Endocrine Regulation of Early Sexual Maturation in Male Atlantic Salmon Parr

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Abstract

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This thesis deals with changes in gene expression during activation of the brain-pituitary-gonadal axis at puberty in early maturing male Atlantic salmon (*Salmo salar*) parr. To help elucidate the physiological roles of gonadotropins and their receptors in the regulation of puberty, cDNAs encoding FSH and LH receptors (FSHR and LHR, respectively) of Atlantic salmon were cloned and characterized. Gene expression of the receptors in the testes was analyzed in parallel with pituitary expression of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) β-subunit genes by RT-PCR. In addition, functional genes encoding proteins involved in the steroidogenic pathway and anti-Müllerian hormone (AMH) were studied in the testes, and plasma 11-ketostestosterone levels were measured. One-summer-old male Atlantic salmon parr were sampled from the prepubertal stage in December until spermiation in October. Sequence analysis of the Atlantic salmon FSHR and LHR showed the typical structure features of glycoprotein receptors including a large extracellular domain connected to a G protein-coupled transmembrane domain. Both of these gonadotropin receptors were expressed in immature testis, FSHR more abundantly than LHR. FSHR transcript levels increased in parallel with FSHβ levels from early spermatogenesis onwards while LHR mRNA levels started to increase prior to any major changes in LHβ expression. *De novo* transcription of genes encoding steroidogenic acute regulatory protein, 3β-hydroxysteroid dehydrogenase, cytochrome P450 17α-hydroxylase/17,20-lyase, and 11β-hydroxysteroid dehydrogenase was observed during the initiation of spermatogenesis in parallel with the changes in FSHβ levels. In contrast, AMH expression was downregulated and AMH levels were lowest during spermiogenesis. During spermatogenesis, large increases in the expression of LHR and all of the steroidogenic genes studied occurred concomitantly with the rise in LHβ transcripts. These findings suggest that FSH is involved in regulation of the expression of several testicular genes during the initiation of puberty and LH during the later stages of spermatogenesis. In addition, results of *in vitro* studies using serum-free primary cultures of pituitary cells indicate that IGF-I differentially modulates gonadotropin expression in the pituitary cells. IGF-I may stimulate FSHβ expression levels through interactions with gonadotropin-releasing hormone (GnRH) in immature males while it directly activates LHβ expression.

Key words: Puberty, male Atlantic salmon parr, gonadotropin, gonadotropin receptor, steroidogenic enzymes, growth factors

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Contents

Appendix

This thesis is based on the following papers, which are refereed to in the text by the corresponding Roman numerals:

I. Maugars, G. & Schmitz, M. 2006. Molecular cloning and characterization of FSH and LH receptors in Atlantic salmon (*Salmo salar L*). General and Comparative Endocrinology 149(1):108-117.

II. Maugars, G. & Schmitz, M. 2006. Expression of gonadotropins and gonadotropin receptors genes during early sexual maturation in male Atlantic salmon parr. Submitted manuscript

III. Maugars, G. & Schmitz, M. 2007. Target gene expression profiling during spermatogenesis in early maturing male Atlantic salmon parr testes. Manuscript

IV. Schmitz, M. & Maugars, G. 2007. Effects of insulin-like growth factor I on gonadotropin subunit expression in early maturing male Atlantic salmon parr. Manuscript

Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase
11β-HSD	11-hydroxysteroid dehydrogenase
11-KT	11-ketostesterone
17α,20β-P	17α,20β-dihydroxy-4-pregnen-3-one
20β-HSD	20β-hydroxysteroid dehydrogenase
AMH	anti-Müllerian hormone or Müllerian inhibiting substance (MIS)
bp	base pair
B-P-G	brain-pituitary-gonad
cDNA	complementary DNA
CNS	central nervous system
E2	17β-estradiol
ECD	extracellular N-terminal domain
FSH	follicle-stimulating hormone
FSHβ	FSH β-subunit
FSHR	FSH receptor
GnRH	gonadotropin-releasing hormone
sGnRH	salmon GnRH
cGnRH-II	chicken GnRH-II
GnRH-R	GnRH receptor
GTH	gonadotropin
GSI	gonadosomatic index
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
LH	luteinizing hormone
LHβ	LH β-subunit
LHR	LH receptor
LRR	leucine-rich repeats
RIA	radioimmunoassay
SF-1	steroidogenic factor-1
TMD	transmembrane domain
T	testosterone
T3	triodothyronine
T4	thyroxine
P45011β	cytochrome P450 11β-hydroxylase (CYP11B)
P450arom	cytochrome P450 aromatase (CYP19)
P450c17	cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17).
P450scc	cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A)

Introduction

Puberty is the process whereby an immature animal acquires for the first time the capacity to reproduce. The initiation of puberty in teleost fish is characterized by the onset of spermatogenesis in males (Schulz & Miura, 2002) and vitellogenesis in females (Patino & Sullivan, 2002). Reproductive function in teleosts is regulated by the brain-pituitary-gonad axis (B-P-G) (Fig 1). Gonadotropin-releasing hormone (GnRH) produced in the brain activates production and release of pituitary gonadotropins (GTH), which stimulate sex steroids and gamete production in the gonads. In return, sex steroids exert both positive and negative feedbacks directly at the pituitary level or via the brain.

Atlantic salmon

Atlantic salmon, *Salmo salar* belongs to the teleost order Salmoniformes, family Salmonidae. Atlantic salmon can adopt several life-history strategies (Thorpe, 1994; Fleming, 1996). Anadromous Atlantic salmon spawn in autumn in freshwater (October-November) and the eggs develop over winter and hatch in the following spring. After hatching, the fry stay for one or several years in freshwater and become parr. During spring-early summer, immature parr undergo parr-smolt transformation and migrate downstream to the sea. After spending several years in the sea, the adults return to spawn in their native river. Male Atlantic salmon may mature at one-year-old parr while still living in freshwater (Fleming, 1996). Early maturing male parr compete as "sneakers" with large anadromous males at the spawning sites. It has been estimated that early maturing males could account for about 40% of egg fertilizations (Fleming, 1996). In the following year, early maturing males may either remature or undergo smoltification and migrate to the sea.

Early maturation is observed in both wild and hatchery-reared populations. The age of maturation is influenced by heritable traits, biotic and abiotic factors (Powers, 1986). Various studies have suggested that favourable growth rates and high energy balances increase the incidence of early maturation (Rowe & Thorpe, 1990; Rowe, Thorpe & Shanks, 1991; Shearer & Swanson, 2000).

Activation of the brain-pituitary-gonad axis during puberty

GnRH system in the brain

Signals related to both external and internal factors such as the photoperiod, water temperature and food availability, are integrated in the brain. Gonadotropin-releasing hormone, a decapeptide produced in the brain, regulates the synthesis and release of pituitary gonadotropins. There are multiple molecular types of GnRH in teleosts and several distinct populations of GnRH neurons in the brain. At least two forms of GnRH are expressed in each vertebrate species (for reviews see Lethimonier et al., 2004; Millar, 2005). In salmonids, the specific salmon GnRH (sGnRH) and the highly conserved chicken GnRH-II (cGnRH-II) show distinct

expression patterns. sGnRH is predominant in the olfactory bulb, telencephalon, hypothalamus, optic tegmentum and the pituitary, while cGnRH-II is expressed mainly in the cerebellum and the medulla (Amano et al., 1993; Amano et al., 1997; Amano et al., 2003). The lack of detectable cGnRH-II in the pituitary suggests that sGnRH is the active GnRH involved in gonadotropin regulation. Three types of GnRH receptors (GnRH-Rs) have been characterized in vertebrates containing both subtypes of GnRH-R, and five different GnRH-Rs (R1, R2, R3, R4 and R5) have recently been isolated from masu salmon (*Oncorhynchus masou*) and characterized (Jodo, Ando & Urano, 2003). In male masu salmon transcripts encoding both the R1 and R4 subtypes are upregulated during the prespawning period in the pituitary, suggesting that they, especially R4 are involved in stimulation of the synthesis and release of gonadotropins by GnRH (Jodo et al., 2005).

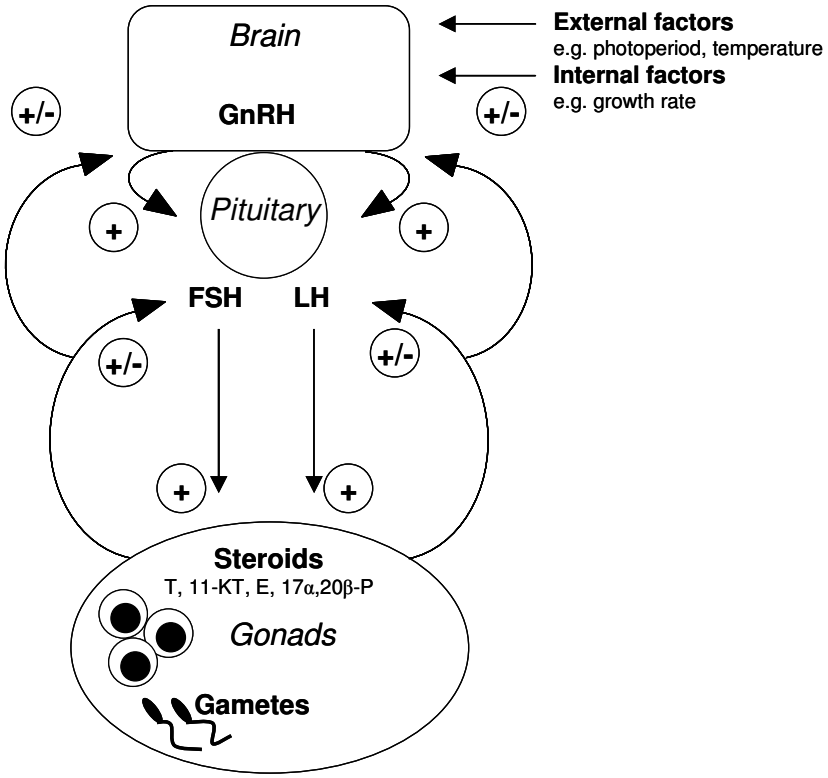

Fig 1: Brain-Pituitary-Gonad axis (B-P-G)

Pituitary

Pituitary gland

The pituitary gland or hypophysis is an endocrine gland that sits in a small, bony cavity, the sella turcica, at the base of the brain and serves as an intermediary between the central nervous system (CNS) and the target organs. The pituitary synthesizes and releases hormones under control of the CNS and, in turn, stimulates other endocrine glands. The pituitary is divided into the adenohypophysis (*pars distalis* and *pars intermedia*) and neurohypophysis (*pars nervosa*). The adenohypophysis contains adrenocorticopic cells, prolactin cells, thyrotrophs, somatotrophs and gonadotrophs. In contrast to mammals, teleosts lack a hypothalamo-hypophysial portal system for the transport of neuropeptides to the adenohypophysis. The adenohypophysis, like the neurohypophysis, receives direct innervation from various parts of the CNS, including the preoptic region, mediobasal hypothalamus, the olfactory system and tegmentum of the midbrain (Peter et al., 1990; Anglade, Zandbergen & Kah, 1993). In contrast to mammals, in which gonadotropins are produced and secreted by the same cells in the pituitary, in teleosts follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are synthesized in different types of cells (Naito et al., 1991; Naito et al., 1993; Naito et al., 1997; Schmitz et al., 2005).

Gonadotropins

As in mammals, two pituitary gonadotropins, FSH and LH, have been isolated and characterized in teleosts (Suzuki, Kawauchi & Nagahama, 1988a; Suzuki, Kawauchi & Nagahama, 1988b; Swanson *et al.*, 1989; Yaron *et al.*, 2003). Complementary DNAs encoding gonadotropin subunits in salmon and several other fish taxa have been cloned and characterized (for a review see Yaron*, et al.*, 2003). Gonadotropins are heterodimeric glycoproteins composed of a common α-subunit, linked non-covalently to a hormone-specific β-subunit that confers the biological activity (Pierce & Parsons, 1981). In salmonids, two α-subunits, α1 and α2, have been described. In chum (*Oncorhynchus keta*) and coho salmon (*Oncorhynchus kisutch*) FSHβ is linked to both α-subunits while LH contains mainly α2-subunits (Suzuki, Kawauchi & Nagahama, 1988a; Swanson *et al.*, 1991). In contrast, in rainbow trout both α1- and α2-subunit are found in both FSH and LH (Govoroun *et al.*, 1997). The glycoprotein α-subunits and each β-subunit are formed by central cystine-knot motifs from which three elongated β-loops extend (Lapthorn *et al.*, 1994; Fox, Dias & Van Roey, 2001). The α- and β-subunits are associated in a head-to-tail arrangement and stabilized by a segment of the β-subunit surrounding the α-subunit like a seat-belt, which is covalently linked by a disulphide bridge between the third and twelfth cysteine residues. The “seat-belt” has been suggested to be involved in the receptor specificity of the β-subunit (Dias, Zhang & Liu, 1994; Moyle *et al.*, 1994; Grossmann *et al.*, 1997). Phylogenetic analyses have indicated that the FSH β-subunit has evolved more rapidly than the LH β-subunit in the lineage leading to the teleosts (Querat *et al.*, 2004). Thus, the position of twelve cysteine residues in the LH β-subunit is highly conserved through the vertebrate lineage. In contrast, the structure of FSH β-subunits differs in several teleost species (Querat, Sellouk & Salmon, 2000; Yaron*, et al.*, 2003). In salmonids and percomorphs, the FSH β-subunits lack the third

highly conserved cysteine residue and a potential N-glycosylation site, and have an additional cysteine upstream of the first cysteine.

In salmonids, the physiological roles of gonadotropins have been studied in detail (Swanson*, et al.*, 1989; Prat, Sumpter & Tyler, 1996; Gomez *et al.*, 1999). FSH and LH are secreted differentially during the reproductive cycle. Plasma levels of FSH are low in immature males, increase during the onset of spermatogenesis while plasma LH levels strongly increase later, at spermiation. Since FSH is already detectable in the plasma of immature fish and it can stimulate steroidogenesis and spermatogonial proliferation (Swanson*, et al.*, 1989; Loir, 1999b) it has been suggested that FSH plays a major regulatory role during early stages of gonadal development and gametogenesis in salmon, while LH is mainly involved in the final stages of maturation.

As in mammals, the sex steroids can modulate gonadotropin synthesis and release, either via direct effects on gonadotroph cells in the pituitary or indirectly via the GnRH system in the brain. In juvenile as well as in adult salmonids, steroid feedback action seems to depend on the reproductive state of the fish (Larsen & Swanson, 1997). The castration of male rainbow trout (*Oncorhynchus mykiss*) that have already matured causes increases in plasma "maturational" gonodotropin (LH) levels (Billard & Peter, 1977). In contrast, gonadoctomy in male Atlantic salmon parr induces reduction in pituitary and plasma contents of FSH and LH, suggesting that the steroids have a stimulatory effect on gonadotropin synthesis (Borg *et al.*, 1998). Numerous studies have reported that estradiol and the aromatizable androgens have positive effects on LH synthesis and secretion at the pituitary level in immature fish. In contrast, the steroid feedback effects on FSH appear to be less consistent (Borg*, et al.*, 1998).

Gonadotropin receptors

FSH and LH act at the gonads via the activation of specific G protein-coupled receptors. Two gonadotropin receptors have been identified in coho salmon on the membrane surface of somatic cells in both the ovary and testis by *in vitro* ligand autoradiography. As in mammals, the FSH receptor (FSHR) in the testis was observed on Sertoli cells and the LH receptor (LHR) on Leydig cells (Heckert & Griswold, 1991; Yan, Swanson & Dickhoff, 1992; Miwa, Yan & Swanson, 1994; Dankbar *et al.*, 1995). In the ovary, the FSH receptor is expressed on the thecal cells, the granulosa cells and in interstitial connective tissue, while the LH receptor is expressed on granulosa cells (Camp, Rahal & Mayo, 1991; Yan, Swanson & Dickhoff, 1992; Miwa, Yan & Swanson, 1994). The duality of the gonadotropin receptors was first confirmed in amago salmon (*Oncorhynchus rhodurus*), through cloning of two gonadotropin receptor cDNAs (Oba *et al.*, 1999a; Oba *et al.*, 1999b), and later in other salmonids (Maugars & Schmitz, 2006) and several other fish taxa: (Bogerd *et al.*, 2001; Kumar, Ijiri & Trant, 2001a; Kumar, Ijiri & Trant, 2001b; Laan *et al.*, 2002; Vischer & Bogerd, 2003). The FSH and LH receptors belong to the rhodopsin receptor subfamily of G protein-coupled receptors. They are characterized by the presence of a particularly large extracellular N-terminal domain (ECD), primarily responsible for hormone recognition, joined to a transmembrane domain (TMD) and an intracellular C-terminal domain coupled to

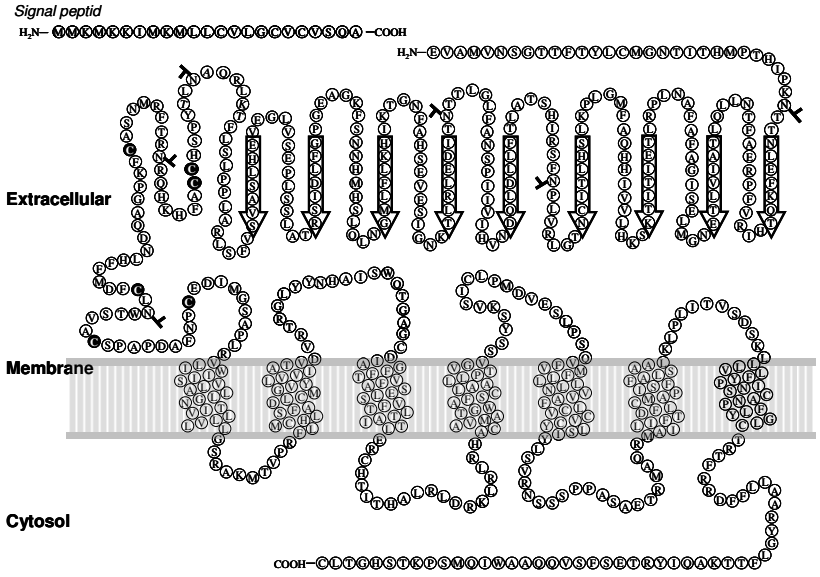

Fig 2: Schematic representation of Atlantic salmon FSH receptor showing the large extracellular domain (ECD), the transmembrane domain and the cytoplasmic tail. The arrows indicate the leucine rich repeats (LRR) in the ECD and the cysteine residues of the C-terminal cysteine-rich domain are indicated in black. The Y indicates potential N-glycosylation sites.

a G protein (Ji, Grossmann & Ji, 1998; Vassart, Pardo & Costagliola, 2004) (Fig 2).

The activation of gonadotropin receptors via interactions with their ligands leads to the induction of two major signalling pathway: activation of adenyl cyclase and the inositol phosphate cascade (Remy *et al.*, 1993; Segaloff & Ascoli, 1993; Vischer & Bogerd, 2003; Donadeu & Ascoli, 2005; Jeoung *et al.*, 2007). In contrast to mammals, where both FSHR and LHR are highly specific for their cognate hormones, studies in teleosts indicate that the specificities of the piscine gonadotropin receptors are less apparent. In coho salmon, binding studies using chum salmon gonadotropin have shown that FSHR preferentially binds salmon FSH, but also binds salmon LH (albeit less strongly), whereas LHR binds only salmon LH (Yan, Swanson & Dickhoff, 1992; Miwa, Yan & Swanson, 1994). In addition, functionality studies using recombinant amago salmon gonadotropin receptors expressed in mammalian cells have revealed that chum salmon FSH preferentially activates FSHR, and has limited interactions with LHR, while chum salmon LH activates only LHR (Oba*, et al.*, 1999a; Oba*, et al.*, 1999b; Oba *et al.*, 2001). In African catfish (*Clarias gariepinus*) and Zebrafish (*Danio rerio*), the putative LHR is highly selective for LH while the FSHR receptor is less discriminative and is activated by both FSH and LH, with a preference for FSH (Vischer *et al.*, 2003; Vischer & Bogerd, 2003; Kwok *et al.*, 2005; So, Kwok & Ge, 2005). A recent study on the expression of the gonadotropin receptors during

the spermatogenesis in rainbow trout found that the expression of FSHR and LHR appears to be related to different stage of the testicular development (Kusakabe*, et al.*, 2006).

Gonads

Testes

In most teleosts, the testes consist of compact elongated paired organs, located along the abdominal cavity. The testes are extended at the posterior edge by the vas deferentia which jointly form a common sinus leading into the urogenital papilla, at the front of the urinary orifice (Hurk, Peute & Vermeij, 1978). Depending on the arrangement of the germinal compartment, the testis is organized either in anastomosing tubules or branching lobules (Grier, 1981; Grier, 1993; Schulz & Miura, 2002). Based on the distribution of spermatogonia, testes with both types of organisation can be further divided into unrestricted and restricted types. Atlantic salmon, like other salmonids, have an unrestricted spermatogonial type of testis with an anastomosing network of tubules (Parenti & Grier, 2004). The germinal compartments contain spermatogenic cysts formed by one germ cell or isogenic clones of developing germ cells at the same developmental stage enclosed by one or several Sertoli cells, while in the interstitium various somatic cells are present including Leydig cells. The spermatogenesis process takes place in the spermatogenic cysts or spermatocysts.

Spermatogenesis

Spermatogenesis starts with spermatogonia undergoing successive mitoses. Aynchronous proliferation leads to the formation of undifferentiated spermatogonia (As, Apr, Aal). A spermatogonia divide synchronously several times to produce B spermatogonia, which enter into meiosis. During the intensive mitotic division phase the numbers of spermatogonia increase exponentially (Loir, 1999a; Ando *et al.*, 2000). Soon after the first meiotic division, secondary spermatocytes start the second meiotic division and develop into small round haploid spermatids. The spermatids transform into spermatozoa during spermiogenesis. This transformation is marked by elongation, associated with the formation of the spermhead with a condensed nucleus, a mid-piece and a flagellum. After completion of spermatogenesis the connections between the Sertoli cells and spermatozoa are broken, the spermatozoa are released and stored either in the tubular lumen, in the efferent ducts or in seminal vesicles. In some species, including salmonids, sperm acquire motility and full fertilization capacity during their passage through the sperm duct (for a review see Miura & Miura, 2003).

The number of Sertoli cells per testis is one of the most important factors determining the quantity of sperm produced in teleosts, which is similar to findings in mammals (Berndtson & Thompson, 1990; Hess *et al.*, 1993; Schulz *et al.*, 2005). Sertoli cells provide a favourable microenvironment for the development and maintenance of spermatogenesis. In contrast with mammals, in which no Sertoli cell proliferation has been observed under normal conditions in adults, Sertoli cells proliferate during spermatogenesis in fish, allowing the increase in space required for the development of spermatogenic cysts (Schulz, *et al.*, 2005).

In catfish and tilapia, the proliferation occurs mainly when spermatogonia undergo mitosis and ceases as germ cells enter into meiosis. At this time, Sertoli cells form tight junctions creating a cell barrier (for a review see Nagahama, 1983 and Schulz, et al., 2005).

Sex steroids

Sex steroid are involved in gametogenesis as well as reproductive behaviour and the development of secondary sexual characters (Borg, 1994). In male fish, testosterone (T) and in particular 11-ketostesterone (11-KT) are considered to be the main androgens (Borg, 1994) and together with 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) are known to be important in spermatogenesis and spermiation (Fostier *et al*., 1983). In salmonids, plasma levels of 11-KT and T are highest at prespawning and decline at spawning, whereas 17α,20β-P levels sharply rise prior to final spawning (Mayer *et al.*, 1990b; Amer *et al.*, 2001). 17β-estradiol (E2) is also found at low concentrations in the circulating blood in teleosts (Mayer*, et al.*, 1990b; Amer*, et al.*, 2001). The role of E2 in spermatogenesis is not clear. It has been associated with the early spermatogonial renewal in Japanese eel (*Anguilla japonica*) (Miura *et al.*, 1999), while in male gilthead seabream (*Sparus aurata*) E2 may induce testis regression (Chaves-Pozo *et al.*, 2006).

Several studies have shown that 11-KT plays an important role in spermatogenesis. In eels and catfish, stimulation by 11-KT has been shown to activate all stages of spermatogenesis (Miura *et al*., 1991; Cavaco *et al.*, 1998). In spring Chinook spring salmon (*Oncorhynchus tshawytscha*), it has been assumed that increases in 11-KT levels six months prior to the initiation of spermatogenesis in prepubertal males may indicate commitment to sexual maturation (Campbell, Dickey & Swanson, 2003). However, the effects of 11-KT on spermatogenesis *in vitro* are less pronounced in salmonids. In Japanese huchen (*Hucho perryi*), *in vitro* stimulation of prepubertal testis fragments by 11-KT can induce spermatogonial proliferation leading, after 15 days, to the formation of B spermatogonia (Amer*, et al.*, 2001), while 11-KT had no apparent effect on premitotic spermatogonia obtained from rainbow trout even after six days of culture in a study by Loir (1999b).

Around spermiation a distinct shift in the steroidogenic pathway from 11-KT to 17α,20β-P synthesis occurs in the testes of salmonid species (Baynes & Scott, 1985; Nagahama, 1994; Planas & Swanson, 1995). It has been shown that 17α,20β-P is required in the sperm maturation process, such as acquisition of sperm motility (Miura *et al*., 1992).

Leydig cells are the principal steroid-producing cells in the testis. In salmonids, Leydig cells are poorly differentiated during early spermatogenesis and are distributed in groups of two or three in the interstitium, whereas during mid-spermatogenesis Leydig cells are found surrounding spermatogenic cysts (Kusakabe, Nakamura & Young, 2003). At the beginning of spermiation, Leydig cells differentiate into active steroidogenic cells characterized by numerous mitochondria with tubular cristae and an extensive smooth endoplasmic reticulum, indicating increased synthesizing activity. After spermiation, the density of Leydig cells appears to be higher, and they are often clustered. Little is known to date

about the development of Leydig cells during spermatogenesis in fish. However, studies by Loir (1990) indicate that Leydig cells at different spermatogenic stages have the potency to proliferate *in vitro*. In addition, proliferation of interstitial cell populations that may be precursors of Leydig cells has been observed in seabream (Chaves-Pozo *et al.*, 2005).

Steroidogenesis is a complex process that leads to the conversion of cholesterol into active steroids such as androgens, estrogen, progestin, mineralocorticoids and glucocorticoids. It takes place mainly in the testis, ovary and adrenal tissues (head kidney in fish) as well as in the brain and blood. The biosynthesis of these steroids proceeds through identical steps initially, and requires the coordinated action of several enzymes located in mitochondria or endoplasmic reticulum (Fig 3). In mammals, the rate-limiting step in this process is the translocation of cholesterol from the outer to the inner mitochondria membrane, which is dependent on a sterol carrier protein, the steroidogenic acute regulatory protein (StAR) (for a review see Stocco, 2000). Within the mitochondria, the steroidogenic pathway is initiated by the conversion of cholesterol into pregnenolone, catalyzed by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc, CYP11A) present in the inner mitochondrial membrane. Pregnenolone is then converted by 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) and cytochrome P450 17α-hydroxylase/17,20-lyase (P450c17, CYP17). P450c17 has two catalytic activities: α-hydroxylation and C17-20 lyase activities. The latter catalyzes the conversion of 17α-hydroxyprogesterone to androstenedione, an important step in the synthesis of T and 11-KT. Due to its two different activities, P450c17 is a key enzyme that directs the sex steroid pathway towards either androgen or progestin production. The final step of 11-KT production is the conversion of T by cytochrome P450 11β-hydroxylase (P45011β, CYP11B) and 11β-hydroxysteroid dehydrogenase (11β-HSD). T can be also converted by the activity of the cytochrome P450 aromatase (P450arom, CYP19) in E2. During the steroidogenic shift C17-20 lyase activity is downregulated and the 17α-hydroxyprogesterone is converted into 17α,20β-P by 20β-hydroxysteroid dehydrogenase (20β-HSD). Complementary DNAs encoding these seven enzymes in several teleost species, including salmonids, have been isolated: P450scc by Takahashi *et al.* (1993), von Hofsten *et al.* (2002) and Arukwe (2005), 3β-HSD and P450c17 by Sakai *et al.* (1992, 1993) and von Hofsten *et al.* (2002), P45011β by Kusakabe *et al.* (2002), 11β-HSD by Kusakabe, Nakamura & Young (2003), 20β-HSD by Guan *et al.* (1999) and P450arom by Tanaka *et al.*, (1992) and Montserrat *et al.* (2004). However, little is known to date about the regulation of the steroidogenic enzymes during sexual maturation in fish.

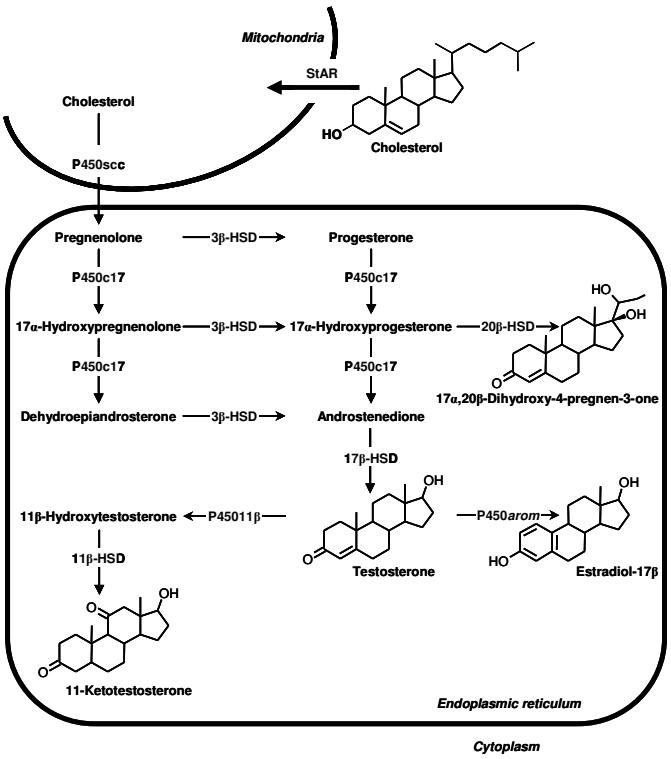

Fig 3: Schematic representation of the gonadal steroid synthesis in fish.

In mammals, the transcription factor steroidogenic factor (SF-1) is a key regulator of endocrine function and sex determination (Sadovsky *et al*., 1995). SF-1, also termed NR5A1 is a member of the orphan nuclear receptor family (NR5A) (Auwerx *et al*., 1999) that regulates genes encoding several steroidogenic enzymes via the regulatory element AGGTCA and various genes that function within the brain pituitary axis such as gonadotropin subunit and FSHR (Lala, Rice & Parker, 1992; Heckert, 2001; Jeyasuria *et al.*, 2004). In teleost, it appears that Ff1b align with NR5A4 and may be an SF-1 ortholog (Hsu, Lin & Chung, 2003). In support of this hypothesis, Ff1b shows an analogous expression pattern to SF-1 and is part of a signalling network that is responsible for sex determination in a similar way to SF-1 (Baron *et al.*, 2005; von Hofsten, Larsson & Olsson, 2005). Thus, in teleosts, Ff1b could play an important role in the initiation of puberty via regulation of steroidogenic enzyme expression.

Anti-Müllerian hormone

Recently, a spermatogenic preventing substance (SPS), expressed by Sertoli cells in immature testis was identified in eels (Miura *et al.*, 2002). SPS cDNA was isolated and shown to be homologous to mammalian anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS). AMH belongs to the transforming growth factor β (TGF-β) superfamily (Cate *et al.*, 1986) and is required in normal sex differentiation and reproductive function in mammals (for a review see Teixeira, Maheswaran & Donahoe, 2001). In eel, the induction of spermatogenesis by hCG or 11-KT treatment is related to the downregulation of AMH homolog expression. *In vitro* treatments of immature testes with AMH antibodies stimulate spermatogonial proliferation, suggesting that an AMH homolog may play a crucial role in initiation of puberty in fish. Recently, cDNAs encoding AMH have also been isolated from Atlantic salmon (Accession No. AY722411) and several other fish species (Yoshinaga *et al*., 2004; Rodriguez-Mari *et al*., 2005; von Hofsten, Larsson & Olsson, 2005; Nakamura *et al*., 2006).

Interactions between growth and puberty

Numerous studies have provided indications that there are direct links between the growth axis and reproductive functions in teleosts. Growth conditions during specific periods of the year appear to be prominent factors influencing commitment to early maturation. Studies in Atlantic salmon have shown that growth rates during the first summer and the opportunity for growth during the period preceding the onset of gonadal development in spring affect the incidence of maturation at one year of age (Berglund, 1995). In salmonids circulating levels of insulin-like growth factor I (IGF-I) produced in the liver under the control of the growth hormone (GH) are highly correlated with growth rates (Beckman *et al.*, 1998; Beckman *et al.*, 2001; Campbell, Dickey & Swanson, 2003). As in mammals, IGF-I can be regarded as a potential link between growth and the initiation of puberty, (for reviews see Funkenstein *et al.*, 1989; Shamblott *et al.*, 1995; Duguay *et al.*, 1996; Butler & Le Roith, 2001).

IGF-I can influence sexual maturation at both the pituitary and gonadal levels (Le Gac *et al*., 1993; Blaise, Weil & Le Bail, 1995; Smith, Chan & Gutierrez, 2005). *In vitro* studies have shown that IGF-I stimulates spermatogonial proliferation in male rainbow trout testes (Loir, 1999b) and influences steroidogenesis in theca and granulosa cells in female coho salmon (Maestro *et al*., 1995). Furthermore, *in vitro* treatments of pituitary cells with IGF-I have been shown to increase cell contents of both FSH and LH, and the potency of GnRH to stimulate FSH release, in coho salmon (Baker *et al*., 2000) and to increase both cell contents and the release of LH in European eels (*Anguilla anguilla*) (Huang *et al.*, 1998; Huang *et al.*, 1999).

Objectives

The aims of the studies underlying this thesis were to investigate changes in gene expression during activation of the brain-pituitary-gonadal axis at puberty in early maturing male salmon parr.

The specific questions addressed were:

1. Are gonadotropin receptors key factors in the initiation of early sexual maturation? More specifically:

- What are the characteristics of the molecular structure of the Atlantic salmon FSHR and LHR? (**I**)
- Does gonadotropin receptor gene expression change during spermatogenesis? (**I**)
- Are changes in gonadotropin receptor gene expression related to the temporal profile of gonadotropin β-subunit expression in the pituitary? (**II**)

2. What are the major changes in functional targeted genes during spermatogenesis in early maturing male Atlantic salmon parr? More specifically:

- Are there differences in the testicular expression of AMH, Ff1b, StAR, P450scc, 3β-HSD, P450c17, P45011β and 11β-HSD between immature and non-maturing parr at the onset of spermatogenesis? (**II**, **III**)

3. What is the potential role of growth stimulating factors as puberty-triggering signals? (**IV**)

Material and methods

Animals

Atlantic salmon (*Salmo salar L.*) were provided by the Norrfors hatchery in northern Sweden (63ºN, 20ºE). The fish were cross-bred progeny between wild and hatchery-reared salmon, and reared under standard hatchery conditions, with through-flowing river water at ambient temperature under natural photoperiods. Maturing males were distinguished by gonadosomatic indices and testis histology (see below).

Samplings

From December 2002 until October 2003, one summer old salmons were sampled monthly with an additional sampling date in each of the months of April, May and June, when sexual maturation is initiated. The fish were anesthetized with metomidate hydrochloride (Aquacalm, Syndel Co.). Body weight and testis weight were recorded and gonadosomatic indices (GSI) were calculated (GSI= gonad weight/body weight*100). Blood was collected for plasma 11-KT assays (**II**). The fish were killed and their testes, pituitary and various tissues were removed, frozen in liquid nitrogen and stored at -80ºC until RNA extraction. Entire testes or the middle section of the testes were fixed in Bouin's fixative to determine their histological development (**II**, **III**).

Histology

The testes were fixed, dehydrated, embedded in paraffin, sectioned (5 µm), and stained with hematoxylin and eosin. Sections were examined by light microscopy and the following five stages of spermatogenesis were identified according to the classification of Schulz (1984): stage I, A spermatogonia; stage II, B spermatogonia and spermatocytes; stage III, spermatids; stage IV, spermatozoa; and stage V, cysts of all the spermatogenic cell types and running milt (**II** to **IV**).

Quantification of gene expression

Primer design

All primers were designed using Primer3 software (Rozen & Skaletsky, 2000) and are based on salmonid sequences available in the GenBank database. For cloning, primers were designed to amplify long, overlapping fragments (**I**, **III**) while for real-time PCR primers were based on exon-junctions or two different exons of Atlantic salmon cDNA sequences to amplify 90-150 bp long fragments (**I** to **IV**).

RNA extraction and cDNA synthesis

Total RNA was isolated from several tissues (brain, gills, heart, intestine, kidney, liver, muscle, pancreas, pituitary, testis and ovary). In immature males, the complete string-like testes were used for RNA extraction while for larger gonads a

weighted cross section from the median part of the testis was extracted. Concentrations of total RNA were measured by spectrophotometry. Generally, total RNAs were DNase-treated in order to avoid genomic contamination and reverse-transcribed using random primers.

Real-time PCR assays

The abundance of transcripts was measured by real-time PCR using an iCycler thermal cycler (BioRad) and Sybr green I dye and specific gene primer sets (**I** to **IV**). Relative transcript abundance was quantified using standard curves prepared from RNA transcribed *in vitro*. Standard curves were generated in duplicate and correlation coefficients were higher than 0.996. 18S RNA was chosen as an internal control gene for data normalization. The specificity of the amplified products was systematically verified by melting curve analysis after the amplification reactions.

Seasonal changes in gene expression were presented either as relative mRNA levels per unit RNA or, to account for changes in gene expression related to organ growth, transcript levels were normalized to the amounts of total RNA per entire organ corrected by body weight.

Isolation and cDNA sequencing

Full-length cDNA of FSHR and LHR were isolated through 5' and 3' rapid amplification of the cDNA ends (RACE) methods (**I**). Complete cDNA sequences were obtained by amplification of overlapping cDNA fragments. Generally, amplified fragments were separated on agarose gels, purified and fragments from several independent PCR amplifications were sequenced either after subcloning into pGEM®-T Easy vector (Promega), or directly after gel purification (**I**, **III**). Fragments were sequenced on both strands using a CEQ TM 8000 sequencer and a dye terminator cycle sequencing kit (both from Beckman Coulter). Full-length cDNA sequences of gonadotropins receptors were compiled by alignment of the different overlapping fragments.

Structural and phylogenetic gene analyses

Sequences homologous to those obtained were sought by BLAST searches (Altschul *et al*., 1990) (**I**, **III**). The nucleotide sequences were translated using the AgiloBio translator program (http://www.justbio.com/translator/index.php/) and the translation initiation ATG was predicted with the ATGpr program (http://www.hri.co.jp/atgpr/) (**I**). Putative signal peptides, potential N-linked glycosylation sites and potential phosphorylation sites were identified using SignalP V3.0 software (http://www.cbs.dtu.dk/services/), and putative transmembrane regions were identified using the HMMTOP method (http://www.enzim.hu/hmmtop/). Searches for motifs and signatures in the sequences were performed by comparison to the Pfam protein families database for sequence patterns (http://www.sanger.ac.uk/Software/Pfam/). Multiple alignments of nucleotide and amino acid sequences were performed using the ClustalW

program (http://www.ebi.ac.uk/clustalw/) at the EMBL-EBI website (**I**, **III**). The phylogeny of the receptor amino acid sequences was inferred by the Fitch-Margoliash distance matrix method using the PHYLIP programV3.6. (http://evolution.genetics.washington.edu/phylip.html) (**I**). An unrooted consensus phylogenetic tree was generated and the robustness of the phylogenetic hypothesis was tested by bootstrapping.

Steroid hormone measurements

Plasma levels of 11-ketotestosterone (11-KT) were measured in individual blood samples using a radioimmunoassay (RIA) previously described by Mayer *et al.* (1990a) (**II**). Due to the small amounts of blood plasma that could be collected from fish sampled in the initial months, plasma 11-KT could only be measured in individual fish from June onward.

Primary cultures of salmon pituitary cells and *in vitro* treatments

Pituitary cells were dispersed using an enzymatic and mechanical procedure as earlier described by Montero *et al.* (1996) and Rousseau *et al.* (1998) with slight modifications (**IV**). Isolated pituitaries were incubated in porcine Type II trypsin dispersion buffer (DB) and then treated with trypsin inhibitor Type I.S with DNAse II**.** They were then mechanically dispersed by repeated passage through a plastic transfer pipette. More than 90% of the dispersed cells were viable according to trypan blue exclusion tests. Cells were cultured on poly-L-lysine-precoated plates in a serum-free medium (CM; medium 199 with Earle's salt, sodium bicarbonate, supplemented with penicillin, streptomycin and amphotericin B) at 16°C under 3% CO_2 and saturated humidity. Cells were plated on 96-well plates at a density of 50,000 cells/well. After incubation for 1 day the culture medium was changed, and treatments were started. Media and treatments were renewed every third day for up to 10 days of culture. Four replicate wells were used for each treatment and each treatment was repeated at least twice (with different cell preparations). Cell cultures were stopped after 1, 3, 6 or 10 days, medium was removed and the cells were washed once with ice-cold PBS. The cells were lysed by adding cold Cells-to cDNA ^{TM} II cell lysis buffer (Ambion) to each sample. The cell lysate was immediately transferred to reaction tubes and incubated at 75°C. The samples were then treated with DNAse I and total RNA from the crude cell lysates was reverse-transcribed using random primers.

Results

I

The full length cDNA of FSHR was 2993 bp long and encoded a predicted mature protein of 635 amino acids while the cDNA of LHR was 2722 bp long and encoded a predicted protein of 701 amino acids (Accession Nos. AJ579790 and AJ567667, respectively). Multiple sequence alignment of Atlantic salmon FSHR and LHR with available gonadotropin receptor sequences of teleosts and representative vertebrates revealed that they had levels of homology with those of other salmonids (97-98% for both receptors) and relatively conserved amino acid identities ranging from 59-67% for FSHR and 47% to 79% for LHR, compared with those of other teleosts, and 50-52% compared with those of other vertebrates. Atlantic salmon FSHR and LH also show characteristic structural features of gonadotropin receptors including a large ECD connected to a TMD consisting of seven membrane-spanning helices and a short cytoplasmic tail. The N-terminal extracellular domains of the Atlantic salmon FSHR and LHR consist of leucine-rich repeats (LRR) followed by a cysteine-rich domain at the carboxy terminal and form the potential recognition sites for the corresponding hormones. Sequence alignments of the ECD showed that the nature of the residues involved in key contacts with the α-subunit were highly conserved (e.g. where substitutions appear to have occurred residues seem to have been replaced by residues with similar characteristics). By contrast, the residues in the interaction sites with the β-subunit highly diverged, especially for FSH. Both FSHR and LHR genes were mainly expressed in the gonads, but expression was also detected, at lower abundance in gills. LHR was also detected in several other extra-gonadal tissues.

II

Both gonadotropin receptor genes were expressed in immature testis with FSHR transcripts being more abundant (8-fold) than LHR in one-year-old male parrs. Expression levels of FSHβ were low during the prepubertal stage in winter and spring and started to increase prior to the onset of gonadal growth at the end of May. Transcript levels continued to increase during early-mid spermatogenesis and declined at spermiation. LHβ mRNA levels were hardly detectable in immature fish, slightly increased during early spermatogenesis and peaked at spermiation. FSHR transcript levels increased in parallel to FSHβ levels from early spermatogenesis onwards, while LHR mRNA levels started to increase prior to any major changes in LHβ expression. Plasma 11-KT levels increased at the beginning of spermatogenesis and peaked at spermiogenesis.

III

Expression of AMH was comparatively high in immature testes of one-year-old male parr during spring and started to decrease when spermatogenesis was initiated, falling to its lowest levels during spermiogenesis. Upregulation of Ff1b,

StAR, 3β-HSD, P450c17 and 11β-HSD transcript levels was recorded during the onset of spermatogenesis in early June. During spermatogenesis, StAR mRNA levels per testis strongly increased, while Ff1b, P450scc, P450c17, P45011β, 11β-HSD mRNA levels per testis strongly increased during spermiogenesis, and 3β-HSD mRNA levels were relatively high in early spermatogenesis, then progressively further increased during spermiation. Correlation analyses of the expression of the genes revealed correlations between FSHβ mRNA levels and StAR, 3β-HSD, P450c17 and 11β-HSD transcript levels from the prepubertal period in March to the initiation of spermatogenesis in early June, but during the later stages of spermatogenesis FSHβ mRNA levels were no longer correlated to testicular gene expression. In contrast, LHβ mRNA levels were related to the expression of several enzymes including P450scc, P450c17, P45011β and 11β-HSD transcripts as well as LHR and plasma 11-KT levels throughout spermatogenesis.

IV

IGF-I mRNA expression in the liver, the primary site of IGF production, increased during the onset of maturation and rose further when spermatogenesis progressed into the final stages of maturation, reaching about 3.3 fold higher than those in immature-nonmaturing males. *In vitro* experiments using serum-free primary cultures of pituitary cells showed that IGF-I increased LH β-subunit expression in a time- and dose-dependent manner, while no effect on FSH β-subunit expression was observed. Comparison of the effects of IGF-I at different reproductive stages revealed that the stimulatory effect of IGF-I was most pronounced in maturing males at the end of July, indicating that the gonadotrophic cells were most sensitive to IGF-1 during this period and that IGF-I plays a role in the upregulation of LHβ mRNA in maturing males at mid-spermatogenesis. Both IGF-I and IGF-II had similar stimulatory potency, while human recombinant insulin was about 100 less potent. The metabolic hormones T_3 and T_4 had no significant effect on either LH β- or FSH β-subunit expression. sGnRH alone effectively stimulated increases in FSHβ mRNA levels, but it had no effect on LHβ expression. Co-administration of GnRH upregulated the expression of both FSH β- and LH β-subunit genes.

Discussion

The Atlantic salmon gonadotropin receptors, FSHR and LHR, were isolated and analysis of the deduced protein sequence revealed that salmon FSHR and LHR have typical characteristics of glycoprotein receptors. Both receptors contain a long N-terminal ECD connected to a TMD typical of G coupled-protein receptors with a short C-terminal cytosolic tail (**I**). Comparison of Atlantic salmon FSHR and LHR sequences with other gonadotropin receptor sequences revealed several highly conserved structural features of the ECD, including a recognition protein motif consisting of nine successive imperfect LRRs and a C-terminal rich-cysteine

subdomain with a conserved cluster of six cysteine residues (**I**). According to structural modelling analyses of the exodomains of gonadotropin receptors, the LRR domain forms a horseshoe-shaped topology, with short parallel β-strands on the concave side with which the cognate ligand may establish multiple key contacts (Wang, Bernard & Moyle, 2000; Kobe & Kajava, 2001; Kajava & Kobe, 2002). The C-terminal cysteine-rich domain is also predicted to contribute to ligand-binding specificity and induction of the signal response (Bernard, Myers & Moyle, 1998; Nakabayashi *et al.*, 2000; Moyle *et al.*, 2004; Moyle *et al.*, 2005). Comparative analysis of the recognition sites in the Atlantic salmon gonadotropin receptors revealed that the nature of the residues involved in the key contacts with the glycoprotein α-subunit are highly conserved (Moyle, *et al.*, 1994; Bhowmick *et al.*, 1996; Fan & Hendrickson, 2005). By contrast, the nature of several key residues involved in the contacts between the human FSH β-subunit with human FSHR differs in the salmon FSHR. Similar differences in the contact residues of the FSH β-subunit have been observed in percomorph FSHRs, which have an additional LRR between LRR1 and LRR2 (**I**). The primary structure of the FSH β-subunit in salmonids and percomorphs differs from that of the tetrapods and may alter the conformation of the FSH β-subunit's "seat-belt" (Fox, Dias & Van Roey, 2001; Swanson, Dickey & Campbell, 2003), and consequently modify key contacts required for the receptor's ligand binding selectivity (Vischer *et al.*, 2004). The chemical and/or physical nature of the residues involved in the ligand binding selectivity in mammalian LHR differs in salmon LHR. Phylogenetic analysis indicated that the ECD of the FSH receptor has evolved more rapidly than the ECD of LHR (**I**). Evidence that FSH has evolved more rapidly than LH has also been observed in the teleost lineage (Querat, Sellouk & Salmon, 2000), suggesting that the gonadotropins and the recognition sites of the gonadotropin receptors have co-evolved in Atlantic salmon. Nevertheless, binding studies have shown that Atlantic salmon FSHR is less discriminative than LHR. Studies using transient expression of the gonadotropin receptors have demonstrated that FSHR can be activated by both FSH and LH, albeit preferentially by FSH, while the recombinant LH receptor is highly selective for LH (Nijenhuis *et al.*, 2004). Similar results have been found in catfish and zebrafish (Vischer*, et al.*, 2003; Vischer & Bogerd, 2003; Bogerd *et al.*, 2005; Kwok*, et al.*, 2005; So, Kwok & Ge, 2005).

During the winter preceding sexual maturation genes encoding both gonadotropin receptors were expressed in immature testes, FSHR genes abundantly than LHR genes (**II**). In coho salmon males, functional FSH receptors have been localized audioradiographically in Sertoli cells and possibly on interstitial cells in immature fish. In contrast, LHRs were detected only during spermiation (Miwa, Yan & Swanson, 1994). While LHR mRNA levels remained low during the prepubertal period a slight increase in FSHR mRNA levels was observed from December to February in the male Atlantic salmon parr we examined (**II**). This indication of enhanced testicular capacity to respond to gonadotropins could play a role in their commitment to sexual maturation. Although FSH was expressed in the pituitary, transcript levels of FSH and FSHR were not correlated during the prepubertal stage (**III**). Furthermore, FSHR transcript levels did not show a clear bimodal distribution, and thus could not be used to distinguish between males that committed or not to sexual maturation. The first significant increase in

gonadotropin receptor transcript levels was observed at the onset of spermatogenesis, after the start of FSH β mRNA upregulation at the end of May, while LH mRNA levels remained very low. Maturing males had significantly higher FSHR levels in early June, while LHR mRNA levels first started to rise significantly (compared to levels in nonmaturing males) in late June (**II**). The increases in FSHR and LHR transcript levels could be related either to an upregulation of gene transcription and/or the proliferation of somatic cells. In tilapia and catfish it has been shown that active Sertoli cell proliferation occurs when spermatogonia intensively divide (Schulz*, et al.*, 2005), and that the stimulation of Sertoli cell proliferation is related to FSH signalling (Schulz, van Dijk & Bogerd, 2003). In contrast, little is known about Leydig cell development at this stage in fish. During spermatogenesis, FSHβ transcript levels continued to increase during early-mid spermatogenesis and declined at spermiation in October, whereas LHβ mRNA levels strongly rose between mid-spermatogenesis and spermiation (**II**). FSHR transcript levels increased in parallel to FSHβ levels from early spermatogenesis onwards, while LHR mRNA levels increased prior to any major changes in LH β expression. Levels of both LHβ and LHR levels were highest during spermiation (**II**). Similar temporal profiles in gonadotropin receptor expression have been reportedly observed in male rainbow trout and yellowtail (*Seriola quinqueradiata*) (Rahman *et al.*, 2003; Kusakabe *et al.*, 2006). In yellowtail, FSHR mRNA and FSH β-subunit mRNA levels increase in parallel during early spermatogenesis and decline during spermiation (Rahman*, et al.*, 2003). The increase in LHR mRNA levels prior to significant rises in levels of its cognate hormone may reflect an increase in testis sensitivity to LH that enables an immediate, strong response to the surge in LH levels at final maturation. These results are consistent with the general belief that activation of the spermatogonial proliferation is triggered by FSH though its receptors and their final maturation by LH. The expression in particular of FSHR in immature testis from December suggests that the immature testes are responsive to FSH and that the changes observed in FSH expression at the end of May and early June are required to trigger spermatogenesis initiation. However, both FSHR and LHR were also expressed in males that did not mature, indicating that failure to commit to sexual maturation may be partly related to insufficient activation of FSH expression during the spermatogenesis initiation period.

Testicular P450scc, 3β-HSD, P450c17, 11β-HSD, P45011β genes were expressed in the testes in both immature and maturing Atlantic salmon parr (**III**). In rainbow trout the presence of P450scc, 3β-HSD, P450c17 transcripts and 3β-HSD activity has been detected in the interstitial Leydig cells in both immature and maturing males (Hurk, Peute & Vermeij, 1978; Kobayashi *et al.*, 1998). Results from our studies showed that StAR and Ff1b transcripts were already abundant in immature testes (**III**). The first significant changes in expression of genes encoding the steroidogenic proteins coincide with the start of spermatogenesis. In early June, StAR, 3β-HSD, P450c17 and 11β-HSD transcript levels were upregulated in the testes showing intensive spermatogonia mitosis, and were correlated to FSHβ mRNA levels. It has been shown that FSH stimulates 11-KT and 17α,20β-P synthesis in testes (Planas & Swanson, 1995). In Japan huchen and two-year-old Atlantic salmon parr, it has been observed that both 11-KT and 17α,20β-P are

produced during early spermatogenesis (Mayer*, et al.*, 1990b; Amer*, et al.*, 2001). A slight increase in circulating 11-KT levels was also observed in the one-year-old maturing parr we examined during this period (**II**). The parallel increases in FSHβ and StAR, 3β-HSD and 11β-HSD mRNA levels in early June in maturing fish suggest that FSH stimulates *de novo* transcription of these steroidogenic enzymes. Since FSH receptors are predominantly expressed in Sertoli cells, the stimulatory effect of FSH could be mediated by the release of regulating growth factor in the Sertoli cells and consequent activation of steroid production in Leydig cells (Lejeune *et al.*, 1996). During the prepubertal stage, transcript factor FF1b mRNA levels did not change (**III**). Nevertheless, in early June maturing males had slightly higher levels than immature males, indicating that Ff1b may be involved in regulation of the expression of genes encoding several actors in early spermatogenesis such as FSHR, StAR and P450scc. However further studies are needed to clarify the functional role of Ff1b in gene regulation in the testis during the course of spermatogenesis, since recent studies indicate that it is homologous to Ff1d in zebrafish and SF-1 in mammals, which are expressed in both the Sertoli cells and Leydig cells (Morohashi *et al.*, 1994; von Hofsten, Larsson & Olsson, 2005). Transcripts of AMH were highly expressed in immature testes until the initiation of spermatogenesis when AMH levels were markedly downregulated, while AMH gene expression remained high in immature-nonmaturing males. AMH expression declined to its lowest levels during final maturation. Downregulation of AMH expression in Sertoli cells during early spermatogenesis has also been observed in Japanese eels, and appears to be essential for spermatogonia proliferation in eels. Interestingly, AMH mRNA levels were negatively correlated to FSHβ mRNA levels during the prepubertal stage, suggesting that expression of AMH is under the control of FSH signalling.

During the course of spermatogenesis, P450scc, P45011β, 11β-HSD and P450c17 transcript levels strongly rose during spermatid maturation when GSI reached its highest levels. Similar significant increases coinciding with strong gonadal growth have been reported in other salmonids (von Hofsten*, et al.*, 2002; Kusakabe*, et al.*, 2006). Transcript levels of these four genes displayed strong correlations with both LHβ as well as LHR mRNA levels. These results are consistent with the report that both FSH and LH show equivalent steroidogenic potency in coho salmon during early spermatogenesis, but LH more potently stimulates 11-KT and 17α,20β-P during spermiogenesis and spermiation (Planas & Swanson, 1995). The expression of Ff1b was highly correlated to that of several steroidogenic enzymes suggesting that Ff1b may be involved in steroidogenic output during spermatogenesis. The transcription pattern of 3β-HSD differed from the other steroidogenic enzymes; transcript levels were relatively high during early spermatogenesis then gradually increased during spermiogenesis-spermiation (**III**). A different temporal profile of 3β-HSD mRNA has also been reported in rainbow trout where 3β-HSD levels peaked during the beginning of spermiogenesis then rapidly declined before spermiation (Kusakabe*, et al.*, 2006). This temporal profile suggests the involvement of factors other than LH in 3β-HSD gene activation in salmonids. Transcript levels of StAR markedly increased during spermiogenesis and rose further during spermiation, when increased levels of 11-KT were recorded (**II**, **III**). Similar testicular StAR expression patterns have been reported in rainbow trout

and Arctic char (*Salvelinus alpinus*), with large increases preceding the production of 11-KT and 17α,20β-P (von Hofsten*, et al.*, 2002; Kusakabe*, et al.*, 2006). StAR mRNA levels did not correlate with LHβ mRNA levels, in contrast to the strong relationship between StAR and LHR mRNA levels we observed in maturing male Atlantic salmon. Similar correlations have been reported in male rainbow trout, suggesting that expression of StAR genes is regulated by LH in the late stage of spermatogenesis (Kusakabe*, et al.*, 2006). Changes in StAR transcript abundance appear to play a prominent role in overall steroidogenic production during spermatogenesis.

Male Atlantic salmon can mature either as small parr in freshwater at one and/or two years of age or later as large males after returning from the sea. Males show a sexual maturation of an "all-or-nothing" type, meaning that individuals either fully mature or remain immature. The incidence of early sexual maturation, observed in both wild and hatchery-reared Atlantic salmon populations is influenced by somatic growth and/or growth opportunity (Berglund *et al.*, 1991; Rowe, Thorpe & Shanks, 1991; Berglund, 1995; Letcher & Gries, 2003). It is assumed that the *decision* to mature depends on the energy stores and size of the fish during the fall, a year prior spawning and/or their growth performance during the following spring. In salmonids growth rates are highly correlated with circulating IGF-I levels (Beckman*, et al.*, 1998; Beckman*, et al.*, 2001; Pierce *et al.*, 2001). In the male Atlantic salmon parr we examined, hepatic IGF-I mRNA levels significantly increased during the onset of maturation and gradually increased further between mid-spermatogenesis and spermiation (**IV**). Similarly, in yearling spring Chinook salmon studied by Campbell, Dickey & Swanson (2003), maturing males tended to have higher plasma IGF-I levels by April than immature fish. Thus, IGF-I appears to be related to both growth rate and maturation. Several lines of evidence indicate that IGF-I acts as a mediator between somatic growth and pubertal activation of the reproductive function. Our *in vitro* studies showed that IGF-I stimulated LH β-subunit expression in a time- and dose-dependent manner, but had no apparent effect on FSH β-subunit expression (**IV**). These findings are consistent with the stimulatory effect of IGF-I on LH cell content and release observed in long-term cultures of eel pituitaries (Huang*, et al.*, 1998; Huang*, et al.*, 1999). In pituitary cells from coho salmon studied by Baker*, et al.* (2000) IGF-I induced increases in FSH and LH contents, and GnRH-mediated FSH release, while basal FSH release was not affected. Comparison of the *in vitro* effect of IGF-I at different reproductive stages in the male Atlantic salmon parr showed that its stimulatory capacity was most pronounced in maturing males at the end of July, indicating that the gonadotrophic cells were most sensitive to it during this period, and that IGF-I plays a role in the upregulation of LHβ mRNA during the later stages of spermatogenesis (**IV**). In addition, comparison of IGF-I action on gonadotropin expression with that of other members of the insulin-like super family showed that IGF-II had similar effects to IGF-I, while human recombinant insulin was about 100-fold less potent. The physiological effects of IGF-I are mediated through high-affinity binding to the type-I IGF receptor. In a recent study, Fruchtman, McVey & Borski (2002) reported the presence of IGF-I receptors throughout the entire pituitary gland in hybrid striped bass (*Morone saxatilis x M. chrysops*), including the proximal *pars distalis* of the adenohypophysis where gonadotrophs are located.

However, further *in situ* hybridization studies are needed to verify the localization of type-I IGF receptors on gonadotropic cells in the pituitary.

Earlier studies in coho salmon and rainbow trout showed that IGF-I can enhance GnRH-mediated FSH-release. The possible role of IGF-I on FSH β-subunit expression through its interaction with GnRH in immature parr was examined by treating pituitary cells with IGF-I and sGnRH, either alone or in combination, in long-term cell cultures. IGF-I slightly enhanced the stimulatory effect of GnRH on FSHβ expression (**IV**). A stimulatory effect of GnRH on gonadotropins and gonadotropin subunit expression has been observed in several species (Yaron*, et al.*, 2003). In salmonids, the action of GnRH on FSHβ and LHβ expression seems to be dependent on the developmental stage (Kitahashi *et al.*, 1998; Ando *et al.*, 2004; Ando & Urano, 2005). These results indicate that IGF-I differentially modulates gonadotropin expression in the pituitary cells. While IGF-I directly exerts a stimulatory effect on LHβ transcription in a time- and dose-dependent manner, which is enhanced during spermiogenesis in the males, IGF-I indirectly modulates FSHβ gene expression by increasing gonadotroph responsiveness to GnRH. It has been suggested that IGF-I may influence sGnRH-induced intracellular signals that lead to activation of GTH synthesis or release (Ando 2006). Other factors such as steroids or gonadal growth factors may also affect the interaction between IGF-I and GnRH. The influence of these factors on the role of IGF-I in modulating gonadotrophic function during the onset and progression of sexual maturation will be examined in further studies.
These results support the view that IGF-I plays an important role in modulation of the gonadotrophic axis during the onset and progression of sexual maturation in teleosts.

Many studies have explored the causes and consequences of variations in the life history of Atlantic salmon (Thorpe *et al.*, 1998). The developmental trajectory is controlled by genetic thresholds that determine the conditions required at critical times for reproductive maturation. It is assumed that the *decision* to mature early depends on the energy stores and size of the fish during the fall, one year prior to reproduction and/or of their growth performance during the following spring (Thorpe*, et al.*, 1998). Results from studies of spring Chinook salmon suggest that the *decision* to initiate maturation is made in the late fall and early winter, approximately 10 months prior to final maturation (Silverstein *et al.*, 1998; Shearer & Swanson, 2000; Campbell, Dickey & Swanson, 2003). In yearling Chinook salmon, B spermatogonia, the first histological signs of the beginning of spermatogenesis, have been observed as early as November-January and their appearance has been correlated with increases in plasma 11-KT levels and both pituitary and plasma contents of FSH (Shearer & Swanson, 2000; Campbell, Dickey & Swanson, 2003). However, in the studied population of Atlantic salmon from northern Sweden, no histological evidence of maturation was observed before June. FSHβ mRNA levels were very low during winter and spring and were first upregulated at the end of May. In addition, no changes in the expression of functional genes in the testes were recorded prior to spermatogenesis initiation in early June (**II**, **III**). Thus, our data do not provide definitive indications about whether or not a preliminary *decision* to mature had been taken during the

preceding fall. It has been theorized that the initiation period is followed by a permissive period in spring, when maturation progresses if growth and energy acquisition stores are sufficient. Our results highlight the importance of the period of enhanced growth in spring on the *decision* to mature. In a recent experiment application of a restricted feeding regime for six weeks prior to the start of spermatogenesis reduced the proportion of early sexual maturation by 50%, supporting the hypothesis that certain growth or energy thresholds have to be reached in late spring in order for maturation to begin.

Conclusions

- The Atlantic salmon FSH and LH receptors show typical structural features of glycoprotein receptors: a long N-terminal ECD forming the potential recognition sites connected to a TMD typical of G coupled-protein receptors with a short C-terminal cytosolic tail. The structures of FSHR and LHR appear to have co-evolved with their cognate hormones (**I**).

- Both FSHR and LHR genes were already expressed in immature testes in December, several months prior to the initiation of sexual maturation and the level of their expression increased from the initiation of spermatogenesis onwards (**II**)

- FSHR gene expression increased in parallel to FSHβ levels during the first spermatogenesis stages while the abundance of LHR transcripts increased prior to any significant increase in the abundance of LHβ transcripts. By contrast, parallel changes in LHR and LHβ were observed during the later stages of spermatogenesis (**II**). These results confirm the differential roles of FSH and LH during sexual maturation, FSH being related to early spermatogenesis and LH to final maturation and spawning. The mechanism responsible for triggering commitment to sexual maturation appears to be dependent on both the presence of FSHR in immature testes and the consequent increase in FSH.

- During the onset of spermatogenesis testicular FF1b, StAR, 3β-HSD, and 11β-HSD transcript levels were higher in maturing males than in non-maturing males. During the course of spermatogenesis, transcript levels of Ff1b, StAR, P450scc, 3β-HSD, P450c17, P45011β and 11β-HSD are highest during spermiogenesis-spermiation and appear to be under the control of LH. AMH transcript levels decreased during early spermatogenesis and were lowest during spermiogenesis (**III**), suggesting that AMH plays a role in puberty in male parr.

- IGF-I mRNA expression in the liver increased during the onset of maturation and rose further when spermatogenesis progressed into the final stages of maturation. This increase in IGF-I could be linked to the enhanced growth observed in maturing males during this period. It can be assumed that elevated liver IGF-I transcription leads to an increase in circulating IGF-I, which in turn can act at different levels of the B-P-G axis to promote the onset of puberty. IGF-I has a direct effect on LH β-subunit transcription in the pituitary (**IV**). This effect was most pronounced in maturing fish at mid-spermatogenesis, suggesting that IGF-I plays a role in the increase in LH during late spermatogenesis. The effect of IGF-I on FSH appears to be more indirect and to be mediated via interactions with GnRH and/or pituitary GnRH receptors.

Swedish summary - svensk sammanfattning

Denna avhandling undersöker förändringar i genuttryck vid aktiveringen av hjärna-hypofysgonad-axeln under puberteten hos tidigt könsmogna hanar hos Atlantlax (*Salmo salar*). cDNA som kodar för FSH- och LH-receptorer (FSHR och LHR) klonades och karakteriserades för att förstå den fysiologiska rollen av gonadotropiner och deras mottagarmolekyler i regleringen av puberteten. Genuttrycket av gonadotropin-receptorer i gonader studerades parallellt med expressionen av follikelstimulerande och luteinizerande hormoner i hypofysen med hjälp av RT-PCR. Samtidigt studerades genuttryck av funktionella gener som kodar för proteiner involverade i syntesen av könshormoner och anti-Müllerian hormon i gonader och 11-ketostestosteron mäts i blodplasman. Ettårig lax, som odlades i Vattenfalls kompensationsodling Norrfors (Umeälven) användes i studierna och förändringar under könsmognadsprocessen studerades från december till spermiering i oktober. Analysen av gensekvenserna visade att FSHR och LHR hos lax har den typiska strukturen av en glykoproteinreceptor med en lång extracellulär domän bunden till G-protein kopplade extracellulära domänen. Båda receptorgenerna är uttryckta redan i gonader av omogna hanar och FSHR är uttryckt i högre grad än LHR. Genuttrycket av FSHR ökar samtidigt som FSHβ-värden från tidig spermieutveckling däremot ökar LHR-uttrycket innan man observerar någon större förändring i LHβ–uttrycket. De novo transkription av gener som koderar för "steroidogenic acute regulatory protein", 3β-hydroxysteroid dehydrogenas, cytochrome P450 17α-hydroxylas/17,20-lyase, och 11β-hydroxysteroid dehydrogenas observerades vid initieringen av spermatogenesen samtidigt med förändringar i FSHβ-värden. I motsats till detta reglerades AMH ner och AMH-värdena är lägst under spermiogenesen. LHR och alla studerade gener involverade i steroidsyntesen ökar under spermatogenesen, samtidigt observerades en stor ökning i LHβ. Dessa resultat tyder på att FSH spelar en viktig roll i regleringen av flera gener i gonaden under de första stegen i spermatogenesen hos laxfiskar och LH reglerar de sista stegen i spermiemognaden. Den direkta effekten av tillväxt och metaboliska hormoner på genuttryck på hypofysnivå studerades med hjälp av ett primärkultursystem för fiskhypofysceller. In vitro-experiment visar att IGF, en tillväxtfaktor som bildas i levern och är känt för att spelar en viktig roll i regleringen av kroppstillväxten, ökar LHβ expression, däremot observerades ingen direkt effekt på FSHβ expression. Effekten på FSHβ sker troligen mer indirekt via en interaction med gonadotropin-releasing hormon.

French summary - résumé

Le sujet principal de cette thèse est l'étude de l'expression de gènes au cours de l'activation de l'axe gonadotrope à la puberté chez le tacon mâle du saumon Atlantique (*Salmo salar*). Pour mieux comprendre le rôle physiologique des gonadotrophins et de leurs récepteurs dans le mécanisme de l'initiation de la puberté et de l'acquisition de la maturité sexuelle, les ADNc codant pour le récepteur à FSH et le récepteur à LH (FSHR et LHR respectivement) ont été isolés et caractérisés chez saumon Atlantique. Puis, nous avons étudié par real-time PCR, l'expression testiculaire des gènes codant pour les récepteurs aux gonadotrophins, pour des protéines de la stéroïdogenèse et pour l'hormone antimüllérienne, en parallèle avec l'expression hypophysaire des sous-unités β de la FSH et de la LH (FSHβ et LHβ, respectivement) et de la concentration plasmatique de la 11-ketotestérone au cours de l'acquisition de la première maturité sexuelle du tacon male.

L'analyse de la séquence du FSHR et du LHR du saumon Atlantique révèle une structure générale conservée typique des récepteurs glycoprotéiques, constituée d'un large extracellulaire domaine lié à un domaine transmembranaire caractéristique des récepteurs couplés aux protéines G. Les transcrits des deux récepteurs sont déjà présents dans les testicules au stade de l'immaturité, le FSHR étant le plus abondant. La quantité relative des transcrits du FSHR et de la FSHβ augmente au cours de l'initiation de la spermatogenèse. La quantité relative de transcrits du LHR augmente au début de la spermatogenèse alors que le taux de transcrits de la LHβ reste faible. *De novo* transcriptions ont aussi été observées pour les gènes codant pour StAR "steroidogenic acute regulatory protein", 3β-hydroxysteroid dehydrogenase, cytochrome P450 17α-hydroxylase/17,20-lyase, and 11β-hydroxysteroid dehydrogenase. A l'opposé, l'expression testiculaire du gène de l'AMH est réprimée et la quantité de transcrits de l'AMH diminue jusqu'à la spermiogenèse.

Le taux de transcrits du LHR et de la LHβ croît fortement au cours de la spermatogenèse. La quantité de transcrits codant pour les protéines de la stéroidogenèse augmente parallèle à l'augmentation de l'expression de la LHβ. Ces résultats suggèrent que la FSH et la LH sont impliquées différentiellement dans la régulation de l'expression testiculaire de gènes, la FSH au cours de l'initiation de la spermatogenèse et la LH dans les phases finales de la spermatogenèse.

Insulin-growth factor I (IGF-I) module *in vitro* l'expression de la FSHβ et de la LHβ dans des cultures primaires de cellules hypophysaires à long terme suggérant l'existence d'un lien via IGF-I entre la croissance et la maturation sexuelle chez le tacon male du saumon Atlantique.

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