General and Comparative Endocrinology 285 (2020) 113293

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Gnrh receptor *gnrhr2bbα* is expressed exclusively in *lhb*-expressing cells in Atlantic salmon male parr

Elia Ciani[a,1,2], Romain Fontaine[a,3], Gersende Maugars[a,4], Rasoul Nourizadeh-Lillabadi[a,5], Eva Andersson[b,6], Jan Bogerd[c,7], Kristine von Krogh[a,8], Finn-Arne Weltzien[a,*,9]

[a] Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Department of Basic Science and Aquatic Medicine, Oslo, Norway
[b] Institute of Marine Research, Bergen, Norway
[c] Utrecht University, Faculty of Science, Department of Biology, Reproductive Biology Group, Utrecht, The Netherlands

ARTICLE INFO

Keywords:
Double fluorescent *in situ* hybridization
Follicle-stimulating hormone
Gonadotropin-releasing hormone receptors
Gonadotropin regulation
Luteinizing hormone
Precocious parr

ABSTRACT

Gonadotropin-releasing hormone (Gnrh) plays a major role in the regulation of physiological and behavioural processes related to reproduction. In the pituitary, it stimulates gonadotropin synthesis and release via activation of Gnrh receptors (Gnrhr), belonging to the G protein-coupled receptor superfamily. Evidence suggests that differential regulation of the two gonadotropins (Fsh and Lh) is achieved through activation of distinct intracellular pathways and, probably, through the action of distinct receptors. However, the roles of the different Gnrhr isoforms in teleosts are still not well understood. This study investigates the gene expression of Gnrhr in the pituitary gland of precociously maturing Atlantic salmon (*Salmo salar*) male parr. A total of six Gnrhr paralogs were identified in the Atlantic salmon genome and named according to phylogenetic relationship; *gnrhr1caα, gnrhr1caβ, gnrhr1cbα, gnrhr1cbβ, gnrhr2bbα, gnrhr2bbβ*. All paralogs, except *gnrhr1caα,* were expressed in male parr pituitary during gonadal maturation as evidenced by qPCR analysis. Only one gene, *gnrhr2bbα*, was differentially expressed depending on maturational stage (yearly cycle), with high expression levels in maturing fish, increasing in parallel with gonadotropin subunit gene expression. Additionally, a correlation in daily expression levels was detected between *gnrhr2bbα* and *lhb* (daily cycle) in immature fish in mid-April. Double fluorescence *in situ* hybridization showed that *gnrhr2bbα* was expressed exclusively in *lhb* gonadotropes in the pituitary, with no expression detected in *fshb* cells. These results suggest the involvement of receptor paralog *gnrhr2bbα* in the regulation of *lhb* cells, and not *fshb* cells, in sexually maturing Atlantic salmon male parr.

1. Introduction

The complex series of morphological and physiological changes occurring during sexual maturation in vertebrates is driven through increased activity in the brain-pituitary-gonad axis. The pituitary gland is responsible for the production of gonadotropins: follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh). These are heterodimeric glycoproteins composed of a common α-subunit and a specific β-subunit conferring the biological activity (Pierce and Parsons, 1981; Swanson et al., 2003). Different from mammals, where both gonadotropins are produced in the same cells (Childs et al., 1986; Liu et al., 1988), Fsh and Lh are produced by different cell types in teleost fish (Kanda et al., 2011; Naito et al., 1993).

The physiological role of gonadotropins in the testes of male teleosts, and salmonids in particular, has been widely studied. Both gonadotropins are capable of inducing, with comparable efficiency, the

* Corresponding author.
E-mail address: finn-arne.weltzien@nmbu.no (F.-A. Weltzien).
[1] Present address: University of Oslo, Faculty of Mathematics and Natural Sciences, Department of Pharmacy, 0316 Oslo, Norway.
[2] Orcid: https://orcid.org/0000-0001-8153-2073
[3] Orcid: https://orcid.org/0000-0003-1123-9773
[4] Orcid: https://orcid.org/0000-0002-2090-6585
[5] Orcid: https://orcid.org/0000-0003-0328-5569
[6] Orcid: https://orcid.org/0000-0002-7864-1014
[7] Orcid: https://orcid.org/0000-0003-1120-7974
[8] Orcid: https://orcid.org/0000-0003-4886-9699
[9] Orcid: https://orcid.org/0000-0002-5111-1558

https://doi.org/10.1016/j.ygcen.2019.113293
Received 14 July 2019; Received in revised form 18 September 2019; Accepted 29 September 2019
Available online 30 September 2019

production of sex hormones (11-ketotestosterone, 11-KT; testosterone, T) by steroidogenic (Leydig) cells in the gonads (Planas and Swanson, 1995; Suzuki et al., 1988; Swanson et al., 1991). In addition, Fsh is involved in the induction of spermatogonial proliferation and spermatogenesis (García-López et al., 2009; Loir, 1999; Mazón et al., 2014), while Lh is involved in the final stages of gamete maturation, regulating spermiogenesis and spermiation (Schulz et al., 2010). In male salmonids, both *fshb* mRNA in the pituitary (Maugars and Schmitz, 2008a; Melo et al., 2014) and Fsh plasma protein levels (Campbell et al., 2003) increase during early stages of maturation. In contrast, *lhb* mRNA and Lh plasma protein levels are low or undetectable during the onset of testes development, but become detectable when germ cells enter meiosis and increase sharply close to spawning season (Breton et al., 1997; Gomez et al., 1999; Prat et al., 1996). In Atlantic salmon male parr, changes in expression of gonadotropins, together with plasma steroid levels and gonad histology were reported in detail in Maugars and Schmitz, (2008a,b). On the basis of early presence in plasma and involvement in steroidogenesis and gametogenesis, Fsh is therefore considered to be the main actor during early stages of maturation while Lh is mainly involved during final stages of maturation and spawning (for review see Levavi-Sivan et al., 2010; Weltzien et al., 2004).

Gonadotropin synthesis and release is considered to be induced mainly by gonadotropin-releasing hormone (Gnrh) in teleosts (Peter, 1983), as in all vertebrates (Conn and Crowley, 1994). Gnrh acts via Gnrh receptors (Gnrhr) belonging to the 7-transmembrane (7TM) G protein-coupled receptor (GPCR) superfamily. Gnrhr exert their biological activity through interaction with two types of G protein, $Gq_{/11}$ and Gs. Coupling to the former activates a cascade involving phospholipase C (PLC), inositol-3-phosphate (IP3), diacylglycerol (DAG), and intracellular calcium (Ca^{2+}), leading to activation of protein kinase C (PKC) (Levavi-Sivan and Yaron, 1989; Naor, 1990). Coupling to the latter increases intracellular cAMP and activates protein kinase A (PKA) (Arora et al., 1998; Liu et al., 2002; Wilson et al., 1994). It is suggested that transduction of the Gnrh signal leading to expression of *lhb* and the common α-subunit is mainly mediated through the PKC cascade, while that leading to *fshb* expression is mediated through the cAMP/PKA cascade (for review see Levavi-Sivan et al., 2010; Yaron et al., 2003).

While in mammals the action of GnRH is mediated through the action of two different receptor sub-types or paralogs (Hapgood et al., 2005), several paralogs have been identified in teleosts. For instance, some species possess up to five Gnrhr paralogs, such as in seabass (*Dicentrarchus labrax*; Moncaut, 2005) and masu salmon (*Oncorhynchus masou;* Jodo et al., 2003). Although multiple paralogs have been identified in teleost species, there is currently no clearly defined consensus nomenclature for these variants. The proposed classification systems divide the Gnrhr in two (Flanagan et al., 2007; Hildahl et al., 2011; Lethimonier et al., 2004; Moncaut, 2005), three (Levavi-Sivan and Avitan, 2005; Millar et al., 2004), or four (Ikemoto et al., 2004; Ikemoto and Park, 2005; Kim et al., 2011) groups, each separated into different subgroups. Recently, two different evolutionary scenarios have been proposed: the first one (Sefideh et al., 2014), based on combined phylogeny and detailed synteny analysis, suggests the existence of two types of Gnrhr divided into three subtypes each. According to this scenario, the two types of Gnrhr result from a local genome duplication prior to the two vertebrate whole genome duplications (1R and 2R), while the six different subtypes result from 1R and 2R. Within one of these subtypes (named GnRHR2ba) additional teleost paralogs have arisen from local duplications subsequent to the teleost-specific 3R. The second scenario (Williams et al., 2014), based on phylogeny and probabilistic protein homology search, argued low evidence of ancestral local duplications and suggests the formation of 3 Gnrhr after 1R and 2R. One paralog duplicated locally in the osteichthyan lineage and a second one in the sarcopterygian lineage leading to the formation of 5 Gnrhr subtypes. Whatever the scenario, the teleosts are present only in two Gnrhr subtypes, each including at least two paralogs resulting from 3R (Sefideh et al., 2014). Considering that salmonids went through a fourth genome duplication (4R), the additional 4R paralogs may have gained new specialized functions.

Regulation of gonadotropin expression is a critical factor for the induction and completion of gonad maturation (Levavi-Sivan et al., 2010; Weltzien et al., 2004), however, the specific role of the different Gnrhr isoforms are still largely unknown. In male Atlantic salmon, sexual maturation shows great plasticity. Maturation may occur after one (grilse) or several years at sea, or at an earlier stage and smaller size as parr during the freshwater phase (Garcia De Leaniz et al., 2007; Hutchings and Jones, 1998; Taylor, 1991). This study investigates the potential role of six *gnrhr* paralogs in the pituitary of male Atlantic salmon parr during gonadal maturation.

2. Materials and methods

2.1. Animals

This study was performed on male Atlantic salmon (*Salmo salar*) parr farmed at the Norwegian Institute for Nature Research (NINA) at Ims, Norway (58°54′N, 5°57′E). Fertilized eggs were produced using first generation broodstock from wild caught salmon from the river Figgjo (58°47′ N 5°47′ E) for three consecutive seasons, hatching on March 13th, 2015 (batch 1), February 22nd, 2016 (batch 2), and March 14th, 2017 (batch 3). After first feeding, fish were reared in outdoor tanks (volume 7.8 m^3) under natural conditions regarding photoperiod and water temperature (range, 5–21 °C). Fish from batches 1 and 2 were 14 months old, while fish from batch 3 were 7 months old at the beginning of the experiment. All experiments were performed according to EU regulations concerning the protection of experimental animals (Directive 2010/63/EU). Appropriate measures were taken to minimize pain and discomfort (FOTS application ID12523).

2.2. Identification of maturing fish

The classification of fish according to their maturational stage is described in details in Ciani et al., 2019b. In brief, gonadosomatic index (GSI = gonad weight/body weight*100) and testes histology (identification of the most advanced germ cell in the tissue) were used to discriminate between two groups: non-maturing (GSI < 0.05; most advanced germ cell = spermatogonia type A) and maturing (GSI < 0.05; most advanced germ cell = from spermatogonia type B to spermatozoa).

2.3. Gnrh receptor identification

The *Salmo salar* Gnrhr were identified from the Atlantic salmon genome assembly (ICSASG v2). For validation, all sequences were cloned and sequenced at GATC biotech (Germany). The cloned sequences were uploaded in NCBI database (Supplementary table 1). Gnrhr sequences were searched for also in the genome of other vertebrate species including representatives of Actinopterygian, Sarcopterygian, and a Chondrichthyan, the catshark (*Scyliorhinus torazame*). Gnrhr sequences were retrieved from GenBank genomic databases using predicted gene annotations or after blast search. Sequences were collected and analysed on CLC Main Workbench 8 (Quiagen). The annotation of non-annotated *gnrhr* genes were performed manually using the Blast alignment of the genomic sequence carrying the putative *gnrhr* with protein sequence of related species *gnrhr*. The genes were named according to the classification of Sefideh et al. (2014) following the HGNC and ZFin recommended conventions. Duplicate receptors arising from 3R were discriminated with the addition of the suffix “a” or “b” after the gene symbol and the ones arising from 4R, with the “α” and “β” suffix. The clade nomenclature was based on the one proposed by Hildahl et al. (2011). “GnRHR” was used to designate the type and subtype. All sequence references and genome assembly information are given in Supplementary table 1.

2.4. Phylogenetic analysis

Multiple alignments of Gnrhr amino acid sequences were performed using Clustal O alignment tool available on CLC workbench and further edited manually. The tree topology was inferred using Maximum Likelihood algorithm using PhyML 3.0, the Smart Model Selection and the Subtree pruning and regrafting (SPR) with topological rearrangement as options on ATGC Montpellier bioinformatic web browser (Guindon et al., 2010; Lefort et al., 2017). Strength of branch nodes was first evaluated by aLRT test and then by bootstrapping using 100 replicates. The consensus tree was plotted using the R package ggtree (Yu et al., 2017).

2.5. Gene expression analysis

All fish were anesthetized with Pharmaq MS222 (Overhalla, Norway; 80 mg/l) and euthanized via quick decapitation prior to tissue sampling. i) To study gene expression during maturation, individual pituitaries were collected from maturing and non-maturing males (n = 6 per group) every two weeks from May to July 2016 (batch 1). This experiment was replicated the following season, anticipating last year's sampling by one month, from April to July 2017, for the investigation of earlier stages of maturation (batch 2), and in under-yearling fish from July to October. ii) In order to study tissue distribution of *gnrhr* expression, the following tissues were collected from batch 2 fish on July 4th, 2017: telencephalon, optic nerves, optic tectum, cerebellum, medulla oblongata/diencephalon, pituitary, eyes, testes, skin (n = 5). iii) To study daily pituitary gene expression, individual pituitaries from non-maturing fish (batch 3) were collected during a 24 h period at 4-hour intervals (at 04.00, 08.00, 12.00, 16.00, 20.00, 24.00) in autumn 2017 (23 October; Sunrise 08.33; Sunset 18.08; n = 6 per time point) and spring 2018 (13 April; Sunrise 06.29; Sunset 20.47; n = 10 per time point). In all cases, pituitaries were collected in 300 µl TRIzol reagent (Invitrogen, Waltham, USA), while other tissues were collected in 1 ml RNA*later* (Sigma-Aldrich, St. Louis, USA). All samples were stored overnight at 4 °C, then frozen at −20 °C until RNA extraction. The collected pituitary samples were also used for gene expression analysis reported in Ciani et al., (2019a,b)

Gene expression analysis, from RNA extraction to qPCR protocols, were performed as described in Weltzien et al., (2005) and Ciani et al., (2019a). In brief, total RNA was isolated from individual pituitaries using TRIzol reagent (Invitrogen) and treated with 2U DNase (TURBO DNA-free kit, Ambion) according to the manufacturer's instructions. RNA quality was measured via Bioanalyzer 2100 (Agilent, Santa Clara, USA). All samples showed RIN values above 8 and 260/280 ratios above 1.8. The concentration of total RNA was measured using NanoDrop (batch 1; Thermo Scientific, Waltham, USA) spectrophotometer or Qubit Fluorometer (batch 2 and 3; Invitrogen). One µg (batch 1) or 170 ng (batch 2 and 3) total RNA was reverse transcribed (RT) from each pituitary using SuperScript III reverse transcriptase (Invitrogen) and 5 µM random hexamer primers (Invitrogen) following the manufacturer's instructions. Specific qPCR primers were designed using Primer-Blast from NCBI website (Ye et al., 2012) (Table 1). To insure target specificity, the primers were designed in divergent regions of 4R paralogs *gnrhr1ca-α, -β* and *gnrhr2bb-α, -β*. Due to the high degree of sequence identity between 4R paralogs *gnrhr1cb-α, -β,* the primer couple used could amplify both transcripts (therefore indicated as *gnrhr1cb)*. Primer efficiency was measured via dilution series, each step in triplicate, of RT product from pooled total RNA and calculated using LightCycler96 (Roche) dedicated software. Thermal cycling was performed using LightCycler96 (Roche) and SYBR Green I master (Roche) kit. Real-time conditions were 10 min incubation at 95 °C followed by 40 cycles at 95 °C for 10 sec, 60 °C for 10 sec and 72 °C for 8 sec. To confirm target specificity, initial qPCR products were sequenced by GATC biotech. Relative gene expression levels were calculated using GenEx software (Mangalam et al., 2001) and normalized against the geometric mean of two reference genes (*rna18s* and *ef1α*) as outlined by Vandesompele et al. (2002). Stability of the housekeeping genes were tested using the online tool RefFinder (Kim et al., 2010).

2.6. Testes histology

Testes were fixed in 4% glutaraldehyde (VWR, Radnor, USA) overnight at 4 °C, then stored in 70% ethanol (EtOH) at 4 °C until histological analysis, performed as described in detail in (Ciani et al., 2019b). In short, tissues were dehydrated in increasing concentrations of ethanol and then embedded in plastic resin (Technovit 7100 (Heraeus Kulzer, Hanau, Germany)). Cured samples were sectioned at 3 µm thickness using a Leica RM2245 microtome (Leica Biosystems, Wetzlar, Germany), at 30 – 60 µm intervals from the periphery to the middle of the tissue. Dried sections were stained with Toluidine Blue O (Sigma-Aldrich). At least five sections per testes were analysed. Testes maturational stages were determined by the most advanced germ cell present in the tissue (Table 2), according to the description by Melo et al. (2014).

2.7. In situ hybridization analysis

Individual pituitaries from maturing fish (batch 2) were collected for *in situ* hybridization analysis of gonadotropin and Gnrhr mRNA distributions. Prior to dissection, cardiac perfusion with 4% paraformaldehyde (PFA) was performed on deeply anesthetized fish (Pharmaq MS222, 80 mg/l) in order to remove blood cells from the tissue. Pituitaries were then fixed overnight in 4% PFA, dehydrated with increasing concentrations of ethanol (from 25% to 100%) and stored in 100% methanol at −20 °C until use. Specific primers were designed via Primer-Blast from NCBI website (Ye et al., 2012) to amplify the transcript of the genes of interest (*fshb*, *lhb*, *gnrhr2bbα, gnrhr2bbβ*; Table 1) by PCR. The products were then isolated after gel extraction using Gel Extraction Kit (Invitrogen), cloned in pCRII Vector (Invitrogen) and sequenced by GATC biotech. Plasmids were linearized by PCR amplification using M13 primers. Antisense and sense cRNA probes were synthetized by *in vitro* transcription on linearized plasmids using T7 or SP6 RNA polymerase (Promega, Madison, Wisconsin) and marked with digoxigenin-11-UTP or fluorescein-12-UTP (Roche). Pituitaries were rehydrated with serial washes with EtOH at decreasing concentrations (from 100% to 25%) and a final wash in phosphate buffered saline (PBS; 1X) (Sigma-Aldrich). Tissues were moulded in 3% agarose and sectioned (60 µm parasagittal sections) in a VT1000S vibratome (Leica, Weltzar, Germany). The fluorescent *in situ* hybridization (FISH) protocol was adapted from Fontaine et al. (2013). During double colour FISH, digoxigenin-labelled probes were marked by an anti-digoxigenin peroxidase-conjugated antibody (Roche Diagnostics) and a custom made TAMRA-conjugated tyramide, while fluorescein-labelled probes were marked by an anti-fluorescein peroxidase-conjugated antibody (Roche) and a custom made fluorescein (FITC)-conjugated tyramide. Cell nuclei were visualized using DAPI-staining (4′,6-diamidino-2-phenylindole; Invitrogen), following manufacturer's instructions. Sections were mounted on slides using Vectashield H-1000 Mounting Medium (Vector, Eurobio/Abcys) after extensive washes in PBS tween 0.1% (PBST; Sigma-Aldrich). Sense probes were used for negative control. All solutions used for FISH were DEPC (Diethyl pyrocarbonate; Sigma-Aldrich) treated to inhibit RNase activity.

2.8. Image acquisition and processing

The 60 µm parasagittal pituitary sections included the entire length of the gland, allowing visualization of both the anterior and the posterior pituitary. Images were acquired from slides derived from at least two different fish and a minimum of two slides per fish were analysed. Images of fluorescently labelled slides were obtained using Zeiss LSM 710 laser scanning confocal microscope (Zeiss, Oberkochen, Germany)

Table 1
Primer sequences used for qPCR and cloning.

Gene	Accession number	Primer Fw (5′–3′)	Primer Rv (3′–5′)	Product size (bp)	Efficiency (%)
qPCR primers					
rna18s	FJ710886.1 †	CTCAACACGGGAAACCTCAC	AGACAAATCGCTCCACCAAC	118	99.5
ef1a	NM_001141909.1	CTTTGTGCCCATCTCTGGAT	ACCCTCCTTACGCTCGACTT	97	99.5
fshb	XM_014126338.1	TACCTGGAAGGCTGTCCATC	TATGCGATCACAGTCGGTGT	101	99.5
lhb	NM_001173671.1	GTCACAGCTCAGAGCCACAG	GACGTCCGCTATGAAACGAT	97	99
gnrhr1caα	KF225728	TGCACCCATTGGATGCG	CGGCTTTGATTGCTCGAAAA	120	98.5
gnrhr1caβ	KF225727	CCGCTGGATGCACTGGAC	CTCTGCTTTGATTGCTCGAAAGA	117	99
gnrhr1cbα	KF225729	ACTCCCACCACCGGAACAA	TGATGGCCCGGAAGATGA	93	98
gnrhr1cbβ	MF073196				
gnrhr2bbα	KF225730 ‡	TCAACCCACTGGCGATCAAT	CGTGATGGTCACACTGTGGAATA	122	100
gnrhr2bbβ	MF073197	ATACCCTCATCTGTTGGCTGAC	TGCTTTCTCACAGCACAAAAGT	60	99.5
Cloning/in situ primers					
fshb	XM_014126338.1	GGACCTGATCTCCTTGTGGA	CTGCTGCAACAGCCTAACTCT	467	
lhb	NM_001173671.1	TGCTGAACCCTTTGAGTCCT	TTACATTGGCAGGCATGTTG	495	
gnrhr2bbα	KF225730	CAGAACACCAGCTGTGAAGC	CGTGATGGTCACACTGTGGAATA	518	
gnrhr2bbβ	MF073197	CTACAGCTGCCCACCTTCTC	CATGTCTGGCTGAATGCAAA	1205	

† from Maugars and Schmitz, (2006), ‡ from Melo et al., (2014).

Table 2
Salmon testis developmental stages, with germ cell identity (cysts) defined according to Melo et al., 2014. SPA: spermatogonia A, either undifferentiated or differentiated; SPB: spermatogonia B; SC: spermatocytes; ST: spermatids; SZ: spermatozoa. Histological sections representative of the different stages are shown in Ciani et al., 2019b

Stage	Observed cysts
I	SPA
II	SPA + SPB
III	SPA + SPB + SC
IV	SPA + SPB + SC + ST
V	SPA + SPB + SC + ST + SZ
VI	With some tubules still immature, or early maturing SZ is dominating. Large lumen in tubules

using the following objectives: Plan-Neofluar 10×/0.3 M27 (Zeiss); Plan-Neofluar 25×/0.8 M27 (Zeiss); C-Apochromat 40×/1.2 (Zeiss). Laser with wavelengths 488 and 564 nm were used for the excitation of FITC and TAMRA, respectively. Channels were scanned sequentially to avoid signal crossover between filters. Images were acquired with ZEN 2009 (Zeiss) and processed with FIJI software (Schindelin et al., 2012).

2.9. Statistical analysis

Statistical analysis was performed using the software JMP pro V14.1 (SAS Institute Inc., Cary, NC, USA). All data were tested for normality using the Shapiro-Wilk W test. When needed, data were log or square root transformed to meet test criteria for normal distribution. After removing outliers using the Quantile Range method (Q = 3; Tail 0.1), potential significant changes in gene expression during maturation were assessed via two-way ANOVA followed by Tukey's HSD test, while changes in daily gene expression and tissue distribution were determined via one-way ANOVA followed by Tukey's HSD test. Correlation analysis were performed to study the relationship between gonadotropin and Gnrhr transcripts, using data obtained from all expression analyses performed in the present study (expression during maturation, daily expression). Due to non-normal distribution, correlation analysis was performed using Spearman's ρ test (non-parametric). For all tests, significance was set at the $p < 0.05$ level.

3. Results

3.1. Phylogenetic analysis

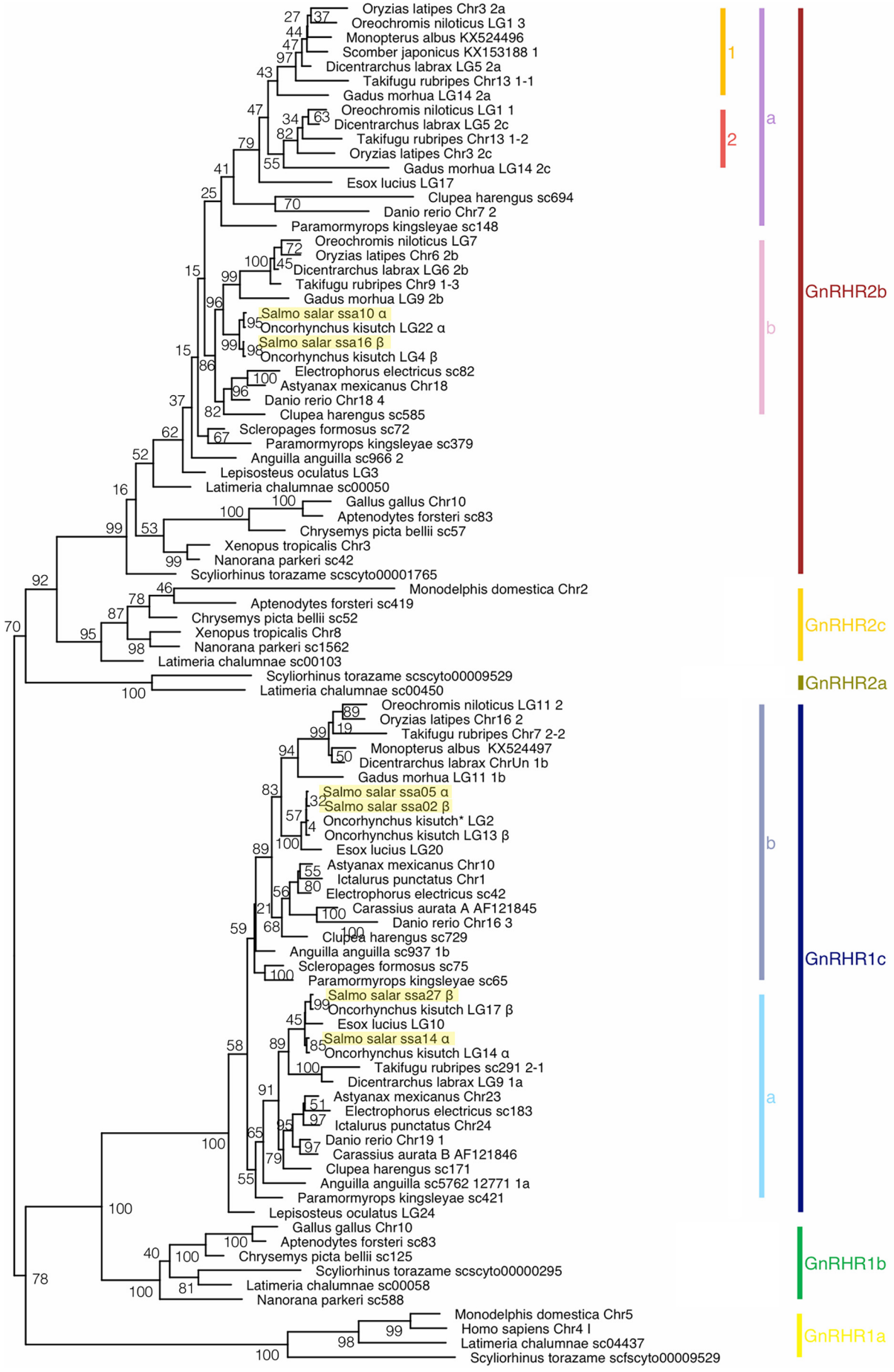
In the different target species genomes, we identified between 1 and 6 paralogous gnrhr genes. The Gnrhr phylogenetic tree is represented in Fig. 1. The Gnrhr are divided into two main types, GnRHR1 and GnRHR2, each divided into three subtypes: GnRHR1a, GnRHR1b, GnRHR1c for the GnRHR type 1, and GnRHR2a, GnRHR2b and GnRHR2c for the type 2. Bootstrap analysis supported this division with a high degree of confidence (96–100%).

The single human receptor was found clustering with an opossum (*Monodelphis domestica*) receptor in the GnRHR1a. The second opossum receptor was from the type 2 and grouped with sauropsid, amphibian, and coelacanth Gnrhr in the GnRHR2c clade. The two other sauropsid and amphibian Gnrhr belonged to GnRHR1b and GnRHR2b subtypes, respectively.

Four Gnrhr were identified in the catshark (*Scyliorhinus torazame)* genome, including two genes found on the same scaffold. Each catshark receptor branched into a different clade (the GnRHR-1a, -1b, -2a and -2b) including the GnRHR2a, comprising only a coelacanth Gnrhr. The actinopterygian Gnrhr were distributed into two clades, one of type 1, the GnRHR1c, and one of type 2, the GnRH2b. In the GnRHR1c clade, only actinopterygian species were represented. One coelacanth receptor was found in each of the five clades beside GnRHR1c. Among the Actinopterygians, the spotted gar branched at the basal position of teleost GnRHR1c. The teleost GnRHR1c were grouped into two clades, GnRHR-1ca and -1cb. Four of the Atlantic salmon Gnrhr belonged to the GnRHR1c: two paralogs clustering in the GnRHR1ca clade (Gnrhr1caα, Gnrhr1caβ) and two in the GnRHR1cb clade (Gnrhr1cbα, Gnrhr1cbβ), with a pair of Coho salmon Gnrhr. While GnRHR1cb paralogs were conserved in all of the teleost species examined, GnRHR1ca was lost in some species, including arowana (*Scleropages formosus*), Atlantic cod (*Gadus morhua*) and medaka (*Oryzias latipes*).

The GnRHR2b subtype included Actinopterygians, Sarcopterygians and a Chondrichthyan. Among the Actinopterygian, the spotted gar branched at the basal position of the teleost GnRHR2b. Teleost GnRHR2b paralogs clustered mainly into two clades, GnRHR-2ba and -2bb. A local duplication formed two additional clusters (named 1 and 2) within the GnRHR2ba. One GnRHR2b paralog from the *Paramormyrops kingsleyae* branched with the single eel (*Anguilla japonica*) and the arowana GnRHR2b at the base of the two teleost GnRHR2b clades while the other paralog branched at the basal position within the GnRHR2bb.

Two Atlantic salmon Gnrhr (Gnrhr2bbα, Gnrhr2bbβ) clustered within GnRHR2bb clade, while the single pike Gnrhr within the GnRHR2ba. Only a few basal teleosts have conserved the GnRHR2ba paralog. In contrast, the acanthopterygian species showed an additional copy of GnRHR2bb dividing into two monophyletic clades. A putative evolutionary scenario for Gnrhr is presented in Supplementary Fig. 1.



(caption on next page)

Fig. 1. Phylogenetic relationship between Gnrh receptors. The tree topology was inferred by Maximum Likelihood algorithm using PhyML 3.0, the Smart Model Selection and the Subtree pruning and regrafting (SPR) with topological rearrangement as options on ATGC Montpellier bioinformatic web browser (Guindon et al., 2010; Lefort et al., 2017). Node support was first evaluated by aLRT test and then by bootstrapping from 100 replicates (indicated as percent). The consensus tree was plotted using the R package ggtree (Yu et al., 2017). Paralogs originating from the salmonid 4R are indicated by suffix α and β. Atlantic salmon sequences are highlighted in yellow. The genomic localization of each receptor is given after the species name. The sequence accession numbers as well as the genome assembly information are given in Supplementary table 1, together with previous designations established by Hildahl et al., (2011) and Peñaranda et al., (2013).

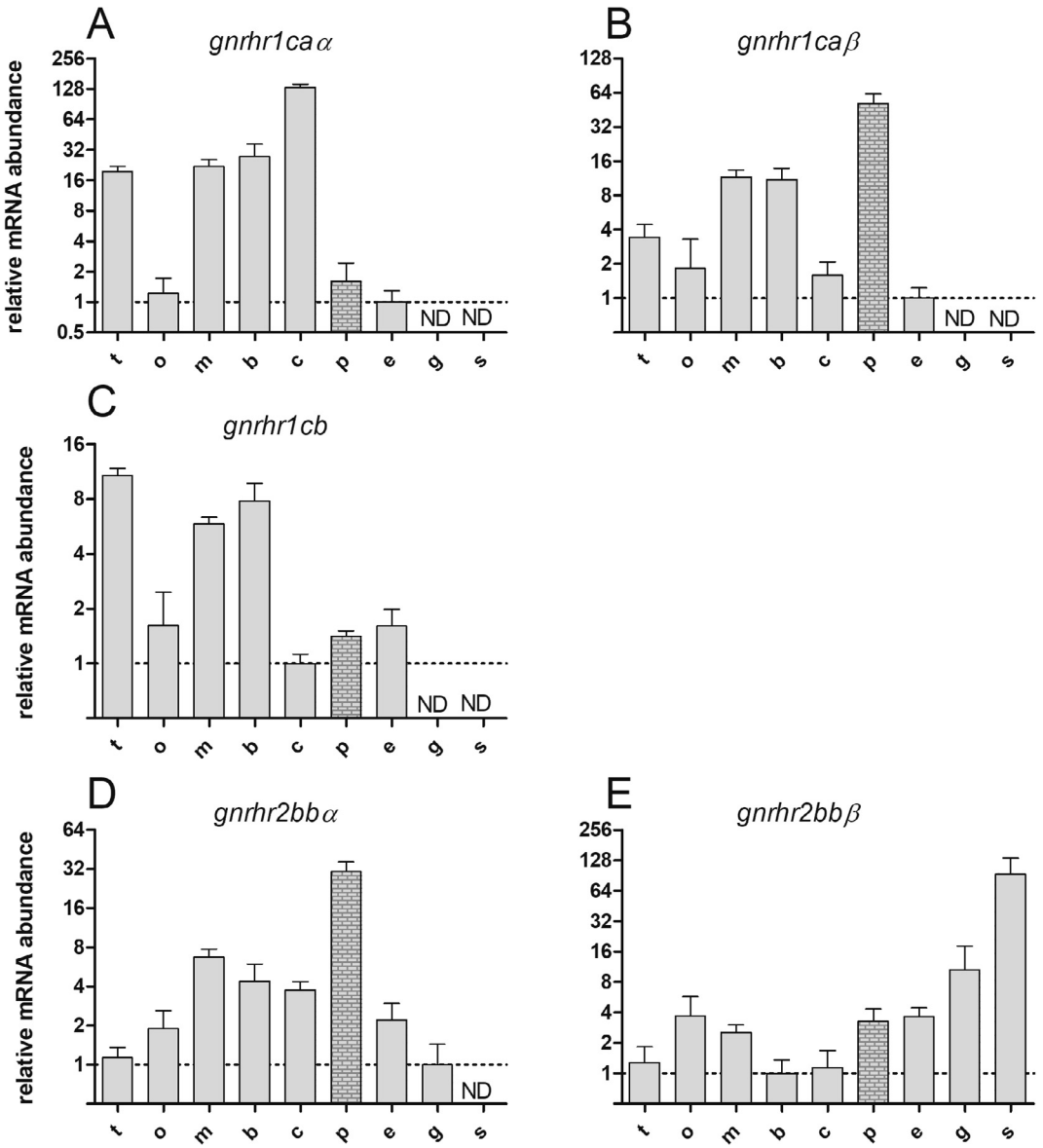


Fig. 2. Tissue distribution of *gnrhr* in Atlantic salmon male parr. Relative mRNA abundance of *gnrhr1caα* (A), *gnrhr1caβ* (B), *gnrhr1cb* (C), *gnrhr2bbα* (D) and *gnrhr2bbβ* (E) in different tissues (t-telencephalon; o-optic nerves; m-optic tectum; b-medulla oblongata and diencephalon; c-cerebellum; p-pituitary gland; e-eyes; g-testes; s-skin). mRNA levels were normalized against *rna18s* and *ef1a*. Data are shown as mean ± SEM (n = 5). Values are graphically expressed as fold change to the lowest expressing tissue (set as value 1). ND = Non-detectable.

Nucleotide and amino acid sequences of Atlantic salmon Gnrhr are shown in Supplementary Figs. 2 to 5.

3.2. Tissue distribution of gnrhr in Atlantic salmon

The expression of Atlantic salmon *gnrhr* (*gnrhr1caα, gnrhr1caβ, gnrhr1cb, gnrhr2bbα, gnrhr2bbβ;* Fig. 2A to E; See Table 1 for accession numbers), was investigated in several neural and peripheral tissues, including telencephalon, optic nerves, optic tectum, medulla/diencephalon, cerebellum, pituitary, eyes, testes and skin.

Messenger RNA from all receptors were detected in the pituitary. One representative for each receptor type (Type 1 and Type 2), *gnrhr1caβ* (Fig. 2B) and *gnrhr2bbα* (Fig. 2E), were highly expressed in the pituitary compared to most other tissues. *gnrhr1caα* (Fig. 2A) showed lower expression in pituitaries compared to the other tissues investigated. The expression of all screened mRNAs was detected in telencephalon, optic nerves, optic tectum, medulla/diencephalon, cerebellum, and eyes. Transcripts of *gnrhr2bbα* and *gnrhr2bbβ* (Fig. 2 D and E) were detected in testes and transcripts of *gnrhr2bbβ* (Fig. 2E) were identified in skin. Comparable expression levels between all examined tissues were detected for *gnrhr1cb* (Fig. 2C) and *gnrhr2bbβ* (Fig. 2E).

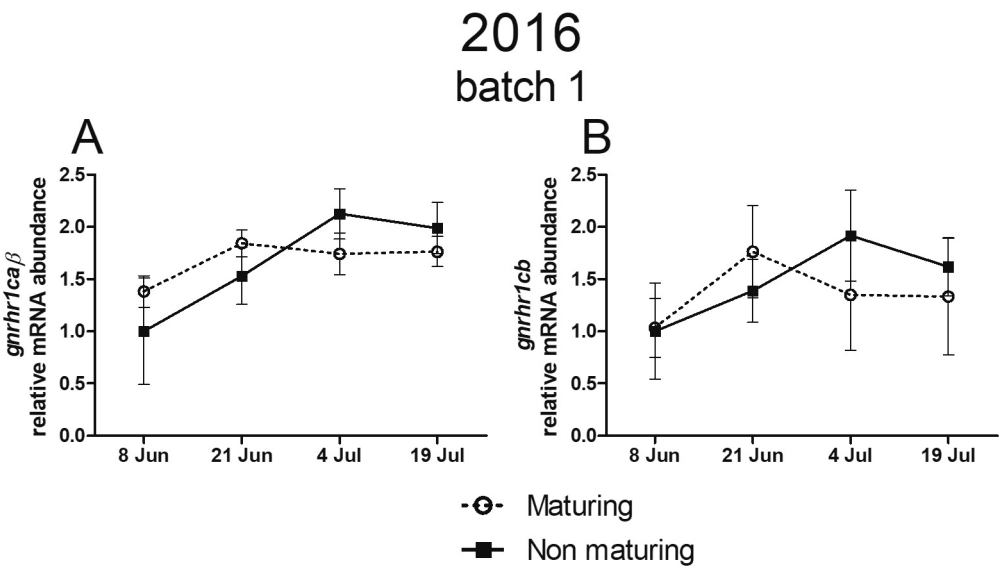


Fig. 3. Relative mRNA expression of *gnrhr1caβ* (A) and *gnrhr1cb* (B) in male parr pituitary during sexual maturation in spring 2016. The other type 1 receptor, *gnrhr1caα*, was not detected. mRNA levels are normalized to *rna18s* and *ef1a*. Maturing fish are represented with dotted lines and open circles. Non-maturing fish are represented with black lines and black squares. Data are graphically expressed as mean fold change to the lowest point (set as value 1) ± SEM (n = 6). No statistically relevant differences ($p < 0.05$) were denoted among groups, analysed via two-way ANOVA followed by Tukey multiple comparison test.

3.3. Gonadotropin and Gnrh receptor gene expression during sexual maturation

Pituitary expression of *gnrhr* (*gnrhr1caα, gnrhr1caβ, gnrhr1cb, gnrhr2bbα, gnrhr2bbβ*) and gonadotropin subunits (*fshb*, *lhb*) during sexual maturation was first measured in spring 2016 and then repeated in spring 2017 (Figs. 3 and 4). Gene expression levels were measured in two groups (maturing and non-maturing) of male parr to identify whether (a) differential expression occurs dependent on maturational stage, and (b) changes in gene expression occur during the season.

Analysing the results from batch 1, it was revealed that type 1 receptors, *gnrhr1caβ* and *gnrhr1cb* (Fig. 3 A and B), showed no differences in expression with regard to either maturational stage or sampling date, while expression of *gnrhr1caα*, was detected only in a sub-set of the sampled fish. Therefore, those genes were not included in the subsequent analyses. For type 2 receptors, batch 1 maturing fish expressed significantly higher levels of *gnrhr2bbα* on June 21st than non-maturing fish (Fig. 4A), while *gnrhr2bbβ* showed no differential expression levels between groups or sampling dates but was characterized by high individual variation (Fig. 4B). In concomitance with the *gnrhr2bbα* expression, higher *fshb* and *lhb* levels were detected in pituitaries from maturing fish on June 21st (Fig. 4C and D). Additionally, maturing fish expressed significantly higher levels of *fshb* than non-maturing fish from June 21st to July 19th peaking on July 4th (Fig. 4D).

To analyse gene expression at earlier stages of maturation, the study was replicated in spring 2017, from April 25th to July 4th, starting one month earlier than the previous year. During the earlier stages, no differences were detected in receptor expression between maturing and non-maturing fish (Fig. 4E and F). Concerning gonadotropins, *fshb* expression decreased in non-maturing fish from May 8th to 23rd, while remaining constant in maturing fish (Fig. 4H), and *lhb* decreased in both groups from April 25th to June 7th (Fig. 4G). Both *fshb* and *lhb* transcripts were more abundant in maturing fish on May 23rd. Due to the lower number of fish available and the higher rate of maturation (49,3% in 2016 vs 66,1% in 2017), only one non-maturing male was available on June 7th and no one on July 4th. Gene expression values from this fish are shown in Fig. 4E to 4H for graphic purposes only and were not included in the statistical analysis.

3.4. Daily pituitary expression of fshb, lhb, gnrhr2bbα and gnrhr2bbβ.

Daily pituitary expression of *fshb*, *lhb*, *gnrhr2bbα* and *gnrhr2bbβ* was measured for 24 h in autumn and spring to investigate the occurrence of different daily rhythms between seasons.

During autumn, the relative gene expression of *fshb*, *lhb, gnrhr2bbα* and *gnrhr2bbβ* in the pituitary showed no significant variation during the 24 h sampling period (October 23rd, Fig. 5A, C, E and G). In contrast, gene expression showed significant daily fluctuations with high levels in the morning and low levels in the evening during spring (April 13th) for *lhb* (Fig. 5D) *gnrhr2bbα* (Fig. 5F) and *gnrhr2bbβ* (Fig. 5H), but not for *fshb* (Fig. 5B). *lhb* (Fig. 5D) and *gnrhr2bbα* (Fig. 5F) showed parallel expression profiles where gene expression levels increased in the morning, with the peak at 08.00, and decreased steadily in the afternoon to reach minimum levels at 24.00. The fold change (mean ± SEM) in relative expression between maximum and minimum point was 3.8 ± 0.4 for *lhb* and 2.5 ± 0.3 for *gnrhr2bbα*. Daily fluctuation in the expression of the second receptor gene, *gnrhr2bbβ*, displayed a much higher induction, with an 83.4 ± 7.8-fold change between minimum and maximum points. Low levels of *gnrhr2bbβ* expression were measured between 16.00 and 20.00. Afterwards, mRNA levels increased from 24.00 onward, with a peak at 08.00 and high level until 12.00. A drastic drop in expression occurred between 12.00 and 16.00 (Fig. 5H).

3.5. Expression of gnrhr in relation to testis development

The plotting of GnRHR2 paralogs (*gnrhr2bbα, gnrhr2bbβ*) relative expression levels versus testis development revealed an increase of *gnrhr2bbα* (Fig. 6A) expression from stage I, when only spermatogonia type A (either undifferentiated or differentiated) are present, to stage VI, where spermatozoa are abundant in the lumen. On the other hand, *gnrhr2bbβ* (Fig. 6B) expression did not vary between different stages of testis development.

3.6. Correlation analysis

Higher correlation coefficients (Spearman ρ) in expression profiles were detected between *gnrhr2bbα* and gonadotropins (*gnrhr2bbα-fshb* $\rho = 0.83$; *gnrhr2bbα-lhb* $\rho = 0.81$) compared to *gnrhr2bbβ* and gonadotropins (*gnrhr2bbβ-fshb* $\rho = 0.25$ *gnrhr2bbβ-lhb* $\rho = 0.49$). Results are summarized in Table 3.

3.7. mRNA localization via fluorescent in situ hybridization (FISH)

While no labelling was detected for *gnrhr2bbβ*, the localization of *fshb*, *lhb* and *gnrhr2bbα* mRNA in the pituitary gland of maturing male parr obtained via FISH is shown in Fig. 7. The two gonadotropins, produced in distinct cell types, showed differential distribution along

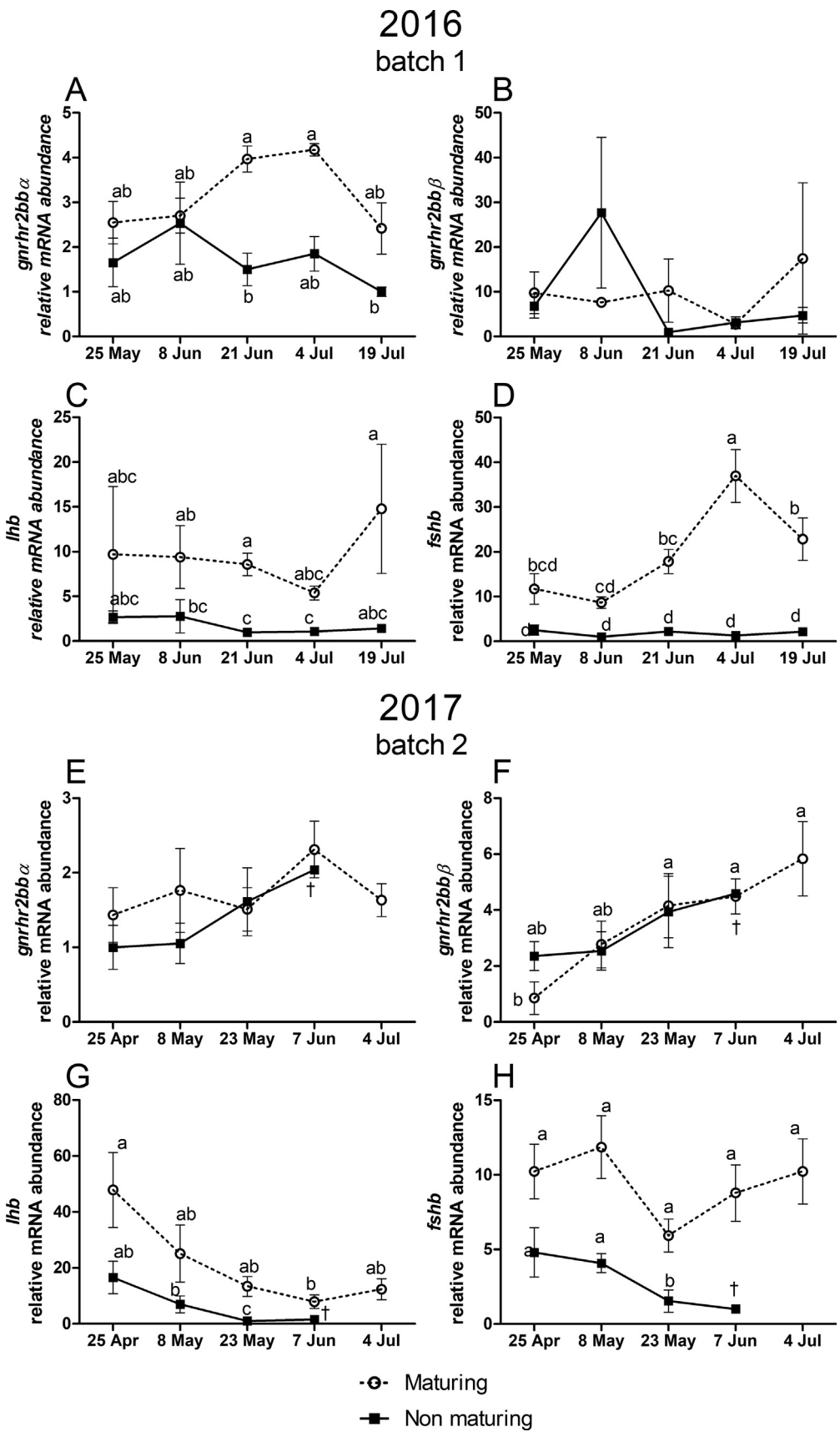


Fig. 4. Relative mRNA expression of *gnrhr2bbα* (A, E), *gnrhr2bbβ* (B, F) *lhb* (C, G) and *fshb* (D, H) in male parr pituitary during sexual maturation, in spring 2016 (A to D) and spring 2017 (E to H). mRNA levels normalized against *rna18s* and *ef1a*. Data are shown as mean ± SEM (n = 6). Maturing fish are represented with dotted lines and open circles. Non-maturing fish are represented with black lines and black squares. Values are graphically expressed as fold change to the lowest point (set as value 1). Distinct letters denote statistically significant differences among groups ($p < 0.05$), analysed via two-way ANOVA, followed by Tukey multiple comparison test. The absence of letters in graphs (B) and (E) denote lack of significant differences between groups. († n = 1, non-maturing 7 Jun 2017, not included in statistical test).

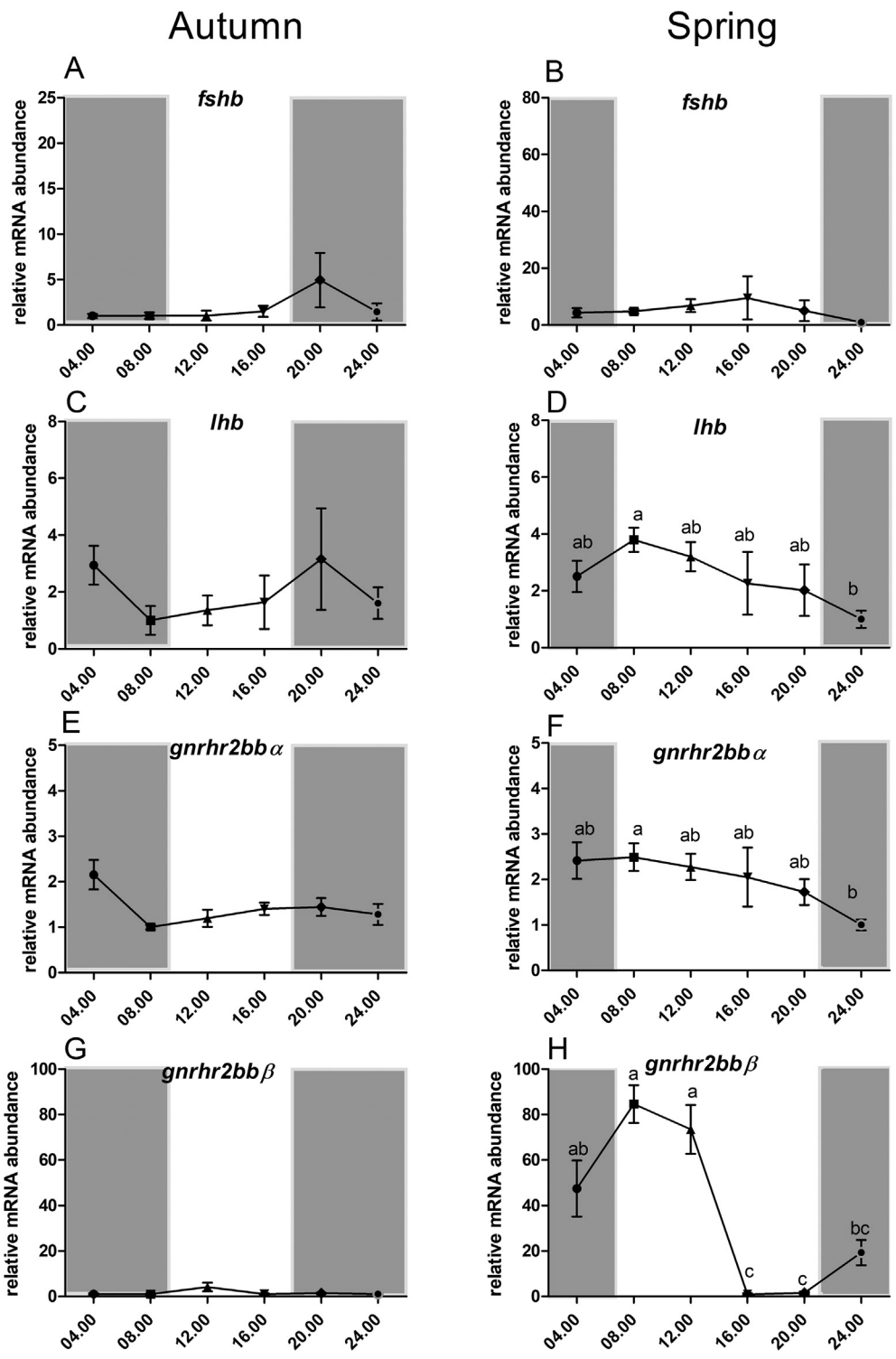


Fig. 5. Relative mRNA abundance of *fshb* (A, B), *lhb* (C, D), *gnrhr2bbα* (E, F) and *gnrhr2bbβ* (G, H) in male parr pituitary in autumn (A, C, E, G; October 23rd, 2017; n = 6 per point) vs spring (B, D, F, H; April 13th, 2018; n = 10 per point). mRNA levels were normalized against *rna18s* and *ef1a*. Sampling intervals every four hours (12.00, 16.00 20.00, 24.00, 04.00, 08.00). Grey column representing dark hours between sunset and sunrise. Data are shown as mean ± SEM. Values are graphically expressed as fold change to the lowest point (set as value 1). Different letters denote statistically significant differences ($p < 0.05$), analysed using one-way ANOVA followed by Tukey multiple comparison test. The absence of letters in graphs (A, C, E, and G) denote lack of significant differences.

the proximal pars distalis (PPD), with *fshb* distributed dorsally to *lhb*.

Double staining for *fshb* and *gnrhr2bbα* (Fig. 7 D, E and F) exposed a distinct localization of the two mRNAs. Double staining for *lhb* and *gnrhr2bbα* (Fig. 7 A, B and C) revealed co-localization of the mRNAs with no visible labelling for the receptor outside *lhb* producing cells, suggesting production of *gnrhr2bbα* exclusively in *lhb*-producing cells. However, a few *lhb*-producing cells presented no labelling for the *gnrhr2bbα*.

4. Discussion

This study reports the phylogenetic relationship of gonadotropin-releasing hormone receptors (Gnrhr) in vertebrates, in combination with tissue distribution of *gnrhr* genes (*gnrhr1caα, gnrhr1caβ, gnrhr1cb, gnrhr2bbα, gnrhr2bbβ*) in neural and peripheral tissues of male Atlantic salmon (*Salmo salar*) parr, their expression in the pituitary gland during sexual maturation, and the co-localization of *gnrhr2bbα* exclusively to *lhb*-producing cells.

The phylogenetic analysis, based on extensive sequence searching in genomic data, brings new information on the evolutionary history of

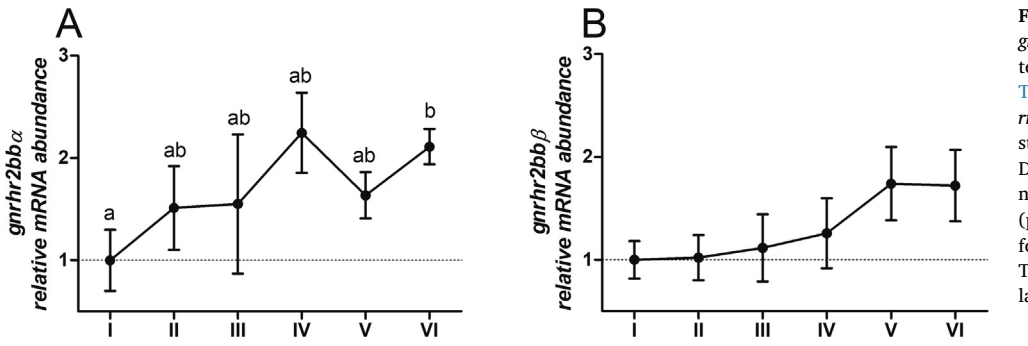


Fig. 6. Change in *gnrhr2bbα* (A) and *gnrhr2bbβ* (B) expression according to the six testicular developmental stages (see Table 2). mRNA levels normalized against *rna18s* and *ef1a*. Transcript abundance of stage I was set as 1 for the representation. Distinct letters denote statistically significant differences among groups ($p < 0.05$), analysed via one-way ANOVA, followed by Tukey multiple comparison test. The absence of letters in graph B denotes lack of significant differences.

Table 3
(ρ) Spearman's rank correlation coefficient between receptors and gonadotropins mRNA; (p) Significance; (n) sample size.

Variable	by Variable	ρ	p	n
gnrhr2bbα	*fshb*	0.83	< 0.0001	205
	lhb	0.81	< 0.0001	205
gnrhr2bbβ	*fshb*	0.25	0.0004	198
	lhb	0.49	< 0.0001	198

Gnrhr in vertebrates. Previous studies conducted from Hildahl et al. (2011), grouped Gnrhr in two main types (named type I and type II from the authors), each divided into two subtypes. According to Hildahl et al. (2011), the two types resulted from the divergence between the two copies of a duplicated gene after the 1R, while the different subtypes were assumed to arise from the 2R and subsequent gene losses. However, the recent discovery of five *gnrhr* in a basal sarcopterygian, the coelacanth (*Latimeria chalumnae*) supports the existence of an additional receptor subtype in Gnathostomes (Sefideh et al., 2014). The comparison of the *gnrhr* synteny with reconstructed vertebrate ancestor chromosomes, revealed 3 ancestral chromosomic regions harbouring each a pair of *gnrhr*, suggesting the existence of 6 extant receptors subtypes. The subsequent evolutionary scenario proposed that a local duplication of an ancestral *gnrhr* occurred before the 2 rounds of vertebrate whole genome duplication, and that eight subtypes were generated during the 1R and 2R. Two subtypes were assumed to be lost after the 2R in the gnathostomes lineage (Sefideh et al., 2014). On the other hand, Williams et al. (2014) suggested the presence of five Gnrhr subtypes in vertebrates, generated by the 1R, 2R, and subsequent local duplications and gene loss. In the present study, the search of *gnrhr* from genomic databases resulted in the identification of four genes in the catshark genome, three of them corresponding to the ones found in the skate (*Leucoraja erinacea*) transcriptomic data by Williams et al. (2014) and the fourth clustering with a single coelacanth gnrhr in the subtype -2a. In catshark genomic assembly (GCA_003427355.1), the *gnrhr2a* was found on the same scaffold than the *gnrhr1a,* thus confirming the presence of an ancient pair of *gnrhr* from the type 1 and type 2. This observation supports that a local duplication of an ancestral gnrhr occurred prior the two vertebrate genome duplications giving rise to the GnRHR type 1 and 2 (Hildahl et al., 2011). Therefore the present study follows the nomenclature proposed by Sefideh et al. (2014), adapting the gene nomenclature to HGNC and ZFin recommendations. The phylogenetic analysis proposed in the present paper, including new basal teleost species, showed that Actinopterygians retained only two out of six Gnrhr subtypes: GnRHR1c and GnRHR2b (named respectively GnRHR1A and GnRHR2B from Hildahl et al., 2011). The teleost 3R

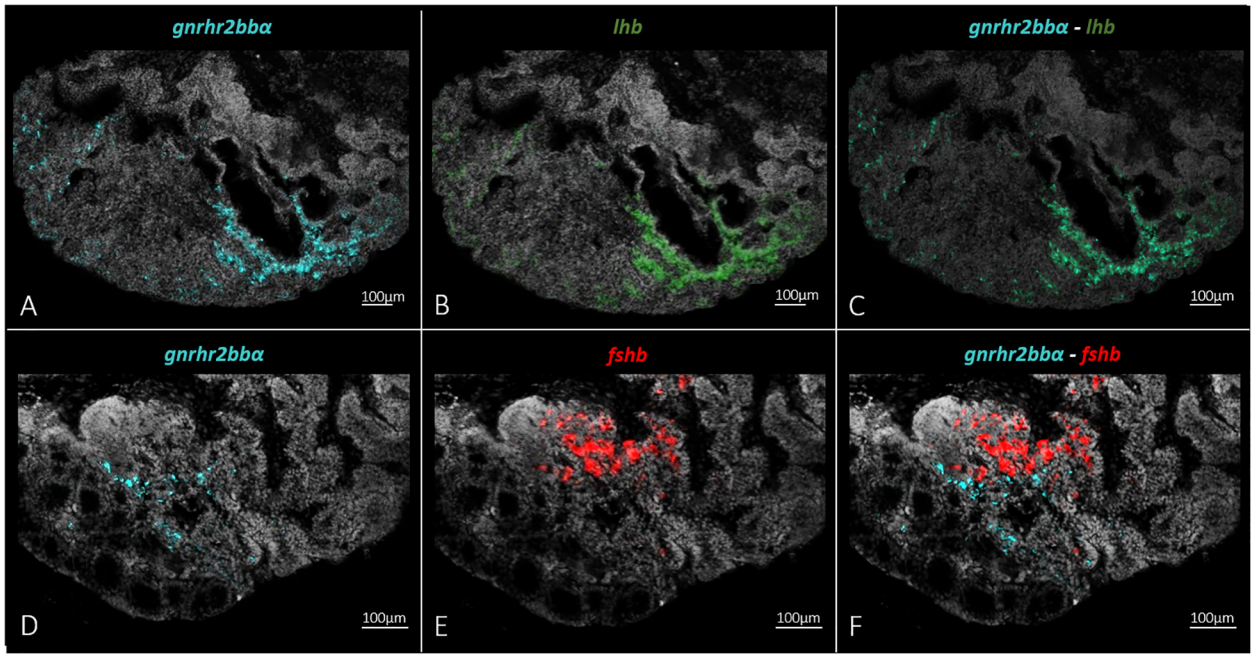


Fig. 7. Fluorescent *in situ* hybridization in parasagittal pituitary sections (anterior to the left) of Atlantic salmon maturing male parr. Confocal pictures of: Double stained section showing *gnrhr2bbα* (cyan) and *lhb* (green) individually (A, B) and together (C); Double stained section showing *gnrhr2bbα* (cyan) and *fshb* (red) individually (D, E) and together (F). Nuclei stained with DAPI are shown in grey. The mRNA localization show expression of *gnrhr2bbα* in *lhb*- but not in *fshb*-producing cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

generated two copies of each GnRHR1c and GnRHR2b paralogs, designated here as *gnrhr1ca*, *gnrhr1cb*, *gnrhr2ba* and *gnrhr2bb*. Only the *gnrhr1cb* was identified in all the teleost genomes examined. For the three other gnrhr, gene losses were observed through the teleost radiation (Supplementary Fig. 1) in agreement with previous studies (Hildahl et al., 2011; Peñaranda et al., 2013). The presence of duplicated *gnrhr2a* on the same chromosomic region confirmed a recent local duplication of the GnRHR2aa in the super order of acanthomorpha (Sefideh et al., 2014; Williams et al., 2014).

In Atlantic salmon, the 4R generated 2 copies of the 3R-paralogs *gnrhr1ca*, *gnrhr1cb* and *gnrhr2ba*. The 4R paralogs were designated as *gnrhr1caα*, *gnrhr1caβ*, *gnrhr1cbα*, *gnrhr1cbβ*, *gnrhr2bbα* and *gnrhr2bbβ*. A similar scenario was detected in coho salmon. The presence of the *gnrhr2bb* in pike (*Esox lucius*), member of a salmonid sister group, suggests the loss of the 4R paralogs -2ba (α, β) during the salmonid radiation. To better understand the role of the six paralogs in Atlantic salmon and to identify candidates involved in gonadotropin regulation during sexual maturation, this study investigated the expression profiles of the different genes in pituitary, brain and peripheral tissues. In mammals, differential regulation of gonadotropin expression from GnRH signal can be achieved by modulation of the frequency of pulsatile GnRH release, while in teleosts, this mechanism seems to be less relevant, or absent (Karigo et al., 2012; for review see Levavi-Sivan and Avitan, 2005). Differential regulation in teleosts may instead be achieved by activation of different intracellular pathways initiated by activation of the Gnrhr, leading to either *lhb* and *gpα*, or *fshb* expression (Yaron et al., 2003). However, the presence of multiple isoforms of Gnrhr in teleosts opens the question whether or not different cell types can be regulated by the presence of specific receptors, in addition to the regulation through different intracellular pathways.

In this study, the analysis of the tissue distribution of the Atlantic salmon *gnrhr* revealed some divergence between 3R- but also between the 4R-paralogs. For instance, observing the 4R paralogs, *gnrhr1caα* showed higher expression in the brain and low level in the pituitary while *gnrhr1caβ* displayed higher expression in the pituitary. Atlantic salmon have conserved also a pair of 4R paralogs belonging to GnRHR type 2. While *gnrhr2bbα* is mainly expressed in the pituitary and in the brain, *gnrhr2bbβ* was mainly found in testis and skin. The transcripts for *gnrhr1cb* were identified in all analysed tissues except gonads and skin. These results suggest that the different paralogs may have acquired function specificity during evolution. Expression of multiple *gnrhr* was detected in all analysed brain regions, including the telencephalon, optic tectum, medulla oblongata, diencephalon and cerebellum. In masu salmon (*Oncorhynchus masou)*, all four GnRHR1c paralogs were found in the brain via qPCR with expression profiles varying according to season (Jodo et al., 2003). The receptor-mediated functions of Gnrh in the teleost brain are still not completely defined. However, the most commonly accepted functions are neuromodulation, control of reproductive behaviour and coupling of olfactory signals to reproduction (Carolsfeld et al., 2000; Temple et al., 2003).

Gnrh may also be involved in the coupling between photoperiod and the endocrine system. All *gnrhr* investigated in the present study were expressed in both eye and optic nerve tissues, which, if translated into protein, may serve as a link for the Gnrh mediated coupling of visual information with reproduction (for review see Okubo and Nagahama, 2008). In European seabass (*Dicentrarchus labrax*), Gnrh2 acts as a melatonin-releasing factor in the pineal gland, probably through receptors Gnrhr1ca and Gnrhr1cb (Gnrhr1b and Gnrhr1a in Servili et al., (2010)). Melatonin is a “time keeping” hormone involved in the control of numerous daily and seasonal rhythms (for review see Falcón et al., 2010). Daily fluctuations in melatonin and melatonin receptors have been reported in brain and pituitary tissue of several teleosts and are associated with the control and synchronization of spawning (Ando et al., 2014; Ciani et al., 2019a; Ikegami et al., 2015; Shahjahan et al., 2010). The retina is also a major component of the teleost circadian system (Falcón et al., 2007). The presence of *gnrhr* has been detected in the eyes and retina of several teleosts including sea bass (*Dicentrarchus labrax;* Moncaut, 2005), green pufferfish (*Tetraodon nigroviridis;* Ikemoto and Park, 2005), rainbow trout (*Oncorhynchus mykiss;* Madigou et al., 2000) and *Astatotilapia burtoni* (Grens et al., 2005).

In the present study, expression of *gnrhr* genes *gnrhr2bbα* and *gnrhr2bbβ* was detected in the testes. Gnrhr mRNA was identified also in rainbow trout testes during sexual maturation (Madigou et al., 2002). In maturing masu salmon males, *gnrhr1caβ* (named GnRH-R5 from the authors) is detected in April in the testis by qPCR (Jodo et al., 2003). These receptors may be responsible for the autocrine/paracrine Gnrh effects in gonads that may include regulation of steroidogenesis and germ cell proliferation (for review see Lethimonier et al., 2004; Ramakrishnappa et al., 2005). The current study also detected transcripts of *gnrhr2bbβ*, in the salmon parr skin. There are few available studies on the potential significance of Gnrhr in fish skin, so implications of the functional role of this finding awaits future investigations.

In teleosts, as in all vertebrates, Gnrh stimulates production and release of gonadotropins from the pituitary via Gnrhr (Conn and Crowley, 1994; Peter, 1983). Therefore, the Gnrhr responsible for direct gonadotropin regulation should be expressed in the pituitary and most likely increase in both transcript and protein levels during critical reproductive periods such as sexual maturation, gametogenesis or spawning. According to the present tissue distribution study, mRNA of all six receptor genes investigated was detected in the male parr pituitaries, with *gnrhr1caa* expressed at the limit of qPCR detection. As individual Gnrhr may play distinct physiological roles, and the pituitary consists of numerous cell types, this finding was not surprising. Indeed, the presence of multiple Gnrhr paralogs has been reported in the pituitary of several teleost species: three (*gnrhr1ca, gnrhr2ba1, gnrhr2bb*) in pufferfish (named R1-1 R1-3 R2-1, respectively in Ikemoto and Park (2005)); three (*gnrhr1ca, gnrhr2ba1, gnrhr2ba2*) in European sea bass (named *dlGnRH1aR1*, *dlGnRHR2a*, *dlGnRHR2c* in Moncaut (2005)); three (*gnrhr1cb*, *gnrhr2ba*1 and *gnrhr2ba2*) in Atlantic cod (*Gadus morhua;* 1b, 2a and 2c in Hildahl et al. (2011); von Krogh et al. (2017)); three in masu salmon (named R1, R3 and R5 respectively, Jodo et al. (2003)).

Among the receptors detected in Atlantic salmon pituitary, only the type 2 receptor gene, *gnrhr2bbα*, showed differential expression dependent on maturational stage in the current work. This is in agreement with the increase of *gnrhr2bba* (*gnrhr4* by Melo et al., 2015, 2014) observed during sexual maturation in post-smolt Atlantic salmon. Despite limited functional data, which makes it difficult to group the multiple receptor variants by function, type 2 receptors are considered to be involved in gonadotropin regulation in several teleost species (González-Martínez et al., 2004; Guilgur et al., 2009; Hildahl et al., 2011; Lin et al., 2010; Lumayno et al., 2017). In the European eel (*Anguilla anguilla*), that has conserved only a single GnRHR2b paralog, *gnrhr2bb* increases during female maturation (Peñaranda et al., 2013). While in eel and salmon the receptor type 2 that may mediate the Gnrh action during reproductive season is the 3R- duplicated -2bb, in acanthomorph species, most of the studies showed that this role is taken from the other 3R paralog, the -2ba. In chub mackerel (*Scomber japonicus*), for instance, *gnrhr2ba1* (named *gnrhr1* by the authors) expression in the pituitary increases in both males and females during sexual maturation, with a positive trend synchronous to *lhb* gene expression (Lumayno et al., 2017). In European seabass, *in situ* hybridization targeted *gnrhr2ba1* (*dlGnRHR-2A* in (González-Martínez et al., 2004)) in all Lh cells and a few Fsh cells, while qPCR measured increased expression during later stages of maturation (González-Martínez et al., 2004). In female Atlantic cod, the expression of *gnrh2ba1* (*gnrhr2a* by the authors) increases in the pituitary in parallel to GSI and is significantly upregulated during sexual maturation (Hildahl et al., 2011). In a successive *in vitro* study, the expression of *gnrhr2ba1* was detected in both *lhb* and *fshb* producing cells via single-cell qPCR (von Krogh et al., 2017). Taken together, these data show a conserved involvement of type 2 receptors in the control of gonadotropin function in teleost

pituitary.

Despite the fact that both type 2 receptors investigated in the present study, the 4R-duplicated *gnrhr2bbα* and *gnrhr2bbβ*, were detected in the pituitary gland, several indications suggest a stronger correlation of *gnrhr2bbα* with maturation stage and gonadotropin expression, particularly with *lhb*: (1) On June 21st, 2016, maturing fish displayed higher *gnrhr2bbα* expression, compared to non-maturing fish, in concomitance with greater *lhb* and *fshb* expression levels. Interestingly, while *fshb* continued to increase in maturing fish in the following days, this was not reflected from *gnrhr2bbα* expression, which, together with that of *lhb*, returned to comparable levels between groups. Similarly, the strong induction of *fshb* expression, detected in underyearling fish maturing in autumn 2017 (additional data), was not coupled with an increase in pituitary expression of *gnrhr2bbα*. No differential expression with regards to maturational stage was detected for *gnrhr2bbβ* at any time point. (2) *gnrhr2bbα* and *lhb* showed similar daily expression profiles, especially during spring, with high levels in the morning (08.00) decreasing toward minimum levels in the night (24.00), while *fshb* was expressed without daily fluctuation in both autumn and spring in immature fish, Also *gnrhr2bbβ* showed daily variations in spring, displaying however slight differences with *lhb* expression. Maximum levels were maintained from 08.00 to 12.00, and minimum levels between 16.00 and 20.00 increasing during the night. Furthermore, *gnrhr2bbβ* showed a much higher induction between minimum and maximum point compared to the other genes. (3) A high correlation coefficient ($\rho = 0.8$) was detected between *gnrhr2bbα* and gonadotropins expression, while *gnrhr2bbβ* displayed a lower coefficient ($\rho = 0.25$ for *fshb* and 0.49 for *lhb*). (4) When analysing gene expression in relationship to testis maturation stage, *gnrhr2bbα* increased during final stages of maturation, while *gnrhr2bbβ* was stably expressed. It is interesting to note that during earlier sampling dates in spring 2017, on May 23rd, maturing fish displayed higher gonadotropin expression compared to non-maturing fish. This was not backed up from higher receptor expression. However, in that period, gonadotropin mRNA was declining in both groups and the differences in gene expression may be interpreted as a faster decrease in non-maturing fish, rather than an induction in maturing, explaining the lack of *gnrhr2bbα* induction.

To further investigate the correlation between *gnrhr2bbα* and gonadotropin gene expression, and to identify the cell type expressing *gnrhr2bbβ,* this study proceeded with localization of the mRNAs in the pituitary gland. Unfortunately, no labelling for *gnrhr2bbβ* could be detected. At the moment of sampling, the results regarding the specific morning peak occurring in mid-April were not yet available, and the samples were collected in different dates and time of the day. This may have resulted in mRNA levels below detection limit of the *in situ* hybridization technique and highlights the importance of the time of the day at which a sampling is performed, in addition to other factors such as season, sex and maturation stage. Two colour fluorescent *in situ* hybridization demonstrated co-expression of *gnrhr2bbα* and *lhb* mRNA in the same cell type. Interestingly, *gnrhr2bbα* mRNA was not detected outside *lhb*-producing cells. In contrast, a small number of *lhb* cells did not label for *gnrhr2bbα*, indicating expression below detection limit, or absence of *gnrhr2bbα* mRNA in these cells. These data corroborates the qPCR results suggesting a correlation between *gnrhr2bbα* and *lhb*.

A key characteristic of the BPG axis is the positive and negative feedback from gonadal hormones to the higher levels of the axis. It is therefore likely that the Gnrhr paralogs involved in gonadotropin regulation would be susceptible to sex steroid influence. Indeed, Melo et al. (2015) demonstrated that testosterone (T) injections increased *lhb* and *gnrhr2bbα* (named there as *gnrhr4*), but not *fshb* mRNA, in pituitary glands of post-smolt Atlantic salmon males prior to maturation. A previous study (Melo et al., 2014), also reported correlation in gene expression between *gnrhr2bbα* and gonadotropins: (1) When observing pituitary gene expression in relation to testes developmental stage (according to the most advanced germ cell present), *fshb*, *lhb* and *gnrhr2bbα* mRNA increase when advancing from spermatogonia type A (either differentiated or undifferentiated) to spermatogonia type B, with *fshb* showing the highest relative change. However, while *fshb* declines at spermiation, *gnrhr2bbα* and *lhb* reach their maximum expression levels. (2) When observing gene expression variation over time, all three genes increase from September to January during smoltification regime, when smoltification was artificially induced. From January to March, when different maturation regimes were applied, both *gnrhr2bbα* and *lhb* expression increase over time in all conditions. Conversely *fshb,* which is highly expressed at initial stages of maturation, decreased in fish kept in freshwater (FW) and constant light (LL); FW and short day photoperiod (LD 12hL:12hD); seawater (SW, 35ppt)-LD; but remain stable in SWLL. In context with the co-localization of *gnrhr2bbα* and *lhb* mRNA presented in the current paper and considering that: (1) Fsh is the main gonadotropin detectable in the plasma prior and during early stages of maturation in salmonids (Breton et al., 1997; Gomez et al., 1999; Prat et al., 1996); (2) Fsh can induce steroid production (Planas and Swanson, 1995; Suzuki et al., 1988; Swanson et al., 1991); and (3) there is a close correlation between *fshb* mRNA and protein plasma level in rainbow trout (Gomez et al., 1999), one could speculate that the early surge in *fshb* transcript and subsequent Fsh release induces androgen production, which feedback to the pituitary, directly stimulating expression of *lhb* and *gnrhr2bbα* and preparing Lh-producing cells for the Gnrh-mediated Lh induction via *gnrhr2bbα*. In the present study, co-expression of *gnrhr2bbα* and *lhb* was confirmed in maturing fish sampled on May 23rd and June 7th. This does not exclude the possibility that the same gene, or a different *gnrhr*, may be expressed in Fsh-producing cells during earlier stages of maturation, considering that Gnrh does stimulate Fsh release from salmonid pituitary *in vitro* (Baker et al., 2000). Alternatively, a paracrine mechanism involving *gnrhr2bbα*-positive cells and Fsh-producing gonadotropes may be operating. However, this remains to be elucidated.

While *gnrhr2bbα* displayed correlation with maturational state and gonadotropin expression in the present study, *gnrhr2bbβ* lacked similar features. An interesting aspect of the *gnrhr2bbβ* expression profile lies in its seasonal fluctuation. It was characterized by intense daily variation in spring, but not in autumn. While this expression profile was not being mirrored by that of the gonadotropins, it does resemble the expression profile of melatonin receptors in the pituitary, detected in the same fish (Ciani et al., 2019a). Since gene expression profiling of *gnrhr2bbβ* excluded any correlation with the gonadotropins and showed a seasonal-specific expression in spring, its role may be linked to other periodic physiological and behavioural functions regulated by the pituitary. It is difficult, however, to speculate about the role of these fluctuations based only to gene expression profiles. Future studies aimed at identifying which cell types express this gene, and quantification of protein availability and functionality, are required for a better understanding of the matter.

Further studies regarding the physiological role of the type 1 receptors are also required. Although highly expressed in the pituitary, the expression profile of *gnrhr1caβ* did not reflect either gonadotropin expression or state of sexual maturity.

5. Conclusion

This study investigates the gene expression of *gnrhr* (*gnrhr1caα, gnrhr1caβ, gnrhr1cb, gnrhr2bbα, gnrhr2bbβ*) in male Atlantic salmon (*Salmo salar*) parr during sexual maturation, in order to identify candidate receptors involved in the direct regulation of gonadotropin synthesis. Pituitary gene expression analysis revealed the presence of five out of six genes during sexual maturation (*gnrhr1caα* was not detected). One gene in particular, *gnrhr2bbα*, showed higher relative gene expression in maturing fish, sharing a similar expression profile with *fshb* and *lhb*, compared with non-maturing fish. The localization of the mRNA via *in situ* hybridization revealed co-expression of *gnrhr2bbα* and *lhb* (but not *fshb*) mRNA in the proximal pars distalis. This advocates for the involvement of receptor paralog *gnrhr2bbα* in the regulation of *lhb*-

cells in sexually maturing Atlantic salmon male parr but requires further studies to explain in more detail the correlation between the increased *gnrhr2bbα* and *fshb* mRNA expression profiles during the initial stages of spermatogenic development.

Acknowledgements

The authors acknowledge the staff at the NINA Aquatic Research Station at Ims, in particular Mr. Knut Aanestad Bergersen, for generously providing and maintaining the fish. We express our gratitude to Dr. Rüdiger Schulz for revision of the manuscript and Dr. Kjetil Hodne for his help during sampling. This project has received founding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 642893 IMPRESS, and the Norwegian University of Life Sciences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113293.

References

Ando, H., Ogawa, S., Shahjahan, M., Ikegami, T., Doi, H., Hattori, A., Parhar, I., 2014. Diurnal and circadian oscillations in expression of kisspeptin, kisspeptin receptor and gonadotrophin-releasing hormone 2 genes in the grass puffer, a semilunar-synchronised spawner. J. Neuroendocrinol. 26, 459–467. https://doi.org/10.1111/jne.12165.

Arora, K.K., Krsmanovic, L.Z., Mores, N., O'Farrell, H., Catt, K.J., 1998. Mediation of cyclic AMP signaling by the first intracellular loop of the gonadotropin-releasing hormone receptor. J. Biol. Chem. 273, 25581–25586. https://doi.org/10.1074/jbc.273.40.25581.

Baker, D.M., Davies, B., Dickhoff, W.W., Swanson, P., 2000. Insulin-like growth factor I increases follicle-stimulating hormone (FSH) content and gonadotropin-releasing hormone-stimulated FSH release from coho salmon pituitary cells in vitro. Biol. Reprod. 63, 865–871. https://doi.org/10.1095/biolreprod63.3.865.

Breton, B., Sambroni, É., Govoroun, M., Weil, C., 1997. Effects of steroids on GTH I and GTH II secretion and pituitary concentration in the immature rainbow trout *Oncorhynchus mykiss*. Comptes Rendus l'Academie des Sci. - Ser III 320, 783–789. https://doi.org/10.1016/S0764-4469(97)85013-5.

Campbell, B., Dickey, J.T.T., Swanson, P., 2003. Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. Biol. Reprod. 69, 2109–2117. https://doi.org/10.1095/biolreprod.103.020560.

Carolsfeld, J., Powell, J.F.F., Park, M., Fischer, W.H., Craig, A.G., Chang, J.P., Rivier, J.E., Sherwood, N.M., 2000. Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinology 141, 505–512. https://doi.org/10.1210/endo.141.2.7300.

Childs, G.V., Hazum, E., Amsterdam, A., Limor, R., Naor, Z., 1986. Cytochemical evidence for different routes of gonadotropin-releasing hormone processing by large gonadotropes and granulosa cells. Endocrinology 119, 1329–1338. https://doi.org/10.1210/endo-119-3-1329.

Ciani, E., Fontaine, R., Maugars, G., Mizrahi, N., Mayer, I., Levavi-Sivan, B., Weltzien, F., 2019a. Melatonin receptors in Atlantic salmon stimulate cAMP levels in heterologous cell lines and show season-dependent daily variations in pituitary expression levels. J. Pineal Res. https://doi.org/10.1111/jpi.12590. e12590.

Ciani, E., von Krogh, K., Nourizadeh-Lillabadi, R., Mayer, I., Fontaine, R., Weltzien, F.A., 2019b. Sexual maturation in Atlantic salmon male parr is triggered in both early spring and late summer under standard farming condition. Submitted for publication.

Conn, P.M., Crowley, W.F., 1994. Gonadotropin-releasing hormone and its analogs. Annu. Rev. Med. 45, 391–405. https://doi.org/10.1146/annurev.med.45.1.391.

Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends Endocrinol. Metab. 18, 81–88. https://doi.org/10.1016/j.tem.2007.01.002.

Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010. Current knowledge on the melatonin system in teleost fish. Gen. Comp. Endocrinol. 165, 469–482. https://doi.org/10.1016/j.ygcen.2009.04.026.

Flanagan, C.A., Chen, C.-C., Coetsee, M., Mamputha, S., Whitlock, K.E., Bredenkamp, N., Grosenick, L., Fernald, R.D., Illing, N., 2007. Expression, structure, function, and evolution of gonadotropin-releasing hormone (GnRH) receptors GnRH-R1 SHS and GnRH-R2 PEY in the Teleost, *Astatotilapia burtoni*. Endocrinology 148, 5060–5071. https://doi.org/10.1210/en.2006-1400.

Fontaine, R., Affaticati, P., Yamamoto, K., Jolly, C., Bureau, C., Baloche, S., Gonnet, F., Vernier, P., Dufour, S., Pasqualini, C., 2013. Dopamine inhibits reproduction in female zebrafish (*Danio rerio*) via three pituitary D2 receptor subtypes. Endocrinology 154, 807–818. https://doi.org/10.1210/en.2012-1759.

Garcia De Leaniz, C., Fleming, I.A., Einum, S., Verspoor, E., Jordan, W.C., Consuegra, S., Aubin-Horth, N., Lajus, D., Letcher, B.H., Youngson, A.F., Webb, J.H., Vøllestad, L.A., Villanueva, B., Ferguson, A., Quinn, T.P., 2007. A critical review of adaptive genetic variation in Atlantic salmon: implications for conservation. Biol. Rev. https://doi.org/10.1111/j.1469-185X.2006.00004.x.

García-López, Á., Bogerd, J., Granneman, J.C.M., van Dijk, W., Trant, J.M., Taranger, G.L., Schulz, R.W., 2009. Leydig cells express follicle-stimulating hormone receptors in African Catfish. Endocrinology 150, 357–365. https://doi.org/10.1210/en.2008-0447.

Gomez, J.M., Weil, C., Ollitrault, M., Le Bail, P.Y., Breton, B., Le Gac, F., 1999. Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 113, 413–428. https://doi.org/10.1006/gcen.1998.7222.

González-Martínez, D., Madigou, T., Mañanos, E., Cerdá-Reverter, J.M., Zanuy, S., Kah, O., Muñoz-Cueto, J.A., 2004. Cloning and expression of gonadotropin-releasing hormone receptor in the brain and pituitary of the European Sea Bass: an *in situ* hybridization study1. Biol. Reprod. 70, 1380–1391. https://doi.org/10.1095/biolreprod.103.022624.

Grens, K.E., Greenwood, A.K., Fernald, R.D., 2005. Two visual processing pathways are targeted by gonadotropin-releasing hormone in the retina. Brain. Behav. Evol. 66, 1–9. https://doi.org/10.1159/000085043.

Guilgur, L.G., Strüssmann, C.A., Somoza, G.M., 2009. mRNA expression of GnRH variants and receptors in the brain, pituitary and ovaries of pejerrey (*Odontesthes bonariensis*) in relation to the reproductive status. Fish Physiol. Biochem. 35, 157–166. https://doi.org/10.1007/s10695-008-9215-4.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321. https://doi.org/10.1093/sysbio/syq010.

Hapgood, J.P., Sadie, H., van Biljon, W., Ronacher, K., 2005. Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. J. Neuroendocrinol. 17, 619–638. https://doi.org/10.1111/j.1365-2826.2005.01353.x.

Hildahl, J., Sandvik, G.K., Edvardsen, R.B., Norberg, B., Haug, T.M., Weltzien, F.A., 2011. Four gonadotropin releasing hormone receptor genes in Atlantic cod are differentially expressed in the brain and pituitary during puberty. Gen. Comp. Endocrinol. 173, 333–345. https://doi.org/10.1016/j.ygcen.2011.06.002.

Hutchings, J.A., Jones, M.E.B., 1998. Life history variation and growth rate thresholds for maturity in Atlantic salmon, *Salmo salar*. Can. J. Fish. Aquat. Sci. 55 (Suppl), 22–47. https://doi.org/10.1139/cjfas-55-S1-22.

Ikegami, T., Maruyama, Y., Doi, H., Hattori, A., Ando, H., 2015. Ultaradian oscillation in expression of four melatonin receptor subtype genes in the pineal gland of the grass puffer, a semilunar-synchronized spawner, under constant darkness. Front. Neurosci. 9, 1–10. https://doi.org/10.3389/fnins.2015.00009.

Ikemoto, T., Park, M.K., 2005. Identification and molecular characterization of three GnRH ligands and five GnRH receptors in the spotted green pufferfish. Mol. Cell. Endocrinol. 242, 67–79. https://doi.org/10.1016/j.mce.2005.07.004.

Ikemoto, T., Enomoto, M., Park, M.K., 2004. Identification and characterization of a reptilian GnRH receptor from the leopard gecko. Mol. Cell. Endocrinol. 214, 137–147. https://doi.org/10.1016/j.mce.2003.10.062.

Jodo, A., Ando, H., Urano, A., 2003. Five different types of putative GnRH receptor gene are expressed in the brain of masu salmon (*Oncorhynchus masou*). Zool. Sci. 20, 1117–1125. https://doi.org/10.2108/zsj.20.1117.

Kanda, S., Okubo, K., Oka, Y., 2011. Differential regulation of the luteinizing hormone genes in teleosts and tetrapods due to their distinct genomic environments – insights into gonadotropin beta subunit evolution. Gen. Comp. Endocrinol. 173, 253–258. https://doi.org/10.1016/j.ygcen.2011.05.015.

Karigo, T., Kanda, S., Takahashi, A., Abe, H., Okubo, K., Oka, Y., 2012. Time-of-day-dependent changes in GnRH1 neuronal activities and gonadotropin mRNA expression in a daily spawning fish, medaka. Endocrinology 153, 3394–3404. https://doi.org/10.1210/en.2011-2022.

Kim, D.K., Cho, E.B., Moon, M.J., Park, S., Hwang, J.I., Kah, O., Sower, S.A., Vaudry, H., Seong, J.Y., 2011. Revisiting the evolution of gonadotropin-releasing hormones and their receptors in vertebrates: secrets hidden in genomes. Gen. Comp. Endocrinol. https://doi.org/10.1016/j.ygcen.2010.10.018.

Kim, M., Gee, M., Loh, A., Rachatasumrit, N., 2010. Ref-finder. In: Proceedings of the Eighteenth ACM SIGSOFT International Symposium on Foundations of Software Engineering - FSE '10. ACM Press, New York, New York, USA, pp. 371. https://doi.org/10.1145/1882291.1882353.

Lefort, V., Longueville, J.E., Gascuel, O., 2017. SMS: smart model selection in PhyML. Mol. Biol. Evol. 34, 2422–2424. https://doi.org/10.1093/molbev/msx149.

Lethimonier, C., Madigou, T., Muñoz-Cueto, J.A., Lareyre, J.J., Kah, O., 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen. Comp. Endocrinol. https://doi.org/10.1016/j.ygcen.2003.10.007.

Levavi-Sivan, B., Avitan, A., 2005. Sequence analysis, endocrine regulation, and signal transduction of GnRH receptors in teleost fish. In: General and Comparative Endocrinology. Academic Press, pp. 67–73. https://doi.org/10.1016/j.ygcen.2005.01.019.

Levavi-Sivan, B., Yaron, Z., 1989. Gonadotropin secretion from perifused tilapia pituitary in relation to gonadotropin-releasing hormone, extracellular calcium, and activation of protein kinase C. Gen. Comp. Endocrinol. 75, 187–194. https://doi.org/10.1016/0016-6480(89)90070-1.

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412–437. https://doi.org/10.1016/j.ygcen.2009.07.019.

Lin, C.J., Wu, G.C., Lee, M.F., Lau, E.L., Dufour, S., Chang, C.F., 2010. Regulation of two forms of gonadotropin-releasing hormone receptor gene expression in the protandrous black porgy fish, *Acanthopagrus schlegeli*. Mol. Cell. Endocrinol. 323, 137–146. https://doi.org/10.1016/j.mce.2010.04.003.

Liu, Y.C., Kato, Y., Inoue, K., Tanaka, S., Kurosumi, K., 1988. Co-localization of LHβ and

FSHβ mRNAs in the porcine anterior pituitary by *in situ* hybridization with biotinylated probes. Biochem. Biophys. Res. Commun. 154, 80–84. https://doi.org/10.1016/0006-291X(88)90652-3.

Liu, F., Usui, I., Evans, L.G., Austin, D.A., Mellon, P.L., Olefsky, J.M., Webster, N.J.G., 2002. Involvement of both G q/11 and G s proteins in gonadotropin-releasing hormone receptor-mediated signaling in LβT2 cells. J. Biol. Chem. 277, 32099–32108. https://doi.org/10.1074/jbc.M203639200.

Loir, M., 1999. Spermatogonia of rainbow trout: II. In vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. Mol. Reprod. Dev. 53, 434–442. https://doi.org/10.1002/(SICI)1098-2795(199908)53:4<434::AID-MRD9>3.0.CO;2-L.

Lumayno, S.D.P., Ohga, H., Selvaraj, S., Nyuji, M., Yamaguchi, A., Matsuyama, M., 2017. Molecular characterization and functional analysis of pituitary GnRH receptor in a commercial scombroid fish, chub mackerel (*Scomber japonicus*). Gen. Comp. Endocrinol. 247, 143–151. https://doi.org/10.1016/j.ygcen.2017.01.027.

Madigou, T., Mañanos-Sanchez, E., Hulshof, S., Anglade, I., Zanuy, S., Kah, O., 2000. Cloning, tissue distribution, and central expression of the gonadotropin-releasing hormone receptor in the rainbow trout (*Oncorhynchus mykiss*). Biol. Reprod. 63, 1857–1866. https://doi.org/10.1095/biolreprod63.6.1857.

Madigou, T., Uzbekova, S., Lareyre, J.J., Kah, O., 2002. Two messenger RNA isoforms of the gonadotrophin-releasing hormone receptor, generated by alternative splicing and/or promoter usage, are differentially expressed in rainbow trout gonads during gametogenesis. Mol. Reprod. Dev. 63, 151–160. https://doi.org/10.1002/mrd.90006.

Mangalam, H., Stewart, J., Zhou, J., Schlauch, K., Waugh, M., Chen, G., Farmer, A.D., Colello, G., Weller, J.W., 2001. GeneX: an open source gene expression database and integrated tool set. IBM Syst. J. 40, 552–569. https://doi.org/10.1147/sj.402.0552.

Maugars, G., Schmitz, M., 2006. Molecular cloning and characterization of FSH and LH receptors in Atlantic salmon (*Salmo salar* L.). Gen. Comp. Endocrinol. 149, 108–117. https://doi.org/10.1016/j.ygcen.2006.04.011.

Maugars, G., Schmitz, M., 2008b. Expression of gonadotropin and gonadotropin receptor genes during early sexual maturation in male Atlantic salmon parr. Mol. Reprod. Dev. 75, 403–413. https://doi.org/10.1002/mrd.20767.

Maugars, G., Schmitz, M., 2008a. Gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes. Gen. Comp. Endocrinol. 159, 178–187. https://doi.org/10.1016/j.ygcen.2008.08.008.

Mazón, M.J., Gómez, A., Yilmaz, O., Carrillo, M., Zanuy, S., 2014. Administration of follicle-stimulating hormone in vivo triggers testicular recrudescence of juvenile European sea bass (*Dicentrarchus labrax*). Biol. Reprod. 90, 6. https://doi.org/10.1095/biolreprod.113.110569.

Melo, M.C., Andersson, E., Fjelldal, P.G., Bogerd, J., Franca, L.R., Taranger, G.L., Schulz, R.W., 2014. Salinity and photoperiod modulate pubertal development in Atlantic salmon (*Salmo salar*). J. Endocrinol. 220, 319–332. https://doi.org/10.1530/JOE-13-0240.

Melo, M.C., van Dijk, P., Andersson, E., Nilsen, T.O., Fjelldal, P.G., Male, R., Nijenhuis, W., Bogerd, J., de França, L.R., Taranger, G.L., Schulz, R.W., 2015. Androgens directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (*Salmo salar*). Gen. Comp. Endocrinol. 211, 52–61. https://doi.org/10.1016/j.ygcen.2014.11.015.

Millar, R.P., Lu, Z.L., Pawson, A.J., Flanagan, C.A., Morgan, K., Maudsley, S.R., 2004. Gonadotropin-releasing hormone receptors. Endocr. Rev. https://doi.org/10.1210/er.2003-0002.

Moncaut, N., 2005. Five gonadotrophin-releasing hormone receptors in a teleost fish: isolation, tissue distribution and phylogenetic relationships. J. Mol. Endocrinol. 34, 767–779. https://doi.org/10.1677/jme.1.01757.

Naito, N., Suzuki, K., Nozaki, M., Swanson, P., Kawauchi, H., Nakai, Y., 1993. Ultrastructural characteristics of two distinct gonadotropes (GTH I- and GTH II-cells) in the pituitary of rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem. 11, 241–246. https://doi.org/10.1007/BF00004571.

Naor, Z., 1990. Signal transduction mechanisms of Ca2+ mobilizing hormones: the case of gonadotropin-releasing hormone. Endocr. Rev. 11, 326–353. https://doi.org/10.1210/edrv-11-2-326.

Okubo, K., Nagahama, Y., 2008. Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. Acta Physiol. https://doi.org/10.1111/j.1748-1716.2008.01832.x.

Peñaranda, D.S., Mazzeo, I., Hildahl, J., Gallego, V., Nourizadeh-Lillabadi, R., Pérez, L., Asturiano, J.F., Weltzien, F.A., 2013. Molecular characterization of three GnRH receptor paralogs in the European eel, Anguilla anguilla: tissue-distribution and changes in transcript abundance during artificially induced sexual development. Mol. Cell. Endocrinol. 369, 1–14. https://doi.org/10.1016/j.mce.2013.01.025.

Peter, R.E., 1983. The brain and neurohormones in teleost reproduction. Fish Physiol. 9, 97–135. https://doi.org/10.1016/S1546-5098(08)60287-3.

Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50, 465–495. https://doi.org/10.1146/annurev.bi.50.070181.002341.

Planas, J.V., Swanson, P., 1995. Maturation-associated changes in the response of the salmon testis to the steroidogenic actions of gonadotropins (GTH I and GTH II) in vitro. Biol. Reprod. 52, 697–704. https://doi.org/10.1095/biolreprod52.3.697.

Prat, F., Sumpter, J.P., Tyler, C.R., 1996. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductivecycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol. Reprod. 54, 1375–1382. https://doi.org/10.1095/biolreprod54.6.1375.

Ramakrishnappa, N., Rajamahendran, R., Lin, Y.M., Leung, P.C.K., 2005. GnRH in non-hypothalamic reproductive tissues. Anim. Reprod. Sci. https://doi.org/10.1016/j.anireprosci.2005.05.009.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Schulz, R.W., de França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411. https://doi.org/10.1016/j.ygcen.2009.02.013.

Sefideh, F.A., Moon, M.J., Yun, S., Hong, S.I., Hwang, J.I., Seong, J.Y., 2014. Local duplication of gonadotropin-releasing hormone (GnRH) receptor before two rounds of whole genome duplication and origin of the mammalian GnRH receptor. PLoS One 9, e87901. https://doi.org/10.1371/journal.pone.0087901.

Servili, A., Lethimonier, C., Lareyre, J.J., López-Olmeda, J.F., Sánchez-Vázquez, F.J., Kah, O., Muñoz-Cueto, J.A., 2010. The highly conserved gonadotropin-releasing hormone-2 form acts as a melatonin-releasing factor in the pineal of a teleost fish, the European sea bass *Dicentrarchus labrax*. Endocrinology 151, 2265–2275. https://doi.org/10.1210/en.2009-1207.

Shahjahan, M., Ikegami, T., Osugi, T., Ukena, K., Doi, H., Hattori, A., Tsutsui, K., Ando, H., 2010. Synchronised expressions of LPXRFamide peptide and its receptor genes: seasonal, diurnal and circadian changes during spawning period in grass puffer. J. Neuroendocrinol. 23, 39–51. https://doi.org/10.1111/j.1365-2826.2010.02081.x.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J.D., Higgins, D.G., 2014. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. 539-539. Mol. Syst. Biol. 7. https://doi.org/10.1038/msb.2011.75.

Suzuki, K., Nagahama, Y., Kawauchi, H., 1988. Steroidogenic activities of two distinct salmon gonadotropins. Gen. Comp. Endocrinol. 71, 452–458. https://doi.org/10.1016/0016-6480(88)90274-2.

Swanson, P., Suzuki, K., Kawauchi, H., Dickhoff, W.W., 1991. Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol. Reprod. 44, 29–38. https://doi.org/10.1095/biolreprod44.1.29.

Swanson, P., Dickey, J.T., Campbell, B., 2003. Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem. 28, 53–59. https://doi.org/10.1023/B:FISH.0000030476.73360.07.

Taylor, E.B., 1991. A review of local adaptation in Salmonidae, with particular reference to Pacific and Atlantic salmon. Aquaculture 98, 185–207. https://doi.org/10.1016/0044-8486(91)90383-i.

Temple, J.L., Millar, R.P., Rissman, E.F., 2003. An evolutionarily conserved form of gonadotropin-releasing hormone coordinates energy and reproductive behavior. Endocrinology 144, 13–19. https://doi.org/10.1210/en.2002-220883.

Vandesompele, J., De Preter, K., Pattyn, I., Poppe, B., Van Roy, N., De Paepe, A., Speleman, R., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 34–41. https://doi.org/10.1186/gb-2002-3-7-research0034.

von Krogh, K., Bjørndal, G.T., Nourizadeh-Lillabadi, R., Hodne, K., Ropstad, E., Haug, T.M., Weltzien, F.A., 2017. Sex steroids differentially regulate fshb, lhb and gnrhr expression in Atlantic cod (*Gadus morhua*) pituitary. Reproduction 154, 581–594. https://doi.org/10.1530/REP-17-0208.

Weltzien, F.A., Andersson, E., Andersen, Ø., Shalchian-Tabrizi, K., Norberg, B., 2004. The brain-pituitary-gonad axis in male teleosts, with special emphasis on flatfish (Pleuronectiformes). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 137, 447–477. https://doi.org/10.1016/j.cbpb.2003.11.007.

Weltzien, F.A., Pasqualini, C., Vernier, P., Dufour, S., 2005. A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. In: General and Comparative Endocrinology. Academic Press, pp. 134–142. https://doi.org/10.1016/j.ygcen.2004.12.019.

Williams, B.L., Akazome, Y., Oka, Y., Eisthen, H.L., 2014. Dynamic evolution of the GnRH receptor gene family in vertebrates. BMC Evol. Biol. 14, 215. https://doi.org/10.1186/s12862-014-0215-y.

Wilson, B.S., Komuro, M., Farquhar, M.G., 1994. Cellular variations in heterotrimeric G protein localization and expression in rat pituitary. Endocrinology 134, 233–244. https://doi.org/10.1210/endo.134.1.8275939.

Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225, 131–185. https://doi.org/10.1016/S0074-7696(05)25004-0.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13, 134. https://doi.org/10.1186/1471-2105-13-134.

Yu, G., Smith, D.K., Zhu, H., Guan, Y., Lam, T.T.Y., 2017. ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol. Evol. 8, 28–36. https://doi.org/10.1111/2041-210X.12628.