Gnrh receptor gnrr2bba is expressed exclusively in lhb-expressing cells in Atlantic salmon male parr

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\textbf{A B S T R A C T}

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 Gnrh 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 (\textit{Salmo salar}) male parr. A total of six Gnrhr paralogs were identified in the Atlantic salmon genome and named according to phylogenetic relationship; gnrhr1ca, gnrhr1cb, gnrhr1ba, gnrhr1bb, gnrhr2ba, gnrhr2bb. All paralogs, except gnrhr1ca, were expressed in male parr pituitary during gonadal maturation as evidenced by qPCR analysis. Only one gene, gnrhr2bb, 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 gnrhr2ba and lhb (daily cycle) in immature fish in mid-April. Double fluorescence in situ hybridization showed that gnrhr2ba was expressed exclusively in lhb gonadotropes in the pituitary, with no expression detected in fshb cells. These results suggest the involvement of receptor paralog gnrhr2bb in the regulation of lhb cells, and not fshb cells. In sexually maturing Atlantic salmon male parr.

\textbf{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 \( \alpha \)-subunit and a specific \( \beta \)-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
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 salmons, both fsb 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 tests 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. Gnrh exert their biological activity through interaction with two types of G protein, G11 and Gs. Coupling to the former activates a cascade involving phospholipase C (PLC), inositol-3-phosphate (IP3), diacylglycerol (DAG), and intracellular calcium (Ca2+), 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 fsb 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 (Happold et al., 2005), several paralogs have been identified in teleosts. For instance, some species possess up to five Gnrh paralogs, such as in seabass (Dicentrarchus labrax; Moncaut, 2005) and masu salmon (Onchorhynchus 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 (Sefidieh 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 GnRHR2a) 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 (Sefidieh et al., 2014). Considering that salmonids went through a four 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 Leanziz 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 Lys, 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 m3) 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 maturation 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 spermatocytes).

2.3. Gnrhr receptor identification

The Salmo salar Gnrhr were identified from the Atlantic salmon genome assembly (ICASAG 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 (Qiagen). 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 Sefidieh 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-a, -b and gnrhr2bb-a, -β. Due to the high degree of sequence identity between 4R paralogs gnrhr1cb-a, -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 ef1a) as outlined by Vandesompele et al. (2002). Stability of the housekeeping genes were tested using the online tool ReFinder (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, gnrhr2bb, gnrhr2bbα, Table 1) by PCR. The products were then isolated after gel electrophoresis using Gel Extraction Kit (Invitrogen), cloned in pCR®II Vector (Invitrogen) and sequenced by GATC biotech. Plasmids were linearized by PCR amplification using M13 primers. Antisense and sense RNA probes were synthesized 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, Wetzlar, 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 pyrocarbontate; 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).
potential signification transformed to meet test criteria for normal distribution. After using the Shapiro-Wilk W test. When needed, data were log or square root transformed (SAS Institute Inc., Cary, NC, USA). All data were tested for normality.

3. Results

3.1. Phylogenetic analysis

Using the following objectives: Plan-Neofluor 10×/0.3 M27 (Zeiss); Plan-Neofluor 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 crosstalk 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 Random 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 Gnhr 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 gnhr genes. The Gnhr phylogenetic tree is represented in Fig. 1. The Gnhr are divided into two main types, GnHR1 and GnHR2, each divided into three subtypes: GnHR1a, GnHR1b, GnHR1c for the type 1, and GnHR2a, GnHR2b and GnHR2c 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 GnHR1a. The second opossum receptor was from the type 2 and grouped with sauropsid, amphibian, and coelacanth Gnhr in the GnHR2c clade. The two other sauropsid and amphibian Gnhr belonged to GnHR1b and GnHR2b subtypes, respectively.

Four Gnhr 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 GnHR-1a, -1b, -2a and -2b) including the GnRHR2a, comprising only a coelacanth Gnhr. The actinopterygian Gnhr were distributed into two clades, one of type 1, the GnHR1c, and one of type 2, the GnHR2b. In the GnHR1c clade, only actinopterygian species were represented. One coelacanth receptor was found in each of the five clades beside GnHR1c. Among the Actinopterygians, the spotted gar branched at the basal position of teleost GnHR1c. The teleost GnHR1c were grouped into two clades, GnHR-1c and -1b. Four of the Atlantic salmon Gnhr belonged to the GnHR1c two paralogs clustering in the GnHR1c clade (Gnrhr1ca, Gnrhr1cb), and two in the GnHR1c clade (Gnrhr1ca, Gnrhr1cb), with a pair of Coho salmon Gnhr. 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 Actinopterygians, the spotted gar branched at the basal position of the teleost Gnrhr2b. Teleost Gnrhr2b paralogs clustered mainly into two clades, Gnrhr-2b and -2b. A local duplication formed two additional clusters (named 1 and 2) within the Gnrhr2b complex clade (Gnrhr2b1a, Gnrhr2b1b), and a pair of Coho salmon Gnrhr. While Gnrhr2b1b paralogs were conserved in all the teleost species examined, Gnrhr2b1a was lost in some species, including arowana (Scleropages formosus), Atlantic cod (Gadus morhua) and medaka (Oryzias latipes).

Table 1

| Gene | Accession number | Primer Fw (5′–3′) | Primer Rv (3′–5′) | Product size (bp) | Efficiency (%) |
|------|------------------|-------------------|-------------------|-------------------|----------------|
| Gnrhr1ca | KF225728 | TGGACATTGGAATGGG | CAGCCTGATTTGCGAAGAGAT | 97 | 98 |
| Gnrhr1cb | KF225729 | ATGCCCATACGACGGAAAG | TATGAGCAGGAGAGAGAT | 117 | 99 |
| Gnrhr2ba | MF073196 | ATACCCTCATCTGTTGGCTGAC | TAAACCCACACGCTGATAC | 60 | 99.5 |
| Gnrhr2bb | MF073197 | CTACAGCTGCCCACCTTCTC | GTCGAAATCAGCTATGGACAAT | 122 | 100 |
| Gnrhr1c | KF225730 | TTACATTGGGAATGGG | TCGTATGGAATGGCAAAAT | 60 | 99.5 |
| Gnrhr1b | MF073198 | CTACAGCTGCCCACCTTCTC | GTCGAAATCAGCTATGGACAAT | 60 | 99.5 |

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

Table 2

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

Table 2

| Stage | Observed cysts |
|-------|----------------|
| I     | SPA            |
| II    | SPA + SPB      |
| III   | SPA + SPB + SC |
| IV    | SPA + SPB + SC + ST |
| V     | SPA + SPB + SC + ST + SZ |
| VI    | SZ is dominating. Large lumen in tubules |

Using the following objectives: Plan-Neofluor 10×/0.3 M27 (Zeiss); Plan-Neofluor 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 crosstalk between filters. Images were acquired with ZEN 2009 (Zeiss) and processed with FIJI software (Schindelin et al., 2012).

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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), gnrhr1caα (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. 2D 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).
3.3. Gonadotropin and GnRH receptor gene expression during sexual maturation

Pituitary expression of <i>gnrhr</i> (<i>gnrhr1ca, gnrhr1cb, gnrhr2ba, gnrhr2bbf</i>) and gonadotropin subunits (<i>fshb, lhb</i>) 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, <i>gnrhr1ca</i> and <i>gnrhr1cb</i> (Fig. 3 A and B), showed no differences in expression with regard to either maturational stage or sampling date, while expression of <i>gnrhr1ca</i>, 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 <i>gnrhr2bb</i> on June 21st than non-maturing fish (Fig. 4A), while <i>gnrhr2bbf</i> showed no differential expression levels between groups or sampling dates but was characterized by high individual variation (Fig. 4B). In concomitance with the <i>gnrhr2bb</i> expression, higher <i>fshb</i> and <i>lhb</i> levels were detected in pituitaries from maturing fish on June 21st (Fig. 4C and D). Additionally, maturing fish expressed significantly higher levels of <i>fshb</i> than non-maturing fish from June 21st to July 19th peakings 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, <i>fshb</i> expression decreased in non-maturing fish from May 8th to 23rd, while remaining constant in maturing fish (Fig. 4H), and <i>lhb</i> decreased in both groups from April 25th to June 7th (Fig. 4G). Both <i>fshb</i> and <i>lhb</i> 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 <i>fshb, lhb, gnrhr2bb</i> and <i>gnrhr2bbf</i>.

Daily pituitary expression of <i>fshb, lhb, gnrhr2bb</i> and <i>gnrhr2bbf</i> 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 <i>fshb, lhb, gnrhr2bb</i> and <i>gnrhr2bbf</i> 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 <i>lhb</i> (Fig. 5D) and <i>gnrhr2bbf</i> (Fig. 5H), but not for <i>fshb</i> (Fig. 5B). <i>lhb</i> (Fig. 5D) and <i>gnrhr2bbf</i> (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 <i>lhb</i> and 2.5 ± 0.3 for <i>gnrhr2bbf</i>. Daily fluctuation in the expression of the second receptor gene, <i>gnrhr2bbf</i>, displayed a much higher induction, with an 83.4 ± 7.8-fold change between minimum and maximum points. Low levels of <i>gnrhr2bbf</i> 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 <i>gnrhr</i> in relation to testis development

The plotting of GnRH2 paralogs (<i>gnrhr2bb, gnrhr2bbf</i>) relative expression levels versus testis development revealed an increase of <i>gnrhr2bb</i> (Fig. 6A) expression from stage I, when only spermatogonia (either undifferentiated or differentiated) are present, to stage VI, where spermatozoa are abundant in the lumen. On the other hand, <i>gnrhr2bbf</i> (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 <i>gnrhr2bb</i> and gonadotropins (<i>gnrhr2bb-fshb</i> ρ = 0.83; <i>gnrhr2bb-lhb</i> ρ = 0.81) compared to <i>gnrhr2bbf</i> and gonadotropins (<i>gnrhr2bbf-fshb</i> ρ = 0.25 <i>gnrhr2bbf-lhb</i> ρ = 0.49). Results are summarized in Table 3.

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

While no labelling was detected for <i>gnrhr2bbf</i>, the localization of <i>fshb, lhb</i> and <i>gnrhr2bb</i> 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).
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...
Gnhr in vertebrates. Previous studies conducted from Hildahl et al. (2011), grouped Gnhr 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 chromosomal 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 Gnhr 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 Gnhr subtypes: GnRHR1c and GnRHR2b (named respectively GnRHR1A and GnRHR2B from Hildahl et al., 2011). The teleost 3R...

Table 3

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

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 ef1α. 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.

Fig. 7. Fluorescent in situ hybridization in parasagittal pituitary sections (anterior to the left) of Atlantic salmon maturing male parr. Confoocal 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 gnrhr2a2 on the same chromosomal region confirmed a recent local duplication of the Gnrh2a2a in the super order of acanthomorpha (Sefiedeh 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 gnrhr1caaa, gnrhr1caab, gnrhr1cb, gnrhr1cbf, gnrhr2aba and gnrhr2bbβ. A similar scenario was detected in coho salmon. The presence of the gnrhr2bb in pike (Esocus 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 gpa, or fshβ 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, gnrhr1caaa showed higher expression in the brain and low level in the pituitary while gnrhr1caab displayed higher expression in the pituitary. Atlantic salmon have conserved also a pair of 4R paralogs belonging to GnRHR type 2. While gnrhr2bba is mainly expressed in the pituitary and in the brain, gnrhr2bbaβ 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 Gnrhr 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).

Gnrhr 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 Gnhr mediated coupling of visual information with reproduction (for review see Okubo and Nagahama, 2008). In European seabass (Dicentrarchus labrax), Gnrhr2 acts as a melanin-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 melanin 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; Ikemoto and Park, 2005). In maturing masu salmon males, gnrhr1caα (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 gnrhr2bββ, 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, Gnrhr 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 gnrhr1caα 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 R3 R2-1, respectively in Ikemoto and Park (2005)); three (gnrhr1ca, gnrhr2ba1, gnrhr2ba2) in sea bass (named dGnRHRaR1, dGnRHRaR2, dGnRHRaR3 in Moncatt (2005)); three (gnrhr1cb, gnrhr2ba1 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, gnrhr2bb, 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; Guigur 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, gnrhr2b increases during female maturation (Peñaranda et al., 2013). While in eel and salmon the receptor type 2 that may mediate the Gnrhr 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 (dGnRHR2-2A 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 gnrhr2ba1 (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 fshβ 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 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 testsis 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 fluctuation in both autumn and spring in immature fish, rather than an induction in maturing fish. While gnrhr2bbα displayed correlation with maturational stage 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 ghnrh2beta and fsh mRNA expression profiles during the initial stages of spermatogenic development.

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Appendix A. Supplementary data

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

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