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Neuroendocrine regulation of pituitary gonadotropins during puberty in Atlantic salmon parr with focus on melatonin and Gnrh systems

Nevroendokrin regulering av gonadotropiner i atlantisk laks parr med fokus på melatonin og Gnrh systemer

Elia Ciani

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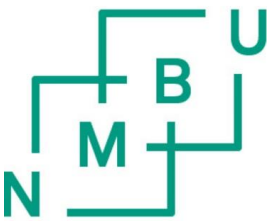

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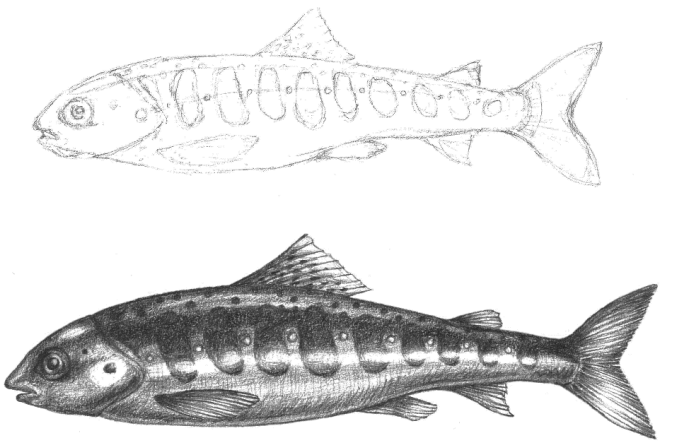
And the earth becomes my throne
I adapt to the unknown
Under wandering starts I've grown
By myself but not alone

Anywhere I roam, where I lay my head is home
(Wherever I may roam – Metallica)



Salmo salar parr.

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Manuscript

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Manuscript

Paper III

Melatonin receptors in Atlantic salmon stimulate cAMP levels and show season-dependent circadian variations in pituitary expression levels

Elia Ciani, Romain Fontaine, Gersende Maugars, Naama Mizrahi, Ian Mayer, Berta Levavi-Sivan, Finn-Arne Weltzien

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Abbreviations

This thesis applies the nomenclature recommendations from MGI (http://www.informatics.jax.org/mgihome/nomen/gene.shtml) and ZFIN (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions), where mammalian genes are italicized, first letter uppercase (e.g. *Fshb*); fish genes are italicized, all letters lowercase (*fshb*); mammalian (or unspecified) proteins are non-italicized, all letters uppercase (FSH); and fish proteins are non-italicized, first letter uppercase (Fsh).

11KT	11-keto testosterone
5-HT	5- hydroxytryptamine (serotonin)
AANAT	arylalkylamine *N*-acetyltransferase
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
BK- K_{Ca}	big conductance calcium-activated potassium channels
cAMP	cyclic adenosine monophosphate
D	darkness
DAG	diacylglycerol
DD	constant darkness
DHP	17α,20β-dihydroxy-4-pregnen-3-one
E2	17β-estradiol
ER	endoplasmatic reticulum
ERK	extracellular signal related kinases
FSHb	follicle-stimulating hormone β subunit
FSHR	follicle-stimulating hormone receptor
GABA	gamma-aminobutyric acid
GAP	GnRH-associated peptide
GH	growth hormone
GnIH	gonadotropin inhibitory hormone
GnRH	gonadotropin releasing hormone
GnRHR	gonadotropin releasing hormone receptor
GPa	gonadotropin subunit α
GtH	gonadotropin hormone
HCG	human chorionic gonadotropin
HIOMT	hydroxyindole-*O*-methyltransferase
BPG	brain-pituitary-gonad axis
IGF-I	insulin-like growth factor
IMPRESS	improved production strategies for endangered freshwater species
IP3	inositol triphosphate
JNK	jun n-terminal kinases
K_{Ca}	calcium-activated potassium channels
L	light
LHb	luteinizing hormone β subunit
LHR	luteinizing hormone receptor
LL	constant light

MAPK	mitogen activated protein kinase
MSH	melanocyte stimulating hormone
Mtnr	melatonin receptors
NPY	neuropeptide Y
PD	*pars distalis*
PI	*pars intermedia*
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PN	*pars nervosa*
PPD	*proximal pars distalis*
PRL	prolactin
RPD	*rostral pars distalis*
T	testosterone
TSH	thyroid stimulating hormone
UTR	untranslated region
VSCC	voltage sensitive calcium channels

Species nomenclature

Throughout this thesis, the common name of a species is followed by the scientific name upon its first use. Only the common name will be given in consecutive use, and in case no common name exist in English, only the scientific name will be used

African catfish	*Clarias gariepinus*
Atlantic cod	*Gadus morhua*
Atlantic croaker	*Micropogonias undulates*
Ayu	*Plecoglossus altivelis*
Bluefin tuna	*Thunnus thynnus*
Chum salmon	*Oncorhynchus keta*
Coho salmon	*Oncorhynchus kisutch*
European eel	*Anguilla anguilla*
European sea bass	*Dicentrarchus labrax*
Goldfish	*Carassius auratus*
Haddock	*Melanogrammus aeglefinus*
Japanese eel	*Anguilla japonica*
Lake whitefish	*Coregonus clupeaformis*
Masu salmon	*Oncorhynchus masou*
Medaka	*Oryzias latipes*
Nile tilapia	*Oreochromis niloticus*
Pacific herring	*Clupea harengus pallasi*
Pike	*Esox lucius*
Rainbow trout	*Oncorhynchus mykiss*
Sea bream	*Sparus aurata*
Senegalese sole	*Solea senegalensis*
Sockeye salmon	*Oncorhynchus nerka*
Spiny dogfish	*Squalus acanthias*
Spotted green pufferfish	*Tetraodon nigroviridis*
White sucker	*Catostomus commersonii*
Zebrafish	*Danio rerio*

Summary

The pituitary gland, gonadotropins, together with neurohormones like gonadotropin-releasing hormone (Gnrh) and melatonin are key players in the control of vertebrate reproduction. In teleosts, despite the numerous studies conducted on the role of these hormones, relatively little is known about the functions of the multiple paralogs of their specific receptors. This study used male parr as a model for investigating the neuroendocrine control of sexual maturation in Atlantic salmon.

First, to identify the onset of maturation and discriminate between maturing and non-maturing fish, testes histology was analysed in combination with gonadosomatic index, gonadotropin expression and plasma steroid levels. The results indicated that sexual maturation can be triggered as early as six months after hatching, in early autumn. Six Gnrh receptor paralogs where found expressed in male parr pituitaries, but only one of them, *gnrhr2b1,* showed a peak in maturing fish in concomitance with higher gonadotropin expression levels. *In situ* hybridization localized *gnrhr2b1* mRNA specifically to *lhb*-producing cells, suggesting this receptor to be involved in the regulation of *lhb*-expression. The third part of this study focused on the characterization of melatonin receptors, considering the importance of melatonin as the "time keeping" molecule in vertebrates. *In silico* studies identified five genes encoding putative functional melatonin receptors, phylogenetically clustered in three subtypes, 1A, 1Al and 1B. Pharmacological characterization of cloned receptors, Mtnr1Aaα, Mtnr1Ab and Mtnr1B, proved their functionality *in vitro.* Three genes, *mtnr1aaβ, mtnr1ab* and *mtnr1b*, were expressed in the pituitary, showing intense daily fluctuations in spring, but not in autumn, indicative of important seasonal differences.

The results presented in this thesis add to our understanding on gonadotropin control from Gnrh, suggesting that, in Atlantic salmon, differential regulation may occur through specific receptor paralogs expressed in different cell types. The detection of melatonin receptors in the pituitary gland, showing variation in expression depending on season, strongly advocates for a direct involvement of melatonin in one (or more) of the seasonal processes regulated from the pituitary gland.

Norsk sammendrag

Gonadotropiner fra hypofysen, folikkelstimulerende hormon (Fsh) og luteiniserende hormon (Lh) regulerer kjønnsmodning hos alle vertebrater. Utskillelse av Fsh og Lh styres hovedsakelig av nevrohormoner fra hypotalamus, med gonadotropinfrisettende hormon (Gnrh) som det viktigste. Et annet nevrohormon, melatonin, har også en sentral rolle men eksakt hvordan det påvirker kjønnsmodningen er ikke kjent.

I PhD arbeidet har jeg benyttet modnende hannfisk (parr) av atlantisk laks for å studere den nevroendokrine kontrollen av kjønnsmodning. For å skille mellom modnende og ikke-modnende fisk ble det foretatt histologiske analyser av testiklene, sett i sammenheng med gonadosomatisk indeks, samt genuttrykk for Fsh og Lh og plasmanivåer av steroidhormoner. Resultatene viste at kjønnsmodningen kan starte allerede seks måneder etter klekking, tidlig på høsten. Det ble videre identifisert seks paraloge gener av Gnrh-reseptorer i hypofysen hos parr, men bare den ene reseptoren, *gnrhr2b1*, viste en signifikant økning hos modnende fisk. Dette resultatet samsvarer også med den observerte økningen i genuttrykket til de to gonadotropinene. *In situ* hybridisering lokaliserte *gnrhr2b1* mRNA spesifikt til *lhb*-produserende celler, noe som kan tyde på at denne reseptoren er involvert i reguleringen av *lhb*-ekspresjon.

Videre karakteriserte jeg laksens melatoninreseptorer, og betydningen av melatonin som en "time-keeper" i forbindelse med kjønnsmodningen. *In silico* studier identifiserte fem gener kodende for potensielle melatoninreseptorer. Disse reseptorene ble fylogenetisk gruppert i tre undergrupper, 1A, 1Al og 1B. Farmakologisk karakterisering av de klonede reseptorene, Mtnr1Aaα, Mtnr1Ab og Mtnr1B, viste at disse er fullt funksjonelle *in vitro*. Tre gener, *mtnr1aaβ*, *mtnr1ab* og *mtnr1b*, var uttrykt i hypofysen, og om våren viste disse reseptorene daglige svingninger i genuttrykk. Om høsten derimot, ble det ikke observert slike svingninger i genuttrykket, noe som indikerer viktige sesongavhengige forskjeller.

Resultatene som presenteres i denne avhandlingen er med på å øke vår forståelse av hvordan gonadotropinene påvirkes av Gnrh i atlantisk laks og indikerer at den ulike reguleringen av gonadotropinene kan skyldes at spesifikke reseptorer er utrykt i ulike celletyper. Identifiseringen av melatoninreseptorer i hypofysen og deres sesongavhengige uttrykksmønstre peker i retning av en mulig rolle for melatonin i

direkte påvirkning av en eller flere av de sesongavhengige prosessene kontrollert av hypofysen.

Introduction

General background

The Atlantic salmon (*Salmo salar*) is an iconic anadromous species, with a complex life cycle divided between rivers and ocean (reviewed in Klemetsen et al., 2003). The bond between salmon and people in Norway is centuries old, as part of the economy and culture of the nation (Fig. 1). In the last decades however, the survival of wild salmon has been threatened from several factors linked in a direct or indirect way to human activities including habitat loss or alterations, overfishing and genetic pollution (Jonsson and Jonsson, 2004; Kennedy and Crozier, 2010; Lundqvist et al., 2008; McGinnity et al., 2003). Despite efforts aimed at improving the quality of wild stocks, the number of individuals returning to the cost of Norway every year has decreased 55% since the 1980s. Norwegian wild salmon populations are estimated to 470.000 individuals, compared to the 1.180.000 tonnes of farmed salmon produced only in 2016 (Thorstad and Forseth, 2017). The decline in wild populations is not restricted to Norwegian stocks, and significant decline has been reported also in Spanish and French stocks (Horreo et al., 2011; Le Cam et al., 2015).

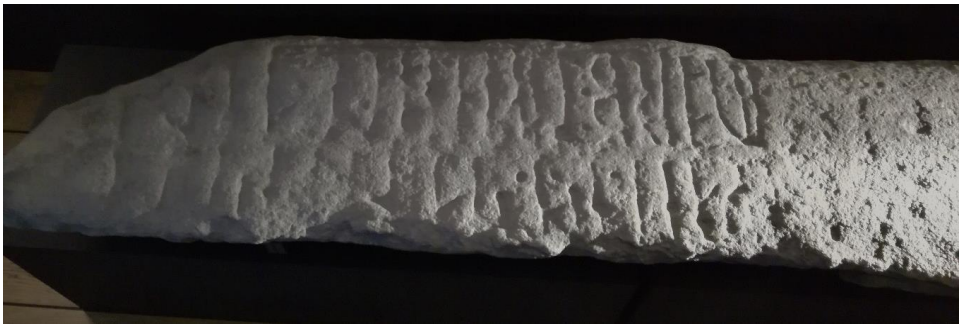

Fig. 1 Figgjo rune stein. This stone, exposed at the archaeological museum of Stavanger (south west Norway) and dated to 1100 AD, carries runic inscription about fishing rights in the "salmon river" Figgjo, testifying the antiquity and the importance of the relationship between people and salmon in Norway. The broodstock used in this thesis derives from wild caught salmon from the same river, Figgjo (photo E. Ciani).

My PhD project is part of an ambitious european project named IMPRESS (Improved production strategies for endangered freshwater species; www.impress-itn.eu), which aims at improving restocking techniques for three threatened European diadromous species: Atlantic salmon, European eel (*Anguilla anguilla*) and Sturgeons (fam. *Acipenseridae*). A deeper knowledge on regulatory mechanisms behind hormonal

and environmental control of reproduction, governed through the brain-pituitary-gonadal (BPG) axis and the circadian system, is necessary to improve the quality of restocking procedures and conservation strategies.

Increased activity of the BPG axis is a key element for the acquisition of reproductive competence and the transition between juveniles and adults in vertebrates, including teleosts fish (Weltzien et al., 2004). The release of gonadotropin-releasing-hormone (Gnrh), stimulate the production of two gonadotropins, luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh) from the pituitary gland, which in turn regulate steroidogenesis and gametogenesis at the level of the gonads (for review see Levavi-Sivan et al., 2010; Schulz et al., 2010; Zohar et al., 2010). Melatonin, the "time keeping" molecule produced from the pineal gland, is involved in the regulation of many seasonal physiological and behavioural processes, including reproduction, acting at different levels of the BPG axis (for review see Falcón et al., 2010). In teleosts however, the details behind the mechanism regulating the differential expression, synthesis and release of Lh and Fsh, as well as the direct effects and cellular target of melatonin in the pituitary, are still not well understood.

Model species: Atlantic salmon

Teleost fish dominate both marine and freshwater environments, represented by almost 30 000 species and accounting for nearly half of all extant vertebrate species (Wootton, 1990). The subfamily Salmoninae comprises about 30 species in seven genera including the genera *Salmo*, *Oncorhynchus* and *Salvelinus* (Klemetsen et al., 2003). Belonging to the genus *Salmo*, the Atlantic salmon (*S. salar*) is characterized by a significant plasticity in its life cycle and reproductive strategies (reviewed in Aas et al., 2011; Behnke et al., 2010; Fleming, 1996; Thorpe et al., 1998).

Atlantic salmon are anadromous fish, migrating from oceans to reach spawning sites in rivers in the autumn. The eggs hatch in the following spring and the newly hatched fish (*alevins*), stay in the gravel for the first 3 to 8 weeks using their yolk sack for nutrition before emerging (as *fry*) to initiate their first feeding. The moment of emersion from the gravel has to be synchronized with food availability, this is therefore one of the environmental parameters shaping the time of spawning in each river (Heggberget, 1988). The freshwater adapted juveniles (*parr,* Fig. 2), remain in rivers for a period ranging from 1 to 8 years, depending on both genetic factors, latitude, and

environmental conditions. Prior migrating to sea, the fish undergo a process of morphological, physiological, and behavioural transformation, named smoltification, preparing it for the life in sea water. At this stage, the smolts may weight from 10 to 80 g and measure from 7 to 30 cm in total length. After entering to sea, those fish face a second critical "bottleneck" for survival, coping with a new environment, different predators and new types of prey (Klemetsen et al., 2003). The *smolts* spend 1 to 5 years at sea in foraging areas over a wide area in North Atlantic Ocean before returning to home rivers for spawning. During this migration, the fish display high variability in body weight within and among population, ranging from 1 to 25 kg of weight and 45 to 135 cm of total length.

Fig. 2 Atlantic salmon parr. Identifiable from the "parr marks", dark camouflaging striped along their sides. The fish in the centre is showing early signs of smoltification having lost the parr marks and displaying a coloration turning to silver.

The spawning season range from September to February, varying with latitude and rivers. Northern populations spawn earlier than southern populations as an adaptation to slower egg development in colder water (Klemetsen et al., 2003). During spawning, females ovulate all their eggs simultaneously, then in a time windows of a few days they dig one or multiple nests where they deposit and cover the eggs after fertilization (de Gaudemar and Beall, 1998). Anadromous males do not participate in the nest construction, they rather invest their energy in aggressive competition for

access to fertile females (Fleming, 1996; Fleming et al., 1997, 1996) and courting behavior (de Gaudemar et al., 2000; de Gaudemar and Beall, 1999).

A different breeding strategy is applied from some male parr that achieve sexual maturation in freshwater, prior migrating to sea. They are known as “precocious parr” or “sneaky spawners”. As the name suggests, those fish adopt an elusive breeding strategy given their smaller size compared to adult smolt. Avoiding physical competition with bigger smolt males and courtship with females, they dart into the gravel nest at oviposition to ejaculate. It has been estimated that precocious parr can contribute up to 40% of total egg fertilization (Fleming, 1996). While maturation in freshwater occur in rare cases in female Atlantic salmon (Klemetsen et al., 2003), early maturation may occur from 10 to nearly 100 % of the male population (Baum et al., 2004; Heinimaa and Erkinaro, 2004; Myers et al., 1986). Precocious parr can mature again in the following season or undergo smoltification and migrate to sea to continue their life cycle (Fleming, 1996).

Salmon males compete for fertilization of the eggs. Given the external fecundation, sperm competition is a common phenomenon among salmon, where the ejaculates of two or more males compete to fertilize a female’s ova, especially in the case of sneaky spawners (Mjølnerød et al., 1998; Stockley et al., 1997). Mature parr invest more in gonads and sperm quality, compared to anadromous males, showing higher gonadosomatic index (GSI 5-10% versus 2-6%; Fleming, 1998), spermatozoa concentration and mobility and longer spermatozoa lifespan (Daye and Glebe, 1984; Gage et al., 1995; Vladić and Järvi, 2001). Differently from Pacific salmon, the Atlantic salmon is iteroparous, meaning that it is not genetically programmed to die after spawning. Some individuals may return to the ocean after the breeding season and repeat this cycle several times during their life span. However, due to the high mortality rate caused from predators, exhaustion and diseases, most fish survive to spawning only once or twice (Fleming, 1996).

While salmon farming is a prosperous industry that produced approximately 1.180.000 tonnes of fish in 2016, wild salmon population are facing a constant decline since 1980s, due to a variety of factors, including habitat destruction, genetic pollution, sea lice infection, acid rainfalls and environmental changes. Despite the efforts aimed to improve the quality of wild populations, the number of salmons that has returned annually from the sea to the coast of Norway has more than halved from 1983-1986 to

2012-2015 (55% reduction) with an estimated number of 470.000 wild individuals in 2016 (Thorstad and Forseth, 2017). As recently reported from a quality assessment on 148 Norwegian wild stocks (Thorstad et al., 2017), only 29 (20%) were classified as good quality stocks. Among the remaining, 42 (28%) were classified as moderate, 14 (9%) as bad, and 63 (43%) as very bad, according to the quality standard for wild salmon adopted under the Nature Diversity act in 2013.

The brain-pituitary-gonad (BPG) axis

Puberty is the process of morphological, physiological, and behavioural changes through which an individual becomes capable for the first time of sexual reproduction. In male teleosts, it is marked by the onset of spermatogenesis (Schulz et al., 2010; Schulz and Miura, 2002). The regulation of sexual maturation is under control of the brain-pituitary-gonad (BPG) axis (Christensen et al., 2012; Weltzien et al., 2004; Zohar et al., 2010) (Fig. 3). Activation of the BPG axis is characterized by increased release of gonadotropin-releasing hormone (Gnrh), which stimulates synthesis and release of the two gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) from the pituitary gland. These two hormones activate steroidogenesis and gametogenesis in the gonads (Schulz et al., 2010). This event is influenced by genetic factors and energy status (Bromage et al., 2001; Thorpe, 1989) as well as environmental cues like temperature and photoperiod (Bromage et al., 2001; Taranger et al., 2010).

The various environmental and physiological messages converge in the BPG axes under the form of neuropeptides and hormones. Those signals include leptin, kisspeptin, melatonin, gamma-aminobutyric acid (GABA), neuropeptide Y (NPY), acting directly on Gnrh release or modulating the Gnrh-induced gonadotropin production (Chang et al., 2009; Nakane and Oka, 2010; Navarro and Tena-Sempere, 2012; Oakley et al., 2009; Trudeau et al., 2000; Yaron et al., 2003; Zohar et al., 2010). The stimulatory action of Gnrh is countered by the inhibitory effects of dopamine in several teleost species (Dufour et al., 2010). Additionally, gonadotropin inhibitory hormone (GnIH) has been proven to decrease gonadotropin release in mammals and birds (Tsutsui, 2009), although stimulatory effects has been demonstrated in teleosts (Biran et al., 2014a). The gonadotropin production may also be influenced by a number of neurotransmitters and hormones directly at the pituitary level trough positive and negative feedback mechanisms.

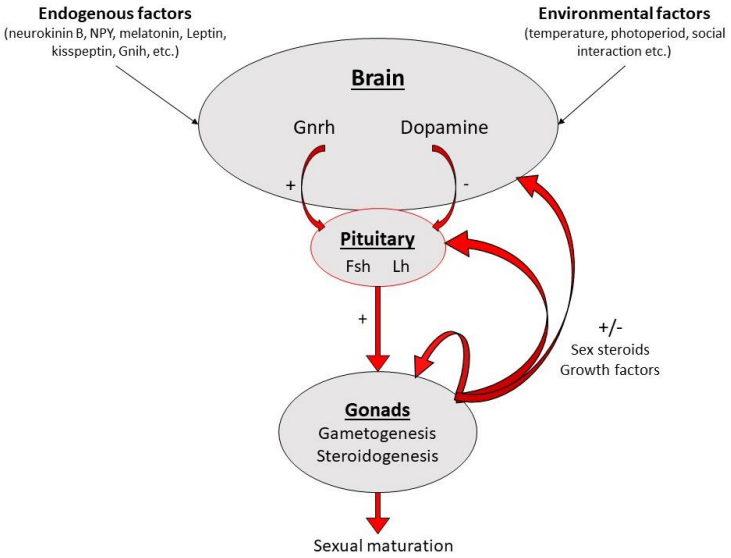

Fig. 3 Brain-pituitary-gonadal (BPG) axis. The integrated signals originating from environmental and endogenous factors may activate the production and release of gonadotropin-releasing hormone (Gnrh). The stimulatory effects of Gnrh are exerted on the pituitary via specific Gnrh receptors leading to the secretion of the gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), while dopamine can inhibit the stimulatory effects of Gnrh. Fsh and Lh, released into the blood stream, trigger the synthesis of steroid hormones (steroidogenesis) and the production of gametes (gametogenesis) after the binding to their specific receptors located in the gonads. Sex steroids affect all levels of the BPG axis through positive and negative feedback mechanisms and stimulate or inhibit the production of gonadotropins.

Once released into the blood stream, the gonadotropins may bind to specific receptors in the gonads (Fshr and Lhr) (Maugars and Schmitz, 2008, 2006; Schulz et al., 2010) activating the production of sex steroids (steroidogenesis) and mature gametes (gametogenesis). The sex steroids, including testosterone (T), 11-keto-testosterone (11KT), 17β-estradiol (E2), 17α,20β-dihydroxy-4-pregnen-3-one (DHP) can exert positive or negative feedback on all levels of the BPG axis depending on species, sex, reproductive status, and period of the year (Nagahama, 1994).

Pituitary morphology

The pituitary gland (Fig. 4), or hypophysis, is an endocrine organ responsible for the production of hormones involved in the control of different physiological and behavioural processes, such as growth, reproduction, migration, and maintenance of

homeostasis (Weltzien et al., 2004). It is divided in two main compartments: the adenohypophysis (or anterior pituitary), originating from the Rathke's pouch, an ectodermal up-growth from the anterior roof of the embryonic oral cavity, and the neurohypophysis (or posterior pituitary), originating from a down-growth of the floor of the diencephalon (Wingstrand, 1966). According to the nomenclature proposed from Green, (1951), the teleost adenohypophysis can be divided into the anteriorly located *pars distalis* (PD) and the posteriorly located *pars intermedia* (PI). The PD can be further parted into the *rostral pars distalis* (RPD) and the *proximal pars distalis* (PPD). In addition to hormone producing cells, the adenohypophysis includes also non-hormonal cells such as folliculostellate cells (Fauquier et al., 2001). The neurohypophysis comprises the *pars nervosa* (PN) and is composed of nerve terminal from hypothalamic neuroendocrine cells, but also supportive cells named pituicytes (Ferrandino and Grimaldi, 2008). In mammals, the PN is located posterior to the adenohypophysis, while in teleosts it is often dorsally located to the adenohypophysis (Norris and Carr, 2013). Differently from mammals, where a hypothalamo-hypophysial portal system is responsible for the transport of neurohormonal messages, in teleosts neurons directly connect the pituitary through the hypophysial stalk and the PN (Ball, 1981; Pogoda and Hammerschmidt, 2007). Whereas hormone-producing cells in the adult tetrapod pituitary are disposed in a mosaic pattern (Doerr-Schott, 1976; Voss and Rosenfeld, 1992), in teleost the different cell types are located in distinct pituitary portions, preserving the embryogenic compartmental organization (Pogoda and Hammerschmidt, 2007; Schreibman et al., 1973). The PPD hosts the gonadotropes (producing Fsh- or Lh), somatotropes (producing growth hormone, Gh) and thyrotropes (producing thyroid stimulating hormone, Tsh), while the RPD hosts the corticotropes (producing adrenocorticotropic hormone, Acth) and lactotropes (prolactin producing cells, Prl). The PI is the location of melanotropes (producing melanocyte stimulating hormone, α-Msh) and somatolactotropes (producing somatolactin, Sl) (Levavi-Sivan et al., 2010; Weltzien et al., 2004; Zohar et al., 2010).

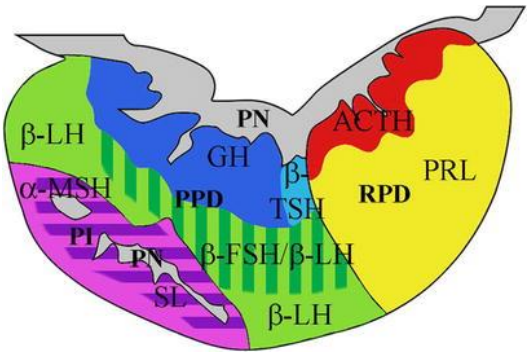

Fig. 4 Schematic representation of pituitary hormone localization in a teleost (Nile tilapia). The rostral pars distalis (RPD) shows regions producing adrenocorticotropic hormone (Acth; *red*) and prolactin (Prl; *yellow*). The proximal pars distalis (PPD) produces growth hormone (Gh; *dark blue*), thyroid-stimulating hormone β-subunit (Tshb; *light blue*), luteinizing hormone β-subunit (Lhb; *light green*) and follicle-stimulating hormone β-subunit (Fshb; *dark green*). The pars intermedia (PI) produces melanocyte-stimulating hormone (α-Msh; *pink*) and somatolactin (Sl; *dark violet*). The pars nervosa (PN) is represented in *grey* (reprinted with permission from Kasper et al., (2006)).

Early light microscopy studies (Olivereau, 1976) showed in PPD of salmonids the presence of two distinct gonadotrope cell types; conversely to tetrapods, where both hormones are produced from the same cells (Childs et al., 1986; Liu et al., 1988). Later studies confirmed the presence of two distinct cell types producing Lh and Fsh in salmonids, via immunohistochemistry and *in situ* hybridization (Naito et al., 1993, 1991; Nozaki et al., 1990). This organization was confirmed in numerous teleosts including Bluefin tuna (*Thunnus thynnus*; Kagawa et al., 1998), Nile tilapia (*Oreochromis niloticus*; Parhar et al., 2002) Atlantic halibut (*Hippoglossus hippoglossus*; Weltzien et al., 2003) and Medaka (*Oryzias latipes*; Kanda et al., 2011). A minor portion of gonadotrope cells producing both Lh and Fsh have been reported in teleost (Kasper et al., 2006; Pandolfi et al., 2006; Pilar García Hernández et al., 2002), as well as gonadotrope cells producing only one hormone in mammals (Pope et al., 2006; Schulz et al., 2006) suggesting a certain level of plasticity in gonadotrope cells.

GnRH

The decapeptide gonadotropin-releasing hormone (GnRH) was first characterized in mammals in 1971 (mGnRH; Amoss et al., 1971; Matsuo et al., 1971) with the primary structure identified as **pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2**.

Approximately ten years later, the first non-mammalian GnRH was identified in chicken (cGnRH-I and cGnRH-II; Miyamoto et al., 1982; Miyamoto and Hasegawa, 1984). In 1983, the first teleost Gnrh was isolated in Chum salmon, (*Oncorhynchus keta;* sGnrh; Sherwood et al., 1983). Many forms of Gnrh were, since then, characterized in teleosts, including medaka (mdGnrh; Okubo et al., 2000), seabream (*Sparus aurata*; sbGnrh Powell et al., 1994); African catfish (*Clarias gariepinus*; cfGnrh Bogerd et al., 1992); Pacific herring *(Clupea harengus pallasi*; hrGnrh; Carolsfeld et al., 2000) and spiny dogfish (*Squalus acanthias*; dfGnrh; Lovejoy et al., 1992). All the GnRH described to date, are decapeptides with perfectly conserved residues (1-Glu, 4-Ser, 9-Pro, and 10-Gly), well conserved residues (2-His and 3-Trp) and residues variable between the different forms (For review see Weltzien et al., 2004).

In vertebrates, the genes encoding GnRH share a common structure composed of four exons and three introns. The second, third and a section of the fourth exon encode for the GnRH prepro-hormone, consisting of a signal peptide (21-23 amino acids), a cleavage site (Gly-Lys-Arg), the GnRH decapeptide, and a GnRH associated peptide (GAP, 40-60 amino acids). The 5'- and3'-UTR are encoded in exons 1 and 4 respectively (Alestrom et al., 1992; Chow et al., 1998; Fernald and White, 1999). The signal peptide allows the transport of the protein to the Golgi apparatus (after cleavage by a signal peptidase), where the peptide is converted in the mature form and concentrated in secretory granules. Together with the GAP, is transported to the axon terminal for its release (Andersen et al., 1988; Rangaraju et al., 1991). The GAP of the different GnRH is the most divergent region both within and among species (Roch et al., 2011).

The different forms of GnRH were initially named accordingly to the species they were first isolated, despite the fact that they can be present in other species and, as later discovered, multiple forms can be present in the same species. A new classification of the GnRH variants was proposed on the basis of phylogenetic analysis and localization of the site of expression (Fernald and White, 1999; White et al., 1995). Phylogenetic analysis clustered the different GnRH form in three groups named GnRH1, GnRH2 and GnRH3. The GnRH1 lineage includes mGnRH, cGnRH-I, and several forms isolated in fish, such as mdGnrh, hrGnrh, sbGnrh, cfGnrh and dfGnrh. The second lineage include the form found in all vertebrates, the cGnRH-II, while the GnRH3 group includes the sGnrh form. The three groups also show characteristic localization in the brain. In

teleost fish, the localization of Gnrh neurons was clarified in European seabass (*Dicentrarchus labrax*), where Gnrh1 and Gnrh3 neurons are located in olfactory bulb, ventral telencephalon and preoptic area, and Gnrh2 neurons are restricted to the midbrain tegmentum (González-Martínez et al., 2002, 2001). This organization was later confirmed in other species including lake whitefish (*Coregonus clupeaformis;* Vickers et al., 2004), *Cichlasoma dimerus* (Pandolfi et al., 2005), Atlantic croaker (*Micropogonias undulates;* Mohamed et al., 2005) and medaka (Okubo et al., 2006).

The expression of two different forms of GnRH was detected in the brain of mammals, birds, reptiles (Millar, 2003), eels (Dufour et al., 1993), and more ancient teleosts including salmonids (Okuzawa et al., 1990), while three different forms are expressed in modern teleosts (Gothilf, 1996; Powell et al., 1994) and amphibians (Yoo et al., 2000). Exceptions have been detected in an ancient teleost evolving before the salmonids, the Pacific herring (Carolsfeld et al., 2000) and in a basal salmonid, the lake whitefish (Adams et al., 2002; Vickers et al., 2004), expressing three distinct Gnrh. Those findings suggest the loss of one form in later evolving salmonids.

In teleosts producing three forms of Gnrh, the first lineage, Gnrh1, is considered to be the main responsible for the production of gonadotropins. Gnrh1 neurons were proven to directly innervate the pituitary gland, ending in the PPD or in proximity to it (where the gonadotrope cells are located) in seabream (Gothilf, 1996), Nile tilapia (Parhar et al., 1998) and European seabass (González-Martínez et al., 2004b). In the pituitary gland of maturing perciform fish, Gnrh1 (sbGnrh) is the most abundant form detected via immunoassays (Gothilf et al., 1997; Holland et al., 1998; Powell et al., 1994). Furthermore, in Pacific herring and seabream, the ability of Gnrh1 to stimulate release of gonadotropins has been shown both *in vivo* and *in vitro* (Carolsfeld et al., 2000; Zohar et al., 1995).

As mentioned before, two forms of Gnrh are generally found in salmonids. Gnrh2 (cGnrh-II) and Gnrh3 (sGnrh) were identified in the brain of masu salmon (*Oncorhynchus masou*, Amano et al., 1991), sockeye salmon (*Oncorhynchus nerka*, Kitani et al., 2003), and rainbow trout (*Oncorhynchus mykiss*, Okuzawa et al., 1990). sGnrh neurons are located in the ventral forebrain from the olfactory nerve to the preoptic area innervating directly the pituitary, while cGnrh-II were found only in the midbrain tegmentum showing no innervation in the pituitary (Amano et al., 1997). Increases in *gnrh* mRNA in the forebrain coincide with increased gonadosomatic index and are

associated with precocious maturation in males (Amano et al., 1997; Ando et al., 2001) and during final gonadal maturation (Onuma et al., 2005). Furthermore, sGnrh was proven to induce Fsh secretion *in vitro* in coho salmon (*Oncorhynchus kisutch*, Dickey and Swanson, 2000). Gnrh3 (sGnrh) is therefore considered the main activator of gonadotropin secretion in salmonids.

GnRH receptors

GnRH acts via specific receptors (GnRHR) belonging to the rhodopsin β sub-family of G-protein coupled membrane receptors (Lethimonier et al., 2004; Strader et al., 1995). The protein structure is composed of seven α-helical transmembrane domains, connected by three intracellular and three extracellular loops where highly conserved amino acids form the ligand binding pocket, interaction sites with G-proteins and glycosylation sites (Millar et al., 2012).

The complexity of the GnRH system is reflected by the multiple forms on GnRHR characterized in vertebrates (Zohar et al., 2010). Despite the numerous studies conducted on GnRHR to date, the nomenclature of the different forms is still missing a clearly defined classification. The proposed systems, divided the GnRHR in two (Flanagan et al., 2007; Lethimonier et al., 2004; Moncaut, 2005), three (Levavi-Sivan et al., 2005; Millar et al., 2004) or four (Ikemoto et al., 2004; Ikemoto and Park, 2005; Kim et al., 2011) types, each composed of several subgroups. All the proposed schemes however, split teleost Gnrhr in two major groups. According to the classification proposed from Hildahl et al., 2011, (Fig. 5) the GnRHR are divided in two types. The Type I forms two subgroups, including mammalian (Type IA) and non-mammalian receptors (Type IB); and the Type II, also forming two subgroups, with fish and frog receptors on one side (Type IIB) and all the other tetrapods on the other side (Type IIA). Mammalian Type IA receptors lack the C-terminal cytoplasmatic tail. The absence of the intracellular tail confer slower desensitization and internalization to the receptors (Hislop et al., 2001); Type II receptors maintain the C-terminal cytoplasmatic tail conferring faster desensitization and internalization to the receptor (McArdle et al., 2002).

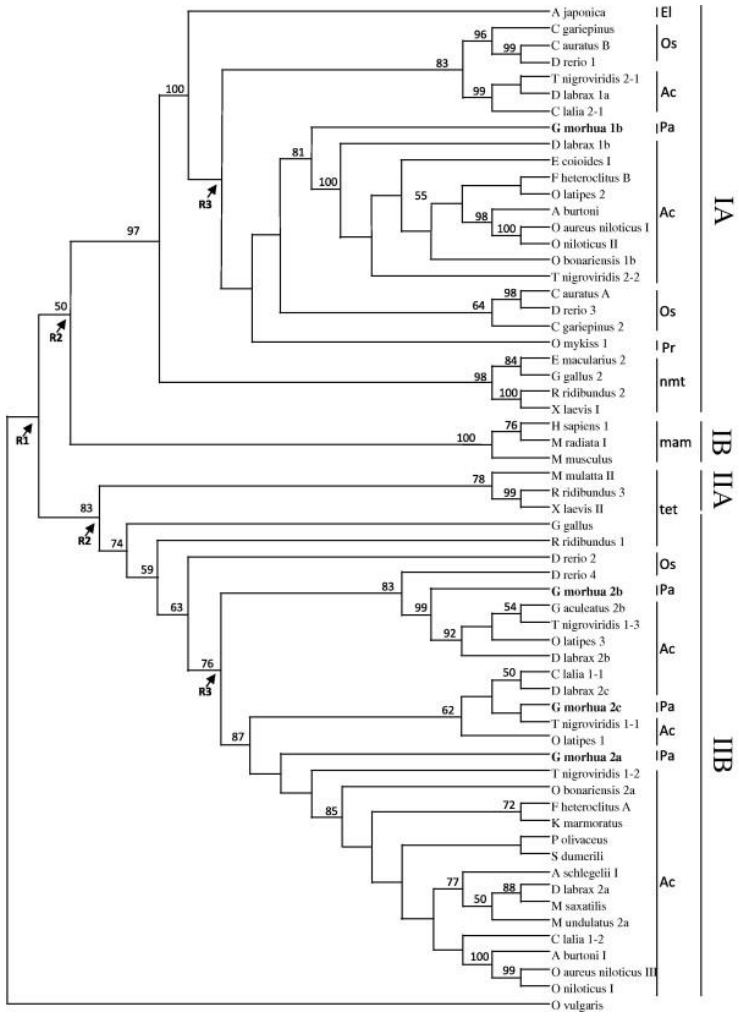

Figure 5 Phylogenetic organization of vertebrate GnRHRs. Proposed from Hildahl and colleagues. The receptors are clustered in four main groups, Type IA, Type IB, Type IIA and Type IIB. Species are identified as mammalian (mam), non-mammalian tetrapod (nmt) and tetrapod (tet) clads. Teleost fish are identified by superorder: Acanthopterygii (ac); Elopomorpha (el); Ostariophysi (os); Paracanthopterygii (pa); Protacanthopterigii (pr). (Printed with permission from Hildahl et al., 2011)

Differently from mammals that have one or two GnRHR (Hapgood et al., 2005), up to five different Gnrhr have been cloned in some teleost fish, such as European seabass (Moncaut, 2005) and spotted green pufferfish (*Tetraodon nigroviridis,* Ikemoto and Park, 2005). Many Gnrhr has been cloned in teleosts species including medaka (Okubo et al., 2001), European eel (Peñaranda et al., 2013), chub mackerel (*Scomber*

japonicus, Lumayno et al., 2017). In teleost pituitary, different Gnrhr may be expressed in the same cell type (Parhar et al., 2005; Strandabø et al., 2013), and the same Gnrhr may be expressed in different cell types (von Krogh et al., 2017). Nonetheless, Gnrhr may also be preferentially expressed in one cell type. For instance, *gnrhr2a* mRNA was identified in all Lh-producing cells and just a small number of Fsh-producing cells in European seabass (González-Martínez et al., 2004a).

Intracellular signaling pathways activated by Gnrh

Gnrhr in teleosts stimulate *fshb* and *lhb* expression through distinct intracellular pathways activated via G-proteins (for review see Chang and Pemberton, 2017; Naor, 2009; Yaron et al., 2003). Although the majority of the studies were conducted in mammals, the teleost Gnrh signal transduction pathways have been studied extensively in goldfish (*Carassius auratus;* Chang et al., 2009, 2000) and Nile tilapia (Gur et al., 2002; Levavi-Sivan and Yaron, 1989).

The coupling with $G_{q/11}$ following ligand binding on the Gnrhr, activates phospholipase C (PLC) that breaks phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in inositol triphosphate (IP_3) and diacylglycerol (DAG). The increase of these two second messengers together with cytosolic Ca^{2+}, activates protein kinase C (PKC). In parallel, activation of G_s stimulates adenylate cyclase (AC) activity, leading to cyclic adenosine monophosphate (cAMP) formation and activation of protein kinase A (PKA). PKA and PKC converge on MAPK/ERK (mitogen-activated protein kinases/extracellular signal-related kinases) pathways, ultimately binding to the promotor region of *gpa* and *lhb* activating their expression. The coupling with G_s protein stimulates AC activity leading to the production of cAMP and activation of PKA. PKA can either trigger *fshb* expression via cAMP responsive element (CRE) promoter on *fshb* gene or phosphorylate other MAPK cascades, including jun N-terminal kinases (JNK) that, in turn, bind AP-1 site on the promoter gene (Yaron et al., 2003) (Fig. 6)

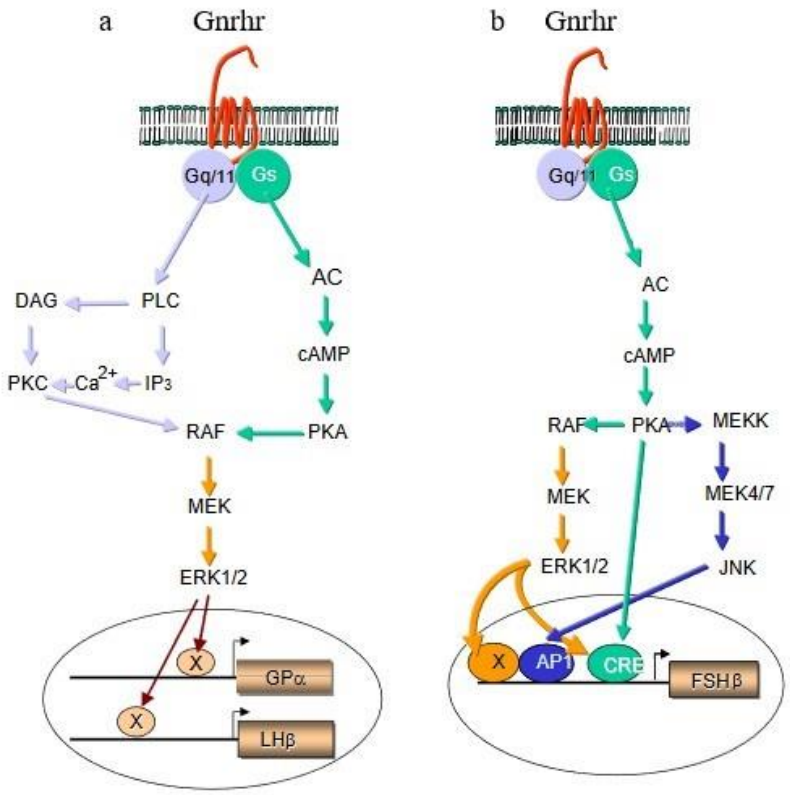

Figure 6 Gnrh signalling pathways in a teleost (tilapia). (a) Proposed pathway for coordinated regulation of tilapia *gpα* and *lhb* following Gnrhr activation. Coupling with G_q stimulates phospholipase C (PLC) to produce inositol trisphosphate (IP3) and diacylglycerol (DAG). Increase of these two second messengers together with cytosolic Ca^{2+} activates protein kinase C (PKC). In parallel, coupling with G_s stimulates adenylate cyclase (AC) activity, increasing intracellular cAMP levels and activating protein kinase A (PKA). Both PKC and PKA converge at the level of (RAF) phosphorylating MAPK kinase (MEK) and sub sequentially the extracellular signal-regulated kinase (ERK). MAPK enters the nucleus acting on transcription factors (X) inducing *gpa* and *lhb* expression. (b) Proposed pathway for tilapia *fshb* regulation. Coupling with G_s stimulating AC activity and cAMP formation. PKA phosphorylate cAMP-response-element-binding protein (CREB) which activate the cAMP responsive element (CRE) on *fshb* promoter or act via MEKK/JNK pathway stimulating the AP1 *fshb* promoter. Furthermore PKA may induce the MEK/ERK pathway acting on CRE or an unknown (X) promoter activating *fshb* expression.

The increase of cytosolic Ca^{2+} activates cascades of intracellular events as well as hormone release by exocytosis from the cells (Zhang et al., 2011). The increase in

intracellular Ca^{2+} is dependent on both extracellular influx and release from endoplasmatic reticulum (ER), regulated trough L-type voltage sensitive Ca^{2+} channels (VSCC; Chang et al., 2000; Hodne et al., 2013; Levavi-Sivan and Yaron, 1989) and Ca^{2+} activated K^+ channels (K_{Ca}; Stojilkovic and Catt, 1995). In medaka, the role of VSCC is only partially responsible for the total Ca^{2+} influx, while the presence of many K_{Ca} channels was detected in *lhb* cells, especially on of the big-conductance (BK) K_{Ca} channel (Strandabø et al., 2013). In Atlantic cod (*Gadus morhua*), Lh cells mainly express small-conductance (SK) K_{Ca} channels, while Fsh cells express mainly BK- K_{Ca} channels (Hodne et al., 2013), indicating both differences among species and within Lh and Fsh producing cell in the same species.

Gonadotropins

The gonadotropins are heterodimeric glycoprotein hormones belonging to the cysteine-knot growth factor superfamily. LH and FSH share a common α-subunit together with thyroid stimulating hormone (TSH) and human chorionic gonadotropin (HCG), non-covalently linked to a specific β-subunit conferring the biological activity (Pierce and Parsons, 1981; Swanson et al., 2003), where each subunit is encoded by a different gene (Fiddes and Talmadge, 1984). The first gonadotropin, LH, was purified in mammals in the late 1950s (Squire and Li, 1959), while FSH was purified almost a decade later in ovine and human pituitaries (Papkoff et al., 1967; Roos, 1968). In teleosts, all the functions ascribed to the gonadotropins, including steroidogenesis, gametogenesis, final oocyte maturation and spermiation, were initially associated to a single gonadotropin, GtH (for review see Burzawa-Gerard and Physiskogig, 1982). The presence of two distinct gonadotropins in teleosts, initially named GtH-I and GtH-II, was confirmed in chum salmon (Suzuki et al., 1988d, 1988b, 1988c) and coho salmon (Swanson et al., 1991). According to the similarity with mammalian counterparts, a resolution was adopted at the Sixth International Symposium on the Reproductive Physiology of fish, Bergen 1999 to use the term Fsh for GtH-I and Lh for GtH-II.

Gonadotropin plasma levels during sexual maturation were extensively studied in male salmonids. Fsh, already detectable in the blood in immature fish, increase significantly during early stages of maturation at the onset of spermatogenesis and again at later stages during spermatogonial proliferation and spermiation. Lh, on the other hand, is very low or undetectable during initial stages of maturation and increases

in later stages, during spermiation (Campbell et al., 2003; Gomez et al., 1999; Planas and Swanson, 1995; Prat et al., 1996; Suzuki et al., 1988a; Swanson et al., 1991).

Gonadotropins exert their biological activity through specific receptors. In mammals Lh regulates Leydig cell sex steroid production, while Fsh regulates Sertoli cell activities, including paracrine support of germ cell development (Huhtaniemi and Themmen, 2005). On the other hand, gonadotropin biological activity in teleosts appear less clearly distinct. Contrary to mammals, where FSHR and LHR are highly specific to their cognate hormones, in teleost this specificity is less apparent. Functional studies were conducted on gonadotropin receptors from several species including African catfish (García-López et al., 2009); Japanese eel (*Anguilla japonica*; Kazeto et al., 2008), coho salmon (Miwa et al., 1994; Yan et al., 1992) and Atlantic salmon (Andersson et al., 2009). A common feature of Fshr is to be activated from Fsh but also to Lh when exposed at high, but still physiological, concentrations. Conversely, Lhr were proved to be highly specific to Lh showing no cross-activation with Fsh at physiological concentrations.

In salmonids, Fsh and Lh stimulate the production of T and 11KT with comparable efficiency (Planas et al., 1993), while Lh is a more potent stimulator of DHP production during final maturation and spawning (Planas and Swanson, 1995). In African catfish and Japanese eel the presence of Fshr has been detected in both Leydig and Sertoli cells, while Lhr was detected only in Leydig cells (García-López et al., 2009; Ohta et al., 2007). In coho salmon Fshr were detected in Sertoli cells but the authors did not exclude a possible localization also in Leydig cells, while Lhr were only detected in Leydig cells (Miwa et al., 1994). Taken together, receptor localization and pharmacological data suggest that Leydig cell steroidogenic activity is regulated by both Lh and Fsh, while the functions of Sertoli cells are mainly regulated from Fsh, although during the spawning season, high concentrations of Lh may activate Fshr.

Melatonin

All living beings have adapted their physiological and behavioural functions to daily and annual fluctuations of environmental cues. Photoperiod, the alternation of light (L) and darkness (D) during the 24 hours, is the main and most reliable of these cues. It is considered as a “noise free” signal, since its variations are consistent, year after year, in the lifespan of an animal. Temperature, water salinity, light spectrum and

food availability are some among other features that can shape biological rhythms. Despite their periodicity, those cues are more subjected to short time variation and therefore considered "noisy" signals (Cowan et al., 2017; Falcón et al., 2010).

A circadian system (Fig. 7) includes all the components by which the photic signal is perceived and converted into a timed hormonal and nervous output (Falcón et al., 2007). In mammals, the photic information is conveyed from the eyes to the suprachiasmatic nuclei of the hypothalamus (SCN), considered as the master clock site, generating the pulse for all circadian rhythms. From here the information is transported to the pineal gland, a vesicle attached by a stalk to the roof of the diencephalon, responsible for the production of circulating melatonin (Simonneaux, 2003). Melatonin (5-methoxy-N-acetyltryptamine) is the main output of the vertebrate circadian clocks, enabling the synchronization of environmental conditions (using photoperiod as the main driver) with physiological, metabolic and behavioural processes, including reproduction (Falcón et al., 2011). In teleosts, the circadian system is organized in a less linear way, as a network of more interconnected circadian units, with retina and pineal having always a central position (Falcón et al., 2007).

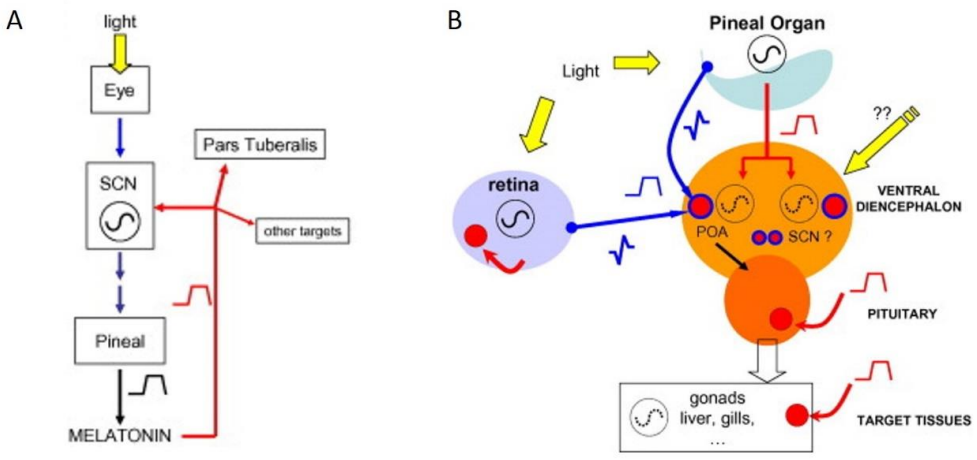

Figure 7 Photoperiodic and circadian regulation of neuroendocrine functions. (A) Schematic representation of the linear flow leading to the rhythmic production of melatonin in mammals. Non-visual information collected from the eyes, travel via the retinohypotalamic tract to the suprachiasmatic nucleus (SCN) of the hypothalamus (blue arrow). The photoperiodic signals influence the synchronization of the circadian activity of the SCN clocks, which in turn influence the cyclic melatonin production from the pineal gland through a multisynaptic pathway (blue arrows). Melatonin feeds back to the SCN and modulates seasonal

neuroendocrine physiological and behavioural functions acting on the pars tuberalis of the pituitary gland, brain regions and peripheral tissues. (B) Photoneuroendocrine regulation in fish. Photoreceptor cells in the pineal and retina perceive light stimulus (yellow arrows) thus synchronizing their internal clocks. Light may also act on other possible photosensitive and circadian structures in the preoptic area (POA) of the ventral diencephalon. The retina and the pineal produce both a neural (blue arrow) and a hormonal (red arrow) response to the photoperiodic information. The neural response reaches the ventral diencephalon via the retinohypotalamic and the pineal tracts. This response provides information regarding day length and variation in ambient illumination. The hormonal response is carried out from melatonin (red arrows) whose production reflects day length and season. Retinal melatonin acts as an autocrine/paracrine factor, while pineal melatonin is released in the blood and cerebrospinal fluid acting through melatonin receptors on specific targets (red circles). Melatonin receptors have been identified on locations, such as the preoptic area (POA), involved in the control of pituitary functions and in the pituitary gland itself (adapted from Falcón et al., 2010 with permission).

Melatonin is an indoleamine hormone produced during the night phase from tryptophan, which is successively transformed into serotonin (5-hydroxytryptamine) from a two-step enzymatic reaction. The conversion from serotonin to melatonin is controlled by two enzymes. The first, arylalkylamine *N*-acetyltransferase (AANAT), is responsible for the formation of *N*-acetylserotonin via acetylation of serotonin. The second, hydroxyindole-*O*-methyltransferase (HIOMT), produce melatonin via *O*-methylation of N-acetylserotonin (Falcón et al., 2011, 2006; Klein et al., 1997). Unlike other vertebrates, two *aanat* genes are expressed in teleosts, *aanat1* and *aanat2*, probably as a result of genome duplication (Falcón et al., 2007). The former is mainly expressed in retina, brain and peripheral tissues and the latter is mainly produced in the pineal (Cazaméa-Catalan et al., 2014, 2012; Falcón et al., 2010; Paulin et al., 2015). Aanat2 is the rate-limiting enzyme in the nocturnal production of melatonin by photoreceptor cells in the pineal gland (Falcón, 1999; Falcón et al., 2011; Ziv et al., 2005), since both Aanat2 mRNA expression and protein activity are inhibited by light (Falcón et al., 2010, 2001; Migaud et al., 2006). While the timing of *aanat2* expression and protein functionality is controlled by photoperiod, the amplitude of Aanat2 activity and therefore melatonin production, is influenced by temperature in combination with other external and internal factors (Falcón et al., 2010). In a number of teleosts species, the maximum Aanat2 activity coincide with the species-specific optimal water temperature interval as a result of evolutionary adaptation (Falcón et al., 2009). Other external factors such as spectral quality of light, salinity, fish species and also fish

populations within the same species may have a strong impact on melatonin production (Bayarri et al., 2002; Brüning et al., 2016; Lopez-Patino et al., 2011; Migaud et al., 2006; Oliveira et al., 2007; Porter et al., 2001; Skulstad et al., 2013; Vera et al., 2005). Furthermore, internal factors such as steroids (Chattoraj et al., 2009; Lopez-Patino et al., 2014) and melatonin itself, can influence melatonin production (Falcón et al., 2011).

The release of melatonin in the plasma during the night phase is a conserved characteristic common to all vertebrates, regardless of a diurnal or nocturnal nature of the animal. Three different types (A, B and C) of plasma melatonin profiles can be distinguished in teleosts, as in vertebrates in general. Type-A profiles are characterized by a peak of melatonin during late dark phase and are typical of gadoid species such as Atlantic cod and haddock (*Melanogrammus aeglefinus*) (Davie et al., 2007; Porter et al., 2000). Plasma melatonin in Type-B profiles peak during the middle of the dark phase, as in Nile tilapia (Martinez-Chavez et al., 2008). Type-C profiles immediately peak at the onset of the dark period, maintaining high levels until morning light. The latter profile is characteristic of salmonid species, and a range of other teleosts (Acuña-Castroviejo et al., 2014; Ceinos et al., 2005; Migaud et al., 2010).

The circadian clock system is a molecular feedback loop where two heterodimers, PER/CRY (repressor) and BMAL/CLOCK (activator) drive the rhythmic expression of a number of genes (including *aanat2*) in phase with solar time, allowing the anticipation of environmental changes (Appelbaum and Gothilf, 2006; Reppert and Weaver, 2002; Zilberman-Peled et al., 2007). In tropical teleosts, adapted to a constant photoperiod throughout the year, the phase of the rhythm is stably locked to 12L/12D cycle, while in temperate teleosts the phase is adjusted daily (Ziv et al., 2005). Many teleost species exhibit circadian rhythms of melatonin release in constant darkness (DD), including zebrafish, pike (*Esox lucius*), white sucker (*Catostomus commersonii*), and ayu (*Plecoglossus altivelis*) (Bolliet et al., 1996; Cahill, 1996; Iigo et al., 2004; Kazimi and Cahill, 1999; Zachmann et al., 1992b, 1992a). Salmonids on the other hand, are lacking this system and show a constant melatonin production under DD (Falcón et al., 2007; Gern and Greenhouse, 1988; Iigo et al., 2007; McStay et al., 2014; Thibault et al., 1993).

Extrapineal melatonin production has been detected in several peripheral tissues such as retina and gut. Interestingly, teleost retinal melatonin production is not limited to the dark phase, as seen in zebrafish and goldfish (Cahill, 1996; Iigo et al.,

1997a) but can also occur during the day (Besseau et al., 2006; Gern et al., 1978; Iigo et al., 1997b). The melatonin produced from the retina is not released into the blood stream, but act as a local autocrine/paracrine signal (Besseau et al., 2006; Falcón et al., 2010; Ping et al., 2008; Sauzet et al., 2008). Nonetheless, light perceptions from the eyes may influence circulating melatonin levels in an indirect way, through stimulation of pineal melatonin release in some teleost (Martinez-Chavez et al., 2008; Martinez-Chavez and Migaud, 2009; Migaud et al., 2007; Nikaido et al., 2009). Melatonin production has been detected in the gastrointestinal tract (GIT) of several teleost species (Isorna et al., 2017) including rainbow trout (Muñoz-Pérez et al., 2016) goldfish, carp and European seabass, likely regulated by feeding time and tryptophan availability (J. Y. Choi et al., 2016; Y. J. Choi et al., 2016; Herrero et al., 2007; Mukherjee and Maitra, 2015). The role of melatonin production in the gut may be related to the regulation of the digestive process (Vera et al., 2007).

Melatonin receptors

Melatonin acts via specific melatonin receptors (Mtnr) belonging to the G-protein coupled receptor superfamily (Brydon et al., 1999). In vertebrates, the receptors are divided in three sub-groups: Mtnr1A (Mel1a or MT1), Mtnr1B (Mel1b or MT2) and Mtnr1C (Mel1c or GPR50). An additional Mtnr1A was reported in some teleost species (Ikegami et al., 2009; Mazurais et al., 1999; Reppert et al., 1995), however the origin of the two teleost Mtnr1A paralogues was not identified.

Mtnr act through different intracellular pathways: Mtnr1A and Mtnr1B activate the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway coupling with G_i-protein. This results in inhibition of adenylyl cyclase (AC) and reduced cAMP formation (Rimler et al., 2006). Mtnr1A and Mtnr1C activate the phospholipase C/protein kinase C (PLC/PKC) pathway via G_q-proteins (Balík et al., 2004), while Mtnr1B activate the cyclic guanosine monophosphate (cGMP) pathway (Huang et al., 2005). Mtnr1C has lost the capacity to respond to melatonin in therian mammals (Dubocovich et al., 2010; Gautier et al., 2018).

The involvement of melatonin in a number of different physiological and behavioural functions is reflected from the wide distribution of Mtnr in vertebrate nervous and peripheral tissues (Witt-Enderby et al., 2003). The presence of Mtnr in the pituitary gland is of particular interest for the purpose of this thesis. Mtnr has been

identified in the *pars tuberalis* (PT) of the mammalian pituitary (Ebisawa et al., 1994; Schuster et al., 2001). In teleosts the presence of either *mtnr* or melatonin binding sites was detected in the pituitary of several species including goldfish (Ikegami et al., 2009), European seabass (Sauzet et al., 2008), Senegalese sole (*Solea senegalensis*; Confente et al., 2010), and salmonids like chum salmon, pike, rainbow trout (Falcón et al., 2003; Gaildrat and Falcón, 2002; Shi et al., 2004), but not Atlantic salmon (Ekström and Vaněcek, 1992). Whether melatonin effects on reproduction in teleosts result from direct effects on gonadotrope cells, indirect effects through other components of the BPG axes, or a combination of both is still not clear, however in some species melatonin was proven to influence gonadotropin levels *in vivo* (Khan and Thomas, 1996; Sébert et al., 2008).

Testes morphology

In teleosts, the testicles are elongated structures covered by a thin connective capsule, the tunica albuginea, located in the body cavity between two lobes divided by a sectum. They share the general structure common to all vertebrates, composed of two main compartments, the tubular and the intertubular compartment (Schulz et al., 2010). The intertubular compartment is composed of connective tissue, blood vessels, fibroblasts and Leydig cells. The Leydig cells are involved in the production of steroids, influencing both spermatogenesis and secondary sexual characteristics (Koulish et al., 2002). Teleosts produce two main androgens: 11KT, acting as a direct activator of spermatogenesis (Borg, 1994; Cavaco et al., 1998; Miura et al., 1991a) and T, influencing reproduction through positive and negative feedback in several tissues, including hypothalamus (Amano et al., 1994; Goos et al., 1986) and pituitary (Dufour et al., 1983; Montero et al., 1995; Xiong et al., 1993). The tubular compartment is composed of a basement membrane enclosing Sertoli cells and germ cells. Sertoli cells provide physical support but also paracrine factors needed for germ cell proliferation and differentiation (DiNapoli and Capel, 2008). Sertoli cells are involved also in phagocytosis of apoptotic germ cells and residual sperm after the spawning season (Almeida et al., 2008; Vilela et al., 2003). During the onset of meiotic division, Sertoli cells form a blood-testis barrier around the dividing germinal cells (Batlouni et al., 2009).

In amniote vertebrates (reptiles, birds, mammals) Sertoli cells arrest their proliferation at puberty and support waves of dividing germ cells at different

developmental stages. In anamniote vertebrates (fish and amphibians) the tubular compartment is organized in spermatocysts where a genetically determined number of Sertoli cells (Matta et al., 2002) surround a clone of germ cells all at the same developmental stage (Billard et al., 1982; Engel and Callard, 2007; Pudney, 1995).

Vertebrate testis can be classified upon the morphology of the germinal compartment and the distribution of the germ cells. In teleosts, two types of spermatogonial distribution have been described. In the first type (restricted), spermatogonia are located in the testis periphery near the tunica albuginea. The cysts migrate towards the spermatic ducts at the centre of the testis during the meiotic cell division. This organization is typical of higher teleosts (Parenti and Grier, 2004). In the second type (unrestricted), spermatogonia are distributed all along the length of the testis and cysts do not migrate during meiotic divisions. This organization is typical of lower teleosts including salmonids (Parenti and Grier, 2004).

Spermatogenesis

Spermatogenesis is a highly coordinated process where diploid spermatogonia differentiate to produce mature haploid spermatozoa. The general process is conserved in vertebrates and can be divided in three main phases: the mitotic or spermatogonial phase, the meiotic phase, and the spermiogenic phase (for review see Schulz et al., 2010)

During the first phase, mitotic proliferation of spermatogonial stem cells lead to the production of new stem cells and differentiated spermatogonia. Following the nomenclature proposed from Schulz et al. (2010, Fig 8), undifferentiated type A spermatogonia (A_{und}) transform into differentiated type A spermatogonia (A_{diff}) followed by type B spermatogonia, with a fixed number of generations for each species (Ando et al., 2000). Primary spermatocytes are formed after the final mitotic division. Simultaneously Sertoli cells undergo a defined number of mitotic divisions (Nagahama, 2000). The germ cell proliferation can be induced in teleosts from 11KT (Miura et al., 1991b; Nader et al., 1999) and insulin like growth factor I (IGF-I; Loir and Le Gac, 1994; Nader et al., 1999), while Sertoli cell proliferation is stimulated by FSH in both mammals (Kumar et al., 1999) and teleosts (Lejeune et al., 1996).

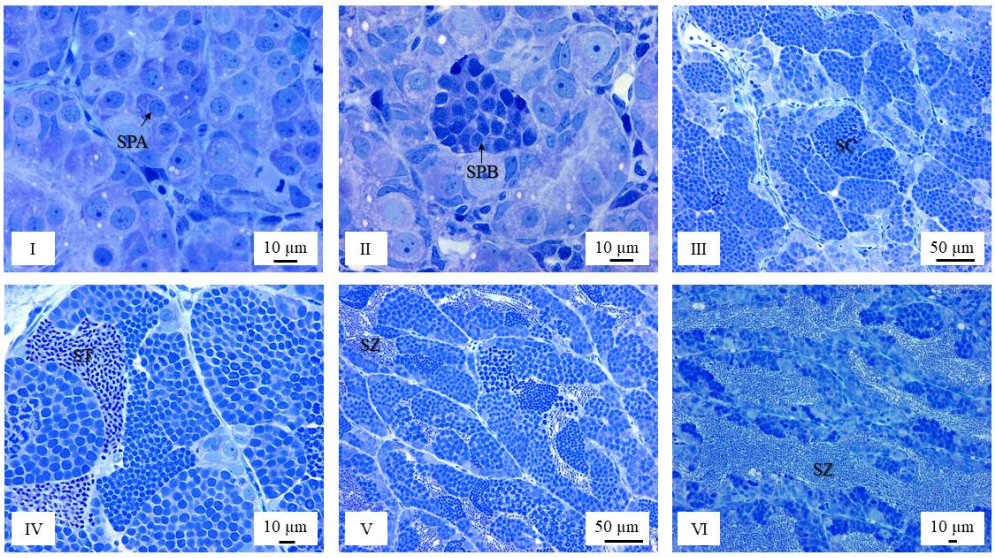

Figure 8 Histological sections of Atlantic salmon testes. The developmental stage (I to VI) is assigned accordingly to the most developed cyst present. SPA spermatogonia A, either differentiated or undifferentiated; SPB spermatogonia B; SC spermatocytes; ST spermatids; SZ spermatozoa (picture from paper I).

During the second phase, primary spermatocytes initiate meiotic division to produce secondary spermatocytes (1st meiotic division) and spermatids (2nd meiotic division). Meiotic division can be induced in teleosts from 11KT (Miura et al., 1991b). The effects of T in teleosts are limited, although in tetrapods T is able to induce meiotic divisions (Hillensjö and LeMaire, 1980; Pati and Habibi, 2000).

During the third phase, named spermiogenesis, haploid spermatids differentiate into flagellated spermatozoa under the regulation of 11KT (Miura et al., 1991a, 1991b). During spermiation the spermatozoa are released into the sperm duct (Nagahama, 1994; Ueda et al., 1985). In salmonids, spermatozoa acquire motility during their passage through the sperm duct (Miura et al., 1992).

Aims of the study

Despite the numerous studies conducted on Gnrh and melatonin on the effects on teleost reproduction, and Atlantic salmon in particular, some knowledge gaps remain in the role of the different forms of their specific receptors. For my PhD project, I had the following three aims:

- Identification of the period of maturation for male parr of the strain in use

- Isolation and characterization of Gnrh receptors involved in gonadotrope cell regulation in male parr

- Isolation and characterization of melatonin receptors and investigation of their pharmacology and pituitary expression profiles in male parr

Methodological considerations

The work in this thesis mainly focused on quantification and localization of gene expression and receptor pharmacology. The methodologies used to answer the main scientific questions, qPCR, fluorescent *in situ* hybridization, and luciferase reporter assay, are discussed in detail in the following chapters.

Histological analysis were performed on salmon testes to characterize the advancement of developmental stage based on the most developed cyst identified according to Melo et al., (2014). Furthermore, blood sample were collected to measure T, 11KT and melatonin plasma levels via radioimmune assay (RIA, Mayer, 2000; Mayer et al., 1990). When performing RIA, a known quantity of radioactive antigens and a specific antibody are mixed together, binding to one another. When a sample is analysed via RIA, the antigen of interest (unlabelled) competes with the radiolabelled antigen for the antibody binding sites. The variation in radioactivity is then measured with a gamma counter, giving a precise measurement of the antigen concentration in the sample.

qPCR

Real-time quantitative PCR (qPCR) was first introduced in early 1990s from Higuchi and co-workers (Higuchi et al., 1993, 1992) and since then qPCR has found broad applications including genotyping (Alker et al., 2004; Gibson, 2006), quantification of viral load (Ward et al., 2004), and study of cancer tissues (Kindich et al., 2005; Königshoff et al., 2003). However, the most common use for this technique is the study of gene expression (Liss, 2002; Livak and Schmittgen, 2001; for review see Farrell, 2010)

Accurate measurement of gene expression can provide valuable information about gene function. For instance it is possible to identify the tissues where a gene is expressed, recognise responses of gene expression in relation to specific stimuli (e.g. hormones, neurotransmitters, chemicals, environmental conditions) and link variation of gene expression to a specific biological state (e.g. maturation status, disease, behaviour). qPCR offers several advantages over other methods, such as northern blot (Alwine et al., 1977; Pall and Hamilton, 2008) and RNase protection assay (Emery, 2007), to study gene expression profiles. Those advantages include highly accurate and

reproducible data, faster protocols, requirement of small amount of samples for the analysis and can be used for both absolute and relative quantification (for reviews see Fraga et al., 2014).

Prior to qPCR analysis, RNA from the tissue of interest is converted to complimentary DNA (cDNA) using retroviral-derived RNA-dependent DNA polymerases (reverse transcriptases). Target DNA sequences are copied during PCR amplification, in a mixture containing a non-specific fluorochrome (SYBR Green is one of the most commonly used) binding to the minor groove of double stranded DNA. The exponential amplification of the target DNA is measured via the fluorescent emission of the fluorochrome (Fig 9A). PCR amplification can be divided in three phases: *exponential*, *linear* and *plateau* (Fig. 9B). During initial cycles (*exponential* phase) the reaction proceeds with efficiency virtually close to 100%, doubling the amount of target at each cycle. Product accumulation and substrate depletion inhibit reaction efficiency over time, resulting in a *linear* amplification of the amplicon after a few cycles. Finally, the reaction will arrest due to product inhibition and substrate depletion entering the *plateau* phase. An indication of the expression level of the gene of interest is given from the "cycle of quantification" (Cq) value. The Cq value is the cycle at which a sample's fluorescent emission reaches an intensity above background levels (threshold line). The lower the Cq value, the higher the expression of the target gene (for review see Glover et al., 2016)

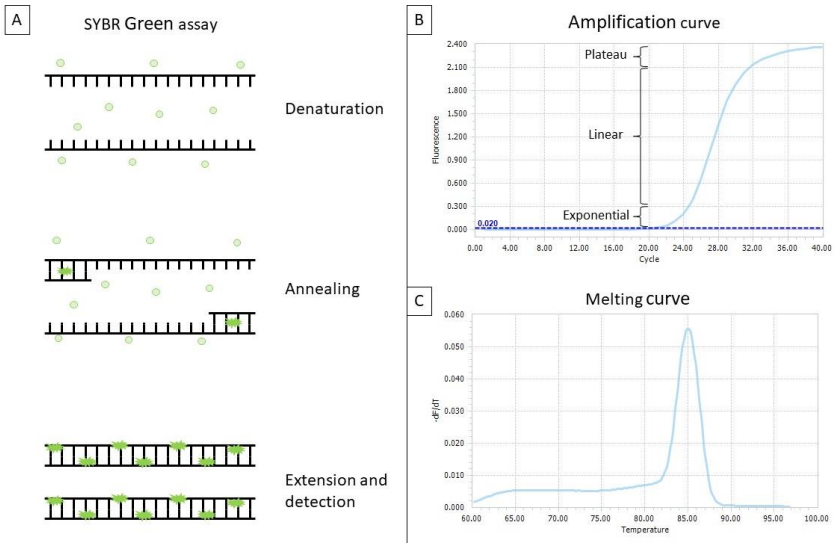

Figure 9 qPCR outline. (a) SYBR Green (green spheres) increases drastically his fluorescence when binding to the minor groove of double stranded DNA (green stars). The intensity of the fluorescent emission, measured at every cycle, is proportional to the number of amplicons (Farrell, 2010; Fraga et al., 2014). (b) Amplification curve of a qPCR reaction showing the *exponential*, *linear* and *plateau* phases. X axis: cycle number; Y axes: amount of DNA measured as fluorescent emission. (c) A melting curve showing a single peak, indicates the absence of unspecific targets and primer dimers. X axis: temperature; Y axes: negative derivate of fluorescence over temperature (-dF/dT)

The efficiency of the overall reaction is highly variable between different primer couples, tissues and qPCR mixtures. Therefore optimization of the protocol and measurement of primer efficiency in every condition is a critical element to produce highly reliable data out of a qPCR study. To ensure a good quality of the assay in my studies, all the RNA samples were treated with DNase to avoid genomic DNA contamination and a constant amount of total RNA was reverse transcribed between the different samples. The quality of the RNA was tested with Nanodrop and Bioanalyzer to test 260/280 and 260/230 ratios (indicative of phenol, carbohydrates or protein contamination) and RNA integrity number (RIN) (indicative of the quality of the RNA). The primers were designed to span exon-exon boundaries as an additional control over genomic DNA contamination and the amplification product was sequenced to ensure specificity of the target. Primer efficiency was measured from serial dilution of pooled salmon pituitary cDNA. A melting curve analysis (Fig 9C) was performed after completion of each qPCR run, thus controlling for primer dimers or unspecific product

formation. Additionally, a non-template control and an interplate calibrator were present in triplicate in each qPCR plate. Relative expression was normalized by geometric averaging of multiple internal control genes (Vandesompele et al., 2002).

Fluorescent *in situ* hybridization

In situ hybridization (ISH) is a technique used for the visualization of specific DNA or RNA sequences on tissues or cell cultures of interest. This methodology was originally developed in the late 1960s using radioactive labels (John et al., 1969; Pardue and Gall, 1969). The technical advancement introduced enzymatic labelling of nucleic acid (Langer et al., 1981) and more recently fluorescent labelling, offering important advantages over radioactive *in situ* hybridization, including reduced health hazard and increased stability of the signal over time (Bartlett, 2004). The principle behind *in situ* hybridization is the capacity of complementary single-stranded DNA or RNA sequences to specifically hybridize, forming double stranded hybrid filaments. First, probes complementary to the target of interest, are labelled enzymatically with modified nucleotides. Then, during the *in situ* protocol, probes are denaturated, and the resulting single filament are allowed to anneal with the target in the tissue/cell culture. After various washing and detection steps, a specific signal is visible at the site of probe hybridization (Haaf, 2006).

In the present work, fluorescent *in situ* hybridization (FISH) has been used for the visualization of transcript of genes of interest in the pituitary gland of Atlantic salmon. The advantage of fluorescent protocols is the possibility to work with multiple colours (Hopman et al., 1998; Speel et al., 1997), allowing the simultaneous visualization of multiple targets required to investigate co-expression of genes from the same cell type as showed in Paper II. Fluorescent labelling may be achieved via direct or indirect methods (for review see Tsuchiya, 2011). In the former, fluorescent reporter molecules are directly incorporated into the probe. A number of different deoxynucleotide triphosphates (dNTPs) can be applied, including Fluorescein-, Cy3-, Rhodamine-dUTP and many more, offering large possibilities for multicolour analysis. Direct methods are faster, compared to indirect methods, since the signal is already visible after the hybridization. The drawback is that probes are prone to photo bleaching during preparation and hybridization procedures, resulting in a possible loss

of signal. In indirect methods, fluorescent antibodies are used to detect the probes, producing a stronger fluorescent signal (Haaf, 2006). In the work showed in Paper II, an indirect method is used in combination with tyramide signal amplification (TSA, Fig 10). The TSA method was introduced by Bobrow et al. (1989) as catalysed reported deposition (CARD). Here, a peroxidase-conjugated primary antibody is used to bind the probe before the addition of tyramides and H_2O_2. The enzyme catalyses the oxidative radicalization of tyramides leading to the deposition of fluorescent intermediates in proximity to the site of reaction further increasing the sensitivity of protocol (Raap et al., 1995; Speel et al., 1999)

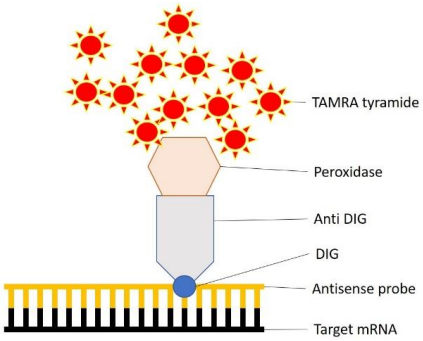

Fig. 10 Schematic view of a FISH reaction. The antisense probe binds to its target mRNA sequence. An antibody/peroxidase complex (Anti DIG-POD in this example) recognises the probe and catalyses the oxidative radicalization of tyramides (TAMRA in this example), leading to deposition of fluorescent intermediates in proximity of the reaction site.

Because of the numerous steps involved, and the necessity of optimization for each tissue and each probe to be used, *in situ* hybridization can be a relatively difficult technique to master. Several of these steps need to be optimized for each tissue and each probe to be used. Critical elements that influence the success and overall quality of FISH results include: control of RNA degradation; tissue fixation; slide preparation and pre-treatment (permeabilization); hybridization and post hybridization conditions.

RNA is very rapidly degraded by ubiquitary RNase enzymes, therefore, in the present work, special attention was dedicated to operating in "RNase free" work spaces. In addition, all the solutions used were treated with diethyl pyrocarbonate (DEPC) to inhibit RNase activity (except solutions containing Tris, which blocks DEPC activity). To

preserve mRNA and tissue integrity, crosslinking fixatives are used in most *in situ* protocols. The application of strong fixatives (i.e. formalin 10%) in combination with embedding of tissue in paraffin, insure excellent morphology but significant mRNA degradation and reduced probe penetration, while weaker fixation and embedding materials, may produce opposite results (Farrell, 2010). Therefore a balanced fixation procedure should ensure a good compromise between hybridization signal and tissue integrity. This was achieved in the present study with the fixation in paraformaldehyde (PFA) 4% and the embedding in agarose 3%. To remove blood cells and reduce autofluorescence, tissues were washed with cardiac perfusion of 4% PFA prior normal fixation procedures.

In order to facilitate the entry of the probes into the cells, the sections are permeabilized by breaking protein-protein crosslink using proteinase treatment. Ideally, this should be achieved with minimal tissue damage, therefore the concentration of the enzyme and the duration of the treatment must be optimized for every different tissue in use (Bartlett, 2004). Hybridization temperature and salt concentration during hybridization and post hybridization washes, are important aspects that influence the specificity and the efficiency of the FISH. Higher temperatures and lower salt concentration increase the specificity of the binding between probe and target. To avoid unspecific labelling and decrease background an RNase treatment was applied in our protocol, that digests all single stranded RNA filaments, removing unspecific labelling and residual probes left in the tissue (Darby et al., 2006; Farrell, 2010).

The use of a confocal microscope allows the acquisition of high quality images with very low background using spatial pinholes that block out of focus light in image formation. The fluorescent signal emitted from the probes is captured sequentially by the microscope. This is particularly useful during co-localization studies where the expression of multiple genes from the same cell type is analysed (Pawley, 2006).

Reporter gene assay

Reporter gene assays are widely used for the characterization of receptor functionality and are commonly used for the study of G-protein coupled receptors (GPCR). In

commonly used protocols, a cell line is transfected with the receptor of interest and a vector containing a specific responsive element and a reporter gene. The cells are then exposed to the ligand and the response of the receptors is measured through the enzymatic activity (e.g. fluorescence, luminescence or colorimetric) of the reporter gene (for review see Dingermann et al., 2004)

Reporter gene assays applied in the study of GCPR uses the ability of receptors to influence gene expression (Cheng et al., 2010). GPCR exert their biological activity through the coupling with intracellular heterotrimeric G proteins (composed of α, β and γ subunits) (Cabrera-Vera et al., 2003). The activation of the receptor stimulates different intracellular pathways, depending on the coupling with specific G_{α} subunits (e.g. $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$) or the $G_{\beta\gamma}$ subunit. The second messengers in turn influence a number of cellular functions, including gene expression via transcription factors and specific response elements that include cAMP response element (CRE), serum response element (SRE), nuclear factor of activated T-cells response element (NFAT-RE), and serum response factor response element (SRF-RE) (Cheng et al., 2010; Dubocovich et al., 2010; Dupré et al., 2009; Millar et al., 2012).

Vectors containing different promoters can be used for the study of the various intracellular pathways (Fig. 11) induced upon receptor activation and to test the efficiency of different ligands. Synthetic vectors may contain for instance CRE or SRE enhancer elements. The enhancer CRE is used to study G_s- and G_i-coupled receptors, where the expression of the reporter gene is regulated from the presence of cAMP. Assays aimed at the study of G_i-coupled receptors are performed in presence of forskolin, a known stimulator of AC, to increase cAMP and make the receptor-mediated inhibition easier to detect (Himmler et al., 1993; Levavi-Sivan et al., 2005). The enhancer SRE is commonly used to study G_q and $G_{\beta\gamma}$ coupled receptors acting through the protein kinase C (PKC)/Ca^{2+} signalling pathway (Biran et al., 2014b).

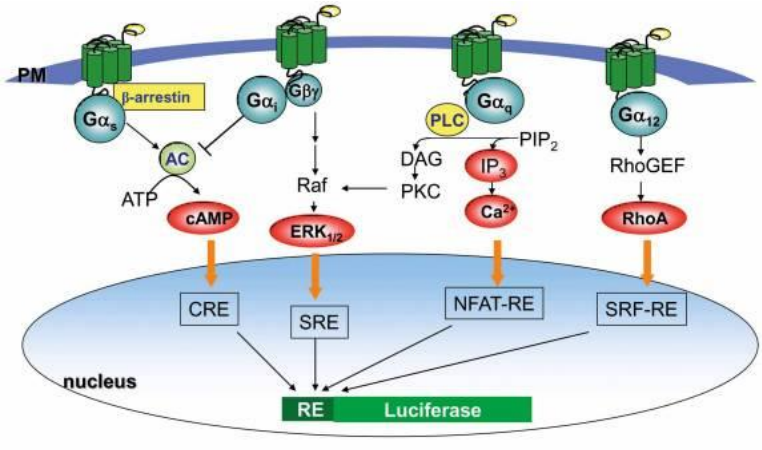

Fig. 11 Luciferase reporter assay. Schematic representation of the major GPCR signaling pathways and their respective responsive elements used in luciferase report assay. Upon receptor activation, Gs-coupled receptors activate adenylyl cyclase (AC) increasing cAMP level; G_i-coupled receptors inhibits AC decreasing cAMP levels; $G_{\beta\gamma}$ subunits activate ERK pathway; G_q-coupled receptors activate phospholipase C (PLC) producing diacylglycerol (DAG) and inositol trisphosphate (IP3) which in turn activate protein kinase C (PKC) and increase intracellular calcium (Ca^{2+}); G12-coupled receptors activate RhoA GTPase increasing cGMP. Different responsive elements (CRE; SRE; NFAT-RE and SFR-RE) promote the expression of luciferase in response to the activation of a specific pathway. (Reprinted with permission from Cheng et al., 2010)

Several reporter gene systems are available, including β-galactosidase, luciferase, and green fluorescent protein. β-galactosidase catalyses the hydrolysis of β-gal sugars and enzymatic activity can be measured via colorimetric- (Chen et al., 1995), fluorescent- (Price et al., 1987) and luminescent-based (Jain and Magrath, 1991) assays. The results are easy to measure, however they may be biased from endogenous activity in some cell types (Dingermann et al., 2004). Luciferase is extensively used because of its dynamic ranges sensitivity, easy quantitation, and lack of endogenous activity (Dingermann et al., 2004). Luciferase are a family of enzymes that catalyses the oxidation of various substrates resulting in light emission (Branchini et al., 2001). Commonly used luciferases include the bacterial luciferase, the renilla (*Renilla reniformis)* luciferase, and the firefly (*Photinus pyralis*) luciferase. Given its heat-labile structure, the bacterial luciferase has limited use as reporter gene in mammalian cells. The firefly luciferase is the most commonly used in GPCR studies (Stratowa et al., 1995). The catalysis reaction is highly efficient resulting in an amount of light directly proportional to the luciferase activity in the sample. The renilla luciferase (Lorenz et al.,

1996) is often used in dual-luciferase assay systems as an internal control for transfection efficiency in combination with firefly luciferase (Parsons et al., 2000). A major disadvantage of the luciferase assay is the necessity of cell lysis prior to analysis. Therefore the cells can't be used for multiple experiments. A valid alternative to the luciferase assay for the investigation of GPCR, is the use of GFP as reporter gene, where the fluorescent emission can be measured in living cells (Dinger and Beck-Sickinger, 2002; Milligan, 1999).

In paper III a CRE-luciferase assay was used to confirm the functionality of cloned salmon melatonin receptors (belonging to the GPCR superfamily) and to test their ability to modulate cAMP in response to melatonin and 2-iodomelatonin in presence, or not, of forskolin. A number of controls were applied to confirm the validity of the assay. The experiment was conducted in COS7 and replicated in CHO cell lines to exclude any possible aberration due to the cell line in use. A tilapia dopamine receptor (D2) previously studied from Levavi-Sivan and colleagues (Levavi-Sivan et al., 2005) was used as an assay positive control. To exclude false positive, the cell lines were exposed to ligand in absence of receptors.

Results

Paper I

This paper aims to correctly identify the period of onset of male parr maturation for the salmon strain in use throughout my PhD project and to produce a valid assay for the identification of maturing males that will be used in Paper II and III. Testis histology in combination with gonadosomatic index (GSI), gonadotropin subunit gene expression and steroid plasma levels indicated that, for the strain in use, sexual maturation can be triggered in both early autumn (end of September) six month after hatching, and in spring from the end of April one year after hatching.

Paper II

After the correct identification and classification of fish according to their maturational state, this second paper focuses on pituitary gene expression, comparing maturing and non-maturing parr. Among the six Gnrhr expressed in male parr pituitary, *gnrhr2b1* showed higher expression in maturing fish during later stages of maturation. Interestingly this gene displayed also daily expression profiles similar to *lhb.* Finally *in situ* hybridization studies displayed co-expression of *gnrhr2b1* with *lhb* but not *fshb* mRNA, suggesting this receptor to be involved in the regulation of *lhb* expression during sexual maturation in Atlantic salmon male parr. A paralogous gene, *gnrhr2b2* displayed no differences with regard to maturation status, but rather linked to season and time of the day, increasing constantly from April to July and showing intense daily fluctuation in spring and constant low expression in autumn.

Paper III

While it is well established that melatonin plays a major role in the control of teleost reproduction, little is known about the role of melatonin receptors in the pituitary gland. *In silico* studies identified five genes encoding putative functional melatonin receptors (Mtnr) in Atlantic salmon, phylogenetically clustered in three different receptors subtypes, 1A, 1Al and 1B. Pharmacological characterization of three cloned receptors, Mtnr1Ab, Mtnr1Aaα and Mtnr1B, conducted in both COS-7 and CHO cell lines, proved their functionality and their ability to respond to melatonin exposure *in vitro.*

Differently from previous studies in vertebrates, activation of salmon Mtnr resulted in induction of intracellular cyclic adenosine monophosphate levels (cAMP). The expression of Mtnr genes was detected at all levels of the BPG axes with three genes expressed in the pituitary, *mtnr1aaβ*, *mtnr1ab* and *mtnr1b*. Expression profiles in the pituitary were characterized by intense daily fluctuations depending on the season, occurring in spring, prior the onset of gonadal maturation but not in autumn, suggesting a direct involvement of melatonin in seasonal processes controlled from the pituitary.

Discussion

This PhD thesis investigates the molecular mechanisms behind the neuroendocrine regulation of gonadotropins, during sexual maturation in Atlantic salmon male parr, with focus on melatonin and Gnrh systems.

Considering the high plasticity characteristic of sexual maturation in male salmon, the first step of this study, exposed in **paper I**, was to correctly identify the period of onset of maturation for the strain in use and to produce a valid assay for the identification of maturing males that will be used throughout the dissertation. Testis histology in combination with gonadosomatic index (GSI), gonadotropin pituitary expression and steroid plasma levels indicated that, for the strain in use, sexual maturation can be triggered in both early autumn, at the end of September six month after hatching, and in spring from the end of April one year after hatching. Histological analysis confirmed the validity of GSI>0.05 as a threshold for the identification of maturing fish. Below that value in fact, no sign of spermatogenetic activation was detected and spermatogonia type A were the only cysts identified.

After the correct identification and classification of fish according to their maturational state, **paper II** focuses on pituitary gene expression, comparing maturing and non-maturing parr. The information regarding the role of the different forms of Gnrhr variants in teleosts, and their involvement in the regulation of different cell types, is still rather fragmentary. Four type I (*gnrhr1a1*, *gnrhr1a2*, *gnrhr1b1*, *gnrhr1b2*) and two type II (*gnrhr2b1*, *gnrhr2b2*) *gnrh* receptor paralogs were identified from the Atlantic salmon (*Salmo salar*) genome, recently released from Lien et al., (2016). Among the six Gnrhr expressed in male parr pituitary, *gnrhr2b1* showed higher expression in maturing fish during later stages of maturation. Interestingly this gene also displayed a seasonal expression profile similar to that of *lhb.* Finally, *in situ* hybridization experiments displayed co-expression in the pituitary of *gnrhr2b1* with *lhb*, but not *fshb* mRNA, suggesting this receptor to be involved in the regulation of *lhb* expression during sexual maturation in Atlantic salmon male parr. A second related receptor gene, *gnrhr2b2* displayed no differences in expression level with regard to maturation status, but rather linked to season and time of the day, increasing constantly from April to July and showing intense daily fluctuations in spring but not in autumn.

The seasonal variation of environmental cues shapes biological activities in virtually all living organisms. In teleosts, changes in photoperiod, the alternation of light and darkness, are perceived throughout the year by the internal biological clock and transformed into a timed nervous and hormonal signal. Melatonin is the main output of the vertebrate's circadian clock system. Melatonin is produced during the dark phase, mainly from the pineal gland, and is involved in the regulation of a number of physiological and behavioural processes including reproduction. While it is well established that melatonin plays a major role in the control of teleost reproduction, and photoperiod management is currently in use to induce or inhibit sexual maturation, little is known about how melatonin signals are mediated to the pituitary gland, and the specific cell types involved. **Paper III** aims at the characterization of high affinity melatonin receptors and their expression in the pituitary gland. *In silico* studies identified five genes encoding putative functional melatonin receptors (Mtnr), phylogenetically clustered in three different receptors subtypes, 1A, 1Al and 1B. Pharmacological characterization of three cloned receptors, Mtnr1Ab, Mtnr1Aaα and Mtnr1B, conducted on COS-7 and CHO cell lines, proved their functionality and their ability to respond to melatonin exposure *in vitro.* Differently from previous studies in vertebrates, activation of salmon Mtnr resulted in induction of intracellular cyclic adenosine monophosphate levels (cAMP). The expression of Mtnr genes was detected at all levels of the HPG axes with three genes expressed in the pituitary, *mtnr1aaβ, mtnr1ab* and *mtnr1b*. Expression profiles in the pituitary were characterized by intense daily fluctuations depending on the season, occurring in spring, prior to the onset of gonadal maturation but not in autumn, suggesting a direct involvement of melatonin in seasonal processes controlled from the pituitary.

Gnrh and melatonin are two key hormones involved in the regulation of the BPG-axis. The results exposed in this dissertation add to our understanding on gonadotropin control from Gnrh, suggesting that, in Atlantic salmon, differential regulation may occur through specific Gnrh receptors expressed in different cell types. The pharmacological characterization of salmon melatonin receptors revealed differences in the intracellular pathways involved, compared to previous studies conducted in vertebrates, opening interesting points for future comparative research. Finally, the detection of melatonin receptors in the pituitary gland showing variation in daily expression between different seasons, strongly advocates for a direct involvement of melatonin in pituitary functions.

General conclusion

The results exposed in this PhD thesis add to our understanding of the role of Gnrh and melatonin receptors in the pituitary gland of early maturing Atlantic salmon parr. The three main scientific question proposed found the following answers:

- Early maturation in male parr of the Figgjo strain, farmed under natural condition of photoperiod and water temperature, occur in both early spring at one year of age, and in late summer six months after hatching

- *gnrhr2b1* is proposed as a regulator of *lhb*-producing cells during sexual maturation. It is specifically expressed in *lhb*-producing cells (but not *fshb*-cells) and shows variation in gene expression in parallel with *lhb* both during yearly and daily cycles.

- Activation of three cloned melatonin receptors; Mtnr1Ab, Mtnr1Aaα, and Mtnr1B following melatonin exposure induces adenyl cyclase activity resulting in increased cAMP levels. The three melatonin receptor expressed in the pituitary; *mtnr1aaβ*, *mtnr1ab*, and *mtnr1b*, all display intense daily fluctuation during spring (but not autumn).

Future Perspectives

The identification of a *gnrhr* specifically expressed in *lhb*-producing cells, together with the detection of five other *gnrhr* in salmon pituitary, open interesting points for future research. The most important follow up of these studies would be the identification of the cell type expressing each *gnrhr* paralog. The *in situ* hybridization used for the localization of *gnrhr2b1* is targeting the mRNA. Therefore the presence of the receptor protein in Lh cells should be validated with the use of a specific antibody and the localization via immunofluorescence to further validate the results presented in this thesis.

Clear circadian fluctuations in gene expression were measured for melatonin receptors *mtnr1aaβ*, *mtnr1ab*, *mtnr1b* in the pituitary gland. A follow up for this finding would be to measure whether or not those fluctuation in mRNA are reflected in variation in each receptor's protein levels. In addition, localization of the cell type expressing the receptors, in combination with measurements *in vitro* of the effect of melatonin exposure on pituitary gene expression, would be an interesting follow up to elucidate the direct role of melatonin in salmon pituitary. Considering that the activation of salmon melatonin receptor in COS7 and CHO cell lines resulted in stimulation of adenyl cyclase, leading to increased intracellular cAMP levels. This is contrary to melatonin receptors in other species studied so far. Future studies should investigate the signalling pathway by salmon melatonin receptors after agonist stimulation in a salmon cell or tissue culture system.

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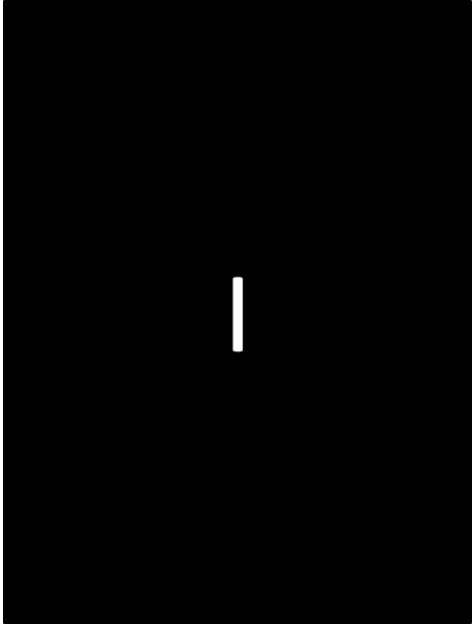
Appendix (my papers)



Sexual maturation in Atlantic salmon male parr is triggered both in early spring and late summer under standard farming condition.

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Keyword

11-ketotestosterone, Atlantic salmon, follicle-stimulating hormone, luteinizing hormone, precocious parr, testis histology, testosterone

Abstract

The life cycle of Atlantic salmon its characterized by a high plasticity with regards to sexual maturation. Atlantic salmon males may mature as parr during freshwater phase, or as post smolts following one to several years at sea. Regardless of strategy, they show the first sign of maturation during spring, with a high degree of variation both within and among populations, depending on both environmental and genetic factors. This paper investigates early stages of maturation in male parr from the Norwegian strain from river Figgjo, with the aim to identify the correct period for the onset of maturation under natural conditions regarding photoperiod and water temperature. Histological analysis, in combination with morphometric measurements, steroid plasma levels, and pituitary gonadotropin gene expression analyses revealed the ability of precocious males to initiate sexual maturation in two periods of the year: either in autumn, six months after hatching, or in spring, at one year of age.

Introduction

Puberty is the process of physical and physiological changes through which an individual become capable of sexual reproduction. In male teleosts, as other vertebrates, it is characterized by the onset of spermatogenesis (Schulz and Miura, 2002). Sexual maturation is regulated by the brain-pituitary-gonad axis, with the pituitary gland being the site of production of the gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh).Fsh and Lh are key hormones involved in the control of reproduction. They are heterodimeric glycoproteins consisting of two non-covalently linked subunits, a common α- and a hormone-specific β-subunit that confers the biological activity (Pierce and Parsons, 1981; Swanson et al., 2003). Different from mammals, where both gonadotropins are produced from the same cell, in teleost, Fsh and Lh are produced from two different cell types, located in the *proximal pars distalis* (PPD) of the pituitary gland (Weltzien et al., 2003). The physiological role of gonadotropins has been extensively studied in salmonids (Gomez et al., 1999; Maugars and Schmitz, 2006, 2008; Prat et al., 1996; Swanson et al., 1989). Gonadotropins are capable of inducing production of androgens, 11-ketotestosterone (11-KT) and testosterone (T) with similar efficacy in males. 11-KT plays a major role in all stages of teleost spermatogenesis, being involved in the induction of spermatogonial proliferation, meiotic division and spermiogenesis (Miura et al., 1991a), while T works through positive feedback mechanisms on the hypothalamus (Amano et al., 1994; Goos et al., 1986) and pituitary (Montero et al., 1995; Xiong et al., 1993), where it can be aromatized to estradiol.

In addition, Lh is a potent stimulator of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) during the last stages of maturation in both sexes (Planas and Swanson, 1995). Plasma Fsh levels increases earlier, during the onset of spermatogenesis, compared to Lh plasma levels, which are undetectable or very low during initial stages of maturation and rises during spermiation. It has been suggested that Fsh, due to its presence in plasma of immature fish and its capacity to stimulate steroidogenesis and spermatogonial proliferation (Loir, 1999), plays a major role during early stages of gonadal development in salmon, while Lh is especially involved in later stages of maturation (for review see Yaron et al., 2003).

Atlantic salmon (*Salmo salar*) shows great plasticity concerning male sexual maturation. Salmon males may mature either during the freshwater phase (parr), from

65 one to four years of age, as small “sneaky spawners/precocious males” (Maugars and
66 Schmitz, 2008), or at a larger size as one- (grilse) or multi-sea-winter individuals
67 (Garcia De Leaniz et al., 2007; Hutchings and Jones, 1998; Taylor, 1991). The occurrence
68 of male parr maturation before one year of age (underyearling or 0+) was reported in
69 French rivers from the Armorican system in a very small percentage of the total parr
70 population (Bagliniere and Maisse, 1985). However, the incidence and characteristics
71 of underyearling maturation in Scandinavian strains is still lacking an in-depth analysis.
72 This study aims to investigate initial stages of maturation in Atlantic salmon male parr
73 measuring morphometry, pituitary gonadotropin expression, steroids plasma levels,
74 and gonadal histology.
75

Material and Methods

Animals

This research was conducted on fourteen month old Atlantic salmon (*Salmo salar*) male parr for two consecutive seasons, in spring 2016 (batch A) and 2017 (batch B), in addition to a batch of underyearling (four months old) parr in summer 2017 (batch C). The broodstock were a farmed first generation from wild specimen caught in river Figgjo (south-west Norway, 58°47' N 5°47' E). The fish were farmed at the Norwegian Institute for Nature Research (NINA) at Ims, Norway (58°54'N, 5°57'E), hatching on March 13th, 2015 (batch A), February 22nd, 2016 (batch B), and March 14th, 2017 (batch C). After first feeding, fish were raired in outdoor tanks (volume 7.8 m^3), under natural conditions regarding photoperiod and temperature (yearly range, 5-21°C). All experiments were performed according to the European Union regulation concerning the protection of experimental animals (Directive 2010/63/EU). Appropriate measures were taken during sampling to minimize pain and discomfort (FOTS application ID12523).

Biometry and identification of maturing fish

Body weight, gonad weight and fork length were measured for all fish. Gonadosomatic index (GSI = gonad weight / body weight * 100) and condition factor (K = 100 body weight / fork $lenght^3$) were calculated from morphometric measurements. GSI was used to discriminate fish in two groups, maturing (GSI > 0.05) and non-maturing (GSI < 0.05) and histological analysis was performed to determine a more precise maturational state (see chapter 2.5).

Gene expression analysis

All fish were anesthetized with Pharmaq (Overhalla, Norway) MS222 (80 mg/l) and euthanized via quick decapitation. Male parr were sampled twice a month from May to July 2016 (batch 1) from April to July 2017 (batch 2) and once a month from July to October 2017 (batch 3). Fish from batch 1 and 2 were 14 months old, while fish from batch 3 were 4 months old at the beginning of the experiment.

All procedures involved in gene analysis, from RNA extraction to qPCR protocol were performed according to Ciani et al. (2018). In brief, individual pituitaries were

collected and stored in 300 µl TRIzol reagent (Invitrogen, Waltham, USA) overnight at 4 °C, then stored at -20 °C until RNA extraction. Total RNA was isolated from individual pituitaries using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To avoid any genomic contamination, extracted RNA was treated with 2 U DNase (TURBO DNA-free kit, Ambion, Foster City, USA).The concentration of total RNA was measured via NanoDrop (Thermo Scientific, Waltham, USA) spectrophotometer for samples from batch 1, and Qubit Fluorometer (Invitrogen) using Qubit RNA BR Assay Kit (Invitrogen) for samples from batch 2 and 3. The 260/280 ratio for all samples was between 1.8 and 2. Quality of RNA samples was measured using Bioanalyzer 2100 (Agilent, Santa Clara, USA). All samples showed RNA Integrity Number (RIN) above 8. SuperScript III reverse transcriptase (Invitrogen) and 5 µM random hexamer primers (Invitrogen) were used, following producer's instructions, to reverse transcribe 1 µg (batch 1) and 170 ng (batch 2 and 3) total RNA from individual pituitaries.

Primer-Blast from NCBI website (Ye et al., 2012) was used to design specific qPCR primer sets for the genes of interest (Table1). Primers were tested for primer dimers and hairpin potential with Vector NTI Express software (Lu and Moriyama, 2004). Gene expression was measured by qPCR using the Light Cycler 96 (Roche, Basel, Switzerland) thermocycler and SYBR Green I master (Roche) kit. Each individual sample was run in duplicate using 3 µl of the same cDNA dilution 1/10. A negative control and an interpolate calibrator were present in triplicate for each qPCR plate. Real-time conditions were 10 min incubation at 95 °C followed by 40 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 8 s. The specificity of the amplified product was verified by running a melting curve and by sequencing of the PCR product. Standard curves were run in triplicates using dilution series obtained from RT products from pooled total RNA of pituitaries. The relative abundance of transcript was determined using GenEx software (Mangalam et al., 2001) through the algorithms outlined by Vandesompele et al. (2002). The internal control genes used for data normalization were *rna18s* and *ef1a*. Stability of the reference genes was tested using the online tool RefFinder (Kim et al., 2010).

Radioimmunoassay (RIA)

Blood samples were collected from the caudal vein with heparinised syringes. Plasma was then isolated by centrifugation and stored at -80 °C. Levels of testosterone

(T) and 11-ketotestosterone (11KT) were measured via radioimmunoassay (RIA) as previously described by Mayer et al. (1990). Standard curves were prepared mixing steroid solutions containing decreasing concentration of the respective, non-radioactive steroid in pH 7.0 RIA buffer (NaH_2PO_4 3.87 g, Na_2HPO_4 10.67 g, Na-Azid 0.05 g, NaCl 9 g, gelatine 1 g, ddH20 1 l). The inter-assay variation for T and 11KT was 13.5 and 14 %, while the intra-assay variation was 5.6 and 4.8 % respectively. Due to the small size of the fish and the insufficient amount of plasma available RIA was not performed in underyearling fish (batch C).

Testes histology

Testes were fixated in glutaraldehyde 4 % (VWR, Radnor, USA) overnight at 4 °C. The samples where then stored in ethanol 70 % at 4 °C until histological analysis. The analysis was performed on 19 fish from batch 1; all maturing and two randomly selected non-maturing fish per date, from batch 2 and 3. Tissues stored in EtOH 70 % were dehydrated with EtOH washes at increasing concentrations (up to EtOH 100 %), each lasting 30 min. The last step was repeated three times. The tissues were then kept at room temperature overnight slowly shaking in preparation solution (100 ml Technovit 7100 added 1 g of Hardener I (Heraeus Kulzer, Hanau, Germany)). Afterwards, tissues were embedded in cold Histoform S (Heraeus Kulzer) added approx. 1 ml preparation solution with 50 µl Hardener II (Heraeus Kulzer) and incubated at 37 ºC. Finally, samples were mounted in Histoblocs using Technovit 3040 (both from Heraeus Kulzer). Sagittal (for small and medium testes) and transverse (for large testes) sections, 3 µm thick, were prepared using a Leica RM2245 microtome (Leica Biosystems, Wetzlar, Germany). Sections were separated by at least 30 µm and collected from the periphery until the middle of the tissue. Dried sections were stained with Toluidine Blue O (Sigma-Aldrich) and mounted with Coverquick 4000 (VWR International, Radnor, PA, USA) before histological analysis. Testes maturational stages were determined by the most advanced germ cell present in the tissue (Table 2). Germ cell stages were defined according to the description by Melo et al. (2014). At least five sections per testes were analysed.

Statistics

All statistical analysis was performed with JMP pro V14.1 software (SAS Institute Inc., Cary, NC, USA). Shapiro-Wilk W test was used for testing normality and Quantile Range method (Q=3; Tail 0.1) was used to identify and exclude outliers. When needed, data were log or square root transformed to meet test criteria. Results are shown as mean ± SEM. Statistically relevant variations during sexual maturation were measured by two-way ANOVA, followed by Tukey's HSD test. While variations in relationship with testes developmental stage were measured by one-way ANOVA, followed by Tukey's HSD test. Due to non-normal distribution, Spearman's ρ (non-parametric) was used to test correlation between variables. In this study, p values <0.05 were considered statistically relevant.

Results

Biometry

In spring 2016, the presence of early maturing males (GSI = 0.56 ± 0.31), was registered already from the first sampling, on May 25th. GSI of maturing fish remained stable until June 21st and increased in July reaching 1.58 ± 0.24 by July 19th (Fig 1A). Despite a constant tendency for longer fork lengths in maturing fish, no significant differences were detected between groups at any time point. On May 25th, maturing fish measured 11.63 ± 0.08 cm, increasing to 12.82 ± 0.36 cm on June 21st (Fig 1D). Maturing fish displayed greater body weight compared to non-maturing ones, from May 25th to June 8th. At this date, maturing fish measured 20.34 ± 2.05 g versus 12.89 ± 0.6 g of non-maturing fish. No significative differences were measured in the following dates (Fig 1G). No differences in condition factor were detected between groups at any time point, remaining stable over time from May 25th to July 19th (Fig 1L).

On the first sampling in spring 2017 (April 25th), early maturing fish with a GSI of 0.24 ± 0.06 could already be distinguished from non-maturing fish. Similarly to the previous year, GSI values remained around 0.5 from May to June rising to 0.85 ± 0.13 on July 4th (Fig 1B). No differences in fork length were detected between groups until early May 8th. However, from that date until July 4th, maturing fish grew from 10.28 ± 0.29 cm to 13.55 ± 0.34 cm, resulting in significant differences in fork length with non-maturing fish on May 23rd (Fig 1E). Condition factor showed no significant differences

between groups at any time point, nonetheless condition factor of maturing fish increased over time from 1.03 ± 0.05 on April 25th to 1.27 ± 0.02 on July 4th (Fig 1M). On June 7th, 2017, due to the lower number of parr available and the high percentage of maturing fish, only one non-maturing individual was found during the sampling. Data from this fish are shown in figures (Fig 1, 4, 5) for graphic purpose only but were not included in statistical analysis. No immature fish were found on July 4th, 2017.

To observe potential variations among consecutive seasons, morphometric values of maturing fish were compared between spring 2016 and 2017 (Fig 1C, F, I, N). The overlap revealed consistent increase in GSI profile (Fig 1C) and fork length (Fig 1F) between seasons. From late June onwards, fish maturing in 2016 (batch A) showed lower body weight compared with fish maturing in 2017 (batch B) (11.83 ± 0.41 g versus 13.55 ± 0.34 g on July 4th, respectively; Fig 1I). Consequently, batch A fish were characterized with lower condition factors from late June onward (Fig 1N).

In Autumn 2017, the first signs of gonadal growth and increased GSI were detectable in some individuals from September 27th, six months after hatching. At this date, maturing fish exhibited GSI = 0.16 ± 0.06. This value increased to 1.71 ± 0.95 on October 23rd (Fig 2A). No statistically relevant differences were measured between groups in condition factor (Fig 2B) and fork length (Fig 2C) , while non-maturing fish displayed greater body weight compared to maturing fish on September 27th (Fig 2D). From July 4th to September 27th, when maturing fish were first detectable, fork length increased from 6.00 ± 0.18 cm to 11.32 ± 0.32 cm in non-maturing and 9.48 ± 0.32 cm in maturing males. Body weight increased from 2.33 ± 0.21 g to 16.19 ± 1.34 g in non-maturing and 8.92 ± 0.87 g in maturing fish, respectively.

In spring 2016, 49.3 % (36 out of 73) of the male parr were classified as maturing according to their GSI. In spring 2017, the ratio increased to 66.1 % (39 out of 59). Among the under yearling males, 33.3 % showed signs of maturation, (excluding the individual sampled before September 27th when early signs of maturation were not yet detectable) (Fig 3A).

Results are summarized in supplementary table S1.

Gonadotropin expression

In spring 2016, no statistically significant differences were detected in *fshb* expression between groups from May 25th to June 8th, while higher levels were

measured in maturing fish in the following dates. In this group, *fshb* expression increased from June 8th to maximum levels on July 4th (Fig 4A). The expression of *lhb* showed no variation over time or between groups, except on June 21st, where higher mRNA abundance was detected in maturing fish (Fig 4C).

In spring 2017, the first differences between groups in *fshb* expression levels were detected on May 23rd with higher levels in maturing fish. This year however maturing fish didn't show a peak of expression on July 4th (Fig 4B). In both groups, *lhb* levels decreased from April 25th onward. Similarly to *fshb*, a higher *lhb* expression was measured in maturing fish on May 23rd (Fig 4D).

Underyearling maturing fish were first identified on September 27th, showing higher *fshb* expression levels compared to non-maturing fish. This divergence was maintained in the following month on October 23rd (Fig 5A), while no differences in expression were measured in *lhb* expression with regard to maturation stage (Fig 5B).

Steroid plasma levels

In spring 2016, no differences between groups were detected for plasma T content, increasing through the season in both groups, from 0.78 ± 0.24 ng/µl on May 25th to 2.62 ± 0.2 ng/µl on July 19th (Fig 6A), 11-KT remained stable under 0.8 ng/µl in both groups until June 21st. While non-maturing fish maintained constant levels in the following dates, maturing fish increased plasma 11-KT content to 2.08 ± 0.34 ng/µl on July 4th and 2.6 ± 0.36 ng/µl July 16th (Fig 6D).

The following year, in spring 2017, maturing fish produced more T compared to non-maturing fish from May 8th (0.96 ± 0.1 vs 0.28 ± 0.11 ng/µl), increasing to 1.72 ± 0.15 ng/µl on July 4th (Fig 6B). 11-KT plasma levels were below the limit of detection (0.1 ng/µl) in all non-maturing fish, while were first detected in maturing fish on May 8th (0.2 ± 0.06 ng/µl). Plasma levels remained below 0.5 ng/µl until June 7th, increasing to 2.07 ± 0.75 ng/µl on July 4th (Fig 6E).

The comparison of steroid levels revealed similar T plasma content between the batches until late June. On July 4th, 2016 maturing fish displayed higher T levels compared to July 4th, 2017 (2.63 ± 0.21 vs 1.72 ± 0.15 ng/µl) (Fig 6C). 11KT levels, on the other hand, did not vary between batches (Fig 6F).

Results are summarized in supplementary table S1.

Testis histology

In depth histological analysis were performed during 2016 and 2017 seasons in both fourteen (batch 1 and 2) and six (batch 3) month old maturing fish. Representatives belonging to all six developmental stages were detected in the analysed tissues (Fig 7). One-year old maturing fish in spring 2016 ranged from stage III to stage VI from the first analysis performed on June 8th (Fig 8A). The following spring, histological analysis was anticipated to April 25th samples to investigate testes development at earlier stages. At this date fish at the onset of maturation (stage II), characterized of proliferation of spermatogonia type B, was identified. On May 8th, despite presence of all germ cell stages (up to stage VI) already detectable, the majority of maturing fish were in stage II and III. The percentage of more advanced stages (IV to VI) increased during the season, and on July 4th, only V and VI were present (Fig 8B).

The presence of a few oogonia within testis tissue was observed in 6 samples (Supplementary file S1, A).

Testis histology from one-year old maturing parr revealed the presence of residual spermatozoa (Supplementary Fig. S1,B) in 5 out of 16 (31.3 %) tissues analysed in 2016 and 6 out of 39 (15.4%) in 2017 (Fig 3B). The presence of residual spermatozoa was identified in all stages except stage I.

First signs of testis maturation in underlyearling males were visible on September 27th, where testes stages spanned from II to V. By October 23rd, they reached full maturation, advancing up to stage V and VI (Fig 8C).

Characterization of gonadal stages

Plotting gonadal stage versus relative gene expression of gonadotropin subunits (Fig. 9A) revealed an increase in *fshb* transcripts from stage I to stage II, and consistent levels between stage II and VI. No significant differences between gonadal stages were detected for *lhb* (Fig. 9B). 11-KT plasma content remained below detection limit (0.1 ng/µl) in most samples from stage I to III, before increasing from 0.67 ± 0.19 ng/µl in stage IV to 1.34 ± 0.4 ng/µl in stage VI samples (Fig. 9C). Plasma T levels, on the other hand, were already detectable from stage I (0.74 ± 0.28 ng/µl). They increased in stage III to 1.39 ± 0.2 ng/µl and reached a peak in stage V at 1.9 ± 0.18 ng/µl. (Fig. 9D). All fish in stage I had GSI ≤ 0.05. This value increased to 0.23 ± 0.02 in stages II to IV and raised to 0.62 ± 0.09 and 0.83 ± 0.1, respectively, in stages V and VI (Fig. 9E).

Statistically significant positive correlations were found between gonadal stage and GSI (ρ 0.79; $p<0.0001$), T (ρ 0.51; $p<0.0001$), 11-KT (ρ 0.61; $p<0.0001$), and *fshb* (ρ 0.33; $p=0.018$) levels, but not between gonadal stage and *lhb* transcript levels.

Discussion

The present study reports the characterization of the onset of puberty in Atlantic salmon male parr. The variables measured or calculated include morphometric parameters [body weight, fork length, gonad weight, gonadosomatic index (GSI) and condition factor (K)], plasma steroids levels (T, 11KT), gonadotropin subunit gene expression levels (*fshb*, *lhb*), and testis developmental stages, in maturing and non-maturing male parr for two consecutive seasons. The aim was to accurately identify the period of first sexual maturation and to characterize morphological and physiological changes during the process. The information deriving from this study will serve as a database for further analysis on the role of the Gnrh and melatonin system in male parr presented in Ciani et al., (2018a, 2018b).

Male sexual maturation in Atlantic salmon is characterized by great plasticity. Precocious male parr can reach puberty after one or several years in freshwater and the percentage of maturing males can vary within and among populations (Garcia de Leaniz et al., 2007; Myers et al., 1986; Taylor, 1991). The first signs of precocious maturation in parr from Scandinavian stocks are generally detectable in spring, at one year of age (Maugars and Schmitz, 2008; Mayer et al., 1990). According to GSI and testes histology, in the present study the onset of maturation was already quite advanced in late April as suggested by the proliferation of spermatogonia type B in the testes, indicating intense mitotic division characteristic of early stages of maturation prior the first meiotic divisions (Schulz et al., 2010). The onset of maturation, for this strain, raised under standard farming condition, is therefore occurring before the end of April, earlier in comparison with other studies conducted on Scandinavian strains raired under standard hatchery conditions and natural photoperiod and water temperature. For instance, Maugars and Schmitz (2008) detected in male parr the first sign of gonadal growth and spermatogonial mitotic divisions in early June, while Mayer et al. (1990) identified maturing fish in July characterized by low steroid plasma levels, with significant increase in September. In the aforementioned studies, salmon parr (Baltic strain, in the study from Mayer) were reared further north compared to our study (63°N vs 58°N). Different photoperiod, water temperature, genetic background or feeding regimes may have influenced earlier maturation, in accordance with the high variability in reproductive strategies typical of salmon maturation (for review see Aas et al., 2011; Behnke et al., 2010; Fleming, 1996; Thorpe et al., 1998).

Additionally, maturing fish were identified also in late September, six months after hatching. In the wild, the presence of parr maturing below the year of age, was detected in rare cases in the wild in rivers from the Armorican system in France (Bagliniere and Maisse, 1985) or under artificial conditions of photoperiod that induces early maturation (Nordgarden et al., 2007). Growth rates, especially during the first summer and the period preceding the onset of gonadal development, are known to affect early parr maturation (Berglund, 1995; Berglund et al., 1991). Underyearling fish, therefore, has already reached adequate size and sufficient energy stores to invest in maturation.

Sexual maturation involves the activation of the brain-pituitary-gonad axis and the release of gonadotropins from the pituitary, which in turn activates steroidogenesis and spermatogenesis modulating the production of hormones and growth factors promoting development of germ cells (reviewed in Levavi-Sivan et al., 2010; Schulz et al., 2010; Zohar et al., 2010).

In salmonids, *lhb* mRNA and Lh plasma protein levels are very low or undetectable during early stages of maturation and increase significantly toward the spawning season (Campbell et al., 2003; Gomez et al., 1999; Planas and Swanson, 1995; Swanson et al., 1991). Accordingly, no peak in *lhb* mRNA was detected during early stages of maturation in neither one year old fish in spring nor underyearling fish in late summer. Furthermore, *lhb* mRNA remained stable also between different stages of gonadal development from stage I to stage VI. Maugars and Schmitz (2008) measured in maturing male parr, induction of *lhb* expression during later stages of maturation in September and October, and steep increase in *lhb* mRNA in stage IV and V parr testes, reaching the highest levels at spermiation. For clarity it is important to mention that the classification systems slightly differ between the two studies: stage IV and V in Maugars and Schmitz (2008a) correspond to stage V and VI in the present study. The inconsistency regarding *lhb* expression with regard to testis developmental stage can be explained by the inclusion of fish at later stages of maturation showing a surge in *lhb* in the study from Maugars, which was not registered in the present study. Lh plasma levels in fact increase drastically when approaching the spawning season (Breton et al., 1997; Campbell et al., 2003; Gomez et al., 1999; Prat et al., 1996).

In salmonids, both *fshb* mRNA and Fsh protein plasma levels increase during early stages of maturation, at the onset of testes development (Campbell et al., 2003; Gomez et al., 1999; Planas and Swanson, 1995; Swanson et al., 1991). In teleosts, Fsh is involved in the induction of spermatogonial proliferation and spermatogenesis influencing both the activity (García-López et al., 2009; Loir, 1999; Mazón et al., 2014) and proliferation (Lejeune et al., 1996) of Sertoli cells. Specific receptors for Fsh (Fshr) were detected in Sertoli cells in several teleost species including African catfish (García-López et al., 2009), Japanese eel (Ohta et al., 2007), and Coho salmon (Miwa et al., 1994). Fshr are already expressed in the testis before sexual maturation and are upregulated in maturing fish from early June at the onset of spermatogonial proliferation (Maugars and Schmitz, 2006; 2008)

Higher *fshb* expression was measured during early stages of maturation in both one year old and underyearling fish. Interestingly, in underyearling fish, the increased *fshb* levels in maturing fish were synchronous to the increase in GSI, while in one year old fish maturing in spring the first statistically relevant differences in *fshb* expression were measured one month after the increase of GSI above 0.05. Histological analysis showed the advancement in the spermatogenetic process in maturing fish, where all types of cysts up to spermatids and spermatozoa were identified before significant differences in *fshb* could be detected between maturing and non-maturing fish. Since Fsh is required for the induction of spermatogenesis (Schulz et al., 2010), and a good correlation was proved between *fshb* transcript levels, Fsh pituitary content, and circulating Fsh plasma levels in rainbow trout (Gomez et al., 1999), a more pronounced induction in *fshb* would be expected in maturing fish. This is found when analysing *fshb* in relation to gonad developmental stage. Interestingly, *fshb* mRNA significantly increase from stage I, when only spermatogonia type A are present in the immature testes, to stage II, when spermatogonial type B are identified in the testis, typical of the onset of spermatogenesis and is maintained in all following stages. At these early stages, it is easier to spot differences in *fshb* expression taking in consideration testis development, and a higher number of biological replicates probably is needed when comparing gene expression between groups at early stages when the differences are less pronounced.

In addition to its effect on Sertoli cells, Fsh promote sexual maturation through the induction of T and 11KT synthesis from steroidogenic Leydig cells in the testes, with similar efficiency as Lh (Kamei et al., 2005; Ohta et al., 2007; Planas and Swanson, 1995; Suzuki et al., 1988; Swanson et al., 1991).

T influence reproduction via negative and positive feedback in different tissues including pituitary (Dufour et al., 1983; Montero et al., 1995; Xiong et al., 1993) and hypothalamus (Amano et al., 1994; Goos et al., 1986). In Atlantic salmon for instance, T injections increase *lhb* expression and the expression of a Gnrh receptor (*gnrhr4*, identical to *gnrh2b1* herein) in maturing smolt preparing the Gnrh-mediated Lh release during final stages of maturation (Melo et al., 2015). The expression of this receptor in *lhb*-cells in maturing parr was recently proved in a recent study from our group (Ciani et al., 2018b). The blood volume that could be sampled from underyearling fish was too low to perform RIA, therefore steroid plasma levels were measured only in one year old fish. T levels showed a variability between the batches. No differences in plasma levels were detected between maturing and non-maturing fish in spring 2016, while differential production was detected in spring 2017. Additionally, plasma levels differed between years, in July 2017 maturing fish showed lower T levels compared to fish maturing the previous year. Mayer et al. (1990) showed a different T profile in Baltic salmon (*Salmo salar*) parr males, characterized by the absence of T in July and increasing levels from September to November, approaching the main breeding period. The rise in T levels as they approach full maturity is in accordance with a previous study (Stuart-Kregor et al., 1981). This show the variability of T plasma levels both between strains and among strains in different years.

11KT act as a direct activator of spermatogenesis (Borg, 1994; Cavaco et al., 1998; Miura et al., 1991a) promoting germ cell proliferation (Miura et al., 1991b; Nader et al., 1999) and differentiation of haploid spermatids into flagellated spermatozoa (Miura et al., 1991b, 1991a). The induction of Fsh on 11KT was particularly evident in spring 2016, when a steep increase in *fshb* mRNA correspond to a higher concentration of plasma 11KT. Despite its role in all the phases of spermatogenesis, 11KT was below detection limit (0.1 ng/ml) in most of maturing fish until May in both seasons. Similarly, when observing 11KT plasma level in relation to testes maturation stage, it was below detection limit in most fish up to stage III, where spermatogonia A, B and spermatocytes

433 are already present. Plasma levels however may not be reflecting local hormonal
434 presence in the testes, where the hormone may be active in inducing germ cell
435 proliferation.

436 In summary, the investigation of early stages of maturation in male parr, exposed
437 in this study, revealed that pubertal activation may occur already in Autumn, six month
438 after hatching. Some of the fish that mature in this period, may undergo maturation a
439 second time in spring, together with newly maturing males.

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648

649 **Table 1** Primers used in the present study. † Primers from Maugars and Schmitz (2006).

Gene	Accession number	Primer FW (5'-3')	Primer RW (5'-3')	Product size (bp)	Efficiency (%)
		qPCR primers			
lhb	NM_001173671.1	5'-GTCACAGCTCAGAGCCACAG-3'	5'-GACGTCCGCTATGAAACGAT-3'	97	99
fshb	XM_014126338.1	5'-TACCTGGAAGGCTGTCCATC-3'	5'-TATGCGATCACAGTCGGTGT-3'	101	99.5
rna18s	FJ710886.1†	5'-CTCAACACGGGAAACCTCAC-3'	5'-AGACAAATCGCTCCACCAAC-3'	118	99.5
ef1a	NM_001141909.1	5'-CTTTGTGCCCATCTCTGGAT-3'	5'-ACCCTCCTTACGCTCGACTT-3'	97	99.5
		In situ primers			
lhb	NM_001173671.1	5'-TGCTGAACCCTTTGAGTCCT-3'	5'-TTACATTGGCAGGCATGTTG-3'	495	
fshb	XM_014126338.1	5'-GGACCTGATCTCCTTGTGGA-3'	5'-CTGCTGCAACAGCCTAACTCT-3'	467	

650

651

652 **Table 2** Salmon testis stage. Defined according to Melo et al. (2014). (SPA)
653 spermatogonia A, either undifferentiated or differentiated; (SPB) spermatogonia B;
654 (SC) spermatocytes; (ST) spermatids; (SZ) spermatozoa.

Stages	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

655

656

Table 3 Correlation matrix between variables. Correlation coefficient (Spearman's ρ) between (11-KT) 11-Keto-testosterone; (T) Testosterone; (GSI) Gonadosomatic Index; (*fshb*) Follicle-stimulating hormone beta subunit; (*lhb*) Luteinizing hormone beta subunit. n = 47-68; * p<0.05; ** p<0.01; *** p<0.001 NS non-significant

	fshb	GSI	T	11-KT	Stage
lhb	0.565***	NS	NS	NS	NS
fshb		0.4078**	0.3146*	0.2994*	0.3379*
GSI			0.4389**	0.5662***	0.7971***
T				0.6052***	0.5139***
11-KT					0.6164***

Figures legend

Figure 1. Morphometric measurements of maturing and non-maturing one-year old male parr during spring 2016 (A, D, G, L), 2017 (B, E, H, M) and overlapped values from maturing fish only, during both years, 2016 and 2017 (C, F, I, N). Gonadosomatic index (GSI= gonad weight/body weight*100; A, B, C); fork length (D, E, F); body weight (G, H, I); condition factor (K=100 W/L^3; where W = Body weight (g) and L = Fork length (cm); L, M, N). Data are shown as mean ± SEM (n=6). Distinct letters denote statistically significant differences among groups ($p<0.05$), analysed via two-way ANOVA followed by Tukey multiple comparison test.

Figure 2 Morphometric measurement [gonadosomatic index (GSI= gonad weight/body weight*100; A); condition factor (K=100 W/L^3; where W=Body weight (g) and L = Fork length (cm); B)Fork length (C); body weight (D);] in maturing and non-maturing underyearling male parr during summer/autumn 2017. Data are shown as mean ± SEM (n=6). Distinct letters denote statistically significant differences among groups ($p<0.05$), analysed via two-way ANOVA followed by Tukey multiple comparison test. mRNA levels are normalized against *rna18s* and *ef1a*.

Figure 3 Percentage of maturing male parr. In 2016, 49.3 % (36 out of 73) of one-year old male parr were classified as maturing according to their GSI. In 2017 this number increased to 66.1% (39 out of 59). 33.3 % (13 out of 39, considering fish from first detection of maturation in September 27th) of underyearling (0+) male parr in 2017 were classified as maturing (A). 27.7 % (5 out of 16) of one-year old maturing parr testes showed residual spermatozoa in 2016 and 15.4 % (6 out of 39) in 2017. Fish maturing for the first time are defined as pubertal (B).

Figure 12 Gonadotropins, *fshb* (A, B) and *lhb* (C, D), relative mRNA expression in maturing and non-maturing one-year old male parr during spring 2016 (A, C) and 2017 (B, D). Data are shown as mean ± SEM (n=6). Distinct letters denote statistically significant differences among groups ($p<0.05$), analysed via two-way ANOVA, followed

692 by Tukey multiple comparison test. mRNA levels are normalized against *rna18s* and
693 *ef1a*. † Only one fish was available at this point. It is represented for graphic purposes
694 only and not included in the statistical analysis.

695

696 **Figure 13** Relative gonadotropin gene expression of A) *fshb* and B) *lhb* in maturing and
697 non-maturing underyearling male parr during summer/autumn 2017. Data are shown
698 as mean ± SEM (n=6). Distinct letters denote statistically significant differences among
699 groups ($p<0.05$), analysed via two-way ANOVA, followed by Tukey multiple comparison
700 test. mRNA levels normalized against *rna18s* and *ef1a*.

701

702 **Figure 6** Testosterone (T, first row) and 11-keto-testosterone (11-KT, second row)
703 plasma levels in maturing and non-maturing one-year old male parr during spring 2016
704 (A, D), 2017 (B, E) and overlapped values from maturing fish only, during both years,
705 2016 and 2017 (C, F). Data are shown as mean ± SEM (n=6). Distinct letters denote
706 statistically significant differences among groups ($p<0.05$), analysed via two-way
707 ANOVA, followed by Tukey multiple comparison test. † Only one fish is available at this
708 point. It is represented for graphic purpose only, but it is not included in statistical
709 analysis.

710

711 **Figure 7** Testicular development stages in Atlantic salmon parr defined according to
712 the most advanced cyst present, as described in Melo et al. (2014). (I-SPA)
713 spermatogonia A, either undifferentiated or differentiated; (II-SPB) spermatogonia B;
714 (III-SC) spermatocytes; (IV-ST) spermatids; (V-SZ) spermatozoa, with some tubules still
715 immature, or early maturing. (VI-SZ) spermatozoa is dominating. Large lumen in
716 tubules. Sections (3 μm) were prepared in plastic resin and stained with Toluidine Blue
717 O. Scale bars; 10 μm (I, II, IV and VI); 50 μm (III and V).

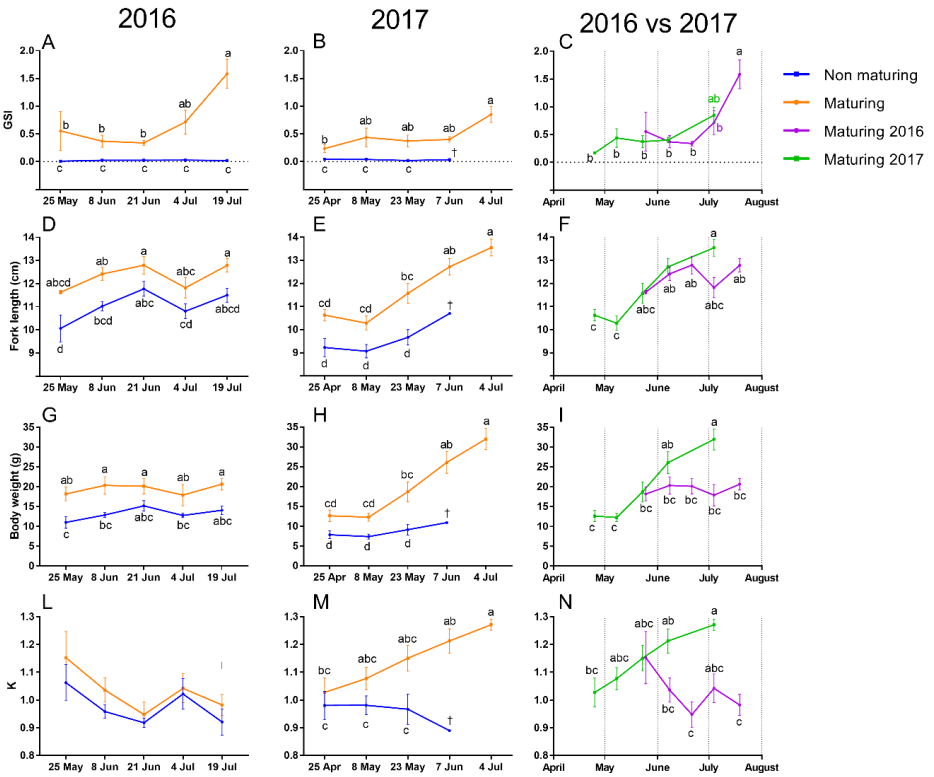
718

719 **Figure 8** Advancement in testicular development stages in one-year old (A, B, D) and
720 under yearling (C, D) male parr in spring 2016 and spring-autumn 2017. Each dot
721 represents the testicular development stages assigned according to the most developed

cyst detected. Testes of all maturing and randomly selected non-maturing males were analysed at each date. NA, not available.

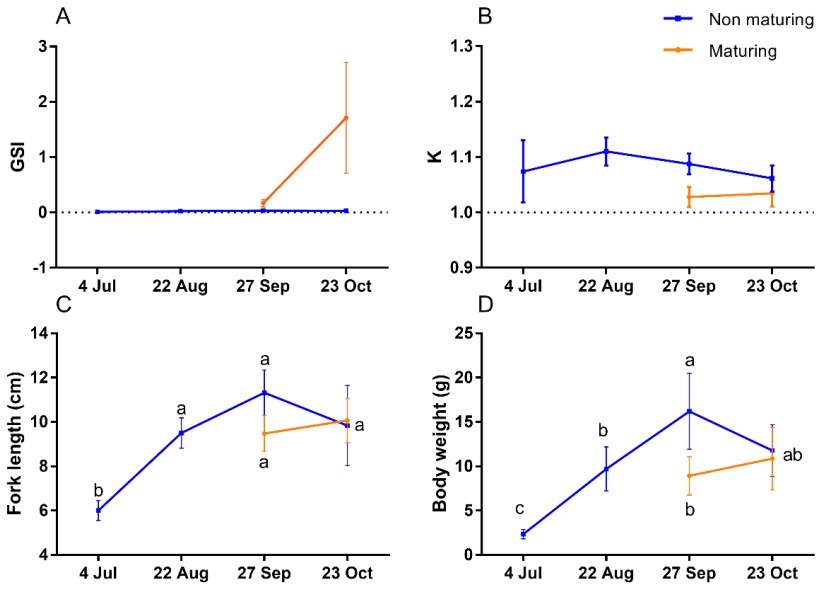
Figure 9 Change in *fshb* (A), *lhb* (B), 11-KT (C), T (D), and GSI (E) according to the six testicular developmental stages. Transcript abundance (*fshb*, *lhb*) of stage I was set as 1 for the representation. Distinct letters denote statistically significant differences among groups ($p<0.05$), analysed via one-way ANOVA, followed by Tukey multiple comparison test. mRNA levels normalized against *rna18s* and *ef1a*.

731 Figure 1



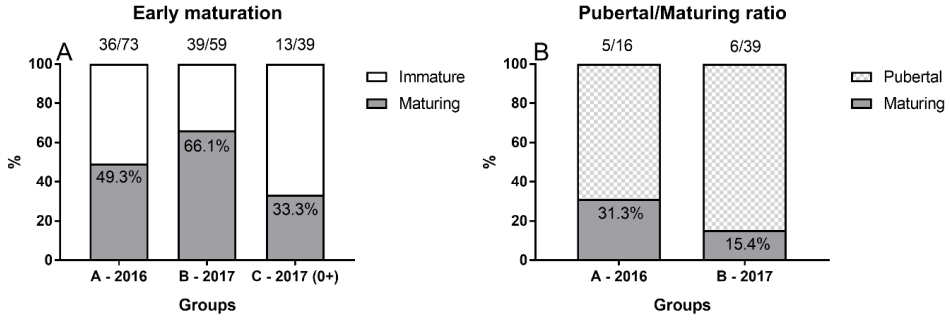
732

733 Figure 2



734

735 Figure 3



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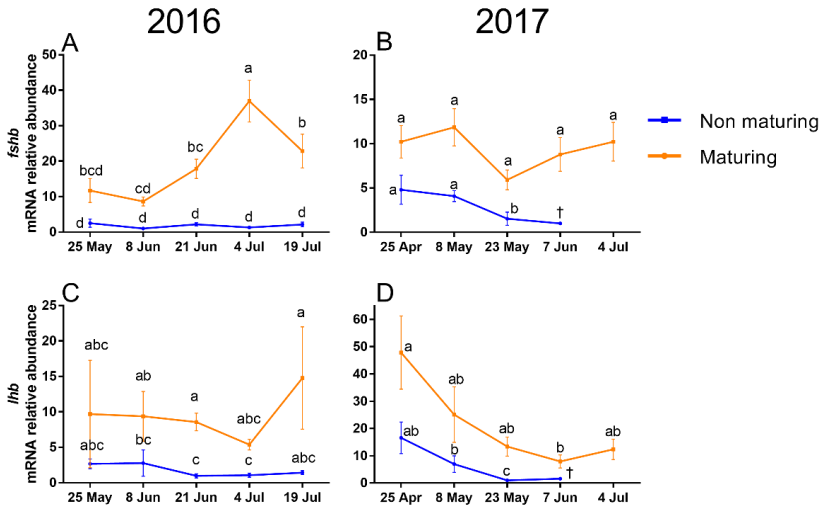
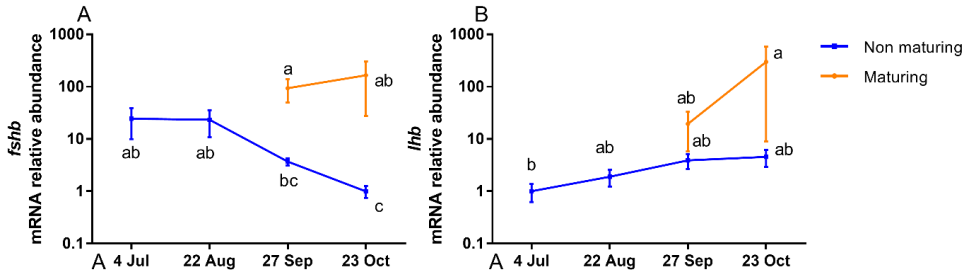
737 Figure 4



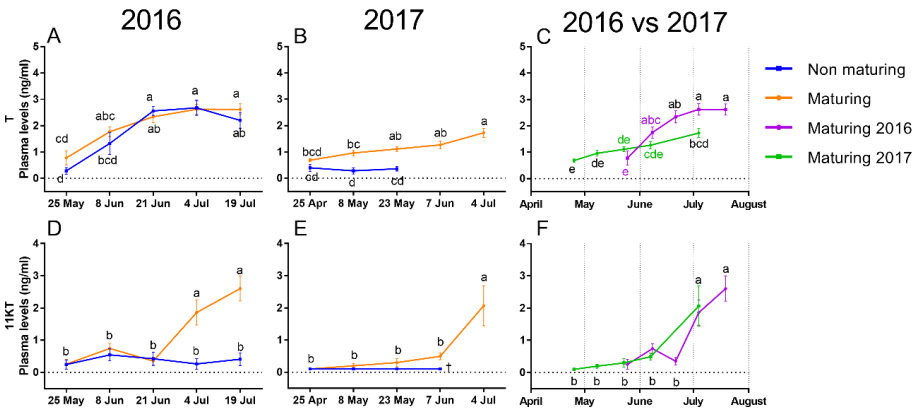
738

739 Figure 5



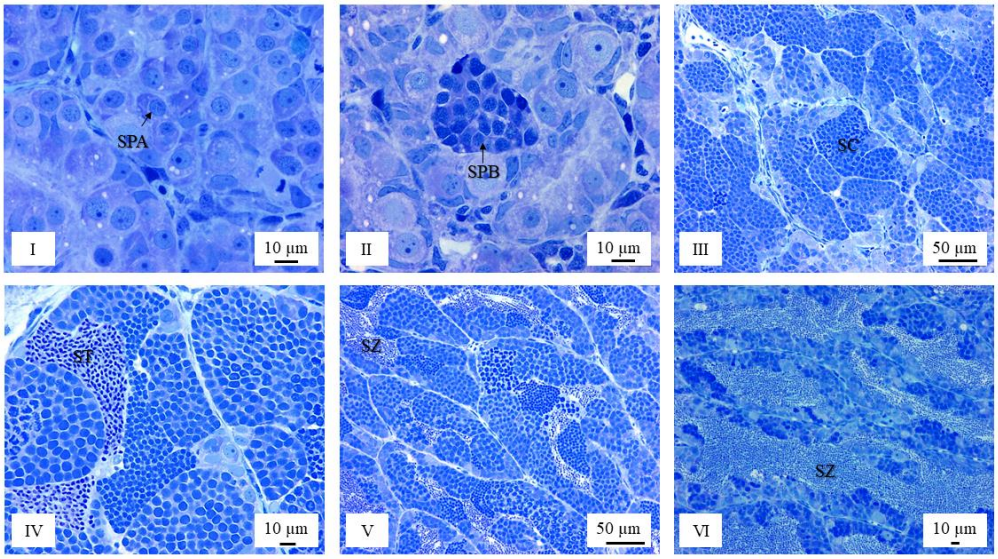
740

741 Figure 6



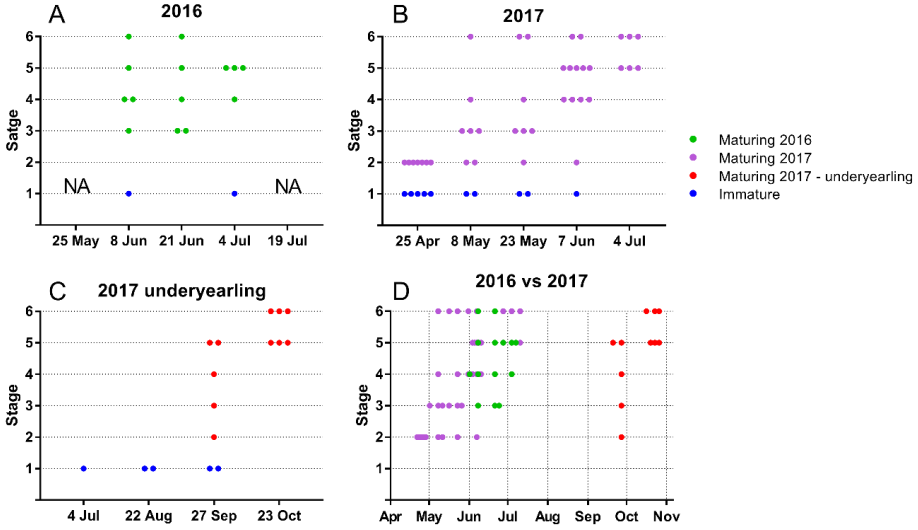
742

743 Figure 7



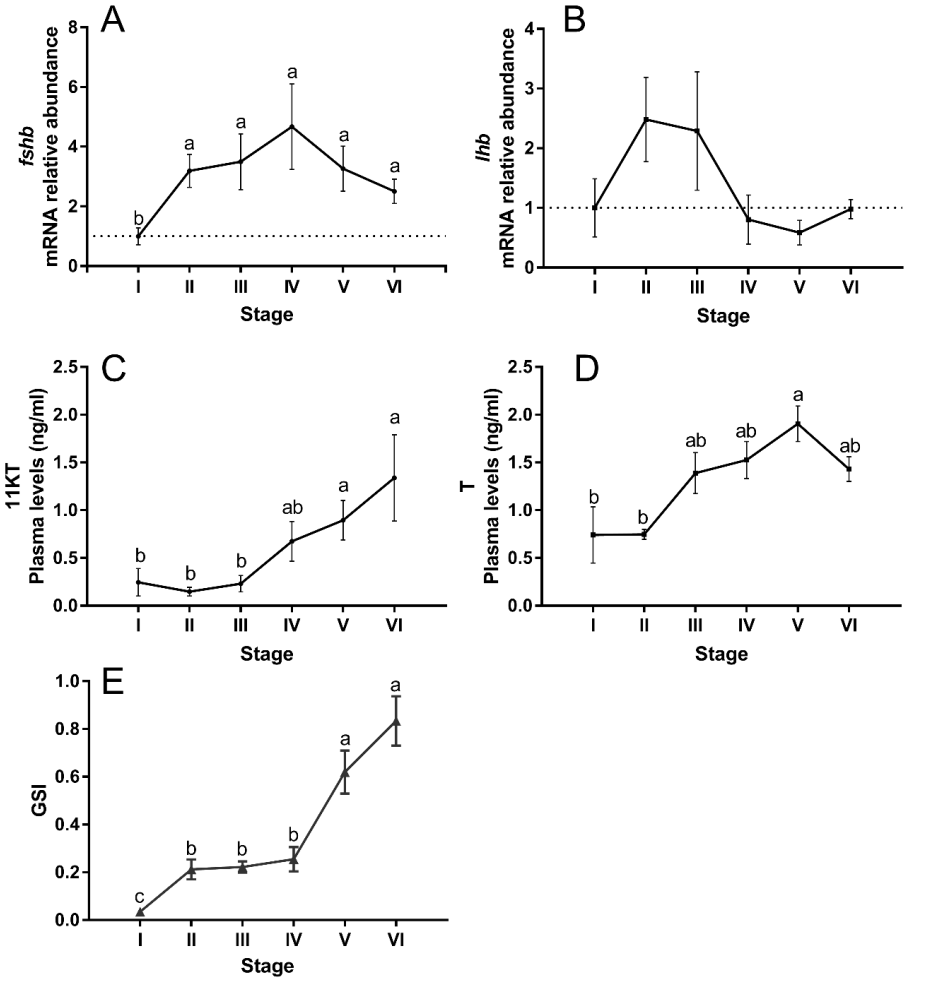
744

745 Figure 8



746

747 Figure 9



748

Supplementary files

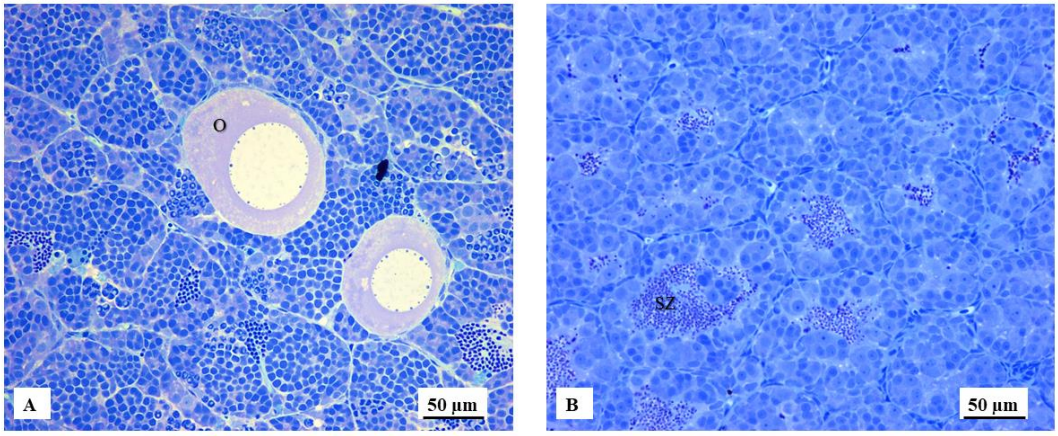
Table S1 Summary of measurements collected in the present study.

		GSI		Fork length (cm)		Body weight (g)		K		T (ng/ml)		11KT (ng/ml)	
Year/batch	Date	Mat	Non-Mat	Mat	Non-Mat	Mat	Non-Mat	Mat	Non-Mat	Mat	Non-Mat	Mat	Non-Mat
2016 Batch A	25 May	0.556±0.314	0.010±0.001	11.63±0.08	10.06±0.53	18.20±1.55	11.01±1.36	1.15±0.08	1.06±0.06	0.78±0.24	0.29±0.11	0.26±0.14	0.24±0.13
	8 Jun	0.371±0.103	0.026±0.002	12.42±0.27	11.02±0.19	20.34±2.05	12.89±0.60	1.04±0.04	0.96±0.02	1.77±0.17	1.33±0.39	0.74±0.15	0.54±0.18
	21 Jun	0.339±0.039	0.025±0.004	12.80±0.36	11.77±0.30	20.13±1.76	15.19±1.20	0.95±0.04	0.92±0.02	2.34±0.21	2.56±0.15	0.35±0.11	0.42±0.19
	4 Jul	0.717±0.202	0.028±0.002	11.83±0.41	10.81±0.30	17.89±2.51	12.77±0.52	1.04±0.05	1.02±0.05	2.62±0.18	2.68±0.26	1.86±0.37	0.26±0.15
	19 Jul	1.587±0.241	0.022±0.001	12.79±0.27	11.50±0.28	20.66±1.39	14.06±0.96	0.98±0.04	0.92±0.04	2.62±0.20	2.20±0.27	2.60±0.36	0.41±0.18
2017 Batch B	25 Apr	0.24±0.06	0.042±0.004	10.63±0.23	9.23±0.37	12.62±1.34	7.82±0.89	1.03±0.05	0.98±0.05	0.68±0.05	0.40±0.13	0.10±0.00	0.10±0.00
	8 May	0.438±0.155	0.040±0.007	10.28±0.29	9.07±0.27	12.27±0.96	7.37±0.58	1.08±0.04	0.98±0.03	0.96±0.10	0.28±0.11	0.20±0.06	0.10±0.00
	23 May	0.375±0.099	0.023±0.007	11.57±0.40	9.67±0.30	18.67±2.34	9.10±1.21	1.15±0.04	0.97±0.05	1.11±0.09	0.36±0.08	0.30±0.12	0.10±0.00
	7 Jun	0.401±0.048	0.037±0.000	12.72±0.35	10.70±0.00	26.10±2.62	10.90±0.00	1.21±0.04	0.89±0.00	1.27±0.14		0.50±0.10	0.10±0.00
	4 Jul	0.852±0.132		13.55±0.34		31.98±2.42		1.27±0.02		1.72±0.15		2.07±0.57	
2017 Batch C	4 Jul		0.010±0.003		6.00±0.18		2.34±0.21		1.07±0.05				
	22 Aug		0.026±0.002		9.50±0.25		9.70±0.93		1.11±0.02				
	27 Sep	0.168±0.059	0.034±0.003	9.48±0.33	11.32±0.32	8.92±0.87	16.19±1.34	1.03±0.02	1.09±0.02				
	23 Oct	1.712±0.945	0.032±0.004	10.07±0.31	9.84±0.60	10.86±1.11	11.77±0.97	1.03±0.02	1.06±0.02				

Supplementary figure legend

Figure S1. Section of testes showing A) testes-ova oocytes (O), and B) residual spermatozoa (SZ)

755 Figure S1



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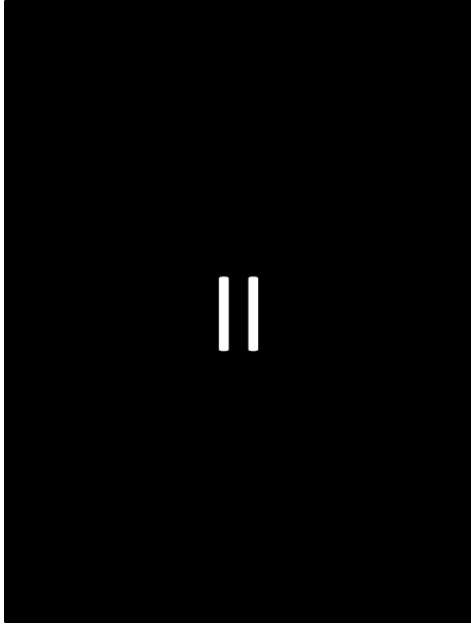

Expression of Gnrh receptor *gnrhr2b1* exclusively in *lhb*-expressing cells in Atlantic salmon male parr

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Abstract

The 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 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 teleost 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; *gnrhr1a1, gnrhr1a2, gnrhr1b1, gnrhr1b2, gnrhr2b1, gnrhr2b2*. All paralogs were expressed in the male parr pituitary during gonadal maturation as evidenced by whole pituitary qPCR analysis. Only one gene, *gnrhr2b1*, was differentially expressed depending on maturational stage (yearly cycle), with high expression levels in maturing fish, increasing in parallel with *gonadotropins* expression. Additionally a similar circadian expression was detected between *gnrhr2b1* and *lhb* (daily cycle) in immature fish in mid-April. Double fluorescence *in situ* hybridization showed that *gnrhr2b1* was expressed exclusively in *lhb* gonadotropes in the pituitary. No expression was detected in *fshb* cells. These results suggest that Gnrh regulation of *lhb* in sexually maturing Atlantic salmon parr is exerted through the receptor paralog *gnrhr2b1*.

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: luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh). 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 production of sex hormones 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 final stages of gamete maturation, regulating spermiogenesis and spermiation (Schulz et al., 2010). In male salmonids, both *fshb* mRNA in the pituitary (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 and their receptors during sexual maturation, together with variations in expression of genes encoding steroidogenic enzymes, plasma steroid levels and gonad histology are reported in detail from Maugars and Schmitz, (2008a, 2008b). 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 in 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 induced from 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), while coupling with 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).

Although multiple *gnrhr* paralogs have been identified in teleost species, there is currently no clearly defined consensus nomenclature for these variants. The proposed classification system divides the Gnrhr in two (Flanagan et al., 2007; 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. In this paper, we adapted the nomenclature of salmon genes according to the classification proposed by Hildahl et al. (2011). In this arrangement, the Gnrhr paralogs are segregated into two types: Type I, divided between mammalian (Type IA) and non-mammalian (Type IB) receptors, and Type II, divided between fish and frog receptors (Type IIB) on one side and all other tetrapod receptors on the other (Type IIA).

Six *gnrhr* paralogs were identified comparing sequences cloned in Atlantic salmon with the recently released *Salmo salar* genome (Lien et al., 2016) (See table 1 for accession numbers). 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 in saltwater adapted smolt 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 the six *gnrhr* paralogs in the pituitary of male Atlantic salmon parr during gonadal maturation.

Materials and methods

Animals

This study was performed on male Atlantic salmon (*Salmo salar*) parr farmed at the Norwegian Institute for Marine Research (NINA) at Ims, Norway (58°54'N, 5°57'E). Fertilized eggs were produced using first generation broodstock from wild caught salmon from 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).

Identification of maturing fish

Maturing fish were identified as described in Ciani et al. (2018b). In brief, fish biometrics (fork length, body and testes weight) were recorded, and gonadosomatic index (GSI = gonad weight/body weight*100) calculated to discriminate between two groups fish, maturing (GSI > 0.05) and non-maturing (GSI ≤ 0.05). In addition, plasma steroid levels were measured and testes development staged based on histological analysis.

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). 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 circadian pituitary gene expression, individual pituitaries from non-maturing fish (batch 3) were collected during a 24 hours 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.

Gene expression analysis, from RNA extraction to qPCR protocols, were performed according to Ciani et al. (2018a). 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 manufacturer's instructions. RNA quality was measured via Bioanalyzer 2100 (Agilent, Santa Clara, USA). All samples showed RIA 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 and Qubit Fluorometer (batch 2 and 3; Invitrogen). One microgram (batch 1) or 170 nanograms (batch 2 and 3) total RNA was reverse transcribed from each pituitary using SuperScript III reverse transcriptase (Invitrogen) and 5 µM random hexamer primers (Invitrogen) following manufacturer's instructions. Specific qPCR primers were designed using Primer-Blast from NCBI website (Ye et al., 2012) (Table1). Thermal cycling was performed using Light Cycler 96 (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. Relative gene expression was calculated using GenEx software (Mangalam et al., 2001) via the algorithms outlined by Vandesompele et al. (2002). Stability of the housekeeping genes were tested using the online tool RefFinder (Kim et al., 2010) and the combination of *rna18s* and *ef1α* was used as internal control genes for data normalization.

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 fixated overnight in 4% PFA, dehydrated with increasing concentration 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 (*lhb*, *fshb*, *gnrhr2b1, gnrhr2b2*; Table 1) by PCR. The products were then isolated after gel extraction using Gel Extraction Kit (Invitrogen) and cloned in pCRII Vector (Invitrogen). Antisense and sense cRNA probes were synthetized by *in vitro* transcription 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 (4',6-diamidino-2-phenylindole; Invitrogen), following manufacturer's instructions. Samples 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.

Image acquisition and processing

The 60 µm parasagittal pituitary sections included the entire length of the gland, allowing the visualization of both anterior and 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) using the following objectives: Plan-Neofluar 10x/0.3 M27 (Zeiss); Plan-Neofluar 25x/0.8 M27 (Zeiss); C-Apochromat 40x/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).

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, circadian 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.

Results

Tissue distribution of *gnrh* receptors in Atlantic salmon

The expression of six *gnrhr* paralogs (*gnrhr1a1, gnrhr1a2, gnrhr1b1, gnrhr1b2, gnrhr2b1, gnrhr2b2;* Fig 1A to F; 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.

Three mRNAs (*gnrhr1b2*, *gnrhr2b1* and *gnrhr2b2*; Fig. 1D,E and F) were detected in the testes and two (*gnrhr1b2* and *gnrhr2b2*; Fig.1 D and F) were identified in skin tissues. The expression of all screened mRNAs was detected in telencephalon, optic nerves, optic tectum, medulla/diencephalon, cerebellum and eyes. mRNA from all six receptors were detected in the pituitary. One representative for each type of receptors (Type I and Type II), *gnrhr1a1* (Fig1A) and *gnrhr2b1* (Fig 1E), were highly expressed in the pituitary compared to most other tissues. *gnrhr1a2* (Fig 1B) showed high Cq values (Cq>30) and lower expression in pituitaries compared to the other tissues investigated. *gnrhr1b1* (Fig 1C), *gnrhr1b2* (Fig 1D) and *gnrhr2b2* (Fig 1F) had comparable expressions levels in all tissues examined.

Gonadotropin and *gnrh* receptor expression during sexual maturation

Pituitary expression of *gnrhr* (*gnrhr1a1, gnrhr1a2, gnrhr1b1, gnrhr1b2, gnrhr2b1, gnrhr2b2*) and gonadotropin subunits (*lhb*, *fshb*) during sexual maturation was first measured in spring 2016 and then repeated in spring 2017 (Fig. 2 and 3). Expression levels were measured in two groups (maturing and non-maturing) of male parr to identify whether (a) differential expression occur dependent on maturational stage and (b) changes in gene expression occur during the season.

Analysing the results from batch 1, it was revealed that three type I receptors, *gnrhr1a1*, *gnrhr1b1* and *gnrhr1b2* (Fig. 2A, B and C), showed no differences in expression with regard to either maturational stage or time, while the expression of the fourth type I receptor, *gnrhr1a2*, was detected only in a sub-set of the sampled fish. Therefore, those genes were not included in the subsequent analyses. For type II receptors, batch 1 maturing fish expressed significantly more *gnrhr2b1* on June 21st than non-maturing fish (Fig 3A), while *gnrhr2b2* showed no differential expression between groups or season but was characterized by high individual variation (Fig 3B).

In concomitance with the *gnrhr2b1* expression, higher *lhb* and *fshb* levels were detected in maturing fish pituitaries on June 21st (Fig 3C). Additionally, maturing fish expressed significantly higher levels of *fshb* than non-maturing fish from June 21st to July 19th (Fig 3D). The expression levels of *fshb* rose from June 8th towards a peak on July 4th.

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 3E and F). Concerning gonadotropins, *lhb* expression decreased in both groups from April 25th to June 7th (Fig 3G) while *fshb* decreased in non-maturing fish from May 8th to 23rd, while remaining constant in maturing fish (Fig 3H). Both *lhb* and *fshb* transcripts were more abundant in maturing fish on May 23rd. Due to the lower number of fish available and the higher rate of maturation, only one non-maturing male was available June 7th. Results from this fish are shown in figures 3E to 3H for graphic purposes only and were not included in the statistical analysis.

Circadian pituitary expression of *lhb*, *fshb*, *gnrhr2b1* and *gnrhr2b2*.

During autumn, the relative gene expression of *fshb*, *lhb, gnrhr2b1* and *gnrhr2b2* in the pituitary showed no significant variation during the 24 hours sampling period (October 23rd, Fig 4A,C,E and G). In contrast, significant daily fluctuations with high levels in the morning and low levels in the evening were measured during spring (April 13th) for *lhb* (Fig 4D) *gnrhr2b1* (Fig 4F) and *gnrhr2b2* (Fig 4H), but not for *fshb* (Fig 4B). *lhb* (Fig. 4D) and *gnrhr2b1* (Fig. 4F) 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 *gnrhr2b1*. Daily fluctuation in the expression of the second receptor gene, *gnrhr2b2*, displayed a much higher induction, with an 83.4 ± 7.8-fold change between minimum and maximum points. Low levels of *gnrhr2b2* expression were measured between 16.00 to 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 to 16.00 (Fig 4H).

Correlation analysis

Higher correlation coefficients (Spearman ρ) were detected between *gnrhr2b1* and gonadotropins (*gnrhr2b1-fshb* ρ=0.83; *gnrhr2b1-lhb* ρ =0.81) compared to *gnrhr2b2* and gonadotropins (*gnrhr2b2-fshb* ρ =0.25 *gnrhr2b2-lhb* ρ =0.49). Results are summarised in Table 2.

mRNA localization via fluorescent *in situ* hybridization (FISH)

While no labelling was detected for *gnrhr2b2*, the localization of *lhb*, *fshb* and *gnrhr2b1* mRNA in the pituitary gland of maturing male parr obtained via FISH is shown in Fig 5. The two gonadotropins, produced in distinct cell types, showed differential distribution along the proximal pars distalis (PPD), with *fshb* distributed dorsally to *lhb* (Fig 5B and D).

Double staining for *lhb* and *gnrhr2b1* revealed co-localization of the mRNAs with no visible labelling for the receptor outside *lhb* producing cells (Fig 5 A, B, C). However, a few *lhb* cells presented no labelling for the *gnrhr2b1*. Double staining for *fshb* and *gnrhr2b1* exposed a distinct localization of the two mRNAs, demonstrating a production of *gnrhr2b1* exclusively in *lhb*-producing cells (Fig 5 D, E, F).

Discussion

This study reports the distribution of six gonadotropin-releasing hormone receptor genes (*gnrhr1a1, gnrhr1a2, gnrhr1b1, gnrhr1b2, gnrhr2b1, gnrhr2b2*) 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 *gnrhr2b1* exclusively to *lhb*-producing cells.

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, expression of multiple *gnrhr*s was detected in all analysed brain regions, including the telencephalon, optic tectum, medulla oblongata, diencephalon and cerebellum. 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*s 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 Gnrhr1b and Gnrhr1a (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 tropical teleosts and are associated with the control and synchronization of spawning (Ando et al., 2014; Ikegami et al., 2015; Shahjahan et al., 2011). 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 retina of several teleosts including rainbow trout (*Oncorhynchus mykiss,* Madigou et al., 2000) and *Astatotilapia burtoni* (Grens et al., 2005).

In the present study, expression of three *gnrhr* genes (*gnrhr1b2, gnrhr2b1 and gnrhr2b2*) was detected in the testes. Gnrhr mRNA was identified also in rainbow trout testes during sexual maturation (Madigou et al., 2002). 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 two *gnrhr*s, *gnrhr1b2* and *gnrhr2b2*, 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 variant 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. As individual Gnrhrs may play distinct physiological roles, and the pituitary consists of numerous cell types, this finding was not surprising. Indeed, the presence of multiple pituitary isoforms of Gnrhr has been reported in several teleost species, including pufferfish (*Tetraodon nigroviridis;* Ikemoto and Park, 2005), European seabass (Moncaut, 2005), Atlantic cod (*Gadus morhua;* Hildahl et al., 2011; von Krogh et al., 2017) and zebrafish (*Danio rerio*; Tello et al., 2008). Of these, only the type II receptor gene, *gnrhr2b1*, showed differential expression dependent on maturational stage in the current work. Despite limited functional data, which makes it difficult to group the multiple receptor variants by function, type II 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 chub mackerel (*Scomber japonicas*), for instance, *gnrhr1* (belonging to Type IIB) 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 *gnrhr2a* 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 *gnrhr2a* 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 *gnrhr2a* 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 II receptors in the control of gonadotropin function in teleost pituitary.

Despite the fact that the type II receptors investigated in the present study, *gnrhr2b1* and *gnrhr2b2*, were both detected in the pituitary gland, *gnrhr2b1* expression seemed to correlate stronger with gonadotropin expression. First, at June 21st, 2016, maturing fish displayed higher *gnrhr2b1* 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 in *gnrhr2b1* expression, which, together with that of *lhb*, returned to comparable levels between maturation groups. No differential expression with regards to maturational stage was detected for *gnrhr2b2* at any time point. Second, while *fshb* was expressed without circadian fluctuation in both autumn and spring in immature fish, *gnrhr2b1* 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). Also, *gnrhr2b2* 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 to 20.00 increasing during the night. Furthermore, *gnrhr2b2* showed a much higher induction between minimum and maximum point compared to the other genes. Third, a high correlation coefficient (ρ=0.8) was detected between *gnrhr2b1* and gonadotropin expression, while *gnrhr2b2* displayed a lower coefficient (ρ=0.25 for *fshb* and 0.49 for *lhb*). It is interesting to notice 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 *gnrhr2b1* induction.

To further investigate the correlation between *gnrhr2b1* and gonadotropin gene expression suggested by the gene expression analysis, and to identify the cell type expressing *gnrhr2b2,* this study proceeded with localization of the mRNA's in the pituitary gland. Unfortunately, no labelling for *gnrhr2b2* could be detected in the pituitary gland. 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 *gnrhr2b1* and *lhb* mRNA in the same cell type. Interestingly, *gnrhr2b1* mRNA was not detected outside *lhb*-producing cells. In contrast, a small number of *lhb* cells did not label for *gnrhr2b1*, indicating expression below detection limit, or absence of *gnrhr2b1* mRNA in these cells. This data corroborates the qPCR results suggesting a correlation between *gnrhr2b1* 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 variant involved in gonadotropin regulation would be susceptible to sex steroid influence. Indeed, Melo et al. (2015) demonstrated that testosterone (T) injections increased *lhb* and *gnrhr2b1* (named there as *gnrhr4*), but not *fshb* mRNA, in pituitary glands of post-smolt Atlantic salmon males prior to maturation. A study from Melo et al. (2014) reported that, when observing pituitary gene expression in relation to testes developmental stage (according to the most advanced germ cell present), *fshb*, *lhb* and *gnrhr2b1* mRNA increase when advancing from spermatogonia type A (either differentiated or undifferentiated are present) to spermatogonia type B, with *fshb* showing the highest relative change. While *fshb* declines at spermiation, *gnrhr2b1* and *lhb* reach their maximum expression levels. Additionally, 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, during different maturation regimes, *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. Both *gnrhr2b1* and *lhb* on the other hand increase over time

in all conditions. In context with the co-localization of *gnrhr2b1* and *lhb* mRNA presented in the current paper and considering that: i) 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), ii) Fsh can induce steroid production (Planas and Swanson, 1995; Suzuki et al., 1988; Swanson et al., 1991), and iii) there is a close correlation between *fshb* mRNA and protein plasma level in rainbow trout (Gomez et al., 1999), it is possible to speculate that the early surge in *fshb* transcript increase and subsequent Fsh release induces androgen production, which feedback to the pituitary, directly stimulating *lhb* and *gnrhr2b1* expression and preparing Lh-producing cells for the Gnrh-mediated Lh induction via *gnrhr2b1*. In the present study, the co-expression of *gnrhr2b1* 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* genes, may be expressed in Fsh-producing cells during earlier stages of maturation considering that Gnrh does stimulate Fsh release from salmonid pituitary (Baker et al., 2000). Alternatively, a paracrine mechanism involving gnrhr2b1-positive cells and Fsh-producing gonadotrophs may be operating. However, this remains to be elucidated.

While *gnrhr2b1* displayed correlation with maturational state and gonadotropin expression in the present study, *gnrhr2b2* lacked similar features. An interesting aspect of the *gnrhr2b2* 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., 2018a). Since gene expression profiling of *gnrhr2b2* 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 I receptors are also required. Although highly expressed in the pituitary, the expression profile of *gnrhr1a* did not reflect either gonadotropin expression or state of sexual maturity. This was also

the case for the other type I receptors, indicating that their functional role lies outside reproductive regulation. A study on coho salmon (*Oncorhynchus kisutch*) found that Gnrh-exposure strongly decreased *gnrhr1* mRNA in a pituitary cell culture from prepubertal fish, while Igf1-exposure increased it significantly (Luckenbach et al., 2010). This suggest a possible involvement of Type I receptors as a link between growth and reproduction in salmonids, but this remains to be elucidated.

Conclusion

This study investigates the gene expression of six *gnrhr* (*gnrhr1a1, gnrhr1a2, gnrhr1b1, gnrhr1b2, gnrhr2b1, gnrhr2b2*) 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 all six genes during sexual maturation. One gene in particular, *gnrhr2b1*, was highly expressed in maturing fish, sharing a similar expression profile with *lhb*. The localization of the mRNA via *in situ* hybridization revealed co-expression of *gnrhr2b1* and *lhb* mRNA in the proximal pars distalis, advocating for the role of this receptor as the main regulator of *lh*-producing cells during sexual maturation in this species.

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779 **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 †	5'-CTCAACACGGGAAACCTCAC-3'	5'-AGACAAATCGCTCCACCAAC-3'	118	99.5
ef1a	NM_001141909.1	5'-CTTTGTGCCCATCTCTGGAT-3'	5'-ACCCTCCTTACGCTCGACTT-3'	97	99.5
fshb	XM_014126338.1	5'-TACCTGGAAGGCTGTCCATC-3'	5'-TATGCGATCACAGTCGGTGT-3'	101	99.5
lhb	NM_001173671.1	5'-GTCACAGCTCAGAGCCACAG-3'	5'-GACGTCCGCTATGAAACGAT-3'	97	99
gnrhr 1a1	KF225727	5'-CCGCTGGATGCACTGGAC -3'	5'-CTCTGCTTTGATTGCTCGAAAGA-3'	117	99
gnrhr 1a2	KF225728	5'-TGCACCCATTGGATGCG -3'	5'-CGGCTTTGATTGCTCGAAAA-3'	120	98.5
gnrhr 1b1	KF225729	5'-ACTCCCACCACCGGAACAA -3'	5'-TGATGGCCCGGAAGATGA-3'	93	98
gnrhr 1b2	MF073196	5'-AACATCACGGTGCAGTGGTA-3'	5'-AGGATGAAGGCTGACGAGTG-3'	92	99.5
gnrhr 2b1	KF225730 ‡	5'-TCAACCCACTGGCGATCAAT -3'	5'-CGTGATGGTCACACTGTGGAATA-3'	122	100
gnrhr 2b2	MF073197	5'-ATACCCTCATCTGTTGGCTGAC-3'	5'-TGCTTTCTCACAGCACAAAAGT-3'	60	99.5
		Cloning/*in situ* primers			
fshb	XM_014126338.1	5'-GGACCTGATCTCCTTGTGGA-3'	5'-CTGCTGCAACAGCCTAACTCT-3'	467	
lhb	NM_001173671.1	5'-TGCTGAACCCTTTGAGTCCT-3'	5'-TTACATTGGCAGGCATGTTG-3'	495	
gnrhr2b1	KF225730	5'-CAGAACACCAGCTGTGAAGC-3'	5'-CGTGATGGTCACACTGTGGAATA-3'	518	
gnrhr2b2	MF073197	5'-CTACAGCTGCCCACCTTCTC-3'	5'-CATGTCTGGCTGAATGCAAA-3'	1205	

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

Table 2. (*ρ)* Spearman's rank correlation coefficient between receptors and gonadotropins mRNA; (p) Significance; (n) sample size.

Variable	by Variable	*ρ*	p	n
gnrhr2b1	*fshb*	0.83	<0.0001	205
	lhb	0.81	<0.0001	205
gnrhr2b2	*fshb*	0.25	0.0004	198
	lhb	0.49	<0.0001	198

Figure legends

Figure 1 Tissue distribution of Gnrh receptors in Atlantic salmon male parr. Relative mRNA abundance of *gnrhr1a1* (A), *gnrhr1a2* (B), *gnrhr1b1* (C), *gnrhr1b2* (D), *gnrhr2b1* (E) and *gnrhr2b2* (F) 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). Data are shown as mean ± SEM (n=5). mRNA levels were normalized against *rna18s* and *ef1a*. Values are graphically expressed as fold change to the lowest expressing tissue (set as value 1). ND = Non-detectable

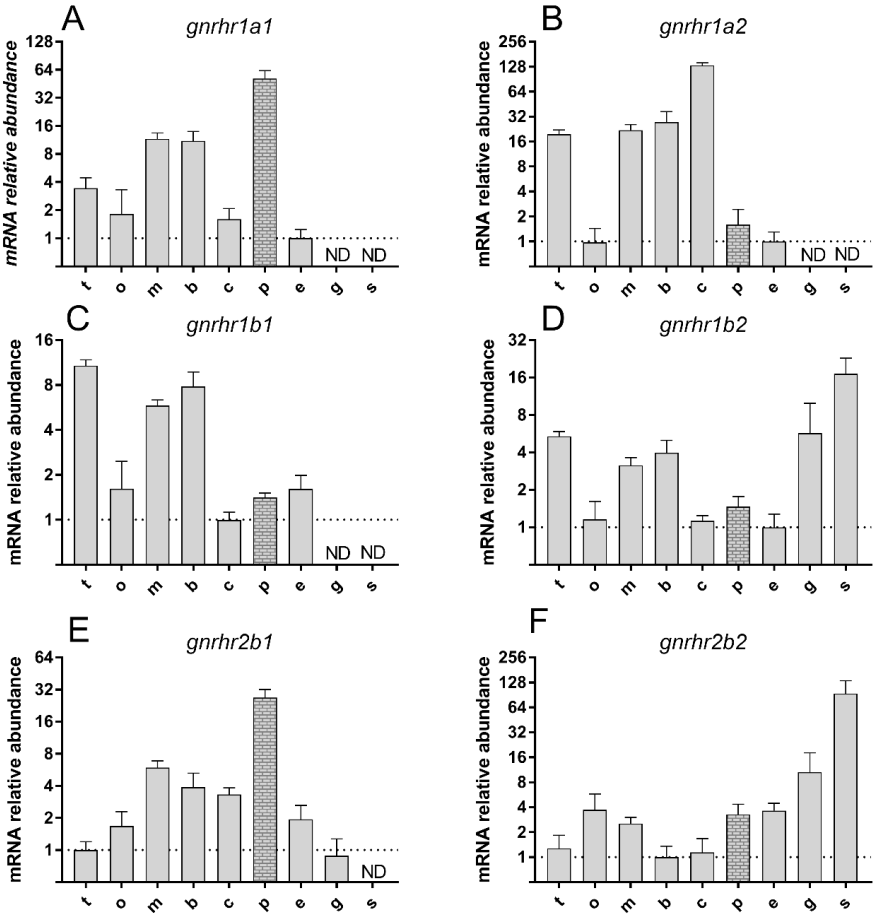
Figure 2 mRNA relative expression of *gnrhr1a1* (A), *gnrhr1b1* (B) *gnrhr1b2* (C) in male parr pituitary during sexual maturation in spring 2016. Maturing fish are represented with dotted lines and open circles. Non-maturing fish are represented with black lines and black squares. mRNA levels are normalized to *rna18s* and *ef1a*. 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.

Figure 3 mRNA relative expression of *gnrhr2b1* (A, E) and *gnrhr2b2* (B, F), *lhb* (C, G), *fshb* (D, H), in male parr pituitary during sexual maturation, in spring 2016 (A to D) and spring 2017 (E to H). 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. mRNA levels normalized against *rna18s* and *ef1a*. 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)

Figure 4 Relative abundance of *fshb* (A, B), *lhb* (C, D), *gnrhr2b1* (E, F) and *gnrhr2b2* (G, H) mRNA 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) expression. 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. mRNA levels were normalized against *rna18s*

817 and *ef1a*. Data are shown as mean ± SEM. Values are graphically expressed as fold
818 change to the lowest point (set as value 1). Different letters denote statistically
819 significant differences ($p<0.05$), analysed using one-way ANOVA followed by Tukey
820 multiple comparison test. The absence of letters in graphs (A,C,E,G) denote lack of
821 significant differences.
822
823 **Figure 5** Fluorescent *in situ* hybridization in parasagittal pituitary sections (anterior to
824 the left) of Atlantic salmon maturing male parr. Confocal pictures of: Double stained
825 section showing *gnrhr2b1* (cyan) and *lhb* (green) individually (A, B) and together (C);
826 Double stained section showing *gnrhr2b1* (cyan) and *fshb* (red) individually (D, E) and
827 together (F). Nuclei stained with DAPI are shown in grey. The mRNA localization show
828 expression of *gnrhr2b1* in *lhb*- but not in *fshb*-producing cells.
829

Figure 1


A
gnrhr1a1
mRNA relative abundance
128
64
32
16
8
4
2
1
ND ND
r o m b c p e g s
B
gnrhr1a2
mRNA relative abundance
256
128
64
32
16
8
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2
1
ND ND
r o m b c p e g s
C
gnrhr1b1
mRNA relative abundance
16
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2
1
ND ND
r o m b c p e g s
D
gnrhr1b2
mRNA relative abundance
32
16
8
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r o m b c p e g s
E
gnrhr2b1
mRNA relative abundance
64
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16
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r o m b c p e g s
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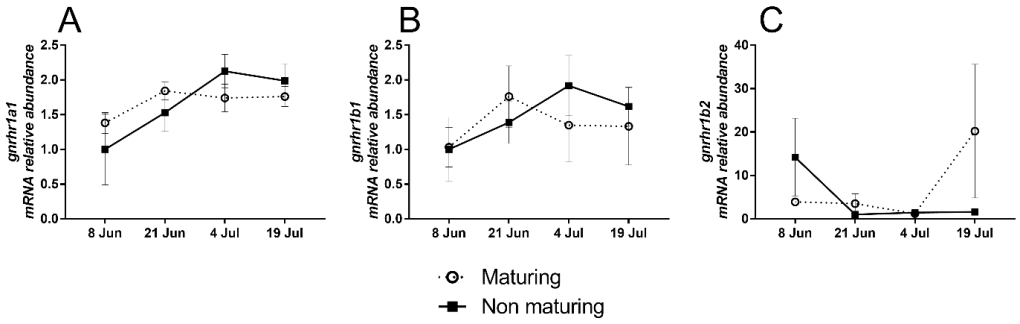
835 Figure 2



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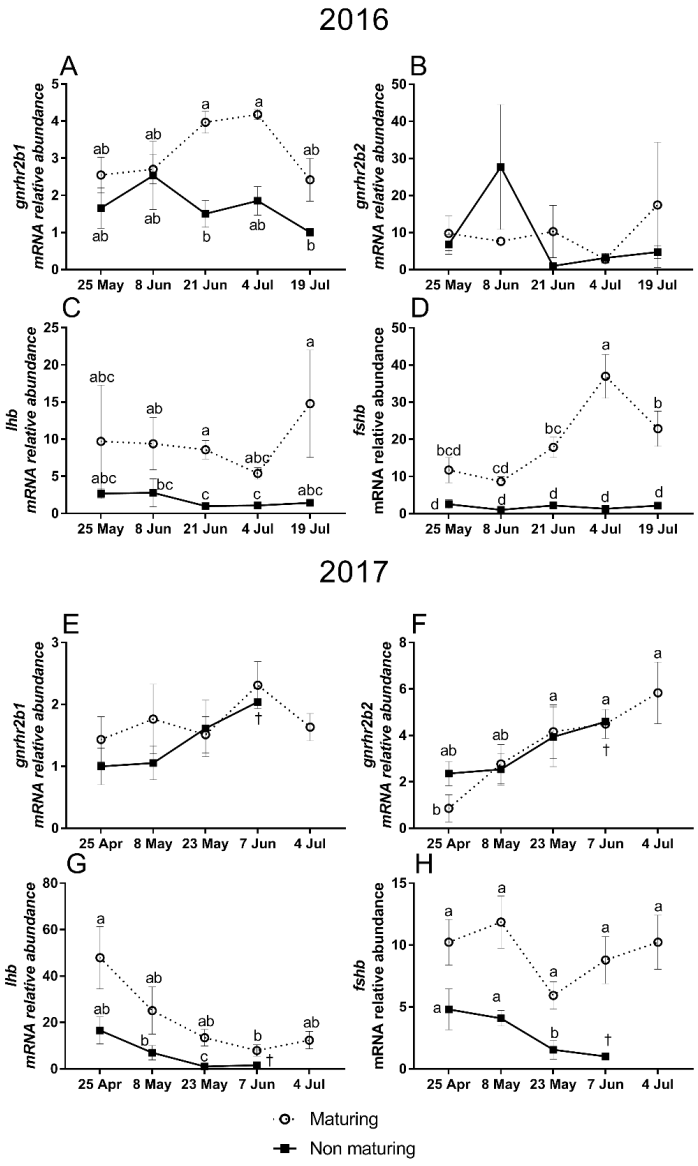
838 Figure 3



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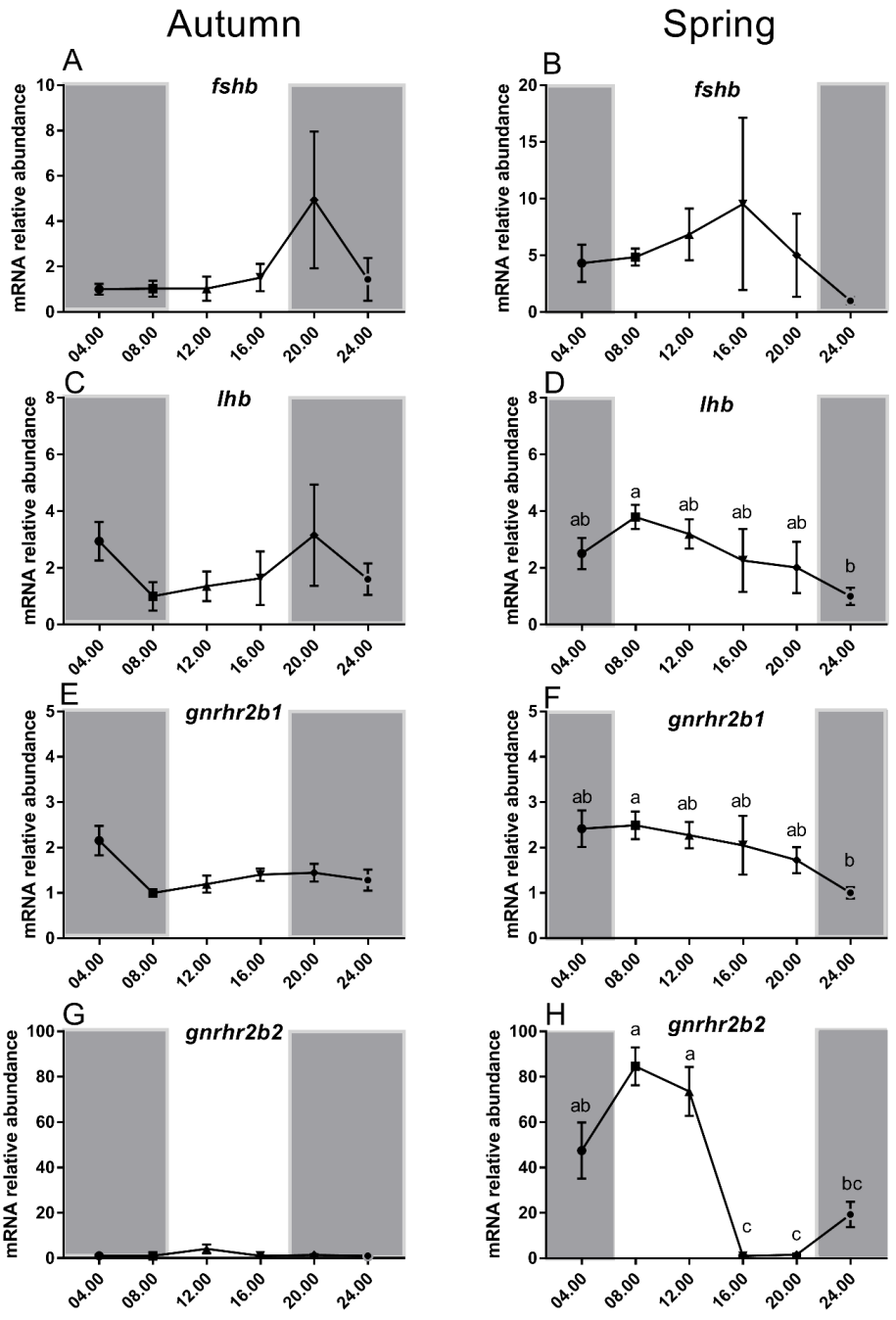
841 Figure 4



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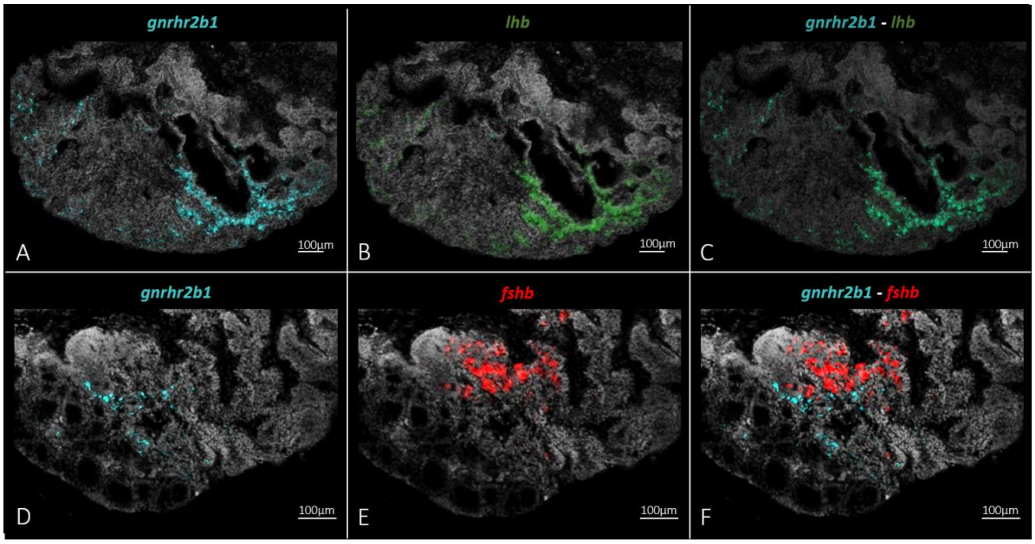
844 Figure 5



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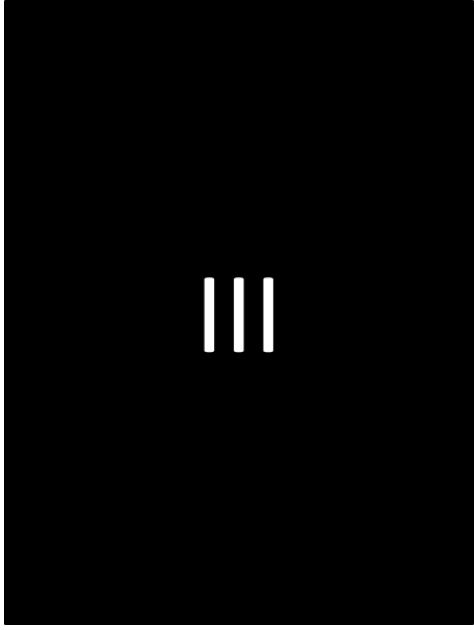

Melatonin receptors in Atlantic salmon stimulate cAMP levels and show season-dependent circadian variations in pituitary expression levels

Running title: Stimulatory melatonin receptors in salmon

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Abstract

The hormone melatonin connects environmental cues, such as photoperiod and temperature, with a number of physiological and behavioral processes, including seasonal reproduction, through binding to their cognate receptors. This study reports the structural, functional, and physiological characterization of five high-affinity melatonin receptors (Mtnr1aaα, Mtnr1aaβ, Mtnr1ab, Mtnr1al, Mtnr1b) in Atlantic salmon. Phylogenetic analysis clustered salmon melatonin receptors into three monophyletic groups, Mtnr1A, Mtnr1Al, and Mtnr1B, but no functional representative of the Mtnr1C group. Contrary to previous studies in vertebrates, pharmacological characterization of three cloned receptors in COS-7 and CHO cell lines (Mtnr1Aaα, Mtnr1Ab, Mtnr1B), showed *induction* of intracellular cAMP levels following 2-iodomelatonin or melatonin exposure. No consistent response was measured after N-acetyl-serotonin or serotonin exposure. Melatonin receptor genes were expressed at all levels of the hypothalamo-pituitary-gonad axis, with three genes (*mtnr1aaβ*, *mtnr1ab* and *mtnr1b*) detected in the pituitary. Pituitary receptors displayed circadian fluctuations in mRNA levels during spring, prior to the onset of gonadal maturation, but not in autumn, strongly implying a direct involvement of melatonin in seasonal processes regulated by the pituitary. To the best of our knowledge, this is the first report of cAMP induction mediated via melatonin receptors in a teleost species.

Nomenclature: We use the following nomenclature: "Mtnr" for protein names and "*mtnr*" for gene names.

Keywords: Circadian expression; Melatonin receptors; Pharmacology; Phylogeny; Pituitary; Sexual maturation; Signaling pathway

Introduction

Melatonin is a highly conserved neurohormone produced in vertebrates by the pineal gland and retina, as well as a number of peripheral tissues, notably the gastrointestinal tract. In all organisms, circulating melatonin levels show a pronounced diurnal rhythm, being high during the night and low during the day; in teleost fishes, the nocturnal rise in plasma melatonin is clearly a function of pineal production. [1] Melatonin is considered the primary hormone that mediates photoperiod information to an organism. Changes in the rhythmic cycle of melatonin release confer photoperiodic information for the control and timing of both circadian and circannual rhythms, including growth and development, and seasonal migration and reproduction (for review see [2,3]). Most, if not all, of these processes are ultimately controlled by altered output from endocrine cells in the pituitary. Although direct effects of melatonin on pituitary cells have been shown both in mammals and teleost fishes, specific details of how melatonin signals affect pituitary cells are still not well understood.

The effects of circulating melatonin, mainly originating from the pineal gland, are mediated through specific melatonin receptors (Mtnr) belonging to the G-protein coupled receptor superfamily. [4] Three sub-groups of Mtnr have been characterized in vertebrates: Mtnr1A (Mel1a or MT1), Mtnr1B (Mel1b or MT2), and Mtnr1C (Mel1c or GPR50). [5] In some teleost species, an additional Mtnr1A has been reported, [5–7] but the origin of the two teleost Mtnr1A paralogs has not been determined.

Mtnr activate different intracellular signaling pathways, including the cAMP/PKA pathway, via G_i-proteins that inhibit adenylyl cyclase and subsequently cAMP formation (Mtnr1A and Mtnr1B), [8] the PLC/PKC pathway via G_q-proteins (Mtnr1A and Mtnr1C), [9] and the cGMP pathway (Mtnr1B). [10] In therian mammals, Mtnr1C has lost the ability to respond to melatonin. [11,12]

Melatonin has widespread effects, as evidenced by the broad distribution of Mtnr in vertebrate nervous and peripheral tissues. [13] Of special interest to the present paper is the pituitary, where Mtnr expression has been detected in the pituitary *pars tuberalis* of mammals. [14,15] The presence of melatonin binding sites has also been detected in the pituitary of teleosts, including the salmonids chum salmon, *Oncorhynchus keta* [16] and rainbow trout, *O. mykiss,* [17] but not, to date, in Atlantic salmon, *Salmo salar.* [18] Whether the effects of melatonin on reproduction in teleosts result from

direct effects on pituitary gonadotropes is not clear, but melatonin has been shown to modulate gonadotropin levels *in vivo* in some teleost species. [19,20]

In the Senegalese sole (*Solea senegalensis*), *mtnr1a* and *mtnr1b* display circannual fluctuations in pituitary expression levels, with higher expression towards the end of the spawning season in June than in the rest of the season. [21] Other studies have reported circadian fluctuations in Mtnr gene expression, peaking either during the daytime or during the night depending on tissue, species, and gene. In the Siberian and Syrian hamster (*Cricetidae)* pituitary, levels of *mtnr1a* mRNA are higher during the daytime and lower at the end of the night. [15] In teleosts, most of the research reporting circadian fluctuations in Mtnr have been conducted in brain. [22,23] At the pituitary level, *mtnr1a* mRNA levels increased during daytime in Chum salmon parr, [16] while no circadian fluctuations were reported in the pituitary of Senegalese sole. [21]

To understand further the effect of melatonin on pituitary cells, particularly the gonadotrope cells controlling gonadal maturation, we have characterized 5 functional Mtnr genes in Atlantic salmon; 3 paralogs of the Mtnr1A sub-group, and single genes of the Mtnr1Al and Mtnr1B sub-groups. Phylogenetic analyses reveal that teleost Mtnr1A paralogs belong to two separate sub-groups. Pharmacological analyses show that, contrary to results reported from other species, Atlantic salmon Mtnr modulate the cAMP/ PKA pathway by *induction* of intracellular cAMP in response to melatonin exposure. Furthermore, the three Mtnr genes expressed in the salmon pituitary (*mtnr1aaβ*, *mtnr1ab*, and *mtnr1b*), all display circadian fluctuations in expression levels in springtime, just prior to initiation of gonadal maturation, but not in the autumn.

Materials and methods

Experimental animals

This study was performed on 1 year old Atlantic salmon (*Salmo salar*) male parr from wild-caught broodstock (Figgjo stock) at the Norwegian Institute for Nature Research (NINA) at Ims, Norway (58°54'N, 5°57'E), reared under natural conditions regarding photoperiod and temperature (yearly range: 5-21°C). 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).

Mntr phylogenetic analysis

Mtnr sequences from 14 vertebrate representatives (Table S1) were retrieved from GenBank, including Atlantic salmon, rainbow trout, and northern pike (*Esox lucius*), the latter belonging to a sister group of the salmonids that diverged before the salmonid-specific genome duplication. Deduced amino acid sequences were aligned using CLC Bio Main Workbench (Qiagen bioinformatic, Hilden, Germany) and manually adjusted. Phylogenetic trees were inferred by Maximum Likelihood algorithm and the AIC model selection [24] using PhyML 3:0 [25] on ATGC Bioinformatic browser. A consensus tree was generated using the SPR algorithm and robustness of the topology was assessed by bootstrapping 1000 replicates. Lancelet Mtnr was used to root the tree. Mtnr nomenclature was based on HUGO, GenBank, and ZFin nomenclatures.

Mtnr pharmacology

All receptor-activation experiments were performed first in COS-7 cells, and thereafter verified in CHO cells. Atlantic salmon *mtnr1aaα*, *mtnr1ab*, and *mtnr1b* inserted into pcDNA3.1 (Invitrogen, Waltham, USA) were obtained from GenScript Biotech (Piscataway, USA) based on sequence information retrieved from GenBank and verified by cloning and sequencing (see PCR primers in Table 1). The procedures for transient transfection of COS-7 or CHO cells and receptor stimulation were according to Levavi-Sivan and Avitan. [26] In brief, COS-7 cells were transfected with luciferase reporter plasmid (3 μg) together with one of the *mtnr* constructs (3 μg). After 48 h, cells were stimulated with increasing concentrations (0, 0.24, 0.98, 3.91, 15.63, 62.50, 250,

1000 nM, each in triplicate) of 4 possible activators: melatonin, N-acetyl-serotonin, serotonin (all Sigma-Aldrich, St. Louis, USA), or 2-iodomelatonin (Santa Cruz Biotechnology, Santa Cruz, USA), either alone or in combination with 20 µM forskolin (used as positive control for cAMP production) (Sigma-Aldrich). Additionally, luzindole, a known inhibitor of mammalian Mtnr (Sigma-Aldrich), was tested at increasing doses (0, 0.01, 0.1, 1, 10, 100, 1000 nM) in combination with either melatonin (100 nM) alone or with melatonin (100 nM) together with forskolin (20 µM). Six hours after stimulation, cells were analysed using GloMax-multi detection system (Promega, Madison, USA). As negative control, COS-7 cells transfected with CRE-LUC reporter only, were exposed to 2-iodomelatonin or melatonin, either alone or in combination with 20 µM forskolin. As an assay function control, COS-7 cells co-transfected with tilapia dopamine receptor D_2, were exposed to quinpirole (0-1000 nM; Sigma-Aldrich), either alone or in combination with 20 µM forskolin. All exposures were performed in at least 3 independent experiments.

Sampling procedure for mtnr gene expression analyses

To study the tissue distribution of *mtnr* expression, the following tissues were collected from 5 male salmon parr (experiment 1) on July 4, 2017: telencephalon, optic nerves, optic tectum, cerebellum, medulla oblongata together with diencephalon, pituitary, eye, testis, and skin.

To study *mtnr* expression during early gonadal maturation (experiment 2), individual pituitaries from maturing and non-maturing males were collected every two weeks from May to August 2016 (N=6 per group). Fish biometry was recorded and gonadosomatic index (GSI= gonad weight/body weight*100) calculated to discriminate between maturing and non-maturing fish.

To study circadian *mtnr* expression (experiment 3), individual pituitaries were collected every 4 hours over a 24-hour cycle in autumn 2017 (October 23; Sunrise 08.33; Sunset 18.08; N=6 per time point) and in spring 2018 (April 13; Sunrise 06.29; Sunset 20.47; N=10 per time point). During night dissections, we used a dim red light to avoid cessation of melatonin synthesis and release (Fig. S1).

In all experiments, fish were treated with an overdose of MS222 (Pharmaq, Overhalla, Norway) and euthanized by decapitation. Pituitaries were collected and stored in TRIzol reagent (Invitrogen) and other tissues were collected in RNA*later*

(Sigma-Aldrich). All samples were stored overnight at 4°C, then frozen at -20°C until RNA extraction.

RNA extraction and cDNA synthesis

For qPCR analyses, total RNA was isolated using TRIzol reagent and DNaseI (Ambion, Foster City, USA) according to the manufacturer's instructions. RNA was quantified using NanoDrop (Thermo Scientific, Waltham, USA) or Qubit (Invitrogen), while RNA quality was checked using Bioanalyzer 2100 (Agilent, Santa Clara, USA). One microgram (experiment 2) or 170 ng (experiments 1 and 3) of total RNA were reverse transcribed using SuperScriptIII and 2.5 µM random hexamers (Invitrogen).

Quantification PCR (qPCR)

qPCR primers (Table 1) for salmon *mtnr* were designed using Primer-Blast (Ye et al., 2012). *mtnr* transcript levels were measured using SYBR Green I (Roche, Basel, Switzerland) on Light Cycler 96 (Roche; Weltzien, Pasqualini, Vernier, & Dufour, 2005). Thermal conditions were 10 min at 95°C followed by 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 8 s. Specificity was verified by melting curve analysis and sequencing. Each sample was run in duplicate using 3 µl cDNA diluted 1:10. Each plate contained triplicates of non-template control and calibrator. Relative expression was determined using GenEx software [28] using algorithms from Vandesomepele and colleagues. [29] *rna18s* and *ef1a* were validated as reference genes using RefFinder [30] and used for data normalization.

Statistical analysis

qPCR results were expressed as mean ± SEM. Statistical differences were determined by two-way (experiment 1) or one-way ANOVA (experiments 2 and 3, and receptor activation experiments), followed by Tukey's HSD test. When necessary, data were log-transformed to meet test criteria. Significance was imparted at $P<0.05$ level. All statistical analyses were performed using JMP pro V.13.0 SAS. Half-maximal effective concentrations (EC_{50}) were calculated from dose-response curves by nonlinear curve fitting (GraphPad Prism 7.04).

Results

Characterization of Atlantic salmon Mntr

The Atlantic salmon GenBank reference genome contains 8 annotated *mtnr1* loci: 4 *mtnr1a*, 2 *mtnr1b*, and 2 *mtnr1c*. Among the *mtnr1a* paralogs, one is located on chromosome ssa04, a second on ssa08, and two on ssa09. All 4 *mtnr1a* paralogs are encoded by two exons. Among the *mtnr1b* paralogs, one is located on ssa09, the other on ssa20. Both paralogs are encoded by three exons, although the one on ssa20 is split, with exon 1 located 6 Mbp apart from the other. Among the two *mtnr1c* paralogs, one is located on ssa04, the other on ssa13. Both paralogs are encoded by two exons, although exon 2 is only partial and includes many frameshifts. The two *mtnr1c* paralogs and the *mtnr1b* paralog on chromosome ssa20 that has a first exon too distant to be translated, are considered pseudogenes and were not further included in our analysis.

Mntr phylogenetic analysis

Phylogenetic analysis clustered the receptors into four monophyletic groups, each containing tetrapod and actinopterygian sequences: Mtnr1A, Mtnr1A-like (Mntr1Al), Mtnr1B, and Mtnr1C (Fig. 1). Teleost Mtnr1A divided into two clades, Mtnr1Aa and Mtnr1Ab, each clade comprising a salmonid cluster branching from pike. Two Atlantic salmon Mtnr1As branched within salmonid Mtnr1Aa and a single sequence within the salmonid Mtnr1Ab. The Mtnr1A paralogs resulting from the teleost-specific third whole genome duplication (3R) were named Mtnr1Aa and Mtnr1Ab, and the Mtnr1Aa paralogs resulting from the salmon-specific whole genome duplication (4R) were named Mtnr1Aaα and Mtnr1Aaβ. Atlantic salmon Mtnr1Al clustered with trout Mtnr1Al, again branching from pike Mtnr1Al. Atlantic salmon Mtnr1B clustered with trout Mtnr1B and pike Mtnr1B within the teleost MtnrB clade.

Mtnr molecular structure

Comparison of deduced amino acid sequences with the human Mtnr1A and Mtnr1B reveals that the five putatively functional salmon Mtnr possess the characteristic features of melatonin receptors, including 7 transmembrane domains (TM) with the typical NRY and NAXXY motifs and conserved residues interacting with G-protein in the TM3 (Fig. S2-4). All the salmon receptors have conserved residues

predicted to form the ligand-binding pocket in different human Mtnr1A and Mtnr1B 3D models (for review see [31–33]). These include the two cysteine residues that form an extracellular stabilizing disulphide bridge. The 3D structure of salmon Mtnr confirm the presence of the extracellular, intracellular, and seven transmembrane domains, together with the ligand-binding pocket and the G-protein interacting site (Fig. 2).

Mtnr pharmacological characterization

Three of the five functional Mtnr genes in Atlantic salmon were pharmacologically characterized in transfected cell lines (COS-7 and CHO). Melatonin and 2-iodomelatonin induced concentration-dependent *increases* in CRE-LUC activity with all tested receptors: Mtnr1Ab, Mtnr1Aaα, and Mtnr1B (Fig. 3A-C). Exposure to N-acetyl-serotonin or serotonin gave no consistent response (data not shown). Luzindole, mammalian Mtnr inhibitor, decreased melatonin-induced CRE-LUC activity (Fig. 3D-F) but had no effect when administered alone (Fig. 3G-I). Mtnr1Ab showed higher sensitivity to melatonin compared with Mtnr1Aaα and Mtnr1B (Table 2; EC_{50} 35.02, 372.03, and 121.76 nM, respectively). Mtnr1Ab and Mtnr1Aaα responded to 2-iodomelatonin at comparable concentrations (EC_{50} 28.88 and 30.23 nM), while Mtnr1B exhibited higher sensitivity to this ligand (EC_{50} 2.89 nM). Mtnr1Ab and Mtnr1Aaα also showed very similar EC_{50} values for luzindole, but higher concentrations were required for Mtnr1B (EC_{50} 10.57, 7.77, and 23.06 nM, respectively). No concentration-dependent increases in CRE-LUC activity were observed in negative controls (Fig. S5). Exposure to forskolin induced cAMP over basal levels and amplified melatonin-induced cAMP stimulation (Fig. S6). Dopamine D2 receptor - that served as a positive control - decreased cAMP levels after exposure to quinpirole together with forskolin (Fig. S7). The fact that salmon Mtnr1Aaα, Mtnr1Ab, and Mtnr1B increased cAMP levels after melatonin exposure was also confirmed in another cell line, namely CHO (Fig. S8)

mntr tissue distribution

mtnr1ab and *mtnr1b* showed a broad tissue distribution, being found in all tissues studied (Fig. 4). The 4R-paralogs, *mtnr1aaα* and *mtnr1aaβ*, showed differential distribution: *mtnr1aaβ* was expressed in all brain parts, pituitary, and skin, whereas *mtnr1aaα* was expressed only in some brain parts (optic nerves, optic tectum, cerebellum) and in eye, testis, skin. *mtnr1al* was expressed in all brain parts and in eye.

Pituitary mntr expression – sexual maturation

The three *mntr* expressed in the pituitary of male Atlantic salmon parr (*mtnr1aaβ*, *mtnr1ab, mtnr1b*) showed no significant differences in transcript levels between maturing and non-maturing salmon during the initial stages of sexual maturation (Fig. 5A-C). When expression profiles were analysed independently of the maturing state (Fig. 5D-F), *mtnr1b* expression remained stable, whereas both *mtnr1aaβ* and *mtnr1ab* expression levels significantly decreased from June onwards. Minimum levels were reached in July (12.9-fold and 6.2-fold decreases, respectively), before tending to increase again later in July.

Pituitary mntr expression – circadian rhythms

Major differences were seen between circadian expression of *mtnr* in the pituitary in the spring and autumn. In autumn, *mtnr1aaβ* and *mtnr1b* expression remained stable and low during the course of 24 hours, while *mtnr1ab* showed a 5-fold increase between 04.00 and 12.00 (Fig. 6). In spring, all receptors displayed strong sinusoidal expression patterns, with highest levels at 08.00 and lowest levels at 16.00 or 20.00 (65-fold, 115-fold, and 238-fold decreases for *mtnr1aaβ*, *mtnr1ab*, and *mtnr1b*, respectively).

Discussion

This study reports the structural, pharmacological, and physiological characterization of melatonin receptors (Mtnr) in Atlantic salmon, with particular focus on pituitary expression in relation to gonadal maturation. An *in silico* search identified five genes encoding putative functional Mtnr. Phylogenetic analysis shows conservation of three GPCR of subtype A (*mtnr1aaα*, *mtnr1aaβ*, *mtnr1ab*) in Atlantic salmon, one of subtype Al (*mtnr1al*), and one of subtype B (*mtnr1b*). Although up to 4 paralogs for each receptor subtype could be expected from the teleost 3R and salmonid 4R, only subtype A shows a high number of functional paralogs, indicating higher functional dependence on this subtype. In contrast, no functional gene of subtype C is conserved in salmonids. *In silico* comparison of primary and tertiary structures of the five Atlantic salmon Mtnr, reveals high conservation of key features known to be involved in receptor binding and activation in mammalian Mtnr (for review see [11,31,32]).

Receptor-activation experiments showed that Mtnr1Ab, Mtnr1Aaα, and Mtnr1B were all activated in a dose-dependent manner by melatonin and 2-iodomelatonin and inhibited by luzindole with the same potencies. This provides *in vitro* confirmation of the functionality of the three salmon Mtnr. Further, activation of the three salmon Mtnr resulted in *increased* intracellular cAMP levels in both COS-7 and CHO cell lines. This result contrasts with the findings from previous *in vitro* studies, in which activation of Mtnr in different vertebrate species led to *decreased* cAMP production; human Mtnr1A and Mtnr1B [34]; chicken Mtnr1A and Mtnr1C [5]; pike Mtnr1B [35]; and medaka (*Oryzias latipes*) Mtnr1B. [36] The validity of our assay is confirmed by (a) the specificity of the response to relevant agonists, (b) the absence of response in cells not transfected with *mtnr*, and (c) the ability of tilapia dopamine D2 receptor, which transduce its signal trough Gi protein [26] and was used as an assay positive control, to decrease cAMP production at the same conditions. Interestingly, some studies have demonstrated that, under specific conditions, Mtnr may activate adenylyl cyclase and increase cAMP levels. For example, Yung and colleagues [37] showed that *Xenopus* Mtnr1C increased cAMP levels in HEK293 cells co-transfected with type II adenylyl cyclase and α_s subunit. Furthermore, mouse Mtnr1A in COS-7 cells co-transfected with adenylyl cyclase VI and G_s protein increased intracellular cAMP levels in response to 2-iodomelatonin. [38] In both those studies, receptor activation decreased cAMP using, respectively, *Xenopus* or mouse intracellular signaling proteins. In contrast, our results showed increased cAMP

levels upon activation of salmon Mtnr using adenylyl cyclase and G proteins endogenous to COS-7 or CHO. This suggest that functional coupling of xenopus and mouse Mtnr may occur with both Gi and Gs proteins, with a much stronger affinity with the former; salmon Mtnr, on the other hand, may transduce their signal via Gs proteins, resulting in overall induction of adenylyl cyclase activity and increased cAMP levels. Future studies should investigate the signaling pathway that is activated by salmon, and other fish species, Mtnr after agonist stimulation in a fish cell or tissue culture system.

Distribution analysis showed that the five receptors are expressed in the eyes and different brain regions, but only *mtnr1aaβ*, *mtrn1ab*, and *mtnr1b* are expressed in the salmon pituitary. In humans, Mtnr1A and Mtrn1B are found in the brain, eyes, pituitary, testis, and skin. [39] The presence of different *mtnr* in the pituitary has been observed in several teleost fish: *mtnr1a*, *mtnr1al* and *mtnr1b* in goldfish, *Carassius auratus,* [40] *mtnr1b* in European seabass, *Dicentrarchus labrax,* [41] *mtnr1a*, *mtnr1b* and *mtnr1c* in the Senegalese sole. [21] For salmonids, *mtnr1a* and *mtnr1b* has been detected by PCR in the pituitary of chum salmon and pike, [16,42] and melatonin binding sites have been observed in trout pituitary, [17] but neither PCR nor autoradiography have previously detected any pituitary Mtnr in Atlantic salmon. [18] The wide and specific tissue distribution of salmon *mtnr* may indicate the array of processes controlled by melatonin. The different distribution patterns of the 3 subtype A receptors indicate functional divergence that may reflect cases of sub-functionalization. In contrast, and differing from other teleosts such as rabbitfish, *Siganus guttatus* [43] and European sea bass, [41] no functional subtype C receptors are conserved in salmon or trout, indicating a pseudogenization or fractionation process in the salmonid lineage.

It is well established that melatonin modulates reproduction in seasonal breeders. [44,45] In mammals and birds, melatonin seems to modulate reproduction via activation of Mtnr1A in the pituitary *pars tuberalis.* [46] In the rat, melatonin inhibits Gnrh-induced Lh release via activation of Mtnr1A. [47] We report the expression of three *mtnr* in the salmon pituitary, two belonging to subtype 1A (*mtnr1aaβ*, *mtnr1ab)* and one to the subtype 1B (*mtnr1b*). The three *mtnr* show different pituitary expression profiles in spring, during the early stages of gonad maturation, suggesting that they have different physiological roles. However, there is no difference in expression between maturing and non-maturing males. Melatonin was reported to regulate growth hormone and prolactin directly in rainbow trout, [17] and to stimulate Lh release in the

Atlantic croaker, *Micropogonias undulatus.* [19] This suggests that different pituitary cell types could express one or more *mtnr*. Further studies are needed to confirm which cell types express which *mtnr*, but this suggests that, in salmon, melatonin can regulate endocrine functions through a direct action at the pituitary level.

Our results show clear circadian fluctuations in expression of the three receptors (*mtnr1aaβ*, *mtnr1ab*, *mtnr1b*) in the salmon pituitary between seasons: expression levels remained low and stable during the 24-hour cycle in the autumn, but showed strong fluctuation in the spring, just around the time when gonad maturation begins. It is well established that photoperiod influences both gonad maturation and smoltification in salmon. [48–50] Differences in pituitary receptivity to melatonin may be involved in determining whether male salmon parr will initiate early sexual (precocious) maturation or not. In addition, these results underline the importance of considering time of day in interpretation of gene expression profiles.

In conclusion, our data add to our understanding of the function and regulation of vertebrate Mtnr. The presence of five functional genes belonging to four distinct phylogenetic clusters, in combination with the wide tissue distribution, is in accordance with the array of processes influenced by melatonin. Pharmacological characterization of salmon Mtnr proved, for the first time in a teleost species, the ability of Mtnr to increase intracellular cAMP levels in response to melatonin exposure. Finally, the identification of Mtnr expression in the salmon pituitary and their clear circadian fluctuation in spring, suggests the involvement of melatonin in neuroendocrine functions through a direct action on the pituitary.

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564 **Table 1**. Sequence of the Atlantic salmon melatonin-receptor primers used for qPCR. †
565 base pairs, ‡ Primers from Maugars and Schmitz [51].

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Gene	Accession number	Primer FW (5'-3')	Primer RW (5'-3')	Product size (bp†)	Efficiency %
mtnr1aaα	XM_014208973.1	5'-CAAGGTGGAGTCGGTGTGA-3'	5'-CTTCCGCCATATTGCTTGTT-3'	120	100
mtnr1aaβ	XM_014195255.1	5'-CAGGCAACATCTTTGTGGTG-3'	5'-GTGGAAGATGGAGGTGAGGA-3'	89	99.5
mtnr1ab	XM_014212815.1	5'-ATGAAAGCGGTCTGACGAAC-3'	5'-AAAGCATCCCAAAGTTGTCG-3'	92	99.5
mtnr1al	XM_014213248.1	5'-TCAGGAACAGGAAACTCAGGA-3'	5'-TAAGGGTAGATCGCCACCAC-3'	85	100.5
mtnr1ba	XM_014215140.1	5'-GTGGATGTCTTGGGCAACTT-3'	5'-CACCACCAGGTCAGCAAAG-3'	111	99
rna18s	FJ710886.1 ‡	5'-CTCAACACGGGAAACCTCAC-3'	5'-AGACAAATCGCTCCACCAAC-3'	118	99.5
ef1a	NM_001141909.1	5'-CTTTGTGCCCATCTCTGGAT-3'	5'-ACCCTCCTTACGCTCGACTT-3'	97	99.5

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Table 2. EC_{50} values (nM) of salmon melatonin receptors transfected to COS-7 cells. Nanomolar (nM) half maximal effective concentration values (EC_{50}) of Mtnr1Aaα, Mtnr1Ab, and Mtnr1B exposed to increasing concentrations (0 to 1000 nM) of 2-iodomelatonin; melatonin or increasing concentrations (0 to 1000 nM) of luzindole in combination with melatonin 100 nM. Each value is given as mean ± SEM and N corresponds to the number of independent experiments.

	Mtnr1Aaα	Mtnr1Ab	Mtnr1B
EC_{50} CRE-Luc (nM)			
2-iodomelatonin	30.23±26.63 N=5	28.88±15.25 N=4	2.89±1.3 N=3
Melatonin	372.03±103.95 N=3	35.02±4.39 N=6	121.76±53.83 N=3
Luzindole + Mel	7.77±2.94 N=3	10.57±5.12 N=3	23.06±4.63 N=3

Figure legends

Figure 1. Phylogenetic relationship between melatonin receptors (Mntr). Tree topology was inferred by Maximum Likelihood from an amino acid sequence alignment using PhyML 3:0 combined with the substitution model selection (SMS) algorithms. Node support was estimated by bootstrapping from 1000 replicates and are indicated as percent. *Branchiostoma belcheri* Mtnr-like was assigned as tree root. Different color backgrounds indicate the four main Mtnr clades: MtnrA, MtnrAl, MtnrB, and MtnrC/Gpr50. Salmonid Mtnr are surrounded by dotted lines. Teleost Mtnr paralogs from the teleost whole genome duplication (3R) are indicated by suffixes a and b, and salmonid Mtnr paralogs from the salmonid whole genome duplication (4R) are indicated by suffixes α and β. Mtnr references are given in Table S1.

Figure 2. 3D-modelling of Atlantic salmon Mtnr. Three-dimensional structures were obtained via web-browser I-Tasser [52] and coloured using Swiss-Pdb v4.1. [53] Transmembrane domains are in yellow, extracellular loops and N terminal domain are in green, intracellular loops and C terminal domains are in blue. Red spheres indicate putative residues forming the ligand-binding pocket. Magenta spheres represent residues, possibly involved in binding G-proteins; Light blue spheres represent conserved cysteine residues forming a disulphide bridge between extracellular loops 1 and 2.

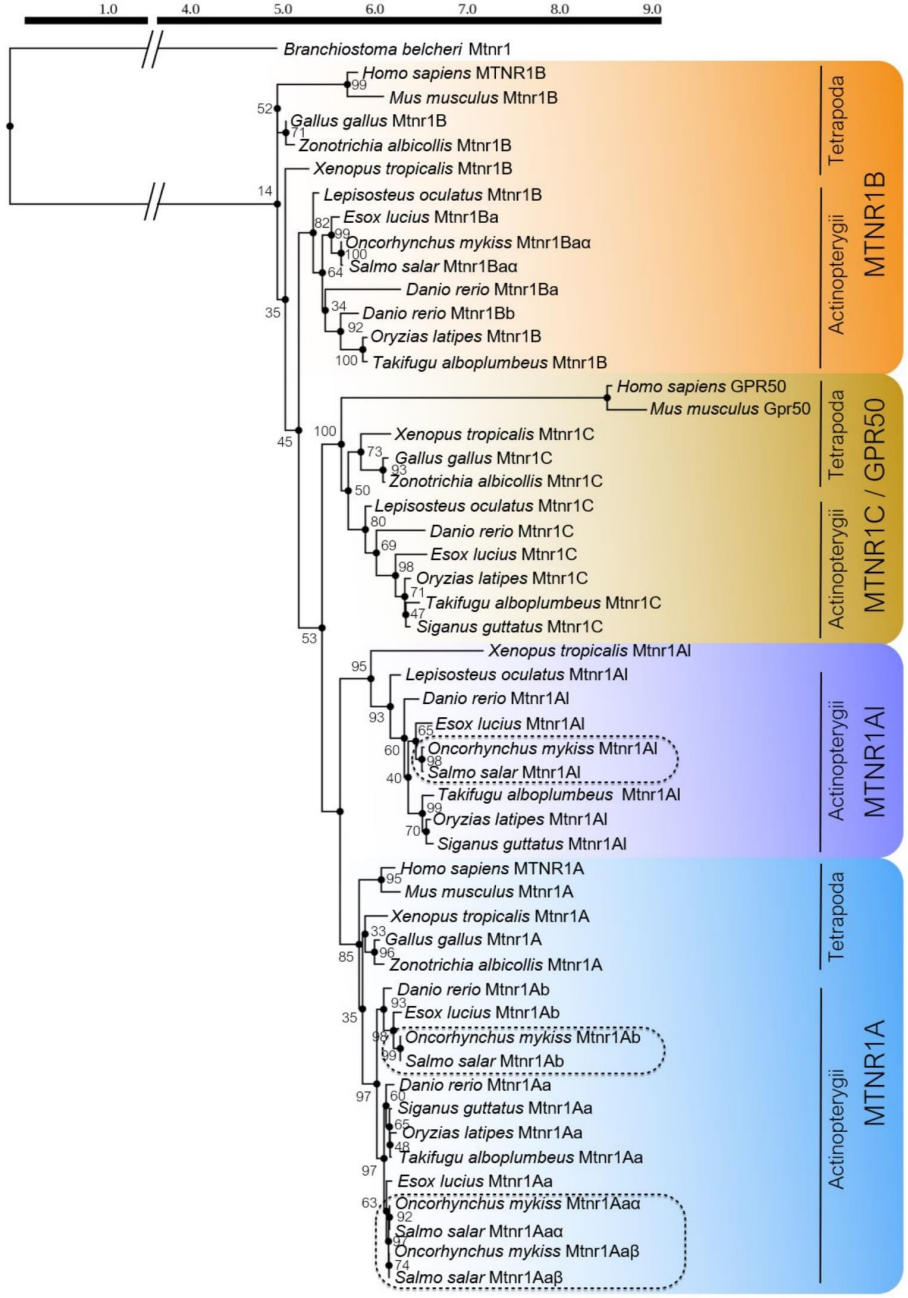
Figure 3. Ligand selectivity of Atlantic salmon Mtnr. COS-7 cells co-transfected with CRE-Luc plasmid and either *mtnr1aaα* (A, D, G), *mtnr1ab* (B, E, H), or *mtnr1b* (C, F, I). Transfected cells exposed to increasing concentrations (0 to 1000 nM) of melatonin (Mel, red lines), 2-iodomelatonin (2im, blue lines) (A, B, C). Transfected cells exposed to increasing concentrations of luzindole (Luz) in combination with Mel 100 nM (D, E, F, black lines). CRE-Luc activity under basal conditions, and after exposure to Mel 100 nM, Luz 1 μM, and a combination of Mel (100 nM) plus Luz (10 nM (G, I); 1 nM (H)). Data are expressed as fold induction of luciferase activity over basal level. Each point was determined in triplicate and is given as mean ± SEM. Different letters denote statistically significant differences among groups ($p<0.05$), analyzed using one-way ANOVA followed by Tukey multiple comparison test (G, H, I). Reference numbers: *mtnr1aaα* (XP_014064448.1); *mtnr1ab* (XP_014068290.1); *mtnr1ba*: (XP_014070615.1).

Figure 4. Tissue distribution of Atlantic salmon *mtnr*. Logarithmic representation of the relative mRNA abundance of *mtnr1aaα* (A), *mtnr1aaβ* (B), *mtnr1ab* (C), *mtnr1al* (D) and *mtnr1b* (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) from male salmon parr (N=5). mRNA levels are normalized to *rna18s* and *ef1a*. Error bars indicate mean ± SEM. Values are expressed as fold-change to the lowest expressing tissue (set as value 1). ND = Non-detectable

Figure 5. Relative expression of pituitary *mtnr* in male Atlantic salmon parr during gonad maturation. Relative abundance of *mtnr1aaβ* (A, D), *mtnr1ab* (B, E) and *mtnr1b* (C, F) mRNA in maturing (orange line, N=6 per point), non-maturing (blue line, N=6 per point), and both grouped together (black line, N=12 per point). mRNA levels were normalized against *rna18s* and *ef1a.* Error bars indicate mean ± SEM. Values graphically expressed as fold change to the lowest point (set as value 1). Different letters denote statistically significant differences among groups ($p<0.05$), analyzed using two-way (A, B, C) or one-way (D, E, F) ANOVA, followed by Tukey multiple comparison test.

Figure 6. Circadian pituitary expression of mtnr in male Atlantic salmon parr in spring and autumn. Relative abundance of *mtnr1aaβ* (A, B), *mtnr1ab* (C, D) and *mtnr1b* (E, F) mRNA in pituitaries of male parr over a 24-h cycle in autumn (A, C, E; 23 October 2017; N=6 per point) and in spring (B, D, F; 13 April 2018; N=10 per point). Samplings were performed every four hours (04.00, 08.00, 12.00, 16.00, 20.00, 24.00). Grey column represents dark hours between sunset and sunrise. mRNA levels are normalized to *rna18s* and *ef1a*. Error bars indicate mean ± SEM. Values are graphically expressed as fold change to the lowest point (set as value 1). Different letters denote statistically significant differences among groups ($p<0.05$), analyzed using one-way ANOVA followed by Tukey multiple comparison test.

641 Figure 1



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643 Figure 2

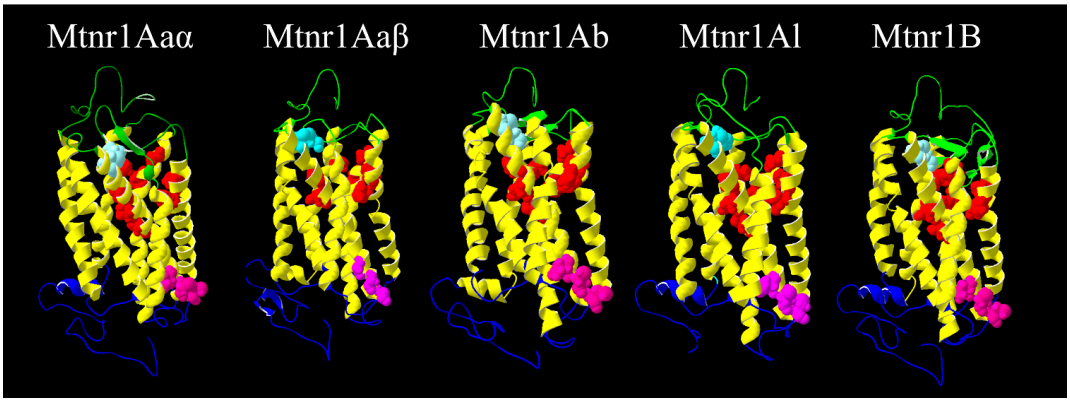

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Figure 3

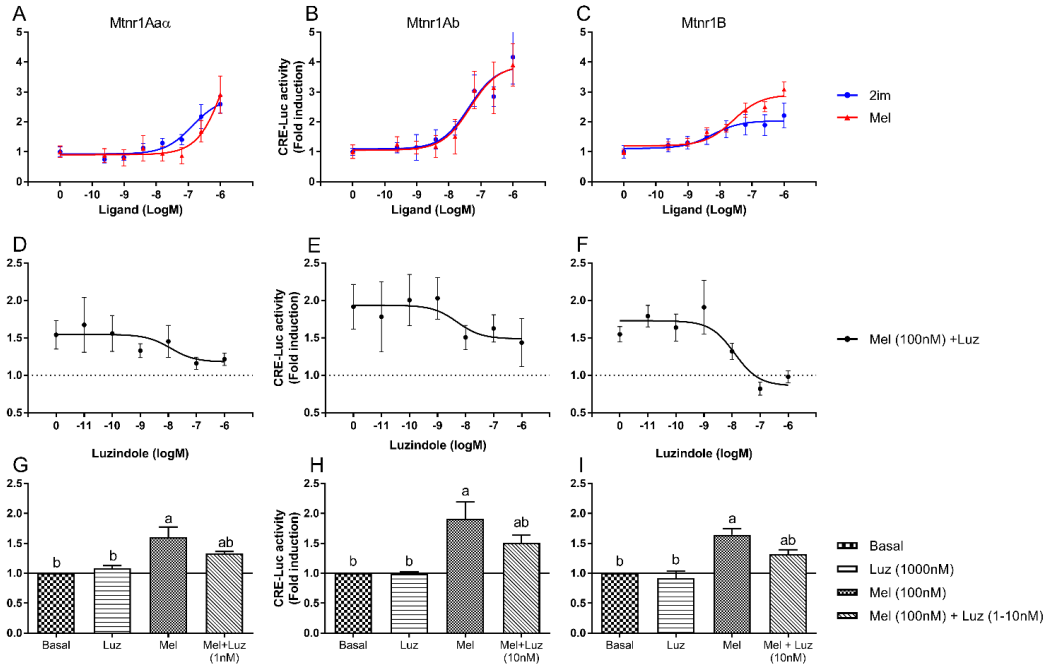
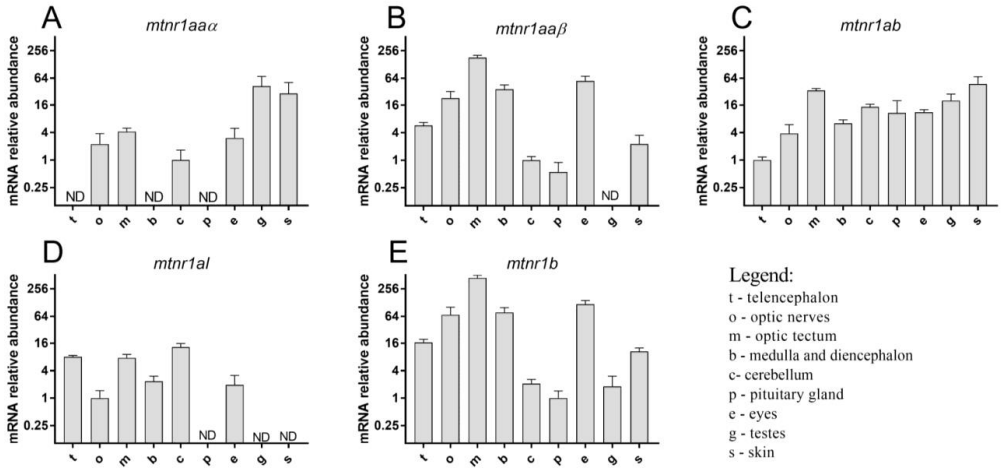
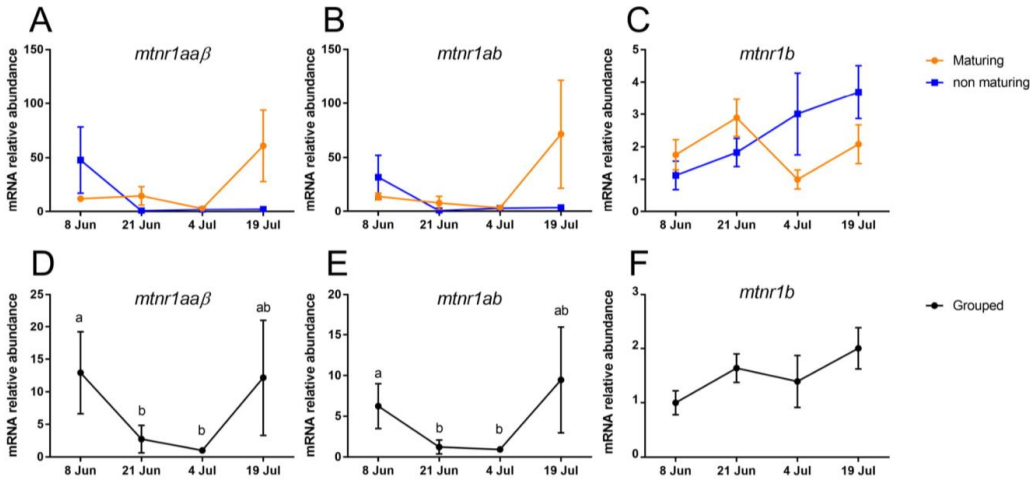

A
Mtnr1Aa
B
Mtnr1Ab
C
Mtnr1B
CRE-Luc activity (Fold induction)
Ligand (LogM)
2im
Mel
D
E
F
Luzindole (logM)
Mel (100nM) +Luz
G
H
I
a
b
ab
Basal
Luz
Mel
Mel+Luz (1nM)
Mel+Luz (10nM)
Mel + Luz (10nM)
Basal
Luz (1000nM)
Mel (100nM)
Mel (100nM) + Luz (1-10nM)

Figure 4


A
mtnr1aaα
B
mtnr1aaβ
C
mtnr1ab
D
mtnr1al
E
mtnr1b
mRNA relative abundance
256
64
16
4
1
0.25
ND
t
o
m
b
c
p
e
g
s
Legend:
t - telencephalon
o - optic nerves
m - optic tectum
b - medulla and diencephalon
c- cerebellum
p - pituitary gland
e - eyes
g - testes
s - skin

650 Figure 5



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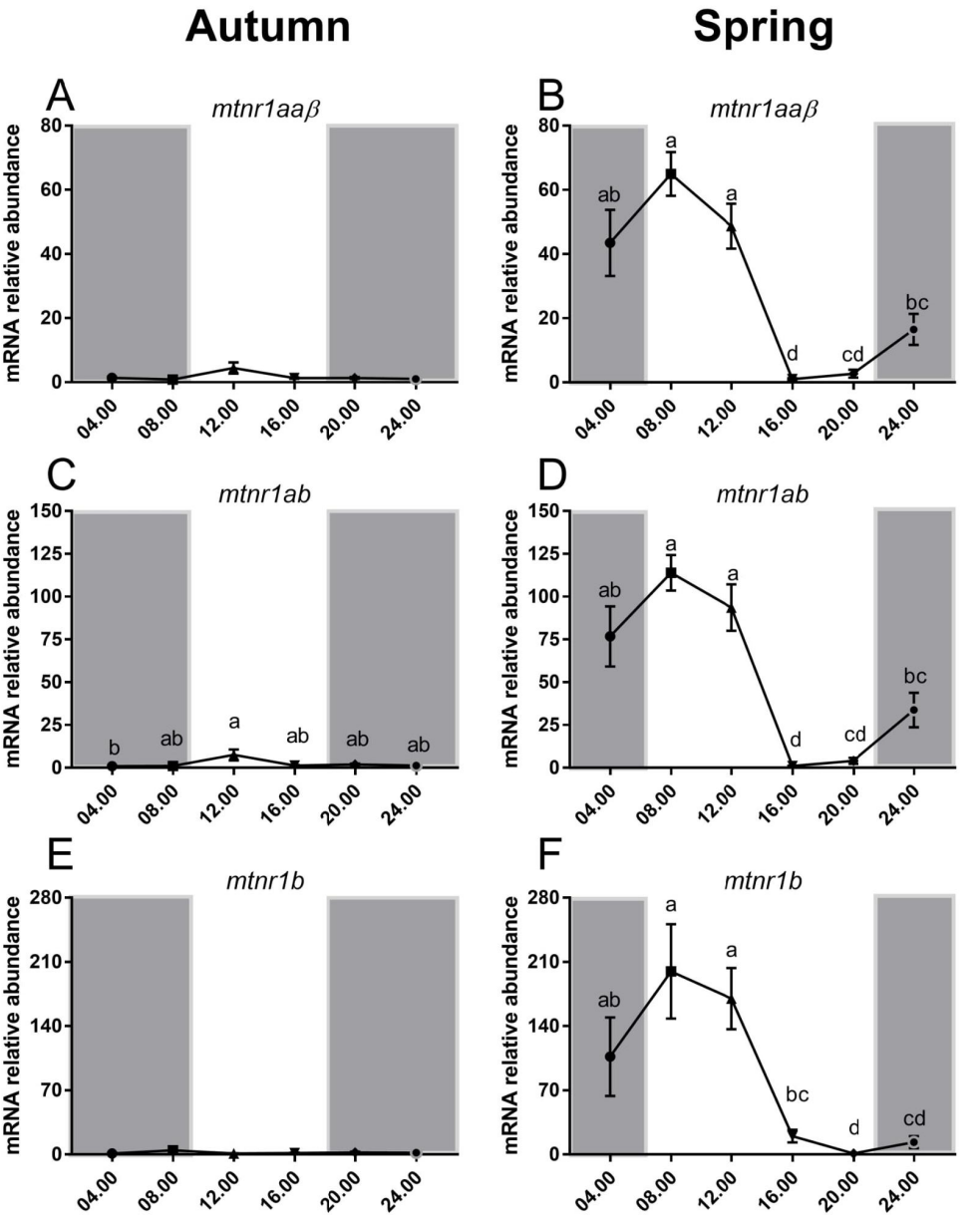
653 Figure 6



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Supplementary data

Table S1 References of Mtnr used for phylogenetic analysis.

Species	Locus ID	Type	Accession no.	Annotation
Branchiostoma belcheri	FQTN01001599	Mtnr		Complement join 829096; 829582; 829670; 830352
Danio rerio	LOC30667	Mtnr1Aa	NP_571468.1	
	LOC565718	Mtnr1Ab	XP_694081.2	
	LOC30660	Mtnr1Al	NP_001153381.1	
	LOC30669	Mtnr1Ba	NP_571470.1	
	LOC30668	Mtnr1Bb	NP_571469.1	
	LOC30661	Mtnr1C	NP_001154956.1	
Esox lucius	LOC105022859	Mtnr1Aa	XP_010889900.1	
	LOC105026623	Mtnr1Ab	XP_010896496.1	
	LOC105030033	Mtnr1Al	XP_010901968.1	
	LOC105026906	Mtnr1Ba	NP_001290778.1	
	LOC105026650	Mtnr1Bb	XP_019900858.1	
	LOC105026775	Mtnr1C	XP_010896763.1	
Gallus gallus	LOC396319	Mtnr1A	NP_990693.1	
	LOC396338	Mtnr1B	NP_001280032.1	
	LOC396318	Mtnr1C	NP_990692.1	
Homo sapiens	LOC4543	Mtnr1A	NP_005949.1	
	LOC4544	Mtnr1B	NP_005950.1	
	LOC9248	Mtnr1C	NP_004215.2	
Lepisosteus oculatus	LOC102685528	Mtnr1Al	XP_006631980.2	
	LOC102696369	Mtnr1B	XP_015196706.1	
	LOC102688516	Mtnr1C	XP_006632834.2	
Mus musculus	LOC17773	Mtnr1A	NP_032665.1	
	LOC244701	Mtnr1B	NP_663758.2	

	LOC14765	Mtnr1C	NP_001295430.1	
Oncorhynchus mykiss	LOC110497458	Mtnr1Aaα	XP_021429243.1	
	LOC100135888	Mtnr1Aaβ		Complement join 56308959; 56309827; 56370732;56370915
	LOC110504716	Mtnr1Ab	XP_021439178.1	
	LOC110504543	Mtnr1Al	XP_021438982.1	
	LOC110504126	Mtnr1B	XP_021438630.1	
Oryzias latipes	LOC101162342	Mtnr1Aa	XP_004066003.1	
	LOC101173872	Mtnr1Al	XP_004073660.2	
	LOC101174522	Mtnr1B	XP_023818482.1	
	LOC105357260	Mtnr1C	XP_023815044.1	
Salmo salar	LOC30667	Mtnr1Aaα	XP_014064448.1	
	LOC106602557	Mtnr1Aaβ	XP_014050730.1	
	LOC106612027	Mtnr1Ab	XP_014068290.1	
	LOC106612252	Mtnr1Al	XP_014068723.1	
	LOC106613152	Mtnr1B	XP_014070615.1	
	LOC106603297	Mtnr1C	XP_014052266.1	
Siganus guttatus	DQ768087	Mtnr1Aa	ABG77572.1	
	DQ522314	Mtnr1Al	ABF67976.1	
	DQ768088	Mtnr1C	ABG77573.1	
Takifugu alboplumbeus	AB492764	Mtnr1Aa	BAI39598	
	AB492763	Mtnr1Al	BAI39597.1	
	AB492765	Mtnr1B	BAI39599.1	
	AB492766	Mtnr1C	BAI39600.1	
Xenopus tropicalis	LOC100488908	Mtnr1A	XP_002940910.1	
	LOC100486065	Mtnr1Al	XP_012809776.1	

	NC_030678	Mtnr1B		Complement join 162859891; 162860759; 162957711; 162957936
	LOC100485432	Mtnr1C	XP_004916939.1	
Zonotrichia albicollis	LOC106629248	Mtnr1A	XP_014120373.1	
	LOC102065760	Mtnr1B		Complement join 38982; 39850; 60116; 60332
	LOC102063887	Mtnr1C	XP_005484544.1	

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Table S2. Sequence of PCR primers used for cloning the Atlantic salmon melatonin receptors. † base pairs (A) Advantage 2 PCR Kit (Takara Bio, Shiga, Japan) (P) Platinum Taq polymerase mix (Invitrogen)

Gene	Taq	Accession number	Primer FW (5'-3')	Primer RW (5'-3')	Product size (bp†)
mtnr1ab	A	XM_014212815.1	GCAAAGACGGCTACTATGGAGCGCAAACC	ACTAGAGCCGAGCCCATCTGTGGAATGTG	1654
	P	XM_014212815.1	AACCTGGGCTTCATTCACTG	TGGCCCTGACTTCTTGAAAC	1492
mtnr1aaα	P	XM_014208973.1	GCTACCACTTACCCGACACC	AAGCTTGAGCTTCCGCCATA	1390
mtnr1ba	P	XM_014215140.1	CGGCTCACTCTGAGCAAGTT	CGCACCATTCCGAGAGTTCA	1397
	A	XM_014215140.1	CGCGGTGGATTCGCAATTCCGAGCA	CGCACCATTCCGAGAGTTCATTGAGGC	1706

Supplementary figures Legends

Figure S1. Atlantic salmon plasma melatonin levels. Blood samples were collected on 13 April 2018 (experiment 3) at 12.00 and 24.00 (N=8). Plasma was isolated by centrifugation and stored at -80°C until analysis. Due to small amounts of plasma, samples from the same sampling time point were pooled prior to analysis. Melatonin plasma assay was performed according to Mayer, 2000. [1] Plasma level are expressed as pg/ml and data are shown as mean ± SEM. Students t-test was used to identify statistical differences among groups. *** $p<0.001$

Figure S2. Deduced amino acid sequences of *Atlantic salmon* Mtnr1Aaα-1Aaβ-1Ab-and alignment with melatonin receptors from other vertebrate species. The transmembrane domains are underlined (sequentially from I to VII). Amino acids predicted to be important for ligand binding in human Mtnr1A 3D models [31,32] are on a grey background. The specific Mtnr NRY and NAXXY motifs are on a grey background with red letters. The conserved cysteine residues, forming a disulphide bridge between extracellular loops 1 and 2, are on a blue background. Protein multiple alignment was performed using Clustal Omega (Sievers et al., 2014) via the EMBL-EBI Multiple Sequence Alignment tool. Sequence abbreviations: *Homo sapiens* (hsMtnr1A)*, Danio rerio* (drMtnr1Aa; drMtnr1Ab), *Esox lucius* (elMtnr1Aa; elMtnr1Ab), *Salmo salar* (ssMtnr1Aaα; ssMtnr1Aaβ; ssMtnr1Ab), *Oncorhynchus mykiss* (omMtnr1Aaα; omMtnr1Aaβ; omMtnr1Ab), *Oryzias latipes* (olMtnr1Aa). Sequence references are given in Table S1.

Figure S3. Deduced amino acid sequences of *Atlantic salmon* Mtnr1Al and alignment with Mtnr from other vertebrate species. The transmembrane domains are underlined (sequentially from I to VII). Amino acids predicted to be important for ligand binding in human Mtnr1A and Mtnr1B 3D models [31,32] are on a grey background. The specific Mtnr NRY and NAXXY motifs are on a grey background in red letters. The conserved cysteine residues, forming a disulphide bridge between extracellular loops 1 and 2, are on a blue background. Protein multiple alignment was obtained using Clustal Omega [54] via the EMBL-EBI Multiple Sequence Alignment tool. Sequence abbreviations: *Danio rerio* (drMtnr1Al), *Esox lucius* (elMtrn1al), S*almo salar* (ssMtnr1Al), *Oncorhynchus mykiss* (omMtnr1Al), *Oryzias latipes* (olMtnr1Al). Sequence references are given in Table S1.

Figure S4. Deduced amino acid sequences of *Atlantic salmon* Mtnr1B and alignment with melatonin receptors from other vertebrate species. The transmembrane domains are underlined (sequentially from I to VII). Amino acids known to be important for ligand binding in human Mtnr1B 3D models [31,32] are on a grey background. The specific Mtnr NRY and NAXXY motifs are on a grey background in red letters. The conserved cysteine residues, forming a disulphide bridge between extracellular loops 1 and 2, are on a blue background. Protein multiple alignment was obtained using Clustal Omega (Sievers et al., 2014) via the EMBL-EBI Multiple Sequence Alignment tool. Sequence abbreviations: *Homo sapiens* (hsMtnr1B), *Danio rerio* (drMtnr1Ba; drMtnr1Bb), *Esox lucius* (elMtnr1B), *Salmo salar* (ssMtnr1B), *Oncorhynchus mykiss* (omMtnr1B), *Oryzias latipes* (olMtnr1B). Sequence references are given in Table S1.

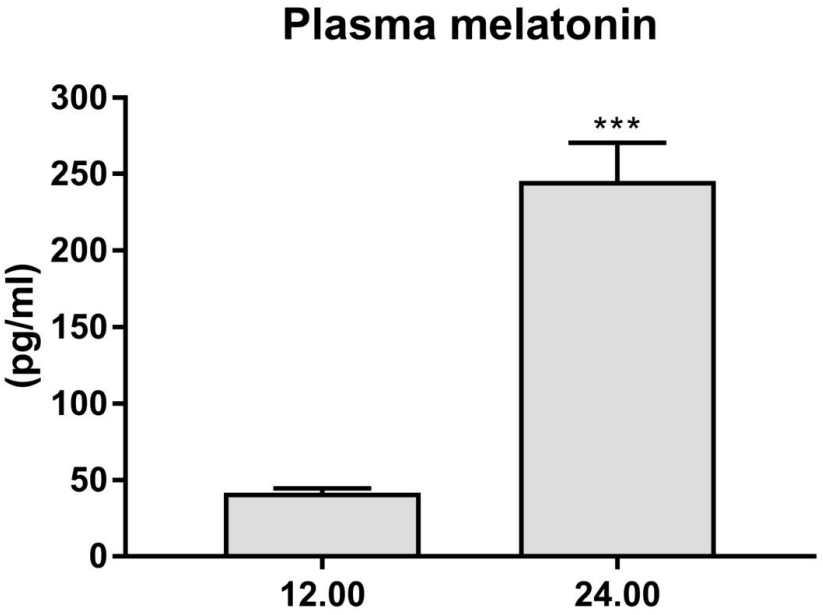
Figure S5. Ligand selectivity, negative control. COS-7 cells transfected with CRE-Luc plasmid alone, exposed to increasing concentrations (0 to 1000 nM) of 2-iodomelatonin (2im) (A) or melatonin (Mel) (B) in combination with forskolin (Fsk) 20 µM or alone. 2im+Fsk (black dots); 2im (grey triangles); Mel+Fsk (red squares); Mel (pink triangles).

Figure S6. Ligand selectivity, positive control for cAMP production. COS-7 cells co-transfected with CRE-Luc plasmid and either Mtnr1Aa*α* (A) Mtnr1Ab (B) or MtnrB (C). CRE-Luc activity under basal conditions and after exposure of forskolin (Fsk) 20 µM, melatonin (Mel) 1µM, and a combination of Fsk 20 µM and Mel 1 µM. CRE-Luc activity expressed as fold induction to basal level. Each point was determined in triplicate and is given as mean ± SEM. Different letters denote statistically significant differences among groups ($p<0.05$), analyzed using one-way ANOVA followed by Tukey multiple comparison test.

Figure S7. Ligand selectivity, positive control. COS-7 cells co-transfected with CRE-Luc plasmid and dopamine receptor (D2) exposed to increasing concentrations (0 to 1000nM) of quinpirole in combination with forskolin (Fsk) 20 µM (black line); quinpirole alone (red squares), and starving medium alone (blue triangles). CRE-Luc activity expressed as absolute values (A) or fold induction to basal level (B).

Figure S8. Ligand selectivity of Atlantic salmon melatonin receptors. CHO cells co-transfected with CRE-Luc plasmid and either Mtnr1Ab (A) Mtnr1Aaα (B) or MtnrB (C). Cells treated with increasing concentrations (0 to 1000 nM) of melatonin (pink line). CRE-Luc activity expressed as fold induction to basal level.

733 Figure S1



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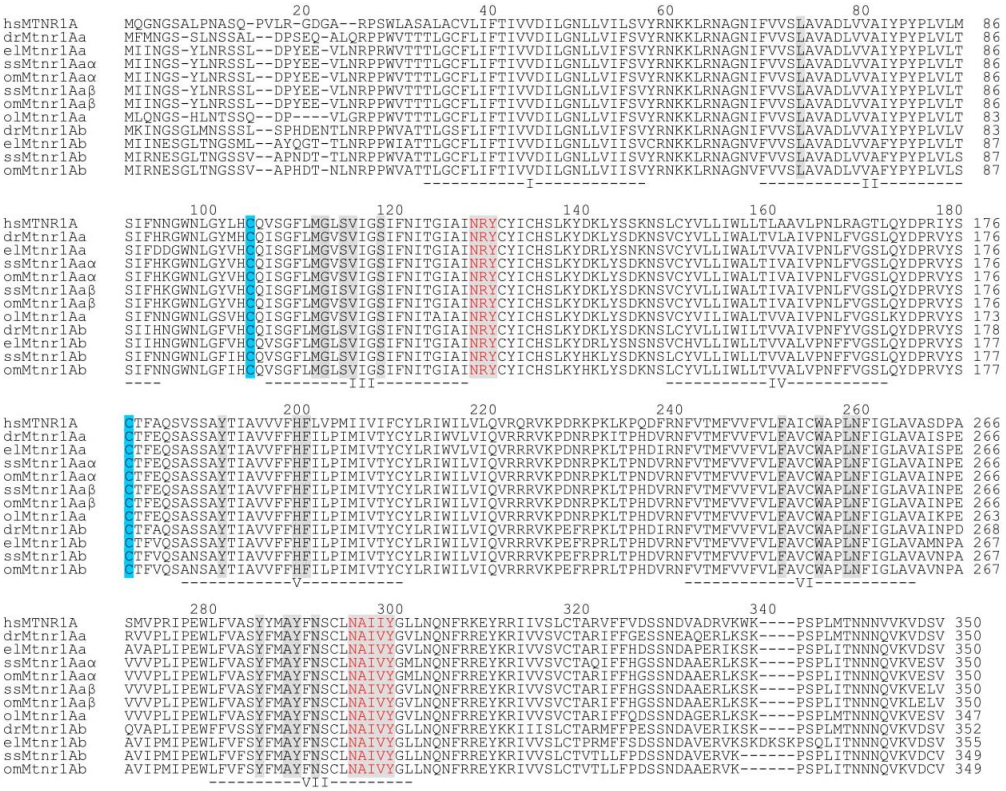

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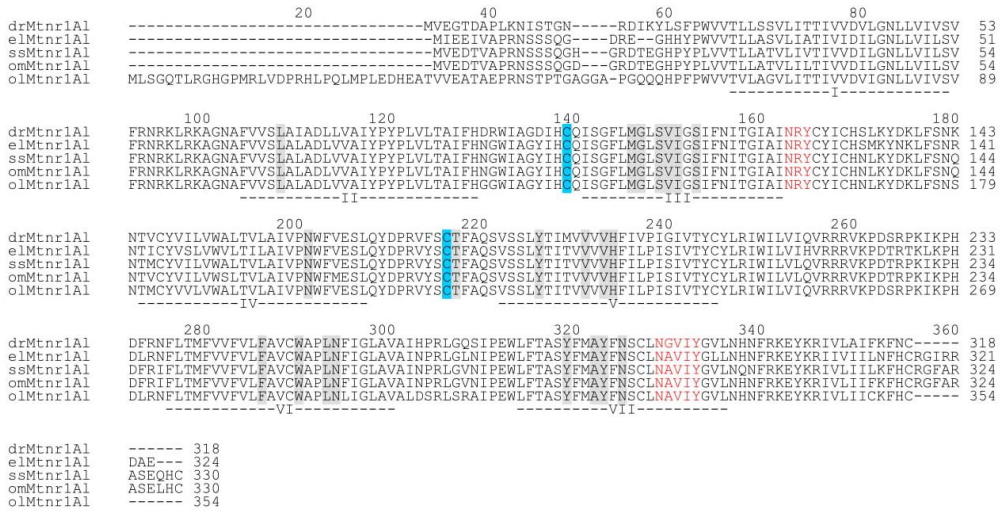

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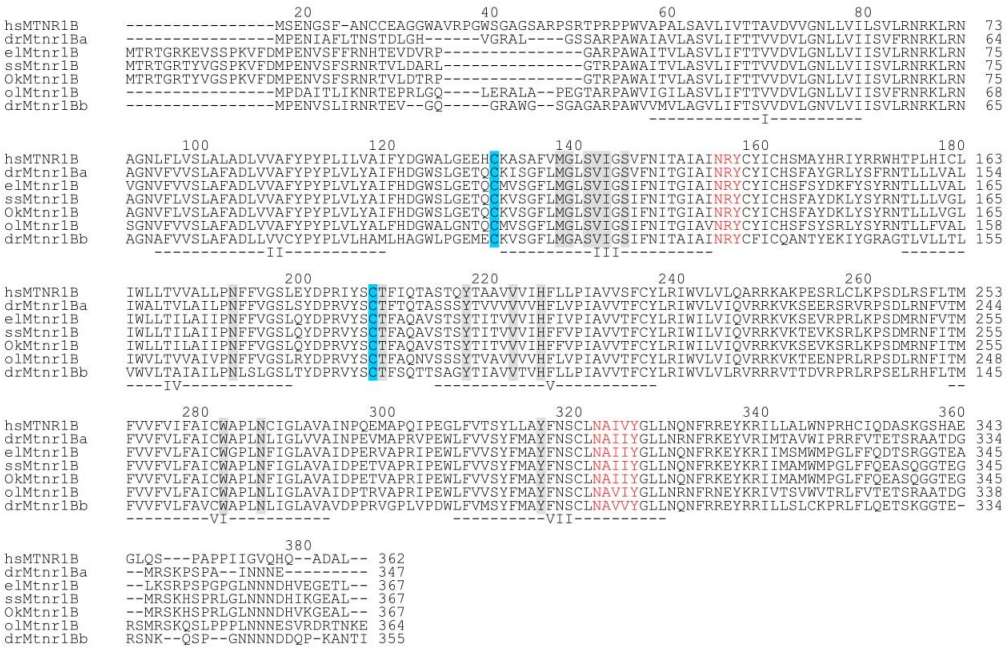
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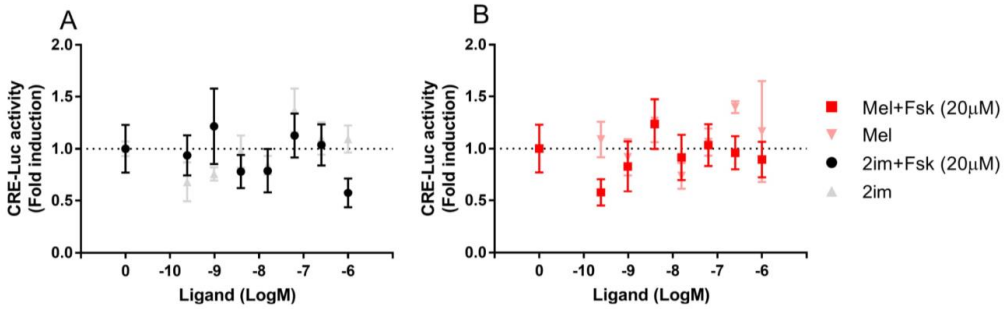

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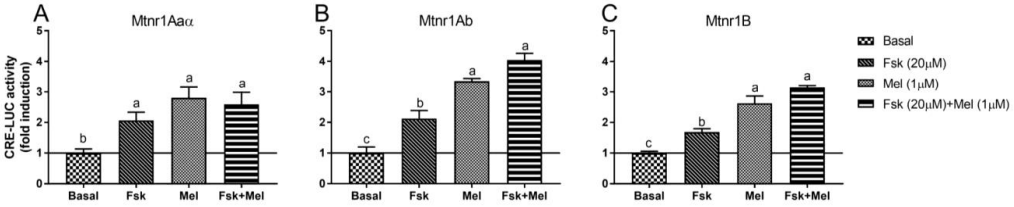

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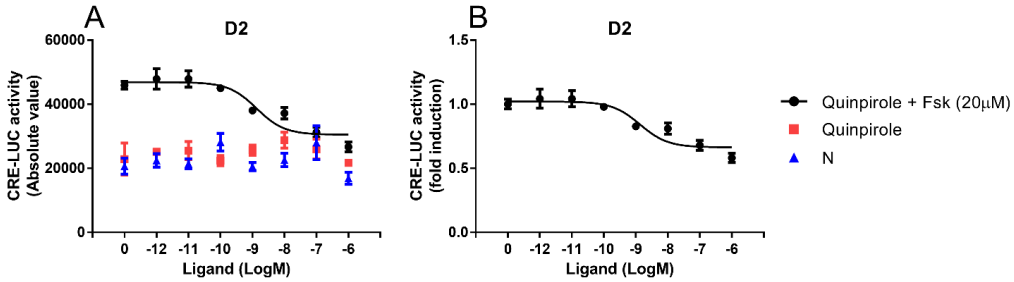

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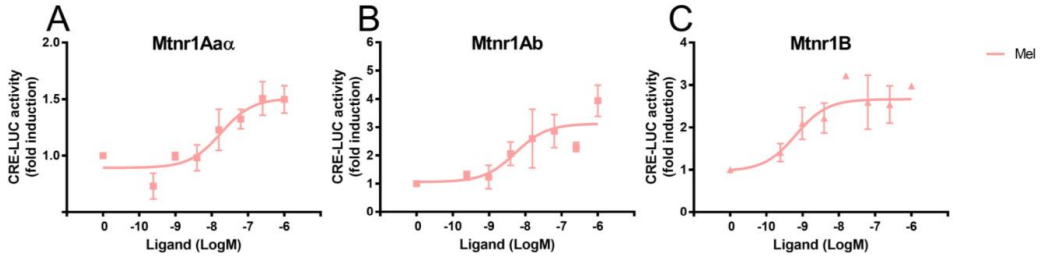

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Errata

Side	Line	Original text	Corrected text
Page 83 onward		The page numeration is continuous throughout the thesis	The page numeration should restart from 1 in each manuscript
Page 91	203	Fig 1H	Fig 1E
Page 92	221	Fig 2B	Fig 2C
Page 92	221	Fig 2D	Fig 2B
Page 92	222	Fig 2C	Fig 2D
Page 93	226	Fig 7C	Fig 6C
Page 94	290	Fig 7B	Fig 8C
Page 111	660		The caption is missing the following text "NS= non significant"
Page 112 to 114		Font size is not consistent	Font size should be adjusted
Page 112	663	Figure 12	Figure 1
Page 112	672	Figure 14 Morphometric measurement [Fork length (A); body weight (B); gonadosomatic index (GSI= gonad weight/body weight*100; C); condition factor (K=100 W/L^3; where W=Body weight (g) and L = Fork length (cm); D)] and gonadotropin relative expression [*fshb* (E); *lhb* (F)]	Figure **2** Morphometric measurement [Fork length (**C**); body weight (**D**); gonadosomatic index (GSI= gonad weight/body weight*100; **A**); condition factor (K=100 W/L^3; where W=Body weight (g) and L = Fork length (cm); **B**)]
Page 112	675	one-year old	underyearling
Page 112	686		The following text should be added at the end of the caption: " (B) "
Page 112	688	Figure 14	Figure 4
Page 113	695	Figure 15	Figure 5
Page 113	696	one-year old	underyearling
Page 115	Fig 1D	lenght	length
Page 116	Fig 2C	lenght	length
Page 124	Table S1		The table must be recentered
Page 138	300	Fig 5 E and G	Fig 5 A, B, C
Page 138	303	Fig 5 F and H	Fig 5 D, E, F
Page 158	800	*fshb* (A,E), *lhb* (B,F), *gnrhr2b1* (C,G) and *gnrhr2b2* (D,H)	*gnrhr2b1* (A,E); *gnrhr2b2* (B,F); *lhb* (C,G) and *fshb* (D,H)
Page 158	810	*lhb* (A,B), *fshb* (C,D)	*fshb* (A,B), *lhb* (C,D)

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