7a* by rLh (Fig. 7E and G), was reduced or suppressed in the presence of MDL and H-89 but not in the presence of TRIL, indicating that these effects were steroid-independent.

Discussion

Differential expression and regulation of gonadotropin receptors

The present work shows that between spermatogenic stages I–II and III, the overall Fshra and Lhcrba protein levels in the sole testis remained unchanged, which is consistent with the absence of *in vitro* testicular *fshra*-induced expression by Fsh and Lh during these stages. In contrast, Lh slightly increased *lhcrba* expression at stages I–II through a steroid-independent mechanism. Since sole Lhcrba is expressed in Leydig cells and medullar mature spermatids free in the tubular lumen, it

is possible that Lh stimulates the expression of its own receptor in these cells. Interestingly, immunohistochemistry revealed that Fshra was no longer expressed specifically in the cortical Sertoli cells at stage III, which was probably masked in analyses of the total protein levels in the testis because of the enlargement of the medullar Sertoli cells, which constitutively express the receptor. This finding indicates that during late spermatogenesis in sole, the effects of Fsh on gene expression in cortical Sertoli cells, including genes that may control Sertoli cell and spermatogonial proliferation, are probably indirect and mediated by steroids produced by Leydig cells. In addition, we noted that the Fshra was no longer N-linked glycosylated at stage III, which can potentially affect negatively the intracellular processing of the receptor (Menon *et al.* 2005). Altogether, our observations indicate that in sole during late spermatogenesis, Fshra is downregulated through translational and post-translational mechanisms.

Convergent effects of Fsh and Lh on gene expression

In the present study, we found that most genes involved in steroid production were significantly upregulated by both Fsh and Lh at stages I–II, which correlated with an increased production of androgens by the testicular

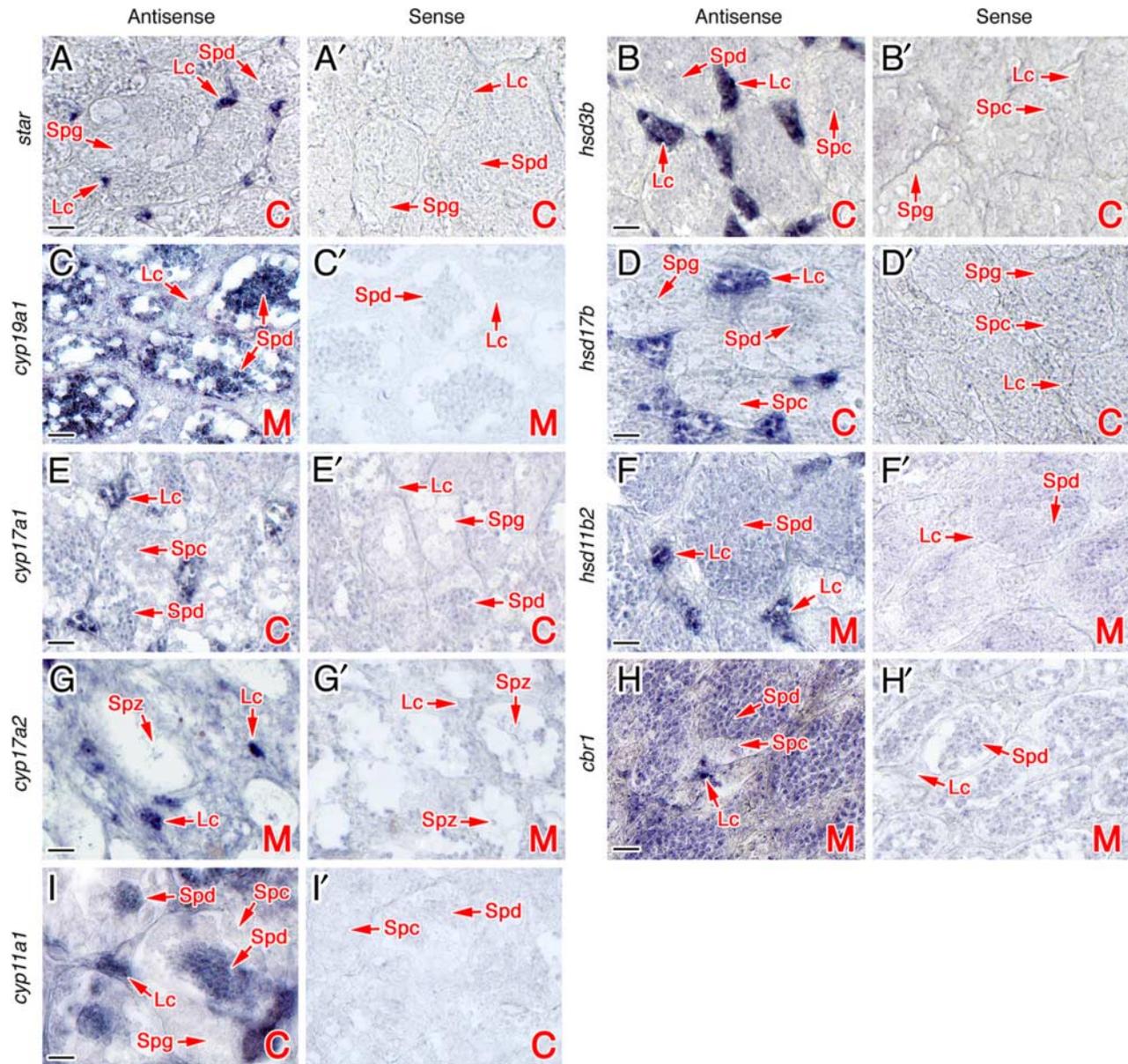


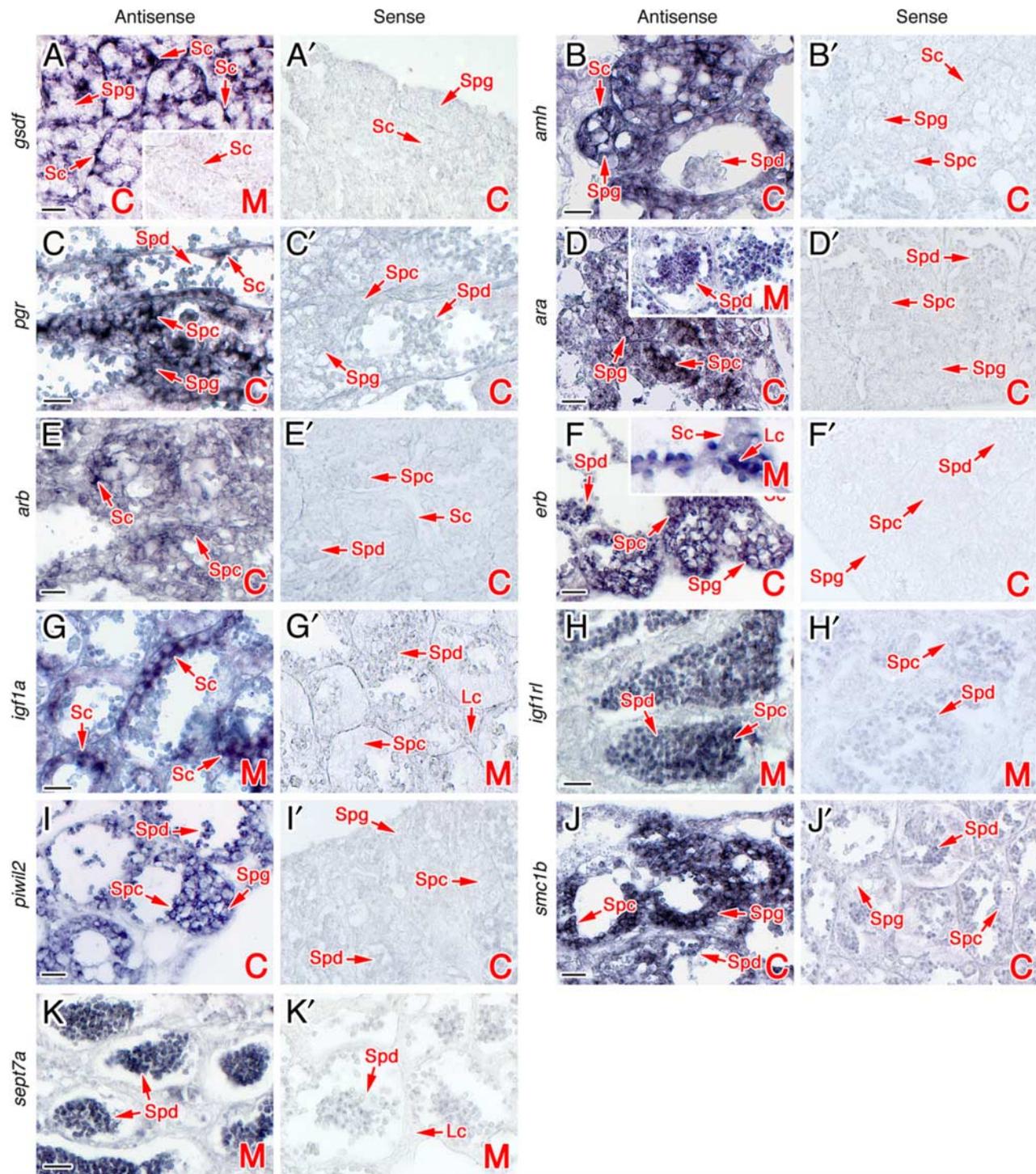
Figure 4

Localization of steroidogenesis-related transcripts in the Senegalese sole testis by ISH. Paraffin sections of testis were hybridized with antisense DIG-labeled riboprobes specific for *star* (A), *hsd3b* (B), *cyp19a1* (C), *hsd17b* (D), *cyp17a1* (E), *hsd11b2* (F), *cyp17a2* (G), *cbr1* (H), and *cyp11a1* (I). The hybridization signal is colored dark-blue to purple. (A', B', C', D', E', F', G', H', and I') Control sections stained with sense probes. The region of

the testis shown in each photomicrograph, cortex (C) or medulla (M), is indicated with red letters. Lc, Leydig cell; Sc, Sertoli cell; Spg, spermatogonia; Spc, spermatid; Spd, spermatid; Spz, spermatozoa. Scale bars, 20 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0087>.

explants at these stages *in vitro*. This is in agreement with previous *in vivo* data for Senegalese sole obtained using hCG (Marin-Juez *et al.* 2011), and is probably mediated by the presence of the Fshra and Lhcgrba in Leydig cells (Chauvigné *et al.* 2012). Our current data indicate, however, that at stages I-II, Fsh preferentially stimulates the expression of enzymes upstream of the steroidogenic

pathway, such as *hsd3b*, *cyp17a1*, and *cyp17a2*, whereas Lh was more potent at promoting the expression of *hsd11b2*, involved in the conversion of testosterone into 11-KT, and of *cbr1*, responsible for the conversion of 17 α -hydroxyprogesterone into 17 α ,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P), a progestin implicated in entry into meiosis and spermiation in teleosts (Miura *et al.* 2006, Scott *et al.* 2010).

**Figure 5**

Localization of transcripts encoding growth factors, nuclear receptors, and meiosis-related markers in the Senegalese sole testis by ISH. Paraffin sections of testis were hybridized with antisense DIG-labeled riboprobes specific for *gsdf* (A), *amh* (B), *pgr* (C), *ara* (D), *arb* (E), *erb* (F), *igf1a* (G), *igf1r1* (H), *piwil2* (I), *smc1b* (J), and *sept7a* (K). The hybridization signal is colored dark-blue to purple. (A', B', C', D', E', F', G', H', I', J', and K') Control sections

stained with sense probes. The region of the testis shown in each photomicrograph, cortex (C) or medulla (M), is indicated by red letters. Lc, Leydig cell; Sc, Sertoli cell; Spg, spermatogonia; Spc, spermatocyte; Spd, spermatid. Scale bars, 20 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0087>.

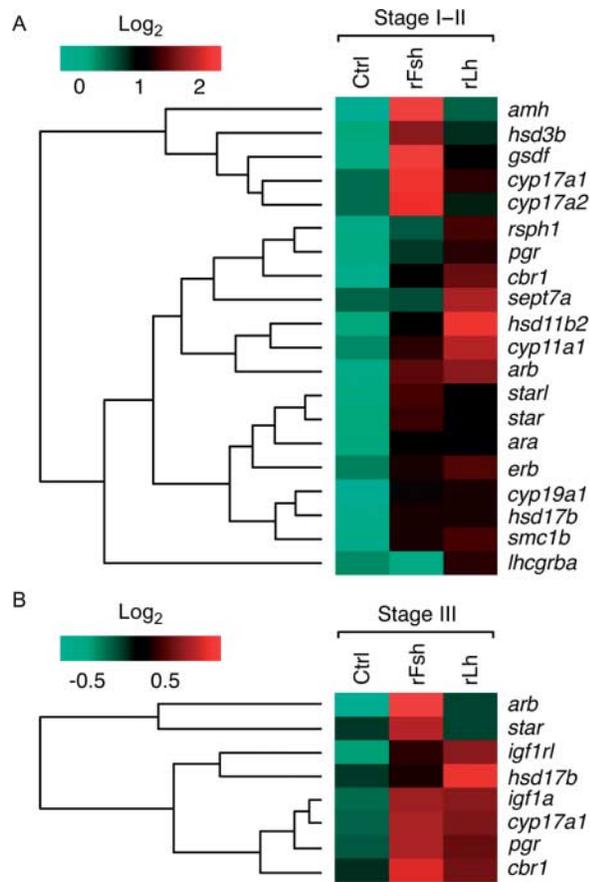


Figure 6 Hierarchical clustering of transcripts that changed significantly ($P < 0.05$) in Senegalese sole testicular explants at stages I–II (A) and III (B) after treatment with rFsh and/or rLh *in vitro*. Distances are measured using euclidean distance. Results are expressed as a matrix view of \log_2 expression levels where rows represent transcripts and columns represent the treatments. For each map, the intensity of each color denotes the standardized ratio between each value and the average expression of each transcript across all treatments. Red pixels correspond to an increased abundance of mRNA, whereas green pixels indicate decreased mRNA levels. The relative mRNA levels of all genes analyzed are shown in Supplementary Figure 2.

However, Lh was also more effective than Fsh at upregulating the expression of *cyp11a1*, an enzyme that initiates steroidogenesis by converting cholesterol to pregnenolone. In contrast, at stage III only *cyp17a1* was upregulated by Fsh and Lh, whereas *hsd17b* transcripts were accumulated only in response to Lh, despite both gonadotropins stimulating the secretion of androgens at this stage. This observation could indicate that at stage III androgens can be produced by Fsh through the stimulation of Cyp17a1-mediated production of the testosterone precursor androstenedione without the activation of downstream genes in the steroidogenic pathway. Similarly, the Lh-specific upregulation of *hsd17b* at this

stage (to the same level than at stages I–II) could explain the increased production of testosterone and 11-KT with respect to that induced by Fsh.

Senegalese sole spermatids also express *cyp11a1*, *cbr1*, and *cyp19a1*, but the physiological significance of this observation remains unclear. In mammals, high transcriptional activity in spermatocytes and spermatids is facilitated by an overall permissive chromatin arrangement in these germ cells that results from extensive chromatin remodeling, but it may be functionally not relevant (Soumillon *et al.* 2013). In zebrafish, however, the presence of Cyp17a1, Cyp19a1, and Cyp19a1b in germ cells has been demonstrated by using specific antibodies (Hinfrey *et al.* 2013). These data therefore indicate that steroids may potentially be produced locally by teleost germ cells.

The activation by Fsh and Lh of the expression of all the steroidogenesis-related genes at stages I–II, except *star*, was dependent on the cAMP/PKA pathway and the production of steroids, which is consistent with the coexpression of Fshra and Lhcgrba in sole Leydig cells and the possible activation of common signaling pathways. However, the steroid-independent positive regulation of *star* by Fsh, but not that by Lh, indicates that gonadotropins can also activate different downstream signaling pathways in somatic cells. These data are in contrast with those reported for teleosts with cystic spermatogenesis such as the rainbow trout, where gonadotropins stimulate the expression of *star*, *hsd3b*, *cyp17a1*, and *hsd11b2* independently from steroids (Sambroni *et al.* 2013a,b), although *star* expression can be downregulated by androgens (Rolland *et al.* 2013). Similarly, treatment of zebrafish testicular explants with Fsh *in vitro* stimulates *star* and *cyp17a1* expression independently from steroids, while Lh has no effect (García-López *et al.* 2010). Altogether, our findings for sole indicate a wider steroid-dependent gonadotropic regulation of genes involved in steroidogenesis compared with other teleosts. However, at stage III most of these genes did not respond to Fsh or Lh despite the high production of androgens at this stage, probably indicating the existence of negative feedback loops of steroids regulating the expression of steroidogenic enzymes during sole late spermatogenesis (Baron *et al.* 2005, Rolland *et al.* 2013). This mechanism might be important for fine-tuning the local regulation of testicular steroid production to sustain spermatogenesis and the continuous differentiation of spermatids.

The expression of Senegalese sole nuclear steroid receptors *ara*, *arb*, *pgr*, and *erb* was upregulated by Fsh

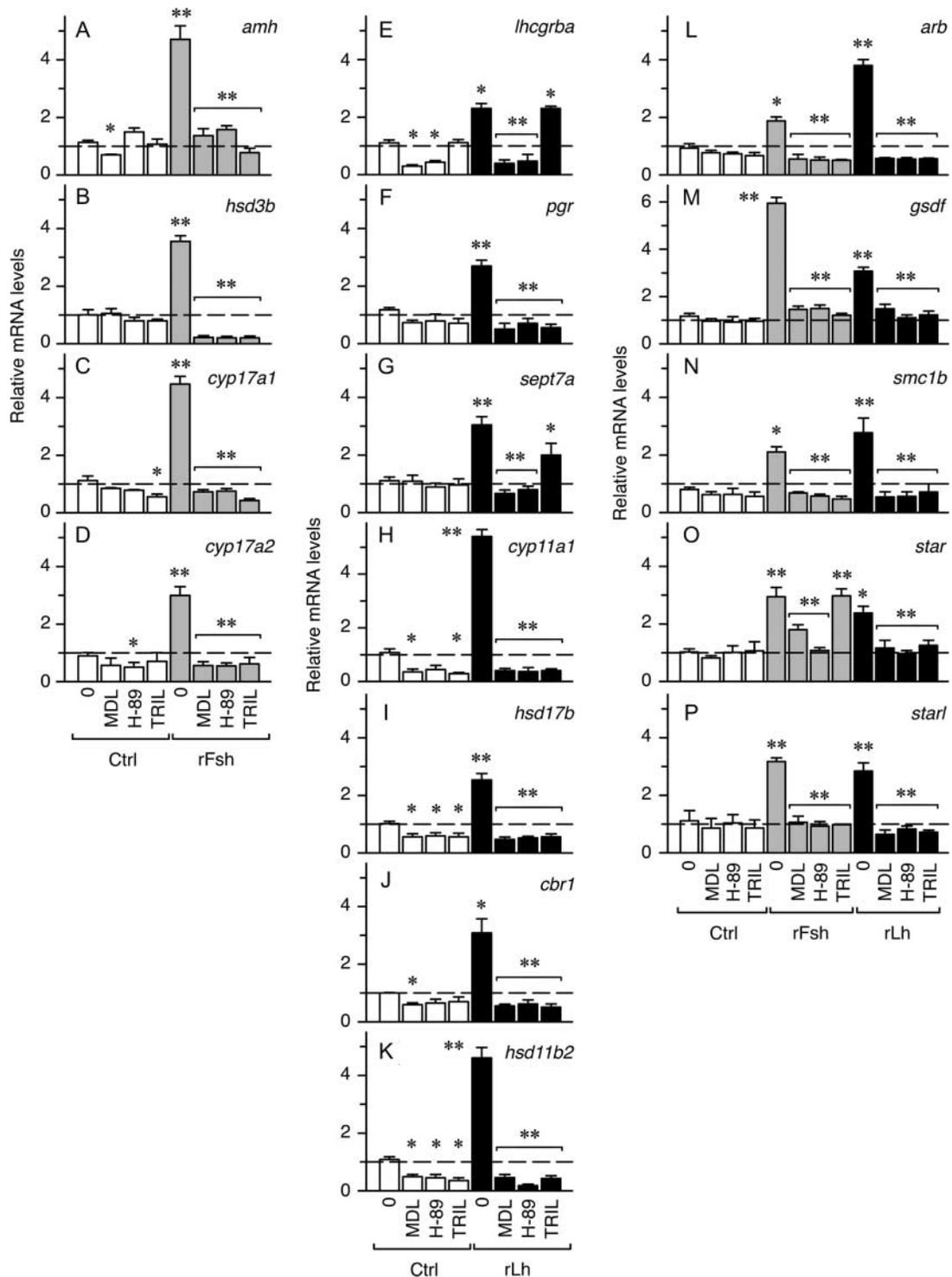


Figure 7

Effect of inhibitors of the cAMP/PKA pathway and of steroidogenesis on transcripts preferentially regulated by rFsh ((A) *amh*; (B) *hsd3b*; (C) *cyp17a1*; and (D) *cyp17a2*), rLh ((E) *lhcrba*; (F) *pgr*; (G) *sept7a*; (H) *cyp11a1*; (I) *hsd17b*; (J) *cbr1*; and (K) *hsd11b2*), or both ((L) *arb*; (M) *gsdf*; (N) *smc1b*; (O) *star*; and (P) *starl*), in testis at stages I-II *in vitro*. Changes in expression were determined by qRT-PCR and normalized to *ef1a1* mRNA expression.

Values (mean \pm s.e.m.) are from three independent experiments, each with three replicates per condition, for three different males. Dashed line at mRNA level 1 indicates no change with respect the control group at time zero. Asterisks denote significant differences (* $P < 0.05$; ** $P < 0.01$) with respect to the untreated control group or the group treated with rFsh alone (brackets).

and/or Lh at stages I–II, and these actions on *pgr* and *arb* were mediated by steroids. At stage III, *arb* and to a lesser extent *pgr* were stimulated only by Fsh. In sole, the expression of *arb* was Sertoli-cell-specific, whereas the other genes were expressed not only in Sertoli cells, or in Leydig cells in the case of *erb*, but also in spermatogonia, spermatocytes, and spermatids. The expression of steroid receptors in the germ cells of the Senegalese sole and other teleosts (Wu *et al.* 2001, Hanna *et al.* 2010, Zhou *et al.* 2010, Pu *et al.* 2013) may indicate a direct action of steroids on germ cell differentiation. However, murine loss-of-function models for some steroid receptors indicate that they are not required for germ cell development and function (Mahato *et al.* 2001), and therefore this mechanism remains to be demonstrated in fish. In sole, the differentiation of free tubular spermatids to spermatozoa is directly triggered by Lh (Chauvigné *et al.* 2014), but whether steroids have a role during early spermatid differentiation, or in the release of spermatids from Sertoli cells into the tubular lumen, is currently unknown.

Finally, at stages I–II both gonadotropins also regulated the expression of the meiosis-related gene *smc1b* in a steroid-dependent manner. This gene was expressed in germ cells enclosed within the Sertoli cells, and is potentially necessary for recombination between homologs during the meiotic prophase (Eijpe *et al.* 2000). Although the expression of the germ-cell-specific *piwil2*, which may also be involved in meiosis progression (Houwing *et al.* 2007), was not significantly regulated by Fsh or Lh during the stages examined, it may be possible that this gene is under gonadotropic regulation at earlier stages.

Fsh mainly controls the expression of growth factors in Sertoli cells

The members of the Tgfb superfamily, such as Gsdf and Amh, produced by Sertoli cells are positive and negative regulators, respectively, of spermatogonial proliferation and early differentiation in adult teleosts (Miura *et al.* 2002, Sawatari *et al.* 2007, Wu *et al.* 2010, Skaar *et al.* 2011, Chen *et al.* 2013). Accordingly, in Senegalese sole, *gsdf* and *amh* were localized exclusively in cortical Sertoli cells enclosing spermatogonia, and both transcripts were upregulated preferentially by Fsh during stages I–II. In zebrafish and trout, Fsh has no effect on testicular *gsdf* expression (García-López *et al.* 2010, Sambroni *et al.* 2013a), but in our study Fsh strongly stimulated (approximately fivefold) the expression of this gene. In sole, the concomitant Fsh-mediated increase in *gsdf* and the apparently antagonist gene *amh* remains intriguing,

since in other teleosts Fsh and androgens strongly inhibit *amh* expression (Miura *et al.* 2002, García-López *et al.* 2010, Mazón *et al.* 2013, Rolland *et al.* 2013, Sambroni *et al.* 2013a). The specific role of *amh* during sole spermatogenesis is currently unknown, and therefore it is possible that this gene is required for germ cell proliferation as has been proposed for medaka (*Oryzias latipes*) during early gonadal differentiation (Shiraishi *et al.* 2008). It is also feasible, that because of the semicyclic and asynchronous nature of the sole testis, *gsdf* and *amh* are activated in Sertoli cells from different compartments. In any event, we found that the gonadotropin-induced expression of *gsdf* and *amh* was steroid-mediated. In zebrafish, progestins can regulate *amh* and *gsdf* expression (Chen *et al.* 2013), which could also be the case in Senegalese sole because *cbr1* and *pgr* expressed in Sertoli cells increased preferentially in response to Lh at stages I–II. Androgens might also be involved in the regulation of *amh* expression since the trout *amh* promoter contains androgen-responsive elements (Rolland *et al.* 2013), and both *hsd17b* and *hsd11b2*, as well as *arb* which is specific to Sertoli cells, are upregulated by Fsh and Lh in sole testes at stages I–II.

We also found an evidence for the differential gonadotropic regulation of the Igf1 pathway in sole, which may play a role during spermatogonial proliferation as shown in rainbow trout (Loir & Le Gac 1994, Le Gac *et al.* 1996). In sole, *igf1a* and *igf1rl* transcripts were detected in Sertoli and late-germ cells (spermatocytes and spermatids), respectively, and both Fsh and Lh appeared to moderately stimulate the expression of *igf1a* at stage III, whereas Lh increased *igf1rl* expression in germ cells. These findings could indicate a paracrine pathway in the sole testis where Igf1a peptides secreted by Sertoli cells control the differentiation of spermatocytes and/or spermatids. However, in trout and Nile tilapia (*Oreochromis niloticus*), results indicate the role of the *igf1b* paralog rather than the *igf1a* in controlling spermatogonial proliferation and gonadal steroidogenesis (Li *et al.* 2012, Sambroni *et al.* 2013a). In Senegalese sole, it is not known whether the *igf1b* gene also plays a role during germ cell development.

Lh directly modulates the expression of spermatozoan differentiation-related genes in spermatids

We have recently shown that ligand-activated Lhcgrba in the free spermatids of Senegalese sole directs the expression of genes involved in spermatozoan differentiation and the progression of spermiogenesis (Chauvigné *et al.* 2014). Using whole-testis explants, we confirmed that Lh preferentially upregulates the transcription of spermatid-

specific genes (*rsph1* and *sept7a*) possibly involved in the formation of the spermatozoan flagellum and the acquisition of motility (Schlecht *et al.* 2004, Lin *et al.* 2011) during stage I–II. The effect of Lh on testicular *sept7a* expression was steroid-independent, which is consistent with our previous findings showing that *sept7a* upregulation is mediated by direct activation of Lhcgrba in spermatids (Chauvigné *et al.* 2014). At stage III, however, the Lh-mediated stimulation of *sept7a* and *rsph1* expression was no longer observed, which was possibly due to the ‘dilution’ of these mRNAs as a result of the increase in differentiating spermatids and spermatozoa in the medulla of the testis at this stage. Interestingly, in the rainbow trout testis, the majority of genes regulated by Fsh encode proteins expressed by somatic cells, whereas ~50% of the genes regulated by Lh belong to the germ line (Sambroni *et al.* 2013a). Lhcgrba is expressed in zebrafish and gilthead seabream (*Sparus aurata*) germ cells (Chauvigné *et al.* 2014), and therefore the modulation of gene expression in these cells through direct activation of Lhcgrba is a mechanism that may also be present in teleosts with cystic spermatogenesis.

Conclusions

As has been recently reported for teleosts with cystic germ cell development, the results of the present study indicate that Fsh and Lh have common and distinct effects on gene expression during the semicystic and asynchronous spermatogenesis of an advanced pleuronectiform teleost. However, our findings also reveal significant differences with respect to previous fish models, which may reflect neofunctionalized gonadotropic regulatory pathways of transcription in the semicystic testis of teleosts. These differences can be summarized as follows: i) differential regulation of the Fshra, both at the translational and post-translational level, in different testicular compartments during late spermatogenesis; ii) broader and steroid-mediated regulation of steroidogenesis and nuclear receptor genes in both somatic and germ cells by Fsh and Lh; iii) steroid-dependent Fsh-positive regulation of genes encoding growth factors with potentially antagonistic effects on germ cell development (*gsdf* and *amh*) in Sertoli cells during early/mid spermatogenesis; iv) preferential regulation of downstream genes involved in 11-KT and 17,20 β -P biosynthesis by Lh; and v) Lh-specific regulation of genes encoding spermatozoa differentiation proteins in haploid spermatids through direct interaction of Lh with Lhcgrba in these cells. These results indicate that in sole during spermatogenesis gonadotropins may

activate alternative regulatory pathways in different testicular compartments to adjust the processes of cell proliferation and/or differentiation, in order to maintain a continuous reservoir of spermatids in the testis for all-year round spermiogenesis. However, in Senegalese sole, as well as in other teleosts with semicystic spermatogenesis, no information is available on the Fsh and Lh plasma levels during gonad maturation, and how changes in the circulating levels of gonadotropins can regulate the spermatogenic mechanisms *in vivo* remains unknown. In addition, since a limited number of candidate genes were studied in this work, it is not known whether in sole the expression of most gonadotropin-dependent genes relies on steroid production. Further studies will be necessary to corroborate this hypothesis and to elucidate the Fsh- and Lh-responsive pathways during semicystic and asynchronous germ cell development in teleosts.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0087>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this research.

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Author contribution statement

F C and J C conceived and designed the study. F C performed the tissue culture experiments, histological analysis, ISH and immunocytochemistry, and immunoblotting. F C, D C, and J V P performed the steroid determination assays, and C Z carried out the real-time PCR analyses. F C and J C analyzed the results. F C drafted the manuscript, and J C and J V P edited the final version. All authors read and approved the final version of the manuscript.

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