

Fsh and Lh have common and distinct effects on gene expression in rainbow trout testis

Elisabeth Sambroni, Antoine D Rolland¹, Jean-Jacques Lareyre and Florence Le Gac

INRA, UR1037, Laboratoire de Physiologie et Génomique des Poissons (LPGP), Campus de Beaulieu, Testicular Physiology and Puberty, Biosit, Biogenouest, F-35000 Rennes, France

¹INSERM, U1085-Irset, Université de Rennes1, Biosit, Biogenouest, F-35000 Rennes, France

Correspondence should be addressed to F Le Gac

Email
florence.legac@rennes.inra.fr

Abstract

The general rules established from mammalian species for the regulation of spermatogenesis by gonadotropins may not be fully relevant in fish. Particularly, Fsh is as potent as Lh to stimulate steroidogenesis and the Fsh receptor is expressed in Leydig cells. In seasonal breeders, Fsh is likely the major gonadotropin involved in spermatogenesis onset and Lh is required to support spermatogenesis progression and gamete release. However, the genes that relay the action of Fsh and Lh have been poorly investigated in fish. The present study was aimed at identifying gonadotropin-dependent genes expressed in the testis during fish puberty. We cultured pubertal trout testicular explants for 96 h, with or without gonadotropin, and analyzed transcriptome variations using microarrays. Fsh and Lh had similar effects on a large group of genes while other genes were preferentially regulated by one or the other gonadotropin. We showed that most of the responsive genes were expressed in somatic cells and exhibited relevant patterns during the seasonal reproductive cycle. Some genes preferentially modulated by Lh could be involved in testicular cell fate (*pvr1* and *bty*) or sperm maturation (*ehmt2* and *racgap1*) and will deserve further examination. Besides Fsh's effects on the steroidogenic pathway, our study demonstrates that Fsh coordinates relevant stimulatory and inhibitory paracrine factors known to regulate early germ cell proliferation and differentiation. Some of these genes belong to major regulatory pathways including the Igf pathway (*igf1b/igf3* and *igfbp6*), the Tgfb pathway (*amh*, *inha*, *inhba*, and *fstl3*), the Wnt pathway (*wisp1*), and pleiotrophin (*mdka*).

Key Words

- ▶ Fsh
- ▶ spermatogenesis
- ▶ *oncorhynchus mykiss*
- ▶ transcriptome

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Introduction

In vertebrates, gametogenesis is mainly controlled by the brain–pituitary–gonad (BPG) axis and Fsh and Lh are the most important mediators regulating gonadal functions. Fsh and Lh biological actions depend on their binding to membrane receptors that belong to the G protein-coupled receptor superfamily (Levavi-Sivan *et al.* 2010). In mammals, each receptor is exclusively activated by its cognate ligand and is exclusively expressed in only one type of

testicular somatic cells, Sertoli cells for FSHR and Leydig cells for LHCGR. In the last decade, the generation of mice lacking gonadotropin or gonadotropin receptors provided unique insight into the roles of gonadotropin signaling in the development and function of the male reproductive axis. In mice lacking FSH or FSH receptor (FORKO), the number of both Sertoli cells and germ cells is reduced, but animals remain fertile. Sperm quality defaults have also

been observed in these mice (Abel *et al.* 2000, 2009, Kumar 2005, Huhtaniemi 2006). By contrast, mice lacking LH or LH receptor (LuRKO) are sterile due to an arrest of spermatogenesis beyond the round spermatid stage (Lei *et al.* 2001, Zhang *et al.* 2001, Kumar 2005). From these models, it is clear that FSH promotes the proliferation of Sertoli cells, increases the number of spermatogonia and enhances the entry of these cells into meiosis. On the other hand, LH is required for Leydig cell proliferation and maturation and is essential for the production of androgens that in turn allow the completion of meiosis and spermiogenesis.

In teleosts, the general scheme of regulation through the BPG axis is conserved but noticeable peculiarities exist regarding the effects of Fsh and Lh on the two main testicular functions, i.e. steroidogenesis and spermatogenesis. Fsh is capable, as well as Lh, of stimulating efficiently steroidogenesis in both immature and maturing testes (Planas & Swanson 1995). With regard to the gonadotropin receptors, the localization and binding or activation properties differ from what is generally admitted in mammals. The presence of Fshr has been described in the tubular compartment but also on Leydig cells in four species so far, i.e. eel, African catfish, zebrafish, and honeycomb grouper (Ohta *et al.* 2007, Garcia-Lopez *et al.* 2009, 2010, Alam *et al.* 2010). These studies have suggested that the steroidogenic potency of Fsh may result from the direct action of Fsh on Leydig cells. Not only does receptor localization differ, but cross-reactivity has also been described in binding assays with purified fish gonadotropins (Yan *et al.* 1992) and in functional studies using mammalian cell lines expressing fish receptors separately. In African catfish (Bogerd *et al.* 2001, Vischer & Bogerd 2003, Vischer *et al.* 2003), zebrafish (So *et al.* 2005), and Atlantic salmon (Andersson *et al.* 2009), a promiscuous activation of Fsh receptors has been reported using high Lh concentrations. However, in two salmonid species, amago salmon and rainbow trout, only Fsh was able to activate Fshr (Oba *et al.* 1999, Sambroni *et al.* 2007). The physiological relevance of the promiscuity of the piscine gonadotropin receptors therefore remains a matter of debate.

Many teleostean fish species are seasonal breeders and constitute original model species to understand the respective roles of Lh and Fsh in all vertebrates. In the highly cyclic salmonid species, gonadotropins are differentially secreted in the plasma through the reproductive cycle. In rainbow trout, Fsh is the only detectable circulating hormone during the first steps of

gametogenetic development (Prat *et al.* 1996, Gomez *et al.* 1999). This suggests that Fsh may play an important role in the early cellular and molecular events that occur during spermatogenesis onset. Fsh has been suggested to be involved in Sertoli cell proliferation (Schulz *et al.* 2005) and was proposed to take an essential part in the initiation of spermatogenetic maturation through the production of androgens in eel (Ohta *et al.* 2007). In addition, we previously showed that purified Fsh was capable of stimulating spermatogonia proliferation in a co-culture system with spermatogonia and Sertoli cells (Loir 1999b).

The deciphering of FSH molecular targets was previously initiated using a transcriptomic approach in rat primary Sertoli cell culture (McLean *et al.* 2002) or in hypogonadal mice (Sadate-Ngatchou *et al.* 2004, Abel *et al.* 2009). FSH acted directly or indirectly to modulate gene expression in Sertoli and Leydig cells but affected only a few genes expressed in germ cells. To our knowledge, no large-scale analysis of the effect of LH on testicular gene expression has been reported in vertebrates.

The present study was aimed at identifying Fsh- and Lh-dependent genes expressed in trout testis using a transcriptomic approach to understand their respective effects during fish puberty. We demonstrated for the first time that Fsh and Lh have both common and distinct effects on gene expression. Our data indicate that Fsh coordinates different stimulatory and inhibitory pathways that are physiologically relevant for early germ cell development or for sperm maturation/excretion in fish. The literature survey shows that some of these pathways are conserved in vertebrates.

Materials and methods

Animals and *in vitro* organotypic culture

All-male population rainbow trout (*Oncorhynchus mykiss*) were obtained from the INRA experimental fish farm (PEIMA, Drennec, France) and kept in the laboratory facilities at 12 °C under a natural photoperiod. Fish were anesthetized in 0.5% 2-phenoxyethanol and killed by a blow to the head. Testes were removed, weighed, and kept on ice in synthetic L15 media modified by Loir (1999a) until preparation for culture.

According to the macroscopic aspect of the testes and to the calculated gonadosomatic index (GSI), two pools of gonads have been used: one pool obtained from 28 fish with a GSI <0.1% (mean GSI=0.08%) and another one obtained from three fish with a GSI of between 0.2 and

0.6% (mean GSI=0.38%). Testes were chopped into 4 mm³ pieces, then pooled, and mixed. Some testis fragments were directly frozen in 1.2 ml of TRIzol reagent (Invitrogen) at -80 °C (T0 sampling) or fixed in Bouin's fluid to determine the testicular developmental stage, whereas the rest of the fragments were randomly distributed (60–80 mg/well) on Nunc polycarbonate membrane inserts in 24-well plates filled with 300 µl of modified L15 culture medium plus 2% Ultrosor SF (Loir 1999a). Incubation was performed in six replicates in the absence or presence of purified salmonid Fsh or Lh (500 ng/ml for 96 h, at 12 °C). The medium was renewed after 48 h of incubation. At the end of incubation, tissues and culture media were centrifuged for 10 min at 200 g. Tissues were frozen at -80 °C in 2 ml of TRIzol until RNA extraction. Culture media were frozen at -20 °C until steroid RIA.

Tissues fixed at T0 were dehydrated and embedded in paraffin, and 5 µm sections were cut and stained with Regaud Haematoxylin-Orange G-Aniline blue. The maturity stages of the gonads were evaluated based on the presence and on the relative abundance (RA) of the different germ cell types, according to a classification described by Gomez *et al.* (1999). The pool of gonads obtained from fish with a GSI <0.1% contained pre-spermatogenic testes in stage I (only A spermatogonia being present) and testes at stage II corresponding to the first appearance of B spermatogonia and active spermatogonia proliferation. The pool of gonads obtained from fish with a GSI >0.2% mainly constituted of testes in stage III corresponding to meiosis onset with the first appearance of spermatocytes and round spermatids.

Steroid measurement

To denature steroid-binding proteins, media were heated at 60 °C for 20 min and centrifuged at 3000 g, at 4 °C for 15 min. Levels of 11-ketotestosterone (11KT) were measured by specific RIA in culture media according to Fostier *et al.* (1982). Each sample was assayed in duplicates. Assay sensitivity was 80 pg/ml and cross-reactivity with testosterone or adrenosterone was 10% and null with androstenedione.

cDNA microarray experiments

RNA extraction and cDNA target synthesis ▶ Total RNA was extracted using TRIzol reagent and further purified with the NucleoSpin RNA II kit (Macherey

Nagel EURL, Hoerdet, France). RNA concentrations were quantified using the NanoDrop ND-1000 (Thermo Fisher Scientific, Courtaboeuf, France) and RNA quality was determined using the Bioanalyzer 2100 (Agilent Technologies, Massy, France). For cDNA target labeling, 5 µg total RNA were reverse-transcribed for 2 h at 42 °C in the presence of radiolabeled dNTP (30 µCi [α -³³P]dCTP, 120 µM dCTP, 20 mM each dATP, dTTP, and dGTP) using an oligo(dT) primer and 400 units Superscript II reverse transcriptase (Invitrogen). RNA were degraded at 68 °C for 30 min with 1 µl of 10% SDS, 1 µl of 0.5 M EDTA, and 3 µl of 3 M NaOH. The reaction was then equilibrated at room temperature for 15 min and neutralized (10 µl of 1 M Tris-HCl and 3 µl of 2 M HCl).

Hybridization of microarrays and raw data production

▶ cDNA nylon membrane microarrays were generated by CRB GADIE (<http://crb-gadie.inra.fr/>) as described previously (Rescan *et al.* 2007). Prehybridization of the membranes was performed at 65 °C for 4 h in 5× Denhardt's, 5× SSC, and 0.5% SDS buffer. Labeled cDNA targets were denatured at 95 °C for 5 min and incubated with the arrays for 48 h at 65 °C in the same buffer. Membrane arrays were then washed three times for 1 h at 68 °C in the washing solution (0.1× SSC, 0.2% SDS) prior to a 48 h exposure to phosphor imaging plates. The plates were scanned using a FUJI BAS 500 and hybridization signal (Si) acquisition was done with BZscan software (Lopez *et al.* 2004). Each membrane was also hybridized with a ³³P-labeled oligonucleotide (5'-TAATAC-GACTCACTATAGGG-3') that recognizes the vector part of every PCR product to quantify the amount of spotted cDNA (signal vector Vi).

Normalization procedure ▶ Expression data were normalized as described previously (Rolland *et al.* 2009). Briefly, raw data (Si) were corrected for the vector signal (Vi) proportional to the amount of spotted cDNA (Si/Vi). To avoid the bias affecting relative gene expression levels, the corrected signal of each spot was further multiplied by the median vector signal of all arrays for this same spot ((Si/Vi)×medVi). Expression values were then log₂-transformed and subjected to a quantile–quantile normalization using AMEN software (<http://sourceforge.net/projects/amen/>; Chalmel & Primig (2008)). Raw data as well as a normalized expression file are available at the Gene-Omnibus public data repository (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39465>).

Statistical and cluster analyses ▶ Non-informative clones for which too small an amount of cDNA was spotted (oligonucleotide signal less than three times the background level in more than 20% of samples) were removed from the analysis. Gonadotropin-responsive genes were then identified by comparing the control group with each of the treated groups (six replicates per group) at each stage (I–II and III), using the multi-class Limma statistical test with a false discovery rate (FDR) of 5% (Smyth *et al.* 2005). All differentially expressed transcripts, obtained by the union of the two lists of genes, were then subjected to a hierarchical classification (uncentered Pearson's correlation measure) to define groups of correlated gene expression (clusters). Trout cDNAs spotted onto the nylon membrane arrays were annotated based on the EST sequences and the search for orthologs in the sequenced and annotated genomes of four fish species, as described previously (Rolland *et al.* 2009) (column D in [Supplementary information file 1](#), see section on supplementary data given at the end of this article), completed by the search for the best protein homologs based on the contig of EST provided by SIGENAE -som8 version (column G in [Supplementary information file 1](#)).

Meta-analysis ▶ Expression data of six testicular developmental stages and of isolated germ cell fractions (Rolland *et al.* 2009) were used to investigate the developmental profile and the cellular origin of gonadotropin-responsive genes. Samples in this dataset included immature testes in early stages containing only slowly dividing type A spermatogonia (stage I) or growing numbers of actively dividing type B spermatogonia (stages IIa and IIb), maturing testes also containing large numbers of meiotic spermatocytes (stage IIIb) and post-meiotic spermatids (stage V), spawning testes containing essentially mature spermatozoa (stage VIII), and fractions of isolated germ cells enriched in spermatogonia, spermatocytes, or spermatids. Differentially expressed genes identified in this previous study (F's statistic permutation test with a 5‰ FDR) were compared with gonadotropin-responsive genes.

Real-time quantitative PCR experiments

The quantitative PCR (qPCR) technique was used to confirm changes in expression for selected transcripts identified from the microarray analysis or to examine other transcripts of potential interest. Two micrograms of total RNA were subjected to RT using random hexamer primers and 200 units of MMLV reverse transcriptase (Promega) for 75 min at 37 °C in a final volume of 25 µl.

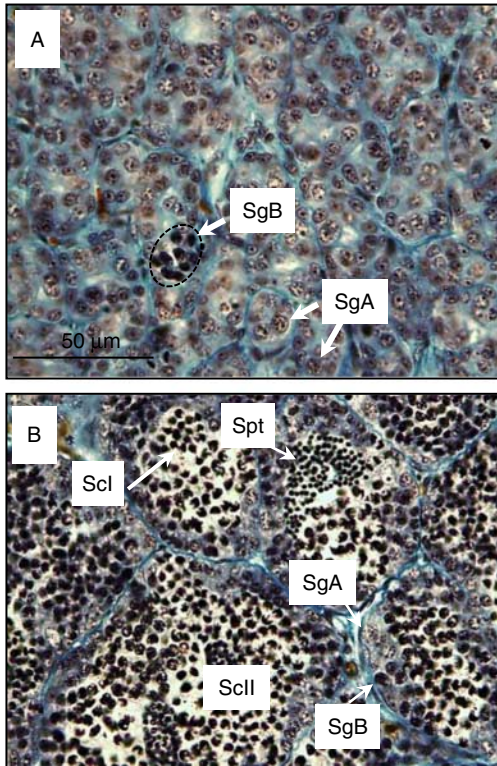
Real-time qPCR assays were performed on the StepOne Real-Time PCR System (Applied Biosystems, Villebon sur Yvette, France) using 4 µl of 1:30 diluted RT products, 1 µl of mixed oligo primers (0.6 µM for both reverse and forward primers), and 5 µl of Fast SYBR Green Master Mix (Applied Biosystems). The amplification program consisted of an initial denaturation step at 95 °C for 20 s, 40 cycles at 95 °C for 3 s, and 60 °C for 30 s. A final progressive increase in temperature (0.5 °C/s) has been carried out from 65 to 90 °C at the end of the amplification for melting curve analysis.

The RA was determined from a standard curve generated by performing serial dilutions of pooled RT products. Relative expression levels were normalized to the reference gene, *rps15* ($RA_{\text{candidate}}/RA_{\text{rps15}}$). The reference gene was chosen on the basis of its invariant expression over the spermatogenic testicular development (Rolland *et al.* 2009) and hormonal treatments. Its expression level also enabled its measurement at the same RT template dilution as selected candidate genes. All gene expression levels were measured in duplicates and statistical analyses were then performed using Statistica software using the non-parametric ANOVA of Kruskal–Wallis followed by the Mann–Whitney *U* test when a statistical difference ($P < 0.05$) was observed between the groups in ANOVA.

Real-time PCR oligonucleotide primers were designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and were verified with the oligoanalyzer 3.1 Web interface (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>) to avoid self- and hetero-dimer formation as well as hairpin structures. They were also systematically matched (BLAST algorithm) against the SIGENAE trout contig collection (som.10 version) to avoid non-specific annealing to other transcripts. Efficiency of PCR amplification was determined using serial dilutions of pooled RT products and was closed to 100%. All primer sequences are provided in [Supplementary Table 1](#), see section on supplementary data given at the end of this article.

Results

We investigated the effects of gonadotropins on the trout testicular transcriptome using testis explants at two stages of early gonadal maturation to focus on the initiation of spermatogenesis. Stages I–II are characterized by the presence of A spermatogonia and a small proportion of B spermatogonia. In stage III testis, B spermatogonia actively proliferates and meiosis progresses with the production of numerous spermatocytes and the appearance of round spermatids (Fig. 1).

**Figure 1**

Histological analysis. Representative histology of the pooled testis explants used in the microarray experiment. (A) Stages I–II and (B) stage III. Tissues were fixed in Bouin's solution, dehydrated, and embedded in paraffin. Five micrometer sections were cut and stained with Regaud Haematoxylin-Orange G-Aniline blue. SgA, A spermatogonia; SgB, B spermatogonia; Scl, primary spermatocytes; Scll, secondary spermatocytes; Spt, spermatids. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-12-0197>.

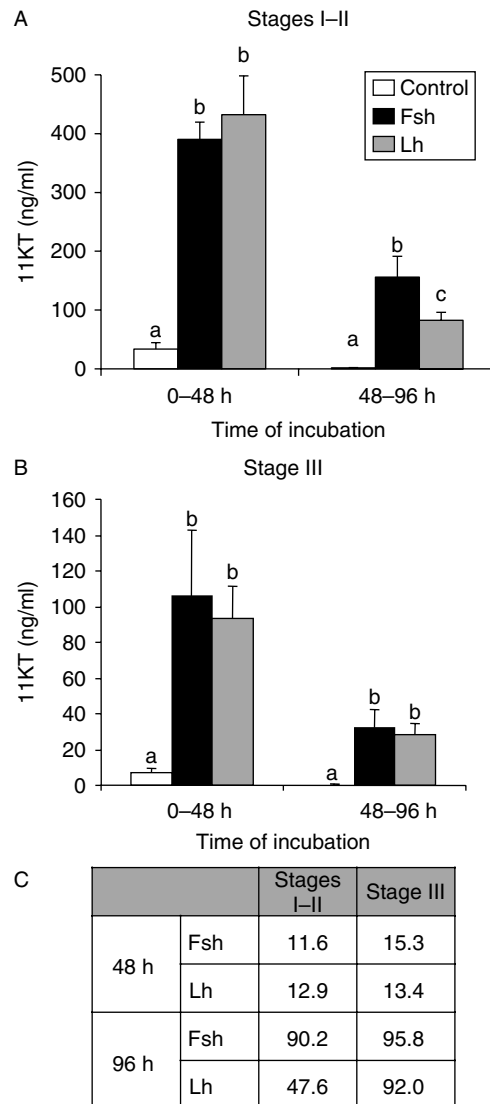
Both Fsh and Lh stimulated 11KT production over the culture period

To demonstrate the functional activities of the gonadotropins over the culture period, we first analyzed their effects on steroidogenesis at both studied stages. At stages I–II, 11KT accumulation in the culture media was highly increased (more than tenfold) when samples were stimulated in the presence of Fsh and Lh for 48 h (Fig. 2A). Although the basal 11KT production was altered after 48 h, gonadotropins still strongly stimulated 11KT production (about 90-fold) during the next 48 h period (Fig. 2A and C). The gonadotropin-induced steroid accumulation at 96 h was higher in the presence of Fsh compared with Lh. At stage III, the amplitude of the gonadotropin response was similar to that observed at stages I–II (Fig. 2B and C). Altogether, our data confirmed that Fsh and Lh efficiently stimulated steroid

production in fish and indicated that the gonadotropin responsiveness of the cultured testes was maintained over the culture period.

Fsh and Lh had common and distinct effects on gene expression in rainbow trout testis

In preliminary experiments, short incubation times (4 and 8 h) did not allow the detection of the

**Figure 2**

11KT production in culture media after 0–48 and 48–96 h of incubation in the absence or presence of purified salmonid Fsh or Lh at 500 ng/ml. (A) Testicular tissue explants at stages I–II; (B) testicular tissue explants at stage III. Culture media were replaced after 48 h. Each bar represents the mean \pm s.d. of six replicates. Different letters indicate that the treatments are significantly different as determined by the non-parametric Mann–Whitney *U* test ($P < 0.01$). (C) Fold changes compared with the control.

gonadotropin-regulated genes. The number of genes and the amplitude of the response increased with the time of exposure (18–96 h incubation). In addition, we analyzed the effect of two doses of Fsh on a subset of the responsive genes (Supplementary Figure 1, see section on supplementary data given at the end of this article). For several of them, the dose of 100 ng/ml appeared suboptimal when compared with the dose of 500 ng/ml. Therefore, we incubated the testicular explants with 500 ng/ml gonadotropins for 96 h.

Microarray experiments provided data for 8175 well-measured clones. Unsupervised hierarchical classification of the stages I–II samples perfectly distinguished between the control and the different gonadotropin-treated samples (data not shown).

A Limma multi-class statistical analysis revealed that the relative abundance of 390 clones, corresponding to 372 non-redundant transcripts (NR), was significantly

modulated following gonadotropin exposure (FDR <5%). In stage III, only 68 clones (65 NR) were found to be statistically differentially expressed and 55 clones (52 NR) were common with stages I–II. For a detailed analysis, we considered the 403 clones (385 NR) statistically differentially expressed in one or the other stage. Their complete list cannot be presented herein but is provided in Supplementary information file 1, together with the corresponding gene annotation, gene ontology (GO)-associated terms, and cluster information. The hierarchical classification carried out with the dataset obtained from the stages I–II samples separated six main clusters of genes with correlated variations (Fig. 3). Overall, an equal number of genes were upregulated or downregulated after the gonadotropin treatments. Interestingly, Fsh and Lh had similar effects for one-third of the regulated transcripts (cluster 2 ‘Up Fsh, Lh’ and cluster 5 ‘Down Fsh, Lh’ in Supplementary information file 1).

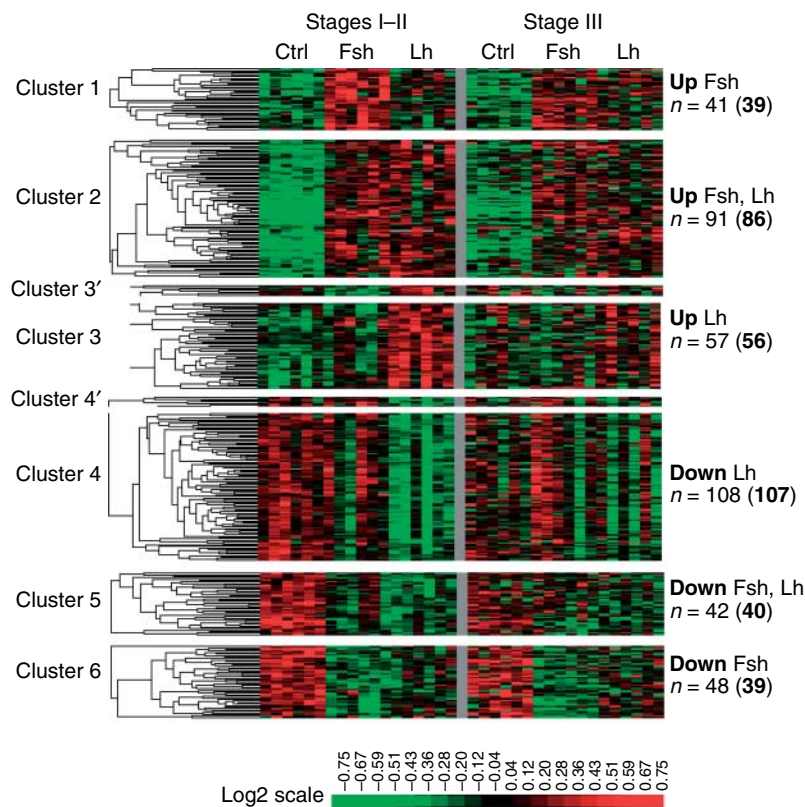


Figure 3

Hierarchical classification of the 403 clones regulated *in vitro* in the absence (Ctrl) or presence of Fsh or Lh at 500 ng/ml; 399 genes segregated into six main groups, corresponding to the genes up- or downregulated by both Lh and Fsh (Fsh, Lh) or preferentially regulated by one gonadotropin. Each

line represents a clone and each column is a sample. Only the stages I–II genes were clustered, whereas the data in stage III are displayed on the same line. *n* is the number of clones in each cluster. The number of non-redundant genes is in bold characters.

Table 1 Selected genes preferentially regulated by Lh with the highest differential between the Lh and Fsh responses. These genes from clusters 3 and 4 were identified as differentially regulated by Fsh and Lh in pairwise comparisons (Limma statistical test, FDR <5%)

Clone name	SwissProt/GenBank accession number	Gene symbol	Annotation/description	Predicted cellular origin
Cluster 3: 23 genes upregulated by Lh				
1RT27H07_B_D04	Q4SL44	<i>anxa11</i>	Annexin A11	–
tcbk0006.h.24	Q7SYB4	<i>pgam1b</i>	Phosphoglycerate mutase 1	–
tcad0003.j.02	P82861	<i>fdxr</i>	NADPH:adrenodoxin oxidoreductase, mitochondrial	–
tcay0006.k.10	Q6DRK0	<i>atp6v1f</i>	ATPase, H ⁺ transporting, V1 subunit F	Germline
tcay0034.c.17	Q4T6N8	<i>ggtl3</i>	Gamma-glutamyltransferase 4 precursor	–
tcbk0003.a.18	Q4RT27	<i>mobk11b</i>	Mps one binder kinase activator-like 1B	Germline
tcba0017.d.24	Q5U3H7	<i>ppm1db</i>	Protein phosphatase 1D	–
tcad0003.h.21	Q9DFH0	–	G-protein B1 subunit	Somatic
tcay0037.j.03	–	<i>atp2a3</i>	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	Somatic
tcba0007.l.12	Q8AVH9	<i>ppp2ca</i>	Serine/threonine protein phosphatase	Gonia A
1RT60M18_C_G09	Q1L035	<i>cox3</i>	Cytochrome c oxidase subunit 3	Somatic
1RT92G03_A_D02	–	<i>fh11</i>	Four and a half LIM domain protein 1	–
tcad0003.i.23	Q5DSV5	<i>btv</i>	Tripartite motif protein 'bloodthirsty-like'	–
tcay0034.d.21	Q6TNT2	<i>ankrd46</i>	Ankyrin repeat domain-containing protein 46	–
tcbk0028.h.02	–	<i>polr3a</i>	DNA-directed RNA polymerase III subunit RPC1	Somatic
tcay0009.o.12	–	<i>copa</i>	Coatomer subunit α (α -coat protein)	Gonia A
tcay0031.f.09	–	<i>frp2</i>	Secreted frizzled-related protein 2 precursor	Somatic
1RT129O10_C_H05	–	<i>serinc5</i>	Developmentally regulated protein TPO1	Germline
tcbk0052.i.13	B2RCV9	<i>slc22a13</i>	Highly similar to <i>Homo sapiens</i> solute carrier family 22, member 13	Somatic
1RT63G13_A_D07	–	<i>smyd1</i>	SET and MYND domain-containing protein 1	–
tcbk0036.m.23	–	<i>pvr11</i>	Poliovirus receptor-related protein 1 precursor	–
tcbk0006.c.09	Q7ZVX9	<i>txndc9</i>	Thioredoxin domain containing 9	Germline
tcba0001.c.18	–	<i>cops5</i>	COP9 signalosome complex subunit 5	Germline
Cluster 4: 20 genes downregulated by Lh				
tcba0006.g.10	P62993	<i>grb2</i>	Growth factor receptor-bound protein 2	–
1RT89F14_D_C07	Q7SYH8	<i>dab2</i>	Disabled homolog 2 (<i>Drosophila</i>)	–
1RT24E14_C_C07	Q8CI59	<i>steap3</i>	Metalloreductase STEAP3	–
tcbk0060.l.19	Q99541	<i>adfp</i>	Adipophilin (adipose differentiation-related protein) (ADRP)	Somatic
tcbk0051.k.09	A9JT06	<i>zgc:110154</i>	<i>Danio rerio</i> Zgc:110154 protein/eukaryotic translation initiation factor 4E	Gonia B
1RT24D22_D_B11	Q7SXT6	<i>msi2</i>	RNA-binding protein Musashi homolog 2 (Musashi-2)	Gonia B
1RT24F14_D_C07	–	<i>rbm28</i>	RNA-binding protein 28	–
tcbk0051.k.17	F1R444	<i>prkca</i>	Protein kinase C α type	Germline
tcba0006.h.12	–	<i>ehmt2</i>	Histone-lysine N-methyltransferase, H3 lysine-9 specific 3	–
tcbk0045.l.11	Q9UDX4	<i>sec14l3</i>	SEC14-like protein 3 (tocopherol-associated protein 2)	–
tcbk0051.n.05	Q7SXY0	<i>vps4b</i>	Vacuolar protein sorting-associating protein 4B (yeast)	–
tcbk0015.e.13	Q9P2R6	–	Arginine-glutamic acid dipeptide repeats protein	Somatic
tcbk0054.b.22	–	<i>pde6h</i>	Retinal cone rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit gamma	–
1RT24G21_A_D11	Q6NV12	<i>slc20a1a</i>	Sodium-dependent phosphate transporter 1	–
tcba0006.i.11	B3DGI9	<i>timm8b</i>	Translocase of inner mitochondrial membrane 8 homolog B	–
tcbk0060.h.01	–	<i>tert</i>	Telomerase reverse transcriptase	–
tcbk0035.i.22	O42200	<i>itk</i>	Tyrosine-protein kinase ITK/TSK	–
1RT24F05_B_C03	–	<i>capn5</i>	Calpain-5	–
tcbk0048.l.08	A0N0D8	<i>creb</i>	cAMP-responsive element-binding protein	–
tcay0032.c.10	–	<i>exoc3l2</i>	Protein 7 transactivated by hepatitis B virus X antigen	Germline

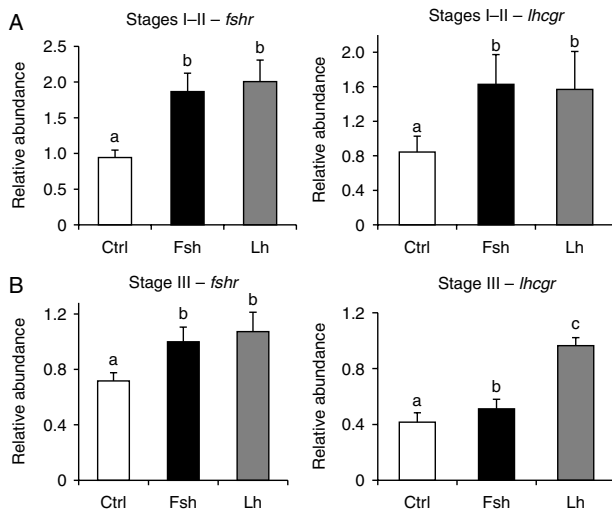
Some of them were further analyzed by qPCR when they belonged to pathways of interest: *star*, *cebpb*, *inha*, *mmp19*, and *vt1*.

Most importantly, we found a greater number of genes preferentially regulated by one or the other gonadotropin. Hence, Lh regulated more specifically the testicular expression of 175 transcripts (cluster 3 'Up Lh' and cluster 4 'Down Lh'). Furthermore, inside each of these two clusters, we noticed a small subgroup of genes whose regulation by Fsh tended to be the opposite to that by Lh (clusters 3' and 4' in Fig. 3 and labeled 'Up Lh, Down Fsh' or 'Down Lh, Up Fsh' in Supplementary information file 1). The genes showing the most differential response

between Lh and Fsh are presented in Table 1. These 'Lh specific' genes were involved in different biological processes such as chromatin remodeling, transcription, regulation of translation, signal transduction, cell proliferation, apoptosis, or cell cycle progression. In turn, Fsh modulated preferentially the expression of 82 distinct transcripts annotated 'Up Fsh' or 'Down Fsh' in Supplementary information file 1. Among these 82 genes, those showing the greatest responsiveness to Fsh compared with Lh are listed in Table 2. The examination of the GO terms of the upregulated genes indicated that they were involved in amino acid metabolism, lipid and steroid metabolism, and cation homeostasis processes.

Table 2 Selected genes preferentially regulated by Fsh with the highest differential between the Lh and Fsh responses. These genes from clusters 1 and 6 were identified as differentially regulated by Fsh and Lh in pairwise comparisons (Limma statistical test, FDR <5%)

Clone name	SwissProt/GenBank accession number	Gene symbol	Annotation/description	Predicted cellular origin
Cluster 1: 22 genes upregulated by Fsh				
tcbk0035.l.03	O95633	<i>flst3</i>	Follistatin-related protein 3 precursor	–
tcad0009.j.11	P48307	<i>tfpi2</i>	Tissue factor pathway inhibitor 2	Somatic
tcba0001.g.14	Q1L692	<i>nr1h5</i>	Farnesoid X receptor FXR β (fragment)	–
tcay0019.j.04	Q9Y6M5	<i>slc30a1</i>	Zinc transporter 1 (ZnT-1) (solute carrier family 30 member 1)	Somatic
1RT41K06_C_F03	–	<i>mdka</i>	Pleiotrophin 1 (midkine-related growth factor)	Somatic
tcbk0024.l.04	–	<i>rrp12</i>	RRP12-like protein	Gonia B
tcbk0035.k.02	–	<i>foxo3b</i>	Forkhead protein FoxO5	Somatic
tcac0003.a.02	Q6F6A1	<i>ctsl</i>	Cathepsin L	Somatic
tcbk0048.o.16	Q29VH6	<i>smtnb</i>	Smoothelin-b	Somatic
tcay0013.g.08	Q92088	<i>cyp2m1</i>	Cytochrome P450 2M1	Somatic
tcba0029.p.03	O95388	<i>wisp1</i>	WNT1-inducible-signaling pathway protein 1 precursor (WISP-1)	Somatic
tcab0002.j.23	–	<i>ldlr</i>	Vitellogenin receptor (fragment)	Somatic
tcav0002.e.20	–	<i>dnajc11</i>	DnaJ homolog subfamily C member 11	–
tcbk0011.i.24	O57656	<i>gpd1</i>	Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic	–
tcay0004.j.03	O95477	<i>abca1</i>	ATP-binding cassette sub-family A member 1	Somatic
1RT145G14_C_D07	–	<i>ggt5</i>	Gamma-glutamyltransferase 5 precursor	Somatic
1RT153E24_C_C12	A5WUN5	<i>abr</i>	Novel protein similar to <i>H. sapiens</i> ABR, active BCR-related gene	–
tcay0029.n.03	P15408	<i>fosl2</i>	Fos-related antigen 2	Gonia B
1RT102H16_D_D08	A1L222	<i>krt12</i>	Keratin 12	Somatic
tcbk0039.m.01	G3NE10	<i>slc40a1</i>	Solute carrier family 40 member 1 (ferroportin-1)	Somatic
tcbk0050.a.09	P24468	<i>nr2f2</i>	COUP transcription factor 2 (COUP-TF2)	–
tcbk0061.a.18	Q9PVQ9	<i>hoxc9a</i>	Homeobox protein Hox-C9	Somatic
Cluster 6: 12 genes downregulated by Fsh				
1RT32D13_B_B07	Q0KFS2	<i>s100A11</i>	S100 calcium binding protein (fragment)	Somatic
tcam0001.o.17	Q9TNN8	<i>onmyUAA-OSU</i>	MHC class Ia heavy chain	Somatic+gonia
tcay0004.c.04	Q4RMF2	<i>ttbk2</i>	Tau-tubulin kinase 2	–
tcad0001.g.06	Q9TNW9	<i>onmyUBA-SP3</i>	Major histocompatibility complex class I protein	Somatic
1RT41E09_A_C05	P06396	<i>gsn</i>	Gelsolin precursor (actin-depolymerizing factor)	Somatic
tcam0002.m.19	Q8JFQ6	<i>krt13</i>	Keratin, type I cytoskeletal 13	Somatic
tcad0007.b.24	G3P658	<i>rd3</i>	Protein RD3	Somatic
1RT134E06_C_C03	O14618	<i>ccs</i>	Copper chaperone for superoxide dismutase	Somatic
tcay0027.n.15	P02452	<i>col1a1</i>	Collagen α -1(I) chain precursor (α -1 type I collagen)	Somatic
tcbk0050.g.24	Q4VBS0	<i>itga2</i>	Integrin α -2 precursor	–
tcam0002.m.19	Q7ZTS4	<i>krt18</i>	Keratin, type I cytoskeletal 18	Somatic
tcbk0010.d.11	Q564J7	<i>pum1</i>	Pumilio homolog 1	Somatic

**Figure 4**

Effect of Fsh and Lh (500 ng/ml) on gonadotropin receptor mRNA levels in the testis explants after 96 h of incubation and according to the testicular stage of development: (A) stages I–II and (B) stage III. Bars represent the mean \pm s.d. of five replicates. Different letters indicate that the treatments are significantly different as determined by the non-parametric Mann–Whitney *U* test ($P < 0.01$).

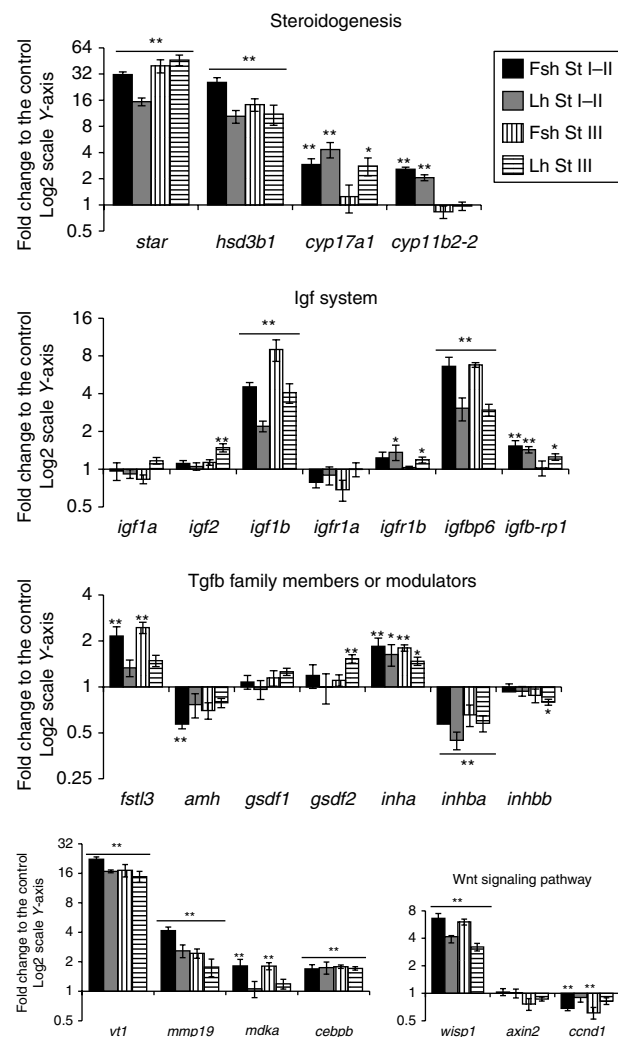
These genes encoded nuclear receptors (*nr2f2* and *nr1h5*), proteases (*ctsL* and *ctsS*), transcription factors (*hoxc9a*, *foxo3b*, and *fosl2*), and zinc/iron transporters (*slc30a1* and *slc40a1*). The Fsh-downregulated genes were mostly involved in tissue remodeling and cytoskeleton organization. These genes included *gsn*, *cnm2*, *des*, *actn3*, *krt8*, *krt18*, and *mapre11*. In the same cluster, we also found several genes that encoded for components of the extracellular matrix including *col1a1*, *col1a2*, *fn1*, *tnc*, and *mmp9*.

In summary, our data show that Fsh and Lh can have either common or independent regulatory effects on gene expression in pubertal trout testis. In stage III, we observed that gonadotropin-regulated transcripts generally displayed similar expression changes as in stages I–II, although less pronounced (Fig. 3).

Gonadotropins regulated remarkable pathways involved in gonadal functions

Taking into account that only Fsh is detected in the blood at the beginning of trout sexual maturation, one major objective of this study was to identify the transcripts that were regulated by Fsh in the testis during the pubertal transition. We therefore focused the data mining on the 226 transcripts (209 non-redundant genes) that were modulated by Fsh either specifically or not specifically

(see Supplementary information file 1). All these genes were considered as possible mediators of Fsh actions during spermatogenesis onset. The examination of gene annotation allowed the identification of signaling pathways and processes of relevant interest for the regulation of spermatogenesis. We performed qPCR validation on several of these interesting candidate genes (Figs 4 and 5). We also examined the gonadotropin responsiveness of additional candidate genes that were of particular interest in the pathways analyzed (Table 3 and Fig. 5).

**Figure 5**

qPCR for the candidate gonadotropin target genes. Testicular tissues were incubated during 96 h in the absence (Ctrl) or presence of Fsh or Lh at 500 ng/ml. After normalization to the reference gene, each individual value was further reported to the mean expression in control samples. Bars represent the mean \pm s.e.m. of five to six replicates. The Y-axis is log₂ scaled and value 1 represents the control level. The asterisks indicate statistical variations of gene expression after the hormonal treatment compared with the control samples (** $P < 0.01$, * $P < 0.05$).

Table 3 List of the additional genes studied using qPCR. These genes were either not present on the nylon membrane array used here (or were spotted in a too low amount to be correctly measured) or were not significantly differentially expressed in the array experiment presented here

SwissProt/ GenBank accession number	Gene symbol	Annotation/description	Preferential gonadotropin response	Stage	Predicted cellular origin
Genes absent from microarray					
–	<i>igf1b/igf3</i>	Insulin-like growth factor 1b	Up Fsh	I–II and III	Somatic
Q3HWG4	<i>igfbp6</i>	Insulin-like growth factor binding protein 6	Up Fsh	I–II and III	–
Q9I8S6	<i>cyp11b2-2</i>	Cytochrome P450 11 β 2	Up Fsh, Lh	I–II	–
Q71MM8	<i>fshr</i>	FSH receptor	Up Fsh, Lh	I–II and III	Somatic
–	<i>inhbb</i>	Inhibin β B chain	Up Fsh, Lh	I–II and III	–
–	<i>gsdf2</i>	Gonadal somatic cell derived factor 2	Up Lh	III	Somatic
AAC16494	<i>igfr1b</i>	Insulin-like growth factor receptor type 1b	Up Lh	I–II and III	–
NM_001165391	<i>ccnd1</i>	G1/S-specific cyclin-D1	Down Fsh	I–II and III	–
Q1HG86	<i>gsdf1</i>	Gonadal somatic cell derived factor 1	None	–	Somatic
AAC16493	<i>igfr1a</i>	Insulin-like growth factor receptor type 1a	None	–	–
Genes present on microarray, badly spotted or not significantly differentially expressed					
P30437	<i>cyp17a1</i>	Steroid 17- α -hydroxylase/17,20 lyase	Up Fsh, Lh	I–II and III (Lh)	Somatic
Q71MM9	<i>lhr</i>	LH receptor	Up Fsh, Lh	I–II and III	Somatic
P08476	<i>inhba</i>	Inhibin β A chain	Up Fsh, Lh	I–II and III	–
Q02815	<i>igf2</i>	Insulin-like growth factor 2	Up Lh	III	Somatic
Q02815	<i>igf1a</i>	Insulin-like growth factor 1a	None	–	Somatic
P57095	<i>axin2</i>	Axin-2	None	–	–

Fsh and Lh modulated gonadotropin receptor transcripts

The control of Fsh and Lh receptor gene expression is an important issue for gonadotropin responsiveness. We observed that Fsh and Lh increased the relative abundance of the two receptor transcripts in testicular tissue after 96 h of incubation. At stages I–II, *fshr* and *lhcr* were upregulated (about twofold) by both gonadotropins. At stage III, Lh was more efficient than Fsh to upregulate its cognate receptor transcripts *lhcr* (Fig. 4A and B).

Fsh and Lh stimulated the expression of genes involved in steroidogenesis

The analysis of the microarray data identified several transcripts encoding proteins involved in lipid metabolism, cholesterol efflux, and steroidogenesis such as *star*, *hsd3b1*, *cyp46a1*, and *abca1a*. We measured the differential expression pattern of some of these genes by qPCR (Fig. 5). We also analyzed the expression of two additional transcripts encoding for key enzymes of 11KT synthesis, *cyp17a1* and *cyp11b2-2* (Fig. 5). The two gonadotropins upregulated these transcripts, although differences were observed depending on the gonadotropin used or the stage of development examined. At stages I–II, Fsh was twice more potent than Lh to stimulate *star* and *hsd3b1* expressions, whereas at stage III, Fsh and Lh had similar

effects. Lh appeared more efficient than Fsh at upregulating *cyp17a1* expression at both stages. By contrast, Fsh was slightly more active than Lh at stimulating the expression of *cyp11b2-2* at stages I–II and no regulation of this transcript was detected in stage III testis.

Fsh and Lh regulated factors involved in germ cell proliferation and/or differentiation

Growth factor signaling pathways are known to be crucial for the control of spermatogenesis. Because of the suspected role of Igf1 as a mediator of spermatogonial proliferation previously reported in our group, we further investigated the Igf system. In trout, we identified three ligands (Igf1a, Igf1b/Igf3, and Igf2) and two receptors (Igf1a and Igf1b). Although the *igf2* gene is duplicated in zebrafish (*igf2a* and *igf2b*), there is no evidence of this duplication in trout and in other known fish genomes. Fsh had a marked upregulatory effect (fourfold) on the expression of the *igf1b* (*igf3*) transcript that encodes for a gonadal paralog of mammalian IGF1. The effect of the gonadotropins was specific to this paralogous gene since no change was observed for *igf1a* and *igf2* transcript expression. Interestingly, the *igfbp6* transcript encoding an Igf-binding protein (Igfbp) was also highly induced (eightfold) by Fsh. The Lh effects on *igf1b* and *igfbp6* were twice lower compared with Fsh. The *igfbp-rP1*

transcript also showed a moderate (1.5-fold) but significant upregulation by Fsh and Lh at stages I–II and by Lh at stage III. Finally, Lh appeared to have a low but significant stimulatory effect on the Igf receptor isoform *igfr1b*. In conclusion, we demonstrate that important members of the Igf regulatory pathway are targets of the gonadotropins in the fish testis. We show for the first time that the gonad-specific *igf1b* is the only Igf paralog regulated by Fsh.

The regulatory effects of the gonadotropins on the genes encoding for members of the Tgfb family were also investigated. *amh* gene expression was repeatedly down-regulated by Fsh, whereas *inha* and *fstl3* were upregulated (Fig. 5). Regarding the transcripts encoding β subunits of activins, we observed that the steady-state level of *inhba* was clearly decreased by both gonadotropins. We extended our investigations to the *gsdf1* and *gsdf2* transcripts. The *gsdf1* gene encodes for a Sertolian factor that stimulates the proliferation of spermatogonia in trout (Sawatari *et al.* 2007). The *gsdf2* gene is a paralog recently described in salmonids only (Lareyre *et al.* 2008). No regulation of these transcripts was observed in the presence of Fsh. Only Lh slightly stimulated *gsdf2* expression at stage III (Fig. 5).

Unexpectedly, the transcript similar to pleiotrophin (*mdka*), encoding a pro-angiogenic cytokine and a growth factor belonging to the neurite growth-promoting factor family, was identified as a specific target of Fsh action. The PCR results confirmed the upregulation of this transcript by Fsh only (about twofold), at both stages of maturation (Fig. 5).

Finally, microarray data analysis showed that genes linked to Wnt signaling, a pathway essential for embryonic development and neurogenesis, were also regulated by the gonadotropins. The *wisp1* and *LOC55552* (*rhou-like*) transcripts were preferentially upregulated by Fsh, whereas

the *faf1* transcript was stimulated by the two hormones. By qPCR, we confirmed the strong upregulation of *wisp1* by Fsh (about eightfold). We also explored other known downstream targets of Wnt/ β -catenin signaling including *axin2* and *ccnd1*. The *ccnd1* gene was significantly down-regulated by Fsh only (about twofold), whereas *axin2* gene expression was not affected by the gonadotropin treatments (Fig. 5 and [Supplementary information file 1](#)).

A subset of gonadotropin-regulated genes exhibited relevant expression patterns during the reproductive cycle

The physiological relevance of the *in vitro* regulation of testicular genes by gonadotropins and the predictive cellular origin of the corresponding transcripts were examined by mining the expression data obtained from trout testes collected at different stages of gonadal development and from isolated germ cell fractions, as described in Materials and methods (Rolland *et al.* 2009). Among the gonadotropin-responsive transcripts, 228 were previously identified as differentially expressed in the testis during the reproductive cycle, suggesting that they have a specific role to play during this process. Only 14 genes were predicted as testis or gonad specific (Rolland *et al.* 2009). A first observation is that a majority of these genes ($n=158$, 69.3%) were predicted to be preferentially expressed in somatic cells (Table 4; [Supplementary Figure 2](#), see section on supplementary data given at the end of this article; [Supplementary information file 1](#), column ‘testicular expression profile’). This observation is in keeping with the expression of *Lhcgr* and *Fshr* in the somatic cells of the testis. The second striking result, illustrated in Fig. 6, is that more than 70% of the somatic genes upregulated by gonadotropins *in vitro* were highly expressed at

Table 4 Predicted cellular origin of the 228 gonadotropin-regulated transcripts deduced from their expression in different stages of testis development and in isolated germ cell fractions enriched in spermatogonia or in more differentiated germ cells (Rolland *et al.* 2009). Data are expressed as a percentage of the total number of genes clustered according to their response to the gonadotropin treatments. The highest values are presented in boldface.

	Response to gonadotropin treatment					
	Up Fsh	Up Fsh, Lh	Up Lh	Down Lh	Down Fsh, Lh	Down Fsh
Testicular expression profile	30	66	38	36	20	38
Somatic expression	83.3	80.3	55.3	38.9	75.0	78.94
Somatic and spermatogonia	6.7	3.0	5.2	8.3	5.0	10.53
Germ cell expression	10.0	16.7	39.5	52.8	20.0	10.53

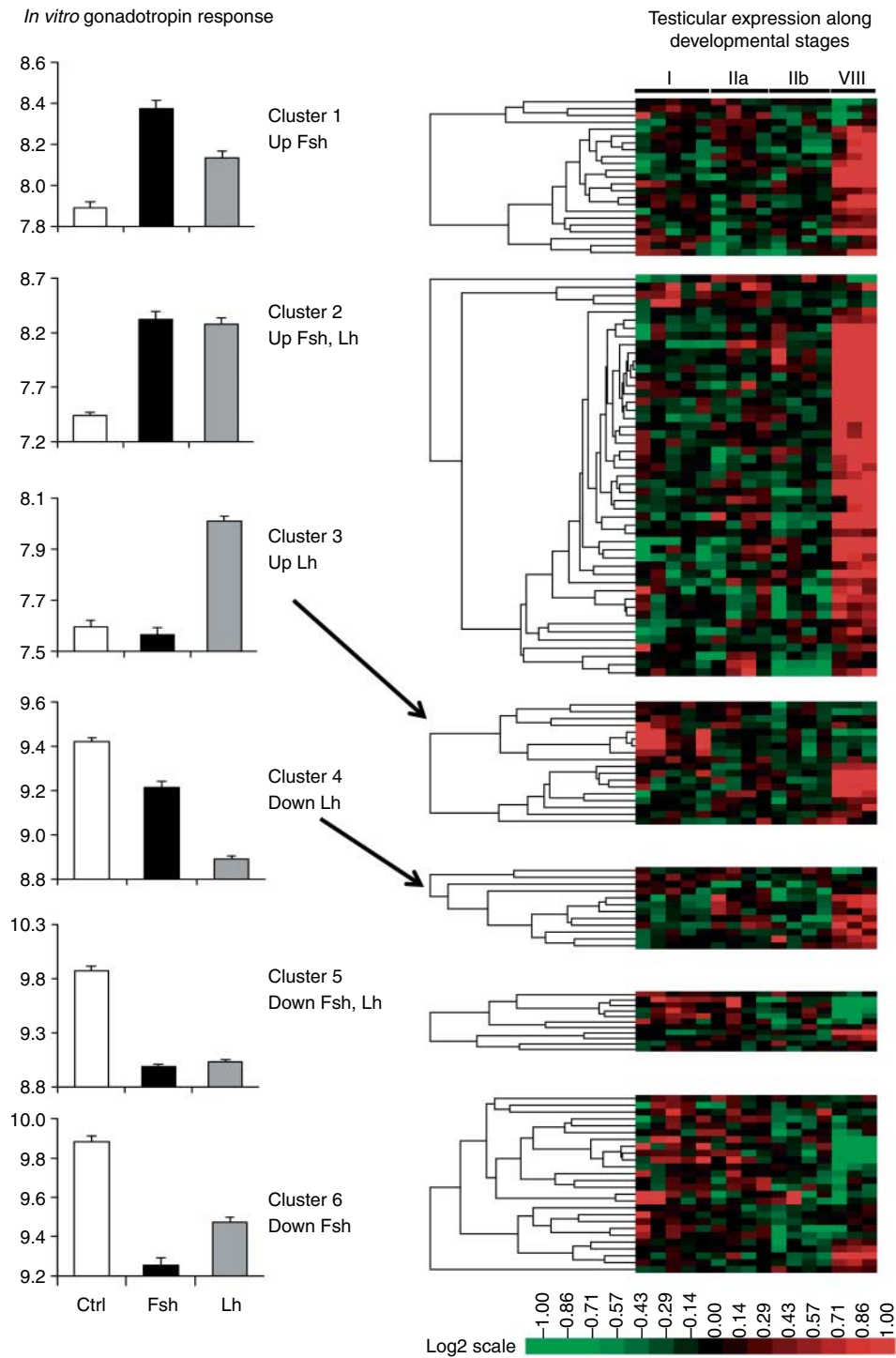
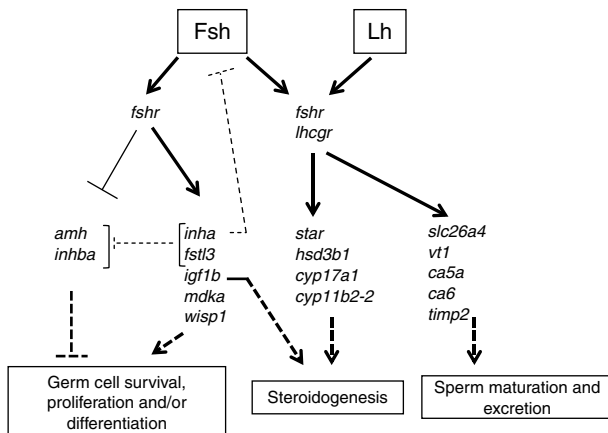


Figure 6

Expression profile of the somatic gonadotropin-regulated genes in the whole testis at different developmental stages. Right: Heatmap representation of the 132 gonadotropin-regulated genes that predicted the origin as somatic. Expression data were obtained from the testes at various developmental stages and from the enriched fractions of isolated germ cells. Roman numerals (I, IIa, IIb, and VIII) indicate the testicular

developmental stages (Rolland *et al.* 2009). Log2 transformed signal intensities are shown according to the scale bar. Left: Median expression, at stages I–II, of the 132 gonadotropin-regulated genes in clusters 1–6 that predicted the origin as somatic. Ctrl, no hormonal treatment; Fsh and Lh, treatment with Fsh or Lh (500 ng/ml) during 96 h.

**Figure 7**

Schematic representation of the predicted function of Fsh- and/or Lh-dependent genes. Fsh and Lh stimulate the gonadotropin receptor genes. Activation of downstream signaling pathways results in the stimulation (arrows) or inhibition (broken line) of the genes. Fsh modulates the genes that have been shown to regulate germ cell survival, proliferation, and/or differentiation. Genes involved in steroidogenesis and sperm maturation/excretion are regulated by both gonadotropins. Besides a role in the paracrine regulation of germ cell proliferation, the positive regulation of *inha* by Fsh (and Lh) suggests that inhibin could exert a negative feedback on the Fsh release as demonstrated in mammals.

stage VIII. Some of these genes were involved in pH regulation, ion transport, tissue remodeling, hydration, and/or smooth muscle contraction (*slc26a4*, *ca5a*, *ca6*, *timp2*, and *vt1*). This suggests that these genes may have a physiological role in sperm maturation or excretion at the end of the reproductive cycle.

Unexpectedly, 56 gonadotropin-regulated transcripts were predicted to be expressed in germ cells. Among the genes regulated by Lh or Fsh, we noticed genes encoding for a cyclin (*ccnb1*), two ATPases involved in proton transport (*atp6v1f* and *atp6ap2*), an apoptotic factor (*ddit4*), and other proteins (*mmp19*, *mina*, *odc1*, and *casp8*) involved in gonadal or germ cell development. Remarkably, Lh affected the expression of a greater number of genes predicted to be expressed in the germ cells compared with Fsh (Table 4).

Discussion

Fsh and Lh gonadotropins modulate the expression of common and distinct genes

Using trout cDNA arrays, we identified the transcripts that were regulated *in vitro* by gonadotropins in testicular explants of pubertal trout after 96 h of exposure.

An important proportion of the transcripts were preferentially up- or downregulated by one or the other

gonadotropin. This observation is consistent with the fact that in trout, Fsh and Lh activate preferentially their cognate receptor at the concentration used here (Sambroni *et al.* 2007). Among the genes preferentially regulated by Fsh, we found the transcripts that were previously shown to be expressed in the Sertoli cells, such as *amh* in fish (Skaar *et al.* 2011) and *Wisp1*, *Abca1*, *Smtn*, *Krt18*, *Slc40a1*, *Col1a1*, *Ccnd1*, and *Fstl3* in mice (Chalmel *et al.* 2007). This is in agreement with the fact that the Fsh receptor would be the only gonadotropin receptor expressed in the salmonid Sertoli cells (Miwa *et al.* 1994). The known or putative function of the Fsh-dependent genes will be detailed below.

We also identified 'somatic' genes that were preferentially modulated by Lh, among which several could be of interest concerning the testicular cell fate, such as *pvr11* encoding a poliovirus receptor-related protein 1 precursor (nectin-1), involved in intercellular adhesion and cell migration, or a tripartite motif protein similar to blood-thirsty, a *trim* gene required for erythropoiesis in zebrafish (Yergeau *et al.* 2005). Many more genes whose function in the gonad remains elusive deserve further examination. Moreover, a noticeable proportion of these genes were predicted to be expressed in germ cells, probably reflecting Lh function in germ cell differentiation or survival. Finally, opposite effects of the two gonadotropins on the expression of a few genes were observed, which reinforces the reality of the differential effects.

More surprisingly in reference to what is described in mammals, our data indicated that one-third of the regulated transcripts were similarly regulated by both Fsh and Lh gonadotropins. Furthermore, among the genes preferentially regulated by Fsh, we found genes known to be regulated by LH in humans or mice as, for example *hsd3b1* or *odc*, the rate-limiting enzyme of polyamine synthesis (Osterman *et al.* 1983). This could be explained by the fact that both receptors, Fshr and Lhcgr, are expressed in the interstitial cells (likely Leydig cells) of the fish testis (see Introduction and Schulz *et al.* (2010)) and could activate common downstream signaling pathways. Furthermore, both receptors were detected in zebrafish seminiferous tubules. Besides, in fish, one could hypothesize that both gonadotropins activate the same receptor type in the target cells. However, as mentioned above, such promiscuous action of Lh on Fsh receptor activity appears unlikely because we previously showed that trout gonadotropins used at 500 ng/ml did not cross-activate their reciprocal receptor (Sambroni *et al.* 2007). Finally, the common effects of the gonadotropins could, at least in part, be mediated through indirect actions resulting from the stimulation of sexual steroids. Indeed,

we found a stimulation of 11KT production by both gonadotropins, together with a positive regulation of mRNA abundance of several key actors involved in steroid metabolism (*star*, *hsd3b1*, *cyp17a1*, and *cyp11b2-2*). This latter observation is in agreement with previous data obtained *in vitro* in eel (Kazeto *et al.* 2008), zebrafish (Garcia-Lopez *et al.* 2010), or female coho salmon (Luckenbach *et al.* 2011). In fact, several genes regulated by both Fsh and Lh were also found to be similarly regulated by androgens *in vivo* (A D Rolland, A Lardenois, A-S Goupil, J-J Lareyre, F Chalmel, R Houlgatte and F Le Gac, unpublished observations).

Gonadotropin-responsive genes exhibited relevant patterns during the seasonal reproductive cycle

When investigating the expression pattern of the gonadotropin-regulated genes during natural testicular maturation in trout, we found that a majority of them were predicted to be expressed in the somatic cells (about 70%). This observation is in agreement with the expression of the gonadotropin receptor genes in the somatic testicular cells described previously in different teleostean fish species including zebrafish, catfish, eel, and coho salmon (Miwa *et al.* 1994, Ohta *et al.* 2007, Garcia-Lopez *et al.* 2009, 2010).

We further identified a number of genes upregulated *in vitro* by both Fsh and Lh that are also found to be naturally and strongly increased at the end of the reproductive cycle, when both gonadotropins and their receptors reach their maximum secretion and expression levels, respectively (Gomez *et al.* 1999). Such a correlation during the reproductive cycle reinforces the physiological significance of our data. It also shows that, in addition to Lh, Fsh may have important functions during the final stages of sperm maturation and transportation.

In the present study, we show that both gonadotropins upregulate *in vitro* the relative abundance of both *fshr* and *lhcr* transcripts after a 96-h incubation time. Similar observations were described in mammals. FSH treatment upregulated *Lhcr* gene expression and steroidogenesis output in the Leydig cells of hypophysectomized immature rats (Vihko *et al.* 1991). In trout, gonadotropins reach their maximum levels just before and during the spawning period at stages VII–VIII (Gomez *et al.* 1999). The positive regulation of the gonadotropin receptor genes observed *in vitro* may explain the dramatic increase in *fshr* and *lhcr* gene expression described *in vivo* at stage VIII (Sambroni *et al.* 2007). The increased availability of the gonadotropins and their receptors may result in the amplification of the gonadotropin effects on

gene expression. In agreement with this assumption, we noticed that a great number of the gonadotropin-responsive genes were highly regulated during the spawning period only (stage VIII).

Molecular and cellular functions underlying the gonadotropin-responsive genes give new insights on the Fsh mechanism of action

The identification of the mediators that relay Fsh actions is of particular relevance in trout since Fsh is the only gonadotropin detected during the pubertal transition and the first stages of testis maturation and is thought to have a major role on early steroidogenesis and spermatogenesis (Gomez *et al.* 1999). However, little is known on the molecular mechanisms of Fsh action. For these reasons, we particularly focus on the genes that were regulated by Fsh.

We showed that Fsh strongly upregulates *igf1b* (*igf3*) and *igfbp6* in the testis. *Igf1b* is a paralog of mammalian *Igf1*, restricted to teleostean fish species and exclusively expressed in the gonads (Wang *et al.* 2008, Zou *et al.* 2009). A recent study showed that hCG increased *igf3* expression in zebrafish ovaries (Irwin & Van Der Kraak 2012). Although another report proposed that *igf1b* might be regulated by the Fsh signaling pathway (Baudiffier *et al.* 2012), the present study is the first demonstration that Fsh upregulates the *igf1b* gene. In our laboratory, we previously demonstrated that rainbow trout spermatogonia harbored type I Igf receptors and that recombinant human or fish IGF1 stimulated their proliferation *in vitro* (Loir & Le Gac 1994, Le Gac *et al.* 1996). In addition, we showed that Fsh stimulated the proliferation of spermatogonia when co-cultured with Sertoli cells (Loir 1999b). Altogether, these observations suggest that Fsh increases Igf1b production by the Sertoli cells and, in turn, Igf1b could act as a direct mediator of spermatogonial proliferation. Besides its involvement in the control of spermatogonial proliferation, it has been demonstrated, in tilapia, that recombinant Igf1b upregulated the transcripts encoding for steroidogenic enzymes and transcription factors (*dmrt1*, *nr5a1*, and *foxl2*) involved in their regulation (Li *et al.* 2012). The authors have concluded that Igf1b could be the primary growth factor involved in the regulation of gonadal steroidogenesis. In addition to *igf1b*, we noted that Fsh stimulated the expression of the *igfbp6* transcript. Igfbp are extracellular Igf transport proteins and are considered to be important modulators of Igf actions. Igfbp can either sequester the Igf away from their receptors or they can increase Igf availability through interactions with matrix components located near their

receptors, thus enhancing Igf activity (Firth & Baxter 2002). Interestingly, no Fsh regulation was observed for the Igf1a and Igf2 ligands. The strong upregulation of *igf1b* and *igfbp6* by Fsh reinforces the idea that the Igf regulatory pathway is a major paracrine pathway that relays Fsh actions on both spermatogenesis and steroidogenesis.

Fsh regulated several genes encoding Tgfb factors, a superfamily known to exert a crucial role in gonadal physiology. They include the *amh*, *inha*, and *inhba* genes. We found that *amh* was downregulated by Fsh in trout. In Japanese eel, Amh (termed spermatogenesis-preventing substance) prevents spermatogonia proliferation and differentiation and is downregulated by 11KT (Miura *et al.* 2002). Recently, in experiments using adult zebrafish testis tissue culture, Amh was shown to inhibit spermatogenesis and was downregulated by Fsh (Skaar *et al.* 2011). Interestingly, during the natural trout reproductive cycle, *amh* mRNA levels are abundant in Sertoli cells in stages I–II and become hardly detectable towards spawning (Rolland *et al.* 2009), when gonadotropins and androgen plasma levels are high (Gomez *et al.* 1999). Thus, the Fsh-induced downregulation of *amh* observed in the present study *in vitro* could reflect the physiological activity of Fsh during the maturation of the testis. In conclusion, there is accumulating evidence that Fsh suppresses the inhibitory action of Amh, allowing the commitment of germ cells to spermatogenesis.

The activins A and B are homodimers of the β subunits $\beta\alpha$ and $\beta\beta$. These subunits are encoded by the *inhba* and *inhbb* genes, respectively. The inhibins are heterodimers of an α subunit (encoded by *inha*) and one of the β subunits shared with the activins. Our data showed that the transcripts encoding for the three subunits (*inha*, *inhba*, and *inhbb*) are regulated independently by Fsh and Lh. *inhba* was downregulated by both gonadotropins, whereas *inhbb* was moderately downregulated by Lh. In rodents, numerous *in vivo* and *in vitro* studies have demonstrated the importance of activin A and its regulators (inhibin and follistatin) in the control of spermatogenesis (de Kretser *et al.* 2004, Barakat *et al.* 2008, 2012). In fetal and postnatal testis, activin A promotes both Sertoli cell and gonocyte proliferation but hampers the differentiation of gonocytes in spermatogonia (Mithraprabhu *et al.* 2010, Fan *et al.* 2012). Meehan *et al.* (2000) hypothesized that a reduction of activin A bioactivity is required for the onset of spermatogenesis in the early postnatal testis, to drive the differentiation of spermatogonial germ cells. Taken together, these observations suggest that Fsh could stimulate spermatogonial differentiation through the inhibition of the $\beta\alpha$ subunit.

Interestingly, the *inha* transcript was upregulated by Fsh and Lh. In addition, follistatin-like 3 (Fstl-3) transcripts were increased by Fsh treatment. Fstl-3 is predominantly expressed in mouse testis (Xia *et al.* 2004) and, similar to inhibin, is able to antagonize the actions of activins (Schneyer *et al.* 2004). In summary, our data suggest that Fsh actions converge to an inhibition of both Amh and activin signaling to finely adjust the balance between germ cell proliferation and differentiation.

Gsdf is another teleostean specific member of the Tgfb family predominantly expressed in the Sertoli cells surrounding spermatogonia (Sawatari *et al.* 2007, Gautier *et al.* 2011). This factor has been involved in the proliferation of primordial germ cells (Sawatari *et al.* 2007), early testicular differentiation (Shibata *et al.* 2010), and sex determination in one fish species (Myosho *et al.* 2012). Surprisingly, our data indicate that Fsh did not regulate *gsdf* gene expression. In agreement with our results, *gsdf* gene expression was not regulated by Fsh in coho salmon ovary (Luckenbach *et al.* 2011) or in the mature male testis of zebrafish (Garcia-Lopez *et al.* 2010). In summary, although Gsdf was proposed as a stimulatory factor of germ cell proliferation, there is no evidence that its expression is regulated by Fsh in fish.

Pleiotrophin mRNA levels were exclusively upregulated by Fsh. Pleiotrophin is a secreted heparin-binding cytokine that regulates numerous functions, including mitogenesis, angiogenesis, and cell differentiation. Its role in the testis is poorly understood, except that a dominant negative mutation led to an increase in germ cell apoptosis in mice (Zhang *et al.* 1999). Considering pleiotrophin (*mdka*) functions, this growth factor specifically regulated by Fsh appears as a new strong candidate gene regulating germ cell survival in trout.

Our study showed that Fsh regulates the steady-state level of several mRNAs connected with the Wnt pathway (*wisp1*, *LOC555552*, and *faf1*). The activation of the Wnt pathway was involved in the proliferation and self-renewal of mouse and human spermatogonia (Boyer *et al.* 2008, Tanwar *et al.* 2010). In the present study, we observed that Fsh negatively regulated the *ccnd1* gene. Interestingly, a similar negative regulation has also been described in mice (Meachem *et al.* 2005). In this species, the expression of cyclin D1 is detected only in proliferating gonocytes and spermatogonia, suggesting a role in the G(1)/S phase transition (Beumer *et al.* 2000). One could hypothesize that the high levels of Fsh observed at stage VIII could suppress cyclin D1 expression and maintain the germ stem cells in a quiescent status.

Interestingly, the expression of genes encoding components or remodeling enzymes of the extracellular matrix

was also found to be regulated by Fsh (*mmp19*, *mmp9*, *timp2*). In the gilthead seabream, *mmp9* and *timp2* are expressed in the testis and are differentially expressed during the reproductive cycle, in association with testis remodeling (Chaves-Pozo *et al.* 2008). In rats, metalloproteinases are expressed in Sertoli cells and have been involved in the changes in the Sertoli cell cytoskeleton elicited by FSH (Longin & Le Magueresse-Battistoni 2002). Metalloproteinases could also participate in the regulation of the Sertoli cell junction dynamic that is important for the function of the hemato–testicular barrier (Siu *et al.* 2003). This suggests that, in addition to the stimulation or inhibition of paracrine growth factors, Fsh could be involved in the dramatic remodeling of the testicular cystic structure that occurs during the pubertal transition. Further investigations will be required to determine whether the components of the extracellular matrix or their modifications are involved in the differentiation of Sertoli cells and/or germ cells.

In conclusion, we showed for the first time on a large scale that Lh and Fsh have similar but also preferential or specific regulatory effects on numerous genes in the fish testis. We confirm that both gonadotropins stimulate the production of androgens and the expression of several steroidogenic enzyme genes. Further investigations will be required to determine to what extent the similar effects of Fsh and Lh are mediated through the stimulation of sexual steroid production. Our data suggest that Fsh acts through multiple convergent regulatory pathways to finely adjust the balance between germ cell quiescence, proliferation, and/or differentiation. These regulatory pathways include the stimulation of the Igf system and the inhibition of Amh and activin pathways. Finally, the present study identifies new gonadotropin-responsive pathways or genes and provides a solid foundation for further studies on the physiological relevance of Fsh and Lh in fish models.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-12-0197>.

Declaration of interest

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

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Author contribution statement

E S performed the tissue culture experiments, the microarray data analyses, and the real-time PCR validations, and drafted the manuscript. F L G and J-J L supervised the study. E S, A R, and F L G participated in the analyses of the microarray data, and F L G and J-J L polished the manuscript. All authors read and approved the final version of the manuscript.

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