Growth hormone and somatolactin function during sexual maturation of female Atlantic salmon

AKADEMISK AVHANDLING

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av

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numbers:

I. Benedet S, Johansson V, Sweeney G, Galay-Burgos M and Björnsson BTh 2005. Cloning of two Atlantic salmon growth hormone receptor isoforms and *in vitro* ligand-binding response. *Fish Physiology and Biochemistry* 31: 315-329

II. Benedet S, Björnsson BTh, Taranger GL and Andersson E 2008. Cloning of somatolactin alpha, beta forms and the somatolactin receptor in Atlantic salmon: Seasonal expression profile in pituitary and ovary of maturing female broodstock *Reproductive Biology and Endocrinology* 6:42

III. Benedet S, Andersson E, Mittelholzer C, Taranger GL and Björnsson BTh 2008. Concurrent measurement of pituitary growth hormone mRNA and protein content in sexually maturing female Atlantic salmon: Correlation analysis with plasma GH levels. *Manuscript*

IV. Benedet S, Björnsson BTh, Taranger GL and Andersson E 2008. The growth hormone - insulin-like growth factor I system during sexual maturation of female Atlantic salmon. *Manuscript*

Dissertation Abstract

Benedet Perea, Susana (2008). Growth hormone and somatolactin function during sexual maturation of female Atlantic salmon.

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Background and aims: The growth hormone-insulin-like growth factor I (GH-IGF-I) system is known to act during sexual maturation of female salmonids, but the specific roles are not known. Somatolactin (SL) is a pituitary hormone closely related to GH and is only found in fish. In some species, including salmonids, there are two forms, SLα and SLβ. The SL receptor (SLR) has recently been cloned and phylogenetic analysis shows that it is similar to previously cloned GH receptors (GHRs) of non-salmonids. The ligand-specificity of the GHR/SLR is unclear. Little is known about the role of the SLs in sexual maturation of fish. The aim of this thesis has been to increase our knowledge about the regulatory role(s) of both the GH-IGF-I system and of SLs during sexual maturation in female Atlantic salmon.

Methods: The cDNA sequences of Atlantic salmon GHRs (two isoforms), SLR, as well as SLα and SLβ were obtained with the goals of carrying out a phylogenetic analysis, and of developing molecular tools for analysis of mRNA levels using real time quantitative PCR (RTqPCR). The roles of GH, IGF-I and SL were examined in a 17-month long study on one sea winter Atlantic salmon females. mRNA expression levels of ovarian components of the GH-IGF-I system and SLR and pituitary GH, SLα and SLβ were studied by RTqPCR. Levels of GH and IGF-I in plasma, and of GH in the pituitary were measured by radioimmunoassay.

Results and Conclusions: The phylogenetic analysis (**Paper I** and **II**) of the cloned sequences reveals the placement of Atlantic salmon GHR in the GHR type II clade and SLR in the controversial GHR type I clade (putative SLRs). Concurrent analyses of pituitary GH mRNA levels, GH protein and plasma GH in the same individual fish demonstrates the complex dynamics of the GH system, which is inhibited by a continuous light. **Papers III** and **IV** confirm that there is an active GH-IGF-I-gonad axis in the female Atlantic salmon that appears to be functional at the start of exogenous vitellogenesis, final oocyte growth, spawning and possibly during postovulatory events. Evidence has been found for a photoperiod-driven GH-system activation which is initiated in January with stimulation of GH secretion from pituitary somatotropes. The role of this activation of the GH system in late winter/early spring appears to be the reversal of a prior plasma IGF-I and ovarian IGF-I mRNA downregulation driven by an unknown factor(s). This downregulation in IGF-I is thought to inhibit somatic cell proliferation. The activation of the GH-IGF-I-gonadal system also appears to limit energy allocation to gonadal growth. This series of events involving the GH-IGF-I system appears to take place during the so-called spring window of opportunity and it is the first time this has been described. The GH-IGF-I system also appears to have an important role during final oocyte growth, spawning and post-spawning events. SLα and SLβ are both actively regulated during sexual maturation and could have several roles, such as signaling the status of visceral fat reserves during the spring window of opportunity, signaling lipid metabolic status before the onset of anorexia, involvement in Ca mobilization during vitellogenesis and/or control of lipid metabolism in lieu of GH during the final stages of oocyte growth.

Keywords: Growth hormone, somatolactin, insulin-like growth factor I, GH, SL, IGFI, GHR, SLR, IGFIR, ovary, maturation, reproduction, Atlantic salmon, spawning, pituitary.

Abbreviations

17,20βP	maturation inducing steroid 17α,20β-dihydroxy-4-pregnen-3-one
2R	genome duplication during early gnathostome evolution
3R	genome duplication event in teleosts
4R	genome duplication event in the Salmonidae family
α-MSH	α-melanotropin, α-melanocyte stimulating hormone
β-END	N-acetylated β-endorphin
aa	amino acids
AANAT	arylalkylamine N-acetytransferase
AC	adenylate cyclase
ACTH	corticotrophin
BPG	brain-pituitary-gonad
Ca^{2+}	divalent calcium
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CHO-K1	Chinese hamster ovary K1 cells
ci	color interfere
CI-MPR	cation-independent mannose 6 phosphate receptor
CRH	corticotropin releasing hormone
Ct	threshold cycle
E_2	estradiol-17β
ECD	extracellular domain
Ef1α	elongation factor 1 alpha
ERα	estrogen receptor α
FSH	follicle stimulating hormone
FSH-R	follicle stimulating hormone receptor
GH	growth hormone
GHBP	growth hormone binding protein
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
GnRH	gonadotropin-releasing hormone
GnRH-R	gonadotropin-releasing hormone receptor
GtHs	gonadotropins
ICD	intracellular domain
IGFBP	insulin-like growth factor I binding protein
IGFBP-rP	insulin-like growth factor I binding protein related proteins
IGFI	insulin-like growth factor I
IGFII	insulin-like growth factor II
IL-6	interleukin-6

IP3	inositol 1,4,5-triphosphate
IRR	insulin receptor related receptor
JAK	Janus kinase
LH	luteinizing hormone
LH-R	luteinizing hormone receptor
LL	constant light
MIS	maturation inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one
MTT	3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide
NO	nitric oxide
OD	oil drop
OMC	oocyte maturational competence
PACAP	pituitary adenylate cyclase-activating peptide
PCR	polymerase chain reaction
PI	pars intermedia
Pit-1	pituitary-specific transcription factor
PKA	protein kinase A
PLC	phospholipase C
PRL	prolactin
PRLR	prolactin receptor
PTHrP	parathyroid hormone related protein
PY	primary yolk
RACE	rapid amplification of cDNA ends
RIA	radioimmunoassay
RTqPCR	real time quantitative polymerase chain reaction
SH2	Src homology 2
SL	somatolactin
SLR	somatolactin receptor
SOCS	suppressors of cytokine signaling
SRIF	somatostatin
STAT	signal transducers and activators of transcription
SY	secondary yolk
T	testosterone
T3	triiodothyronine
TSH	thyroid stimulating hormone
TY	tertiary yolk
UPGMA	unweighted pair group method arithmetic mean
UTR	untranslated region
Vg	vitellogenin

Introduction

Atlantic salmon and its life cycle

Atlantic salmon (Figure 1) belongs to the phylum Chordata (the chordates), the class Osteichthyes (the bony fishes), the order Salmoniformes, the family Salmonidae, and the genus Salmo. It was named and described scientifically in 1758 by Carolus Linnaeus, the great Swedish taxonomist and botanist, and given the name Salmo salar L., meaning 'The Leaper'. It was, however, not until the 19th century that it was proven that the silvery, ocean-migrating fish were the same species as the cryptic-colored parr found in rivers.

Figure 1. Atlantic salmon female used in the study.

Atlantic salmon is a teleost species whose natural habitat is the Atlantic coasts of Europe and North America. In recent years it has become a commercially important aquaculture species grown all over the globe as quality fish. Atlantic salmon can live up to 15 years and can reach a length of 150 cm and a weight of up to 50 kg (reviewed by Klemetsen et al. 2003). Atlantic salmon exhibit a remarkable phenotypic plasticity and variations of its life cycle that allow it to adapt to the varied temperate biogeography and seasonal climate. Generally, it is anadromous, i.e. it spawns in fresh water, but spends much of its life at sea. However, there are also landlocked lake/river populations. Spawning takes place in freshwater streams in the autumn, generally between October and December (Figure 2). A female may lay 1,500 eggs or more for each kg of body weight (Thorpe et al. 1984). The eggs are buried in the gravel and hatch early the following spring. The alevins that emerge are about two cm long and hide from predators in the gravel of the streambed. When their yolk sac is used up, the juveniles, usually called fry, leave the gravel and start feeding. When the fry are five to eight cm long, they turn into parr, acquiring vertical camouflage markings on their flank called parr marks. Anadromous salmon parr can remain in fresh water

from one to six years, and some males mature without going to sea (Thorpe 1994). In the spring of the year of their seaward migration, parr go through a series of complex morphological, physiological and behavioral changes called smoltification or parr-smolt transformation, which prepares them for pelagic ocean life. They remain in the ocean for 1 to 4 years, growing and accumulating energy stores, after which the anadromous spawning migration takes place as they return to their native rivers to spawn. If the migration occurs after one sea winter, the salmon are called grilse. If it occurs after two winters at sea, two sea-winter fish and so on. However, a minority of fish can also mature during the first autumn after smoltification and this is called jacking or post-smolt maturation. Adults in fresh water, which are approaching the reproductive stage, stop feeding, living off accumulated fat reserves. Atlantic salmon are iteroparous, i.e. they do not die after spawning, but can return to the sea and mature repeatedly (Schaffer 1974). The reproductive strategy is thus quite plastic and depends on biotic and abiotic indicators for the best outcome in terms of survival and reproductive success.

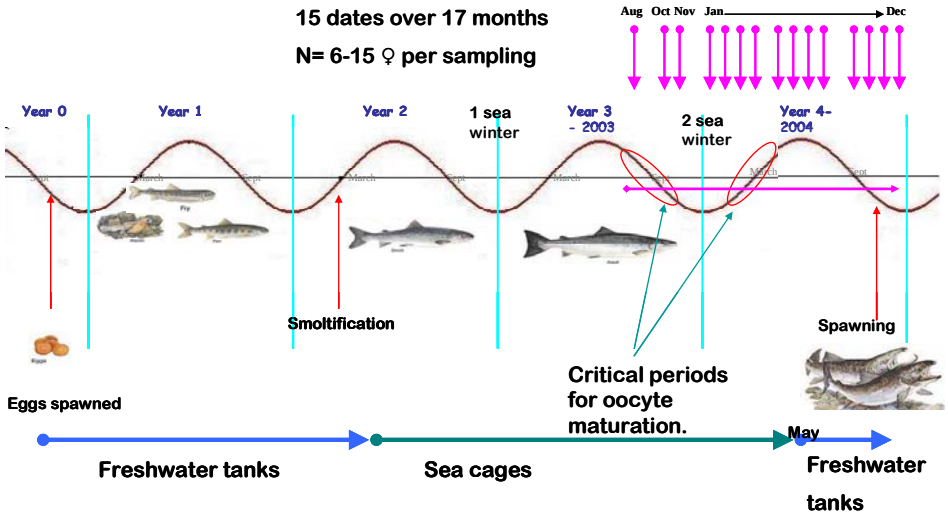

Figure 2. Life cycle and sampling protocol used in **Papers II**, **III** and **IV**. Pink arrows indicate 15 sampling points from August 2003 to December 2004.

Female salmon reproduction

The brain-pituitary-gonad axis in salmonids

The brain-pituitary-gonad (BPG) axis controls most aspects of the reproduction in salmonids, as in the rest of vertebrates (Figure 3). The gonads, ovaries and tests, have two main roles: the production of gametes (gametogenesis) and the production of sex steroid hormones (steroidogenesis). The gonads, in turn, are mainly governed by two distinct gonadotropic hormones (gonadotropins, GtHs), follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are produced by gonadotropes, an endocrine cell type of the pituitary. The gonadotropins are released into the circulation and bind to specific membrane receptors in the gonads: the FSH receptor (FSH-R) and the LH receptor (LH-R). Secretion of the gonadotropins by the pituitary is mainly under the control of the hypothalamus, stimulated by the release of gonadotropin-releasing hormone (GnRH) and inhibited by the release of dopamine. The actions of the BPG axis are subject to a complex control involving extensive feedback systems among the gonads, the pituitary and the brain, and are modulated by other hormonal systems and by environmental cues (reviewed by Peter and Yu 1997).

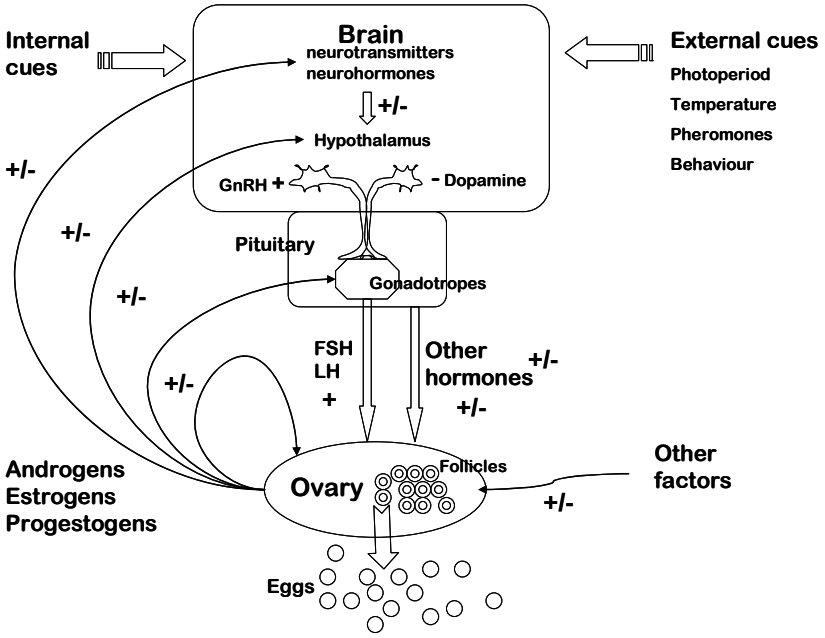

Figure 3. The brain-pituitary-gonad axis (BPG). GnRH (gonadotropin releasing hormone), FSH (follicle stimulating hormone), LH (luteinizing hormone).

GnRH-producing neurons in the brain innervate the pituitary directly and the GnRH secreted binds to GnRH receptors (GnRH-R) on the gonadotropes, which then release GtHs. In each of the many fish species studied, at least two, but often three, forms of GnRH have been identified, while two or three GnRH-R have been found (Peter et al. 2003; Steven et al. 2003; Lethimonier et al. 2003, 2004; Ikemoto et al. 2003; Parhar 2003; Levavi-Sivan and Avitan 2005). GnRHs also seem to regulate the secretion of other pituitary hormones via the different GnRH forms and GnRH receptors which show differential localization in the brain and pituitary (Parhar 2003; Kah et al. 2000; Andersson et al. 2001; Dubois et al. 2001, 2002). GnRH and GnRHR have also been found in the gonads of a number of fish, including salmonids so that GnRH may carry out direct actions at the level of the gonads (Madigou et al. 2000; Lethimonier et al. 2004; Weltzien et al. 2004).

LH and FSH are heterodimeric glycoproteins made up of a common α-subunit and a hormone-specific β-subunit. They are secreted by gonadotropes of the pituitary gland and regulate steroidogenesis and gametogenesis in the gonads, although their specific roles are not yet well understood. In salmonids, studies on the seasonal variation in the levels of the GtHs indicate a role for FSH primarily in regulating the early stages of gametogenesis and vitellogenic processes, whereas LH is thought to act mainly in the final stages of maturation, spermiation and ovulation (Swanson et al. 1989; Prat et al. 1996; Bon et al. 1999; Breton et al. 1998). However, FSH may play an important role in the final stages of maturation as well, as there is a surge in FSH just before ovulation and spermiation in rainbow trout (*Oncorhynchus mykiss)* (Prat et al. 1996; Gomez et al. 1999; Santos et al. 2001).

The effects of LH and FSH on the gonads are mediated by two distinct gonadotropic receptors, LH-R and FS-R (Oba 1999a, 1999b; Bogerd et al. 2001; Vischer and Bogerd 2003), which are expressed on the somatic cell layers that surround the germ cells (Kumar and Trant 2001; Schulz et al. 2001). FSH-R binds both FSH and LH whereas LH-R appears to be more specific to LH (Bogerd et al. 2001; Vischer and Bogerd 2003). FSH and LH stimulate the gonads to produce an array of steroid hormones which, in turn, regulate gametogenesis, secondary sexual characteristics and behaviour.

Cholesterol is the precursor of all the steroid hormones. It is transformed by the activation of different steroidogenic enzymes in multi-step enzymatic reactions (Nagahama 1994). The sex steroids can be classified into androgens, estrogens and progesterones. Testosterone (T) is an androgen which serves as the precursor for 17β-estradiol (E_2), the major sex steroid influencing ovarian development in fish (Nagahama 1994; Peter and Yu 1997). According to the a two-cell model proposed to describe ovarian steroidogenesis (Nagahama 1997; Senthilkumaran et al. 2004), T is produced

in the theca cells under FSH control and converted into E_2 in the granulosa cells by the enzyme aromatase. In vitro studies have shown that aromatase activity in salmonids either proceeds under direct gonadotropic regulation or depends on the presence of a certain mediating factors (Nagahama 1994; Planas et al. 2000; Montserrat et al. 2004). During the early stages of oogenesis, both GtHs can induce steroidogenesis in the follicle (Suzuki et al. 1988; Swanson et al. 1989; Planas et al. 2000), but only plasma FSH levels are elevated during vitellogenesis (Swanson et al. 1989; Bon et al. 1999; Santos et al. 2001).

Final maturation and ovulation in salmonids is marked by a decrease in E_2 and T and a surge in maturation inducing steroid (MIS, 17α,20β-dihydroxy-4-pregnen-3-one, 17,20βP) (Nagahama 1994; Patiño et al. 2001). Low water temperature (6°C) is believed to be a late stage environmental cue for ovulation (Taranger and Hansen 1993).

Oogenesis

Oogenesis is the process by which primordial germ cells (PGC) become ova that can be fertilized (reviewed by Tyler and Sumpter 1996; Patiño and Sullivan 2002). During early embryogenesis in fish, a small pool of primordial germ cells differentiates into oogonia which proliferate by mitosis shortly after sex differentiation (Figure 4). Oogonia also increase in number by mitosis at the start of each reproductive cycle later in life. Some of the oogonia are transformed into primary oocytes when they start meiosis which proceeds into the diplotene stage of prophase I (Devlin and Nagahama 2002). Meiosis is resumed at final maturation, but meanwhile, the primary oocytes start previtellogenic growth at the same time as the ovarian follicle is formed. The follicle consists of the oocyte surrounded by two layers of somatic cells, an internal granulosa cell layer and an external theca cell layer, which are separated from each other by a basement membrane (Patiño and Sullivan 2002). Previtellogenic growth is characterised by an increase in the oocyte size and intense RNA synthesis which provides most of the RNA for the in advanced oocytes (Brooks et al. 1997; Patiño and Sullivan 2002). Between the oocyte and the granulosa cell layer forms an extracellular membrane called vitelline envelope, zona radiata or chorion. The oocyte develops microvilli that penetrate the zona radiata and contact the granulosa cells.

During the cortical alveoli stage, the oocyte synthesizes glycoproteins and accumulates them in structures around the periphery of the oocyte called cortical alveoli. This is followed by an oil drop stage in which oil droplets increasingly accumulate in the oocyte. During fertilization, the cortical alveoli release their contents into the perivitelline cavity in what is known as the cortical reaction (Patiño and Sullivan 2002).

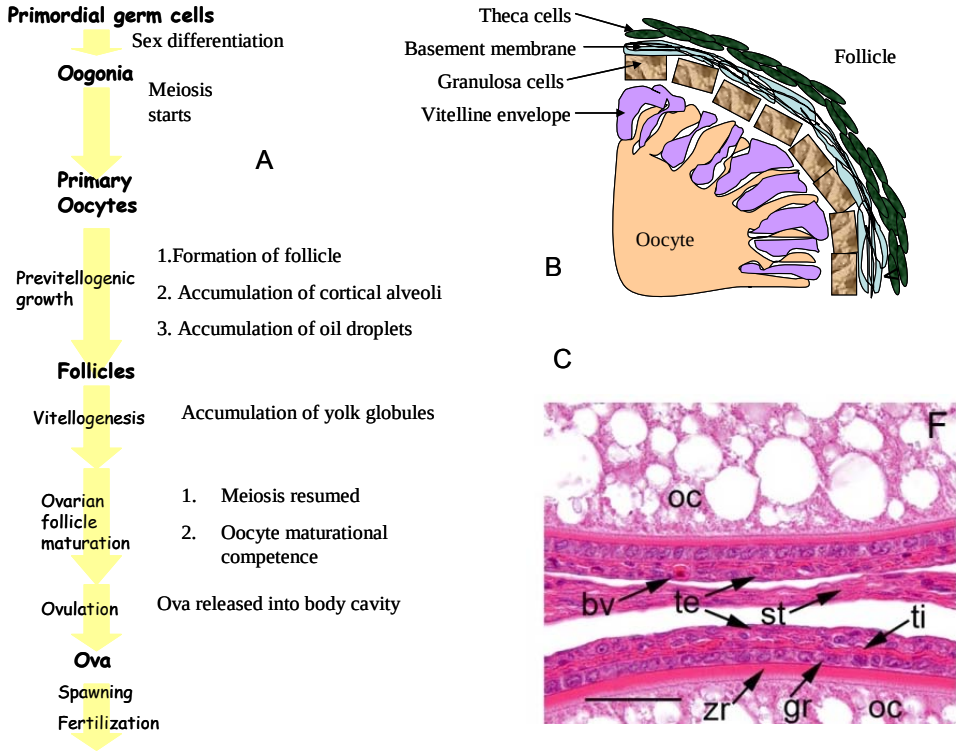

Figure 4. A. Oogenesis. B. Structure of the fish follicle. C. Histology section of Atlantic salmon follicle showing the somatic cell layers surrounding the oocyte. Oc (oocyte cytoplasm), bv (blood vessels), te (external theca), ti (internal theca), zr (zona radiate=vitelline envelope), gr (granulosa cells), st (stromal tissue). Preparation and photo by Eva Andersson (Andersson et al. submitted).

During vitellogenesis, most of the increase in size of the oocyte takes place as the oocyte sequesters vitellogenin (Vg) from the circulation by receptor-mediated endocytosis. Vg is a glycophospholipoprotein which is synthesized by the liver upon stimulation by E_2 and secreted into the circulation. It constitutes the main source of yolk proteins, lipids and certain vitamins and minerals for the developing embryo. There are multiple Vg genes in rainbow trout and at least two Vgs in other teleosts (Patiño and Sullivan 2002; Matsubara et al. 2003) which are differentially regulated. Vg is enzymatically cleaved into separate yolk proteins, mostly lipovitellin and phosvitin (Brooks et al. 1997; Patiño and Sullivan 2002). Phosvitin has phosphate covalently linked to it and calcium ionically bound. Other components of a viable egg such as lipids, vitamins, minerals and hormones are also

deposited into the growing oocytes (Patiño and Sullivan 2002; Sullivan et al. 2003).

After the completion of vitellogenesis, the process of ovarian follicle maturation takes place during which the follicle goes through several LH driven maturational processes necessary for later fertilization and survival of the embryo (Patiño et al. 2001; Patiño and Sullivan 2002; Sullivan et al. 2003). The most important event in ovarian follicle maturation is that the oocyte acquires the capacity to resume meiosis in response to MIS (17,20βP in salmonids) what is called oocyte maturational competence (OMC).

During OMC, the nucleus, called germinal vesicle, migrates toward the periphery of the oocyte near the micropyle, a small opening in the egg membrane where a sperm enters during fertilization. The germinal vesicle breaks down and meiosis is resumed and after the first meiotic division, a large secondary oocyte is formed and a first polar body is expelled. The second meiotic division continues until metaphase II and is there arrested until fertilization (Kalinowski et al. 2004).

The final step of oogenesis is ovulation when the oocyte, now called ovum, separates from the follicle and is released into the body cavity, while the follicular layers rupture and degrade. The ova can now be fertilized, and if this happens, meiosis will be completed and the second polar body expelled. The cortical reaction then takes place and the contents of the cortical alveoli are released into the perivitelline space, causing the vitelline envelope to harden and form the protective shell.

Growth and reproduction in salmon

In salmon, as in other temperate teleost species, seasonal timing of sexual maturation and spawning has evolved to make use of optimal environmental conditions and food availability for survival of the offspring. As in most species, reproduction entails a mobilization of resources towards the production of young. In this respect, sexually maturing salmon grow faster than non-maturing fish during the late winter and early spring prior to maturation (Hunt et al. 1982; Youngson et al. 1988; McLay et al. 1992), monopolizing the feed (Kadri et al. 1996) until they voluntarily become anorexic and stop feeding (Kadri et al. 1995, 1996) while returning to the spawning grounds. As sexual maturation proceeds during the summer, the salmon acquire their secondary sexual characteristics. Their skin changes from silvery to mottled brown in both sexes and the males develop their characteristic hooked jaw, the kype. When anorexia starts, somatic tissues are mobilized towards gonad growth and metabolism, and muscle is depleted of lipids, carotenoids and proteins (Aksnes at al. 1986). This brings about a deterioration of flesh quality which can cause problems in aquaculture production.

The model proposed for salmonid commitment to sexual maturation has two major components. The first is a threshold level for either a critical size-at-age/growth rate or level of accumulation of energy stores that must be attained prior to the start of maturation (Rowe and Thorpe 1990a; Rowe et al. 1991; Kadri et al. 1996; Silverstein et al. 1998; Shearer and Swanson 2000). The second component implicates an endogenous clock mechanism which may be under the control of two or more separate oscillators (Duston and Bromage 1991; Randall et al. 2000). This can be summarized by the "gating model" where fish mature in a particular year only if they reach a threshold of fitness at the same time as the circannual clock is at a specific "gate open" stage of its cycle ("the window of opportunity").

There is also significant genetic control on the choice of reproductive strategy and age at puberty which like other traits runs in families originally adapted to particular river systems (Naevdal 1983; Silverstein and Hershberger 1995). However, considering the great plasticity observed in reproductive strategy, it is now thought that if there are in fact maturational thresholds for body size, specific growth rate, adiposity or rates of accumulation of lipids, these must be interrelated and accommodating of other factors (Iwamoto et al. 1984; Skilbrei 1989; Hankin et al. 1993). In the model proposed by Thorpe (1998) for salmonid commitment to smoltification and sexual maturation, two critical windows for maturation were identified, in November and in April. At those times, the parameters believed to be gauged are body size, adiposity and their rate of change with respect to genetically determined thresholds. The resulting model thus takes into account two critical periods, the static and dynamic physiological state of the animal, plasticity in the interaction between biotic and abiotic factors and flexible genetically determined threshold levels for critical parameters.

The annual cycles of growth and maturation are known to be brought about by environmental cues such as light and temperature, but it is not known how these cues translate into growth and reproductive events. Photoperiod is considered an important environmental cue involved in diurnal and circannual timekeeping, affecting maturation, spawning time and development in salmonids. Photoperiod manipulation can be used to advance or delay ovulation in salmonids (Bromage et al. 2001). There is a lack of evidence supporting the physiological role of melatonin in fish reproduction (Mayer et al. 1997), and the molecular link between photoperiod and reproduction could involve other mechanisms. Light is believed to entrain endogenous oscillation of clock genes (Roenneberg and Merrow 2003) which, in turn, regulate other genes that require rhythmic daily expression. Recently, the clock gene, clock, has been implicated with spawning timing in rainbow trout (Leder et al. 2006). Clock genes have also been

implicated in the regulation of pulsatile secretion of GnRH in the mouse, suggesting an effect in reproductive rhythms (Chapell et al. 2003).

There is evidence for a strong growth component in influencing the incidence of sexual maturation. In juvenile chinook salmon, the main factor influencing the onset of male sexual maturation is growth (Shearer et al. 2006). In Atlantic salmon, incidence of sexual maturation correlates with both growth rate (Thorpe et al. 1990; Rowe and Thorpe 1990a,b) and size-at-age (Skilbrei 1989; Kadri et al. 1996; Duston and Saunders 1999). In a study on yearling coho salmon, body growth during the fall determined the advancement of oocytes in the following spring (Campbell et al. 2006). In this study, plasma IGF-I was the second determining factor. It is easy to see the link between IGF-I and growth as IGF-I is part of the GH-IGF-I growth promoting system and plasma IGF-I levels often correlates with growth (Beckman et al. 2004; Duan 1998; Pérez-Sánchez et al. 1995). IGF-I is considered to be a signal integrating season, temperature and day length (Larsen et al. 2001; Mingarro et al. 2002) and is believed to have an important role with respect to reproductive recruitment with respect to growth (Taylor et al. 2008). IGF-I is considered one of the stronger candidate molecules, along with leptin (Peyon et al. 2003), which signals metabolic status and growth to the reproductive control centers in the brain (Shearer and Swanson 2000; Furukuma et al. 2008). However, there is now evidence that the relationship between the GH-IGF-I system and the BPG axis is bidirectional as not only does growth (presumably through IGF-I) affect the BPG axis, the BPG axis can also affect growth. The transition between somatic and gonadal growth during gonadal recrudescence in tilapia (*Oreochromis mossambicus*) is believed to be signaled by E_2 via the estrogen receptor α (ERα) in the liver which then downregulates the GH-IGF-I system at the level of the liver (Davis et al. 2007, 2008).

As mentioned before, evidence suggests that the onset of puberty in fish is also related to absolute levels or rate of lipid store accumulation (Rowe and Thorpe 1990a, b; Rowe et al. 1991; Silverstein et al. 1998; Shearer and Swanson 2000). As in the lipostatic model in mammals, appetite in fish appears to be under negative feedback control from adipose tissue (Shearer et al. 1997; Silvertein and Plisetskaya 2000: Jobling et al. 2002; Johansen et al. 2003). Leptin, a metabolic hormone from the same family as GH (and SL), is considered to be the lipostat in mammals, the molecule that signals the metabolic status from the adipocytes to the brain, and thus regulating feed intake (Mácajová et al. 2004; Zieba et al. 2005). Fish leptin has recently been identified in several fish species including putative Atlantic salmon leptin (GenBank: DY802078) and a putative receptor has been cloned (Kurokawa et al. 2005; Wong et al. 2007). As in mammals, leptin in fish is also believed

to be one of the candidate signals for exerting nutritional control of the onset of puberty (Peyon et al. 2001; Weil et al. 2003; Volkoff et al. 2005). As in mammals, (mammalian) leptin was able to stimulate and modulate direct and indirect gonadotropin hormone release in European sea bass (*Dicentrarchus labrax*) (Peyon et al. 2001) and rainbow trout (Weil et al. 2003).

Recently, it has been established in mammals that the Kiss1 receptor (Kiss1r, also called GPR54) that binds kisspeptins coded by the Kiss1 gene plays a crucial role in initiating puberty (reviewed by Popa et al. 2008). Kiss1r is highly expressed in brain areas which express GnRHs. Kisspeptins are potent secretagogues for GnRH and believed to trigger the initial GnRH cascade at puberty. Kiss1r and Kiss1 genes have recently been cloned in several fish species (van Aerle et al. 2008; Filby et al. 2008; Martinez-Chavez et al. 2008) and have been shown to increase in expression in the brain at the onset of puberty. A regime of continuous light delays the surge in the Kiss1/Kiss1r system at puberty in Nile tilapia (Martinez-Chavez et al. 2008). In later vertebrates, melatonin regulates Kiss1r (Greives et al. 2007; Revel et al. 2006) and could be a part of a regulatory chain between photoperiod and reproduction. Kiss1 gene is a target for regulation by gonadal steroids (e.g., E_2 and T), metabolic factors (e.g. leptin), photoperiod, and season. Kiss1 neurons have recently been suggested to mediate the effect of leptin on the reproductive system (Blüher and Mantzoros 2007; Morelli et al. 2008), providing a direct link between energy status and onset of puberty. Kiss1 is expressed in other tissues besides the brain such as the gonads, and in mammals, there is evidence that Kiss1/Kiss1r could have other roles in reproduction (Castellano et al. 2006). Some evidence suggests that SL could also play a role of in the nutritional control of the onset of puberty (Peyon et al. 2003).

As mentioned above, it is probably a combination of factors which is signaling nutritional status and growth to the BPG axis to initiate the maturation in fish. Most likely, there will be species-specific differences as fish have highly varying habitats, reproductive and growth strategies, and lifecycles. Despite differences in life history strategies, the salmonids belonging to the Salmo and the *Oncorhynchus* families are phylogenetically closely related, so that endocrine factors controlling reproduction are likely to be similar.

The teleost pituitary and neuroendocrine regulation of pituitary hormones

In fish as in mammals, the pituitary gland consists of a neurohypophysis of neural origin and an adenohypophysis of epidermal origin. The adenohypo-

physis is divided into the pars distalis (or anterior pituitary) and the pars intermedia (Figure 5). There are six types of endocrine cells in the pars distalis and pars intermedia in both mammals and fish: corticotropes which secrete corticotrophin (ACTH), lactotropes which secrete prolactin (PRL), thyrotropes which secrete thyroid stimulating hormone (TSH), somatotropes which secrete GH, gonadotropes which secrete LH and FSH, and melanotropes which secrete α-melanotropin (α-MSH) and N-acetylated β-endorphin (β-END). Fish also have an additional secretory cell type in the pars intermedia called somatolactotropes which secretes somatolactin (Ono et al. 1990; Kaneko et al. 1993). SLα is produced in somatolactotropes in the posterior pars intermedia of the pituitary (Ono et al. 1990; Rand-Weaver et al. 1991; Kaneko et al. 1993; Amemiya et al. 1999; Vega-Rubin de Celis et al. 2003) whereas SLβ is produced by somatolactotropes in the anterior pars intermedia (Zhu et al. 2004).

Unlike mammals, fish lack a hypophyseal portal blood system and their adenohypophysis is directly innervated by hypothalamic nerve fibers which in the case of somatotropes in Atlantic salmon innervate their vicinity (Águstsson et al. 2000). Unlike mammals, fish also have a clear pattern of zonal distribution of endocrine cells in their pituitaries (Figure 5). The histology of the Atlantic salmon pituitary has been extensively described (Fontaine and Olivereau 1949). Somatotropes of Pacific salmon species were first identified in the proximal pars distalis of the adenohypophysis by immunocytochemistry and histophysiological studies (Ball and Baker 1969; McKeown and VanOverbeeke 1971; Wagner and McKeown 1983). These studies also identified isolated somatotropes in the pars intermedia, which are more densely stained than somatotropes in the pars distalis, and in sockeye salmon often have cytoplasmic extensions in contact with the neurohypophyseal tissue (Wagner and McKeown 1983). In sea bream, SLα has been found to co-localize with parathyroid hormone related protein (PTHrP) cells and, in some instances, both hormones are found in the same cell (Ingleton et al. 1998; Abbink et al. 2006). PTHrP is a hypercalcemic factor in fish. In smoltifying Atlantic salmon an increase of 20-30% in the number of somatotropes parallels an increase in somatotropic activity related to longer days in the spring, first in the area adjacent to the neurohypophysis and then in the pars intermedia (Komourdjian et al. 1976). This has also been observed in pars intermedia SL cells and lactotropes. Isolated somatotropes have also been found in the pars intermedia of the sturgeon (Hansen and Hansen 1975) what could indicate a certain level of plasticity in the pituitary, and that in these ancient fish lines, SL and GH cells were in close proximity. It has been suggested that some cells in the pituitary could produce different hormones depending on the life stage of the animal, or pro-

duce two different hormones as is the case for somatolactropes in sea bream (Ingleton et al. 1998; Abbink et al. 2006).

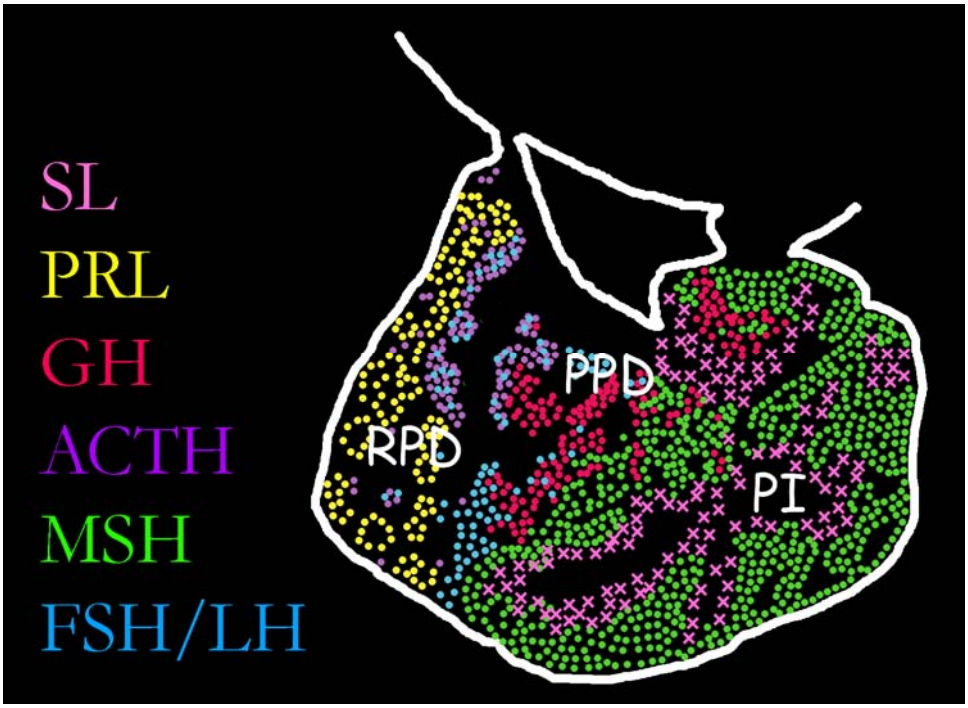

Figure 5. Schematic drawing showing the saggital distribution of hormone producing cells types in the Atlantic salmon pituitary. RPD (rostral pars distalis), PPD (proximate pars distalis), PI (pars intermedia), SL (somatolactin), PRL (prolactin), GH (growth hormone), ACTH (corticotrophin), MSH (melanocyte stimulating hormone), FSH/LH (follicle stimulating hormone/luteinizing hormone). Dots represent localization by immunohistochemistry in parallel sections while crosses (SL) have been placed with a distribution that was very similar to that of MSH. Immunohistochemistry and drawing by Lars O. Ebbesson.

Although different secretory cells in the fish pituitary are physically close, it is not known whether there are physical connections among them forming a 3D-network as is known for somatotropes in the rodent pituitary (Bonnefont et al. 2005). There are some structural similarities in the organization of the mammalian and the teleost adenohypophysis, mainly the presence of a folliculostellate cell network in close relation to the secretory cells (Leather-

land 1970, 1976; Alluchon-Gérard 1978; Abraham et al. 1979; Young and Ball 1983) and a capillary network, albeit with different sources. Follicostellate cells can respond to central and peripheral stimuli such as pituitary adenylate cyclase-activating peptide (PACAP) and estrogens, transport small diffusible molecules and secrete factors and signal molecules such as interleukin-6 (IL-6) and nitric oxide (NO) (Tatsuno et al. 1991; Fauquier et al. 2002). A third network of extracellular spaces in the teleost pituitary (Abraham 1971, 1979) aids the communication between neurosecretory fibers and secretory cells. In mammals, this role, at least for somatotropes, seems to have evolved into an integrated network of secretory cells and a portal system which delivers factors simultaneously to the whole adenohypophysis. Electrical activity and Ca^{2+} influx through voltage-gated channels is important in regulation of pituitary hormone release in teleosts as in mammals. In teleosts, the structure of the adenohypophysis would also seem capable of coordinating some degree of GH pulsatility, as seen in mammals.

In fish, neuroendocrine regulation of GH secretion by somatotropes is multifactorial, and directly controlled at the level of the pituitary. The basal level of GH secretion in many teleost species including salmonids is autonomous (Yada et al. 1991). In rainbow trout, somatotropes can be further stimulated to increase GH secretion, so the basal level is not maximal (Falcón et al. 2003). In salmonids as in other fish, GH release is mainly under inhibitory control by somatostatin (SRIF) (Yada et al. 1991; Yada and Hirano 1992) which inhibits GH release in vitro (Águstsson et al. 2000; Yada and Hirano 1992) and *in vivo* (Diez et al. 1992). In fish, growth hormone releasing hormone (GHRH) appears not to be a major GH stimulating factor and PACAP, which is structurally similar could play such a role (Montero et al. 1998). To date, around 20 factors influencing GH secretion have been identified, and these can be classified into neuropeptides, biogenic amines, excitatory/inhibitory aa, steroids (including sex steroids and corticosteroids), thyroid hormones and growth factors (reviewed by Wong et al. 2006 and Canosa et al. 2007). Neuroendocrine factors relay information on endogenous rhythms, environmental cues as well as negative feedback from physiological states. Both GH and IGF-I exert negative feedback control on GH secretion in salmonids (reviewed by Björnsson et al. 2002). In goldfish, it has been shown that differential sets of neuroendocrine factors stimulate GH release at different stages of sexual maturation (Peter and Marchant 1995) and that convergence may occur at the level of secondary messenger pathways (Chang et al. 1993).

Daily patterns of GH secretion

In mammals, GH release is known to be pulsatile, often follows a diurnal rhythm, and is sexually dimorphic. In male rats, pulses take place every 3-4 hours, with relatively low interpulse levels (200-250 ng/ml GH pulses 3-4h^{-1} and then 2-2.5 h with GH<2 ng ml^{-1}) whereas in female rats, the pulses are relatively lower and the plasma GH levels between pulses are relatively higher (Jansson et al. 1985). T is needed to maintain low interpulse levels in adult males and estrogens elevate basal plasma GH. Growth is more effectively promoted with high pulses with interpulse levels of GH so that part of the growth promotion of androgens is indirect via the GH pulse alteration (Jansson et al. 1985). GH pulsatility promotes specific patterns of hepatic enzyme production, increase in weight gain and somatic growth (Clark et al. 1985; Isgaard et al. 1988; Waxman et al. 1991). Estrogens influence body growth by means independent of the GH pattern, but the female pattern of GH secretion feminizes the liver (Jansson et al. 1985). It is not the magnitude of the pulse, but the minimum 2.5h refractory period between pulses which determines the effects of GH pulsatility in male rats. Because of the high affinity of the GHR for GH, half maximal saturation is reached at 2 ng GH ml^{-1} so that high peaks are not necessary (Waxman et al. 1991). The liver GHR are internalized after a GH pulse and reappear at the surface after about 3 hours (Bick et al. 1996). In mice, GH pulses can be up to 1000 fold basal levels in vivo. Female mice have a more continuous irregular pattern of GH release. In humans, GH release is episodic, with 4-8 pulses a day, but about 2/3 of the total is secreted during sleep (Wajnrajch, 2005). Sheep have also elevated GH levels during their frequent naps.

Initial studies on circadian patterns of GH secretion in rainbow trout found episodic fluctuations in GH secretion (Le Bail et al. 1991; Niu et al. 1993), but also great inter-individual variability. However, the high GH levels measured in certain fish were argued to correspond to pulsatile GH peaks (Boujard and Leatherland 1992; Holloway et al. 1994; Reddy and Leatherland 1994). A study of cannulated immature rainbow trout (Gomez et al. 1996) found that the daily pattern of GH secretion consisted in a very low baseline level (0.32±0.01 ng ml-1) interrupted by irregularly spaced peaks of GH (0-4; average 2.1±0.1 peaks 24 h-1) of varying amplitude (0.5-12 ng ml^{-1}; average 2.0±0.3 ng ml^{-1}) and long duration (1-9 h; average 3.5±0.2 h), which cannot be considered to be due to pulsatile secretion pattern as the increase in GH was relatively slow. Mean GH levels over 24h were low (0.7±0.1 ng ml^{-1}) and no differences between night and day were observed. GH pulsatility as such has only been described in grass carp (Zhang et al. 1994) and goldfish (Marchant and Peter 1986). The patterns of GH secretion in fish are clearly species-specific and perhaps affected by the

stage, sex and physiological state of the fish. In Atlantic salmon, smolts experience daily variations but not parr (Ebbesson et al. 2008). In immature rainbow trout, males exhibit higher GH peaks than the females (Gomez et al. 1996).

GH and SL and their receptors

GH and SL belong to the same family of peptide hormones that also includes prolactin, leptin and mammalian placental lactogens. This family is thought to have emerged through gene duplication events over the past 350 million years (Miller and Eberhardt 1983).

GH and SL structure

The family Salmonidae exhibit an ancestral tetraploidy in their genomes that occurred 25-100 million years ago (reviewed by Phillips and Ráb, 2001). This explains why there are two non-allelic GH genes in Atlantic salmon that are 97% homologous with no apparent functional differences: GH1 and GH2 (Johansen et al. 1989: Male et al. 1992). An additional GH2 gene has been found on the Y chromosome of some salmonid species suggesting further duplication of GH loci (Du et al. 1993). In Atlantic salmon, both mature GH1 and GH2 are 188 amino acids (aa) and about 22 kDa in weight. In teleosts, GH is non-glycosylated with post-translational phosphorylation which could give rise to charge heterogeneity possibly related to GHs functional activity (Skibeli et al. 1990). Atlantic salmon GHs contain four cysteins which make two disulfide bridges with a tertiary structure made by four alpha helices arranged in distinctive anti-parallel manner. Hydrophobic cores are essential for the stability of GH molecule and salt bridges and hydrogen bonds are also important for the binding of the molecule with its receptors (Sami 2007).

SLα and SLβ represent two distinct SL families that arose by genome duplication in teleosts (Zhu et al. 2004). Atlantic salmon SLα is 209 aa whereas SLβ is 206 aa and about 24 kDa in size. Both GHs and SLs they are characterized by having a signal peptide sequence of 22 aa and 23 aa, respectively. The degree of similarity between Atlantic salmon SLα and SLβ peptides is 54%. The similarity of SLα and SLβ with Atlantic salmon GH1 is 26% and 23% and with Atlantic salmon PRL, 19% and 17%, respectively.

Not much is known about regulation of SL secretion but both central and peripheral signals can regulate SL expression in the pituitary. In rainbow trout SL release can be differentially regulated by neurotransmitter like dopamine and neuropeptides such as corticotropin releasing hormone (CRH) and GnRH (Kakizawa et al. 1997). In salmon, SL gene expression can be stimu-

lated by GnRH, estradiol and 11-ketotestosterone (Onuma et al. 2005). Leptin can stimulate SL secretion in sea bass and this depends on the reproductive stage of the fish (Peyon et al. 2003). Like GH and PRL, SL gene expression is controlled in part by the pituitary-specific transcription factor, Pit-1 (Ono et al. 1994) and Pit-1 appears to drive different hormone signaling cascades in the estrogen responses of the SL and GH genes in gilthead sea bream (Astola et al. 2005).

GH and SL receptors and signaling pathways

Two GHR genes have also been identified in Atlantic salmon, and these isoforms, GHR1 and GHR2, are 86% similar. The mature GHR1 and GHR2 are 576 aa and 570 aa long plus 19 and 20 aa signal peptides, respectively and a molecular weight (MW) of about 64 kDa for the mature protein. In rainbow trout, the GHR isoforms are differentially expressed in different tissues (Very et al. 2005). In contrast, only one isoform of salmonid SLR has been identified so far. In Atlantic salmon, this is 633 aa long with a 20 aa long signal peptide and an estimated size of 70.5 kDa.

As their ligands, the GHR and SLR belong to the same family of receptors, the type I cytokine receptor superfamily (reviewed by Kopchick and Andry 2000; Brooks et al. 2008) which also includes the PRL receptor (PRLR) and the leptin receptor (Tartaglia 1997). Class I cytokines do not have intrinsic tyrosine kinase activity and depend on associated tyrosine kinases for signal transduction.

GHR exists as a constitutive dimer which is activated by the reorganization of the receptor subunits when one ligand molecule (GH) binds to two receptor molecules (Gent et al. 2002; Brown et al. 2005). One of the receptors binds with strong affinity to site 1 of the GH molecule while the other binds to the weaker site 2. This ligand-induced signaling activates the classical JAK (Janus kinase)-STAT (signal transducers and activators of transcription) signaling pathway, but there is evidence that GHR can also signal though other pathways independent of JAK. Also, recent findings suggest that some membrane receptors such as GHR and PRLR may signal by dissociating from the plasma membrane and translocate to the nucleus, where they direct the transcriptional machinery, especially with regard to cell proliferation (Swanson and Kopchick 2007).

Both GHR and SLR are transmembrane receptors with an extracellular domain made by two fibronectin type III β sandwich domains with three disulfide bonds, connected to the intracellular domain by a rigid single pass helical transmembrane domain. The intracellular domain consists of the Box 1 and Box 2 motifs, which can bind the tyrosine kinase JAK2, and several tyrosine residues which can be phosphorylated by JAK2, thus becoming

binding sites for SH2 (Src homology 2) domain proteins, specifically STAT5a and 5b. The STATs 1, 3, 5a and 5b are then phosphorylated by JAK2, dimerize, translocate to the nucleus, and bind to STAT response elements and activate transcription (Ihle and Gilliland 2007). Termination of the JAK2 signaling involves phosphatases, SOCS (suppressors of cytokine signaling) proteins and receptor downregulation (Waters et al. 2006). The half-life of mammalian GHR is around 1 h as it is continuously degraded. This turnover of GHR is carried out by ligand-induced endocytosis and receptor degradation (van Kerkhof et al. 2007) and cleavage of the extracellular domain by a metalloprotease that leads to production of growth hormone binding protein (GHBP) (Brooks et al. 2008).

There is evidence that PACAP stimulates SLα and SLβ mRNA expression and secretion via the activation of pituitary PAC-I receptors through differential coupling to overlapping and yet distinct signaling pathways (Jiang et al. 2008a,b) that have in common the AC/cAMP/PKA (adenylate cyclase / cyclic adenosine monophosphate / protein kinase A) and PLC/IP3 (phospholipase C / inostitol 1,4,5-triphosphate) cascades and subsequent rise in intracellular Ca2+ levels and calmodulin (CaM) activation.

Little is known about how GH induces the transcription of IGF-I. It is known that one of the transcription factors which regulate GH-stimulated IGF-I expression is STAT5b (Davey et al. 2001; Woelfle et al. 2003), but other factors may act as well.

IGFs and their receptors

The insulin-like growth factor (IGF) system in teleosts consists of two ligands, IGF-I and IGF-II, transmembrane receptors type I and type II, and six binding proteins (Kamangar et al. 2006). In rainbow trout, the peptide sequences of IGF-I and IGF-II. are only 43% similar and the two forms originate from separate genes (Shamblott and Chen 1992) believed to have originated early in vertebrate evolution. Unlike in mammals, where IGF-II acts mainly during fetal development, IGF-II in bony fish is highly expressed from the early stages of embryonic development until the adult stage and is produced in virtually all tissues, indicating that in fish IGF-II might have just as important physiological role as IGF-I (Palamarchuk et al. 2002).

IGF-I can bind not only to the IGF-I receptor (IGF-IR), but also to the insulin receptor and the orphan insulin receptor related receptor (IRR) (Adams et al. 2000). IGF-II can bind to the IGF-IR and to the IGF-IIR, which is structurally different (Méndez et al. 2001).

The IGF-IR belongs to the tyrosine kinase receptor family. The IGF-IR is a transmembrane receptor made up by four subunits, two α units which are extracellular and contain the ligand-binding site, and two β units which

go pass through the membrane and have a tyrosine kinase domain. Both subunit types arise from a single preproreceptor molecule and are linked by disulphide bonds (Ullrich et al. 1986). There is a high degree of sequence conservation among vertebrates, especially of those domains responsible for catalytic activity and the transduction pathway. In rainbow trout and coho salmon, two forms of the IGF-I receptor cDNA have been found (Chan et al. 1997; Greene and Chen 1999), reflecting the tetraploid nature of the salmonid genome (Chan et al. 1997).

The IGF-II receptor (IGF-IIR) is a single chain glycoprotein which is not structurally related to the IGF-I and insulin receptors. It is identical to the cation-independent mannose 6 phosphate receptor (CI-MPR) so it is also called IGF-II/M-6-P receptor. It has a short cytoplasmic tail and no tyrosine kinase activity (Méndez et al. 2001).

The liver is the main site of IGF-I and IGF-II production and accounts for up to 75% of plasma IGF-I in mouse (Sjögren et al. 1999). In rainbow trout, IGF-II transcripts are more abundant in the liver than IGF-I transcripts (Shamblott and Chen 1992). GH is a potent stimulator of IGF-I and IGF-II (Shamblott et al. 1995) which mediate some of its actions whereas IGF-I exerts a negative feedback control on GH secretion. Extra-hepatic IGF-I is believed to act mainly in a local, paracrine fashion. IGF-I receptors, like IGFs, have been detected in a variety of fish tissues including retina, liver, skeletal and red muscle, heart, ovary, testis, gill arch and adipose tissue (Gutiérrez et al. 1993; Planas et al. 2000; Párrizas et al. 1994 Otteson et al. 2002). In Japanese flounder (Nakao et al. 2002) and in Atlantic salmon (**Paper IV**) IGF-I gene expression was strongest in the gonads and heart.

GH treatment induces an increase in liver IGF-I expression (Duan 1997) and IGF-I plasma levels (Niu et al.1993; Moriyama 1995), and IGF-I treatment stimulates growth in coho salmon (McCormick et al. 1992).

Binding proteins

GH binding proteins

In many vertebrate species, a protein corresponding to the extracellular domain of the GHR has been identified in plasma as a soluble receptor acting as a high-affinity GH binding protein (GHBP). In mammals, GHBP has been estimated to up to 60% of circulating GH (Baumann et al. 2001). In the rat and other mammals, GHBP is found not only in the plasma where it prolongs the half-life of GH during pulsatile GH excretion (circulating hormone reservoir function), but also in many tissue and cell types (reviewed by Lobie et al. 1992). Nuclear localized GHBP is needed for complete transcriptional response to GH through the JAK-STAT pathway (Graichen

et al. 2003). It is thought that GH, GHR and GHBP could interact directly with transcription factors as a mechanism for the action of GH in the nucleus (Mertani et al. 2003).

GHBP has been detected in the plasma of rainbow trout (Sohm et al. 1998) and goldfish (*Carassius auratus*) (Zhang and Marchant 1999). In the goldfish, ligand blotting has revealed multiple forms of GHBPs in plasma and cultured hepatocytes of goldfish, forms which are 25, 40 and 45 Kda in weight (Zhang and Marchant 1999). The affinity of the plasma GHBP and the liver membrane GHR for rcGH are similar, indicating that the GHBP may be protein derived from the GHR by proteolytic cleavage but there is no proof that fish GHBPs are derived from proteomic cleavage. The pattern of hormone specificity is similar for goldfish GHBP and GHR (Zhang and Marchant 1996) and points to a high degree of species-specificity in terms of hormone binding. It is not known why there are such large variations in the binding affinity of teleost GHBPs, but it could be different categories of plasma GHBPs in teleost as in mammals (Zhang and Marchant 1999).

IGF binding proteins

IGF-binding proteins (IGFBPs) bind up to 97% of circulating IGF-I in humans (Jones and Clemmons 1995). They not only prolong the plasma half-life of IGF-I, but modulate IGF-I functions (Kamangar et al. 2006), as well as having IGF-I independent actions. In rainbow trout, six different IGFBPs have been sequenced and these show differential tissue expression (Kamangar et al. 2006). In addition to the six IGFBPs, there are IGFBP-related proteins (IGFBP-rP) which are similar to IGFBPs, but do not bind IGFs, but act in an IGF-independent fashion, regulating cell proliferation and differentiation (Hwa et al. 1999; Kamangar et al. 2006). In rainbow trout, IGFBP2, 3, 4, 5 and 6 are implicated in oocyte maturation whereas IGFBP1 is only found in the liver (Kamangar et al. 2006). In contrast to GHBP, which in mammals is splice variant of the GHR gene or a proteolytic cleavage product of the GHR, IGFBPs are encoded by individual genes.

Roles of GH

As the focus of this thesis is the interplay between the GH- IGF-I system, SL and the BPG axis in female salmon sexual maturation, the roles of the GH- IGF-I system and of SL in growth and metabolism will be described in some detail.

GH, also called somatotropin, was first isolated in fish in 1954 (Pickford 1954) and IGF-I in 1977 (Shapiro and Pimstone 1977). GH mediates many

of its actions by stimulating the production both IGF1 and IGF2, mostly from the liver (Shamblott et al. 1995). Since then, much research has been carried out on these two hormones (reviewed by Reinecke et al. 2005) and today, GH and IGF-I are known for its many and diverse roles in fish physiology, parallel to those in other vertebrates.

In fish as in other vertebrates, GH targets different tissues producing multiple effects. GH is the main growth-promoting hormone, and an important regulator of metabolism. Other important actions of GH in fish include osmoregulation (Sakamoto et al. 1993), immune function (Yada et al. 1999), metamorphosis (Hildahl et al. 2007, 2008), behaviour (Björnsson et al. 2002) and reproduction. Many of the actions of GH are mediated by IGF-I and IGF-II induced in the liver and other tissues in what is sometimes referred to as the GH- IGF-I axis. However, it is increasingly obvious that the regulatory functions of GH and IGF-I are not only along a simple pituitary-hepatic "axis", but also include non-pituitary control of IGF-I secretion, regulation of binding proteins, and local IGF-I production and action. Thus, it is more appropriate to refer to the GH-IGF-I system (Björnsson et al. 2002).

The GH-IGF-I system in growth

Growth regulation involves many components, but the principal regulator of growth is the GH-IGF-I-system (Figure 6). Other hormones such as thyroid hormones, insulin, sex steroids and glucocorticoids also play a role in regulating growth and metabolism.

Unlike other vertebrates, most fish undergo indeterminate growth, growing throughout their lifetime. Fish are able to store energy in protein in a larger proportion than terrestrial vertebrates. GH treatment increases the specific growth rate in length and weight, but decreases the condition factor as the fish become leaner (Sumpter 1992; McLean and Donaldson 1993; Peter and Marchant 1995). GH has a strong stimulatory effect on length growth even during periods of starvation (Johnsson and Björnsson 1994). Also germ line GH-transgenic strains of Pacific salmon and trout (*Oncorhynchus kisutch, O. tshawytscha, O. mykiss, O. clarki*) show higher growth rates when compared with non-transgenic strains (Devlin et al. 1995). Weight growth represents chiefly growth of soft tissues such as muscle, adipose and gonads, and is thus partly reversible, e.g. during periods of starvation and sexual maturation and spawning. Length growth, which primarily represents skeletal growth, is on the other hand, relatively permanent.

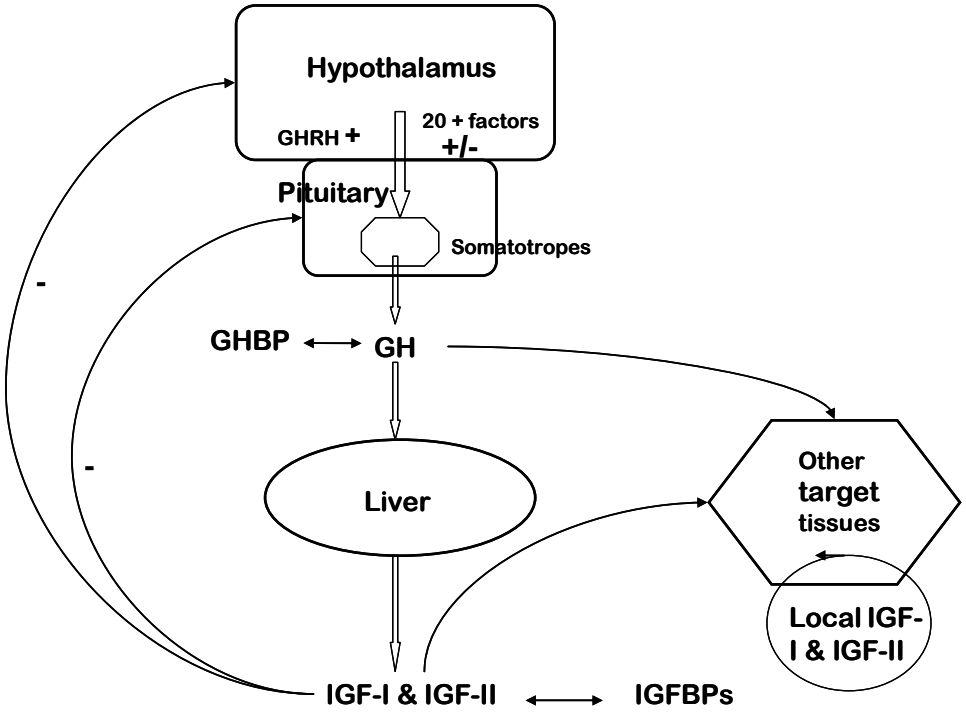

Figure 6. The GH-IGF-I system. GH (growth hormone), GH-BP (GH binding protein), IGF-I (insulin-like growth factor I), IGF-II (insulin-like growth factor II), IGF-BP (IGF-binding protein), GHRH (growth hormone releasing hormone).

GH improves feed conversion during growth and this is one of the proposed mechanisms whereby GH might increase weight in fish (McLean et al. 1991; Farmanfarmaian and Sun 1999). Another proposed explanation is a GH induced increase in appetite, and thus increased feed intake (Johnsson and Björnsson 1994).

In general, there seems to be no correlation between plasma GH levels and growth rates and low GH plasma can be associated with rapid growth (Stefansson et al. 1991; Nordgarden et al. 2003) though some data indicate otherwise (Björnsson et al. 1995). The reason for this lack of correlation could be that a relatively small increase in plasma GH levels may trigger a large increase in growth rate (Björnsson et al. 1995), and that the plasma GH levels do not reflect GH secretion rate or the tissue density of GH receptors. A high stimulation of GHR or a high occupancy rate may maintain plasma GH levels low, even if there is an increase in the metabolic clearance rate of the hormone (Sakamoto et al. 1991; Sakamoto and Hirano 1991). Con-

versely, a downregulation of GHR densities in target tissues is probably the cause of the high plasma GH levels in stunted (Bolton et al. 1987; Gray et al. 1990) and starved salmon (Sumpter et al. 1991b; Gray et al. 1992; Leatherland and Farbridge 1992) with reduced growth rates.

Thus, while GH plasma levels cannot be used as an index of growth rate, other components of the GH-IGF-I system could possibly be used. IGF-II levels correlate well with nutritional status of rainbow trout as well as correlating positively with triiodothyronine (T3) levels (Gabillard 2003a, b). Plasma IGF-I levels and specific growth rate correlate positively in numerous species including Pacific salmonids (Beckman and Dickhoff 1998), rainbow trout (Taylor et al. 2005) and Atlantic salmon (Dyer et al. 2004) although not always (Silverstein et al. 1998). Currently, data are lacking on correlation between GHR tissue densities and growth rates, but it is possible that GHR data could be used to predict growth rates.

The model for the GH- IGF-I regulation of growth is the "dual somatomedin hypothesis" that states that locally produced IGF-I at the tissue level is the main promoter of growth while the function of liver-derived IGF-I could be to regulate GH secretion by negative feedback on the pituitary and to mediate the effects of GH on metabolism (Ohlsson et al. 2000). There is mounting evidence showing that GH, as a cytokine, could also have the capacity to carry out actions independently of IGF-I. It is becoming clear that GH might exercise its different actions through the mediation of a variety of growth factors and their receptors, instead of via IGF-I alone. In this respect, in Atlantic salmon GH has been shown to have independent as well as IGF-I mediated effects on growth (Wargelius et al. 2005, Nordgarden et al. 2006).

At temperate latitudes, spring growth in postsmolt Atlantic salmon takes place between January and June. It is characterized by plasma IGF-I levels that peak in early February and sometime precede the GH peak in late February (Wargelius et al. 2005; Nordgarden et al. 2006). With respect to length growth in Atlantic salmon, GH is thought to have an initial role in stimulating vertebral growth, whereas IGF-I stimulates growth in late spring in a paracrine manner (Nordgarden et al. 2006). In white muscle, GH also partially stimulates growth or metabolism independently of IGF-I.

The role of GH in regulating metabolism

The GH-IGF-I system provides integrated signals for regulating growth and metabolism, two intimately related physiological processes. In fish as in mammals, GH stimulates protein synthesis, amino acid uptake and lipid mobilization chiefly from adipose tissue and liver (Mommsen 2001). GH increases white muscle and whole body protein accretion and increases

muscle hyperplasic growth (Fauconneau et al. 1996).GH stimulates lipolysis though stimulating triacylglycerol lipase activity (Sheridan 1986), thus elevating plasma levels of free fatty acids (Leatherland and Nuti 1981; O'Connor et al. 1993). Further, GH stimulates glycogen breakdown and increases gluconeogenesis in the liver. In rainbow trout, however, GH does not appear to affect basal metabolic rate, and probably stimulates food intake directly in the hypothalamus (Johansson et al. 2005) and not indirectly through increased basal metabolism as was thought before (Fauconneau et al. 1996; Herbert et al. 2001).

GH plays an important role during episodes of fasting. Starvation leads to a rise in plasma GH levels whereas hepatic IGF-I mRNA expression and plasma IGF-I levels decrease. The elevation of plasma GH levels could be the result of a lack of negative feedback by IGF-I, although elevated GH levels in stunted coho salmon have been explained by downregulation of tissue GHR density (Fukada et al. 2004; Pierce et al. 2005b; Small et al. 2006). The role of GH during starvation appears to be to mobilize energy sources such as fatty acids and glycerol from adipose tissue. The lipolytic effects of GH are not mediated by IFG-I and this may spare proteins for growth purposes in fed fish or preserve lean body mass in fasted fish.

Despite all the evidence for the role of IGF-I in lipid metabolism in adipose tissue in fish, no direct effects on lipogenesis have been demonstrated. However, IGF-I has a hypoglycemic effect and in fish the functions of insulin and IGFs partially overlap with insulin promotes growth (Plisetskaya 1995).

Effects of temperature on plasma GH and IGF-I levels

There is a correlation between seasonal temperature and variations of growth and plasma GH levels in goldfish (Marchant and Peter 1986) rainbow trout (Gabillard et al. 2003a). In rainbow trout, temperature elevates plasma GH levels, independently of nutritional status, whereas pituitary GH content depends more on nutritional status (Gabillard et al. 2003a).

Seasonal plasma IGF-I levels correlate strongly with water temperature in rainbow trout (Taylor et al. 2008). IGF-I is believed to be a signal that integrates season, temperature and daylength (Larsen et al. 2001; Mingarro et al. 2002). Plasma IGF-II levels in rainbow trout are 10 times higher than IGF-I levels and not related to growth rate. The role of IGF-II is believed to be more related to metabolic status, exerting control on GH by negative feedback as in mammals. Temperature promotes liver IGF-I gene expression probably through its direct effect on GH secretion, but only if fish are under optimal nutritional status (Gabillard et al. 2003b). The growth-promoting effect of temperature is not mediated by autocrine/paracrine expression of

IGF-I and IGF-II in muscle of rainbow trout (Gabillard et al. 2003b). Complete food restriction induces regulation of IGFR genes (Gabillard et al. 2003b). It should be noted that all studies on the effects of temperature on GH and IGF-I plasma levels have been carried out on rainbow trout, which unlike the Atlantic salmon, is a winter/spring spawner. The links between regulatory axes of growth and reproduction with respect to photoperiod and water temperature could therefore result in different GH and IGF-I profiles in relation to these environmental cues.

Effects of photoperiod on plasma GH and IGF-I levels

Photoperiod is a major environmental cue for regulating plasma GH levels in fish (Marchant and Peter 1986; Björnsson 1997). In rainbow trout, photoperiod advancement can override the temperature effect on plasma IGF-I levels (Taylor et al. 2008), and which respond to manipulations of photoperiod under constant water temperatures.

In salmonids, the pineal gland translates information on daylength by secreting melatonin in a square-wave fashion with high secretion during darkness, and low during daylight hours (Randall et al. 95; Bromage 2001). The diurnal changes in melatonin levels result from the activity of the enzyme arylalkylamine N-acetytransferase (AANAT) which is the penultimate enzyme in the production of melatonin from serotonin. In trout, AANAT is regulated directly by light (Begay et al. 1998; Mizusawa et al. 2000). This would explain the lack of internal circadian regulation of melatonin by the pineal in rainbow trout and masou salmon and that melatonin secretion remains high in Oncorhynchus during long-term darkness (Gern and Greenhouse 1988; Iigo et al. 2007). It is believed that the circadian rhythm in melatonin production was lost during salmonid evolution (Iigo et al. 2007). Melatonin has been found to modulate GH secretion directly at the level of the somatotropes (Falcon et al. 2003). Melatonin stimulated secretion during the dark phase is believed to be the mechanism behind the inhibition of the GH system by constant light (LL) regime found in **Paper III** and **Paper IV**.

The GH-IGF-I system and reproduction

The GH-IGF-I system has long been suspected to have important roles in vertebrate reproduction and there is evidence for interaction between the somatotropic and gonadotropic axes. During the sexual maturation of female salmonids, there appears to be a functional GH-IGF-I system in the ovaries. Both the GHR and the IGF-IR are highly expressed in the ovary and there is also locally produced IGF-I (Gray et al. 1990; Sakamoto and

Hirano 1991;Yao et al. 1991; Gutiérrez et al. 1993; Gomez et al. 1999a; Kajimura et al. 2004; Gioacchini et al. 2005), and these components are up- and downregulated during maturation. In addition, IGF-II, five different IGF-I binding proteins (IGFBPs) and at least one IGFBP related protein (IGFBP-rP) seem to have important roles in regulating locally produced IGF-I which acts in a paracrine/autocrine manner regulating maturation (Le Gac et al. 1996; Kamangar et al. 2006; Filby and Tyler 2007; Taylor et al. 2008).

Several links appear to connect the two systems. Plasma GH could have both direct and indirect roles mediated by locally produced factors, which would include IGF-I, with paracrine/autocrine roles. The presence of GHR in somatic granulosa and theca cells has been confirmed by *in situ* hybridization (Kajimura et al. 2004). GHR is believed to have a potentially important role during oocyte recruitment into vitellogenesis and initiation of growth (Gomez et al. 1999a). GH is also thought to have a role during final follicular maturation (Gomez et al. 1999a) and appears to be necessary for final gonadal growth in *Fundulus* (Singh et al. 1988). GH may stimulate or modulate gonadal steroid production including E_2 (Young et al. 1983; Singh et al. 1988; Van Der Kraak et al. 1990; Le Gac et al. 1992; Singh and Thomas 1993). GH appears to be involved in Vg production in fish perhaps mediated by IGF-I and/or E_2 (Kwon and Mugiya 1994; Mosconi et al. 2002; Carnevali et al. 2005).

IGF-I can act at different levels. Plasma IGF-I can affect hypothalamic GnRH release and pituitary gonadotropin secretion (Baker et al. 2000; Schmitz, 2003). IGF-I has been found to have direct effects on pituitary FSH content (Huang et al. 1999; Baker et al. 2000) and in Atlantic salmon pituitary cell culture, IGF-I stimulates LH β subunit (Schmitz 2003). At the previtellogenic oocyte stage, IGF-I directly stimulates synthesis and release of FSH and LH (Furukuma et al. 2008) IGF-I can stimulate proliferation and function of ovarian oocytes and somatic cells in several fish species (Kagawa et al. 1994a; Weber and Sullivan 2000). IGF-I is also thought to have a role in recruitment of oocytes into secondary oocyte growth (Campbell et al. 2006) and could have a role in increasing oocyte size as demonstrated *in vitro* in *Anguilla australis* (Lokman et al. 2007). IGF-I in fish has been found to be essential for final oocyte maturation which it can induce *in vitro* (Kagawa et al. 1994; Weber and Sullivan 2000, 2005; Weber et al. 2007) and IGF-I and IGF-II mRNA levels increase during maturational competence acquisition and oocyte maturation (Bobe et al. 2003, 2004). During final oocyte maturation, there is a downregulation of IGFBP3, 4 and 5 in the oocyte in response to gonadotropic and 17,20βP stimulation, while gonadotropic stimulation upregulates IGFBP2 and 6 in follicular layers or extrafollicular tissues (Kamangar et al. 2006). IGF-I affects the production

of the MIS (17,20βP) by the granulosa cells of coho salmon (Maestro et al. 1995, 1997) while 17,20βP affects transcript expression of IGFBP1-5 and IGFBP-rP1 (Kamangar et al. 2006).

The brain-pituitary-gonad axis also can affect the GH-IGF-I system. In salmonids, the effect of GnRH on GH gene expression appears to have a seasonal component (Taniyama et al. 2000; Bhandari et al. 2003) which may explain why studies have shown contradictory results (Le Gac et al. 1993; Holloway and Leatherland 1997b; Blaise et al. 1995). In rainbow trout, gonadal steroids stimulate GH secretion (Holloway and Leatherland 1997a), and could have an effect on growth and IGF-I (Shearer and Swanson 2000), and regulate IGFBP in the follicle (Kamangar et al. 2006). At the level of the liver, E_2 has been shown to inhibit GH stimulated IGF-I and IGF-II secretion while GnRH inhibited GH stimulated IGF-II secretion (Carnevali et al. 2005). It appears that the inhibitory effects of E_2 on IGF expression are at the level of gene expression. Clearly, the interplay between the two axes is highly complex and probably includes numerous known and unknown components which interact at different levels. The final resulting interaction will determine whether maturation takes place and to what degree the fish will invest its resources into producing offspring at the same time it ensures its own fitness and survival.

Regulatory roles of SL

SL has been implicated in the regulation of numerous physiological functions, and giving the multifunctional roles of both GH and PRL, such pluripotent role of SL is plausible. However, while many roles have been implicated, comprehensive data are lacking to allow firm conclusions. The function of SL that has most evidence is its role in body coloration. SLα stimulates pigment cell proliferation in medaka (Fukamachi et al. 2004) and adaptation to background (Zhu and Thomas 1995, 1998). SL has also been indicated to have a role in the stress responses (Rand-Weaver et al. 1993), immune function (Calduch-Giner et al. 1998), phosphate (Lu et al. 1995), sodium (Zhu and Thomas 1995) and calcium (Kakizawa et al. 1993) metabolism, as well as in acid-base balance (Kakizawa et al. 1996). Plasma SL levels suggests that it might be involved in salmon smoltification (Rand-Weaver and Swanson 1993), possibly related to metabolic changes during the process (Rand-Weaver et al. 1992; Taniyama et al. 1999; Fukamachi et al. 2005). It is believed SLα and SLβ could have different roles during embryo organogenesis as in zebrafish, silencing of SLβ led to inhibition of swim bladder development but not SLα which had no noticeable effects (Zhu et al. 2007). In medaka embryos, putative SLR mRNA was more strongly expressed than GHR (Fukamachi et al. 2005). SL is also believed to play

important roles in energy mobilization and lipid metabolism (Rand-Weaver et al. 1995) as well as in reproduction (Rand-Weaver et al. 1992; Planas et al. 1992). Plasma SL levels increase with advancing age (Company et al. 2001).

Role of SL in metabolism

SL, as GH, appears to have a role in regulating metabolism (Rand-Weaver et al. 1993; Rand-Weaver et al. 1995). However, unlike GH, SL does not increase IGF-I plasma levels in European sea bass (Vega-Rubín de Celis et al. 2003), and in vivo and in vitro studies in salmon show that SL only affects hepatic IFG-I mRNA expression at very high doses (Duan et al. 1993).

A regulatory role for SL in lipid metabolism has been suggested to be to suppress fat accumulation in different organs. This is based on the high amount of intraperitoneal fat and enlarged liver containing high glycogen and fat in the cobalt rainbow trout mutant which lacks the pars intermedia of the hypophysis (Oguri 1976; Kaneko et al. 1993; Yada et al. 2002). SL deficient mutant medaka color interfere (*ci*) show high triglyceride and cholesterol levels in liver and muscle (Fukamachi et al. 2005). Both SL and GH hormones enhance lipid catabolism and inhibit hepatic activity of acetyl–coA carboxylase (Vega-Rubín de Celis et al. 2003). The role of SL in metabolism under normal conditions appears to be the regulation of energy resources into fat reserves and/or signaling the replenishment of these (Rand-Weaver et al. 1995a; Company et al. 2001; Mingarro et al. 2002; Vega-Rubín de Celis et al. 2003). However, in salmonids under starvation, SL appears to have an immediate effect on lipid mobilization for about 2 weeks while GH seems to take over after that (Kakizawa et al. 1995). There seem also to be species differences as in rabbitfish (*Siganus guttatus*) that show no alteration in plasma SL during starvation (Ayson et al. 2007). Both under normal growing circumstances and starvation, GH and SL have opposite profiles (Rand-Weaver et al. 1995b; Kakizawa et al.. 1995; Mingarro et al. 2002). While decrease in ration size and reduced nutritive value of diets increases plasma GH levels, increase in ration size increases SL plasma levels (Pérez Sánchez et al. 1995).

Expression studies also support the notion that a major function of SL is regulation of lipid metabolism. In coho salmon SLR mRNA was detected in 11 tissues with highest levels in liver and visceral fat (Fukada et al. 2005). In tilapia GHR type I (putative SLR) also had higher mRNA expression in fat, followed by liver, muscle and skin (Pierce et al. 2007). The cobalt rainbow trout which lacks SL producing cells in pituitary accumulates a large amount of intraperitoneal fat tissue (Kaneko et al. 1993).

Role of SL in reproduction

SL appears to have a role during reproduction in salmonids based on elevated plasma SL levels and pituitary mRNA levels that coincide with final gonadal growth (Rand-Weaver et al. 1992; Rand-Weaver and Swanson 1993; Taniyama et al. 1999). Mature salmonids have higher plasma levels than immature fish (Rand-Weaver et al. 1993, 1995). In salmon, immunohistochemistry indicates an activation of somatolactotropes during sexual maturation and spawning (Olivereau and Rand-Weaver 1994a, b). It is believed to play a role in previtellogenic oocyte growth (Campbell et al. 2006), gonadal maturation (Rand-Weaver et al. 1992, Weaver and Swanson 1993) and in stimulating gonadal steroid synthesis (Planas et al. 1992). Gonad expression of SLR is relatively high in immature (Fukada et al. 2005). However, not all studies in salmonids have found a correlation between plasma SL and final gonadal maturation (Kakizawa et al. 1995) and this is also the case for non salmonids (Zhu and Thomas 1995b; Johnson et al. 1997). The role of SL appears not to be determining for reproduction as the SL-null mutant medaka *ci* can reproduce (Fukamachi et al. 2005).

It has been proposed that the role of SL in reproduction could be the mobilization of Ca during vitellogenesis (Mousa and Mousa 2000) based mostly on circumstantial evidence. Vg correlates with plasma calcium levels (Norberg et al. 1989) and E_2 with SL in salmonids (Rand-Weaver et al. 1992, Rand-Weaver and Swanson 1993). One of the roles of SL could be control of calcium metabolism (Kakizawa et al. 1993) which could be partly mediated by PTHrP which is produced in the vicinity of somatolactotropes (Ingleton et al. 1998; Abbink et al. 2006) and mediates the hypercalcemic effect of E2 (Fuentes et al. 2007). SLR expression is high in the gills but not the kidney of masu salmon (Fukada et al. 2005) perhaps for absorption of calcium from the water. However, like GH, the role of SL may simply be to act as a facilitator of oocyte maturation through its regulation of lipid metabolism.

Scientific Aims

The overall aim of this thesis project has been to increase our knowledge about the pituitary hormones, growth hormone (GH) and somatolactin (SL), in salmonid fish. In particular, the focus has been on structure and phylogenetic relationships of their respective receptors (GHR and SLR), and their regulation of physiological processes during the female sexual maturation.

In order to accomplish this, the following specific objectives were formulated, concerning sequence information, methodological development, and physiological function:

- To obtain the complete cDNA sequences of Atlantic salmon SLα, SLβ, SLR and the two isoforms of GHR (type II).
- To characterize the above mentioned hormones and receptors with respect to their phylogenetic associations.
- To use the sequence information to establish quantitative methods for assessing the gene expression of both hormones and receptors.
- To clarify the role of the two SL forms and the SLR in oocyte maturation of female Atlantic salmon, by assessing their gene expression during the maturational process.
- To clarify the role of the GH-IGF-I system in oocyte maturation of female Atlantic salmon by measuring pituitary, plasma and ovarian components of the system.
- To determine in a long-term temporal study the relationships among pituitary GH mRNA, pituitary GH protein and plasma GH as a paradigm of correlation between a secretory pituitary protein and its mRNA.

Methodological considerations

This section highlights the most important and/or novel materials and methods used in the thesis work, but for further information, the reader may also consult the methodological sections of **Papers I-IV**.

Fish used in reproductive study/setup

Figure 7. Sampling of a 2-seawinter female Atlantic salmon, measuring about 1 m in length, at the Matre station in March. A. Weighing and measuring length. B. Extracting blood from the caudal vein. C. Sampling the pituitary gland pointed by the forceps. D. Piece of ovary showing the lamellae containing follicles. Photos by B Th Björnsson.

The fish used in the studies on female Atlantic salmon maturation (**Papers II-IV**) were raised from fertilized eggs at the Matre Aquaculture Research Station, Matredal, Norway (61ON). After smoltification, in their second spring (1+ aged fish), the mixed population was transferred to seawater net-pens (5x5x8 m). Two-year old females (one sea-winter fish) were sampled on 15 occasions during a 17-month period spanning from August 2003 to

December 2004, a month after they spawned (Figure 7). At the beginning of the experiment, the fish weighed 4.2 kg, reaching their heaviest body weight of 11.8 kg in May, and a post- spawning weight in December of 9.4 kg. Therefore these fish were considerably larger than one sea-winter salmon used in previous studies. The AquaGen strain (AquaGen AS, Trondheim, Norway) has been selected for rapid growth and minimal early maturation (grilsing). In August 2003, all the fish had reached the oil drop (OD) stage of previtellogenic growth, indicating optimal energy reserves and growth rates. It is possible that oocytes of these fish were already recruited into maturation the previous spring, but were arrested at the OD stage until November when vitellogenesis was initiated with the appearance of the first primary yolk (PY) stage oocytes. The passage from OD to vitellogenesis in these fish coincided with the autumn window of opportunity (Thorpe 1998). There is a clear genetic component inhibiting the maturation of these very large fish, which are able to extend their follicular growth phase over the course of two years, when typically for fall-spawning salmonids, primary oocyte recruitment takes place in the fall preceding spawning (Campbell et al. 2006).

Histological analysis of oocyte maturation

The maturational study carried out in **Papers II-IV** was based on 15 sampling dates but at the time of sampling, ovarian samples were taken for histological analysis of oocyte maturation. This allows changes in growth, gene expression and hormone levels to be analyzed in relation to a specific ovarian development stage rather than only on a temporal scale, giving further information about possible regulatory mechanisms at the level of the BPG axis. Analysis was carried out up and until June, when all the ovaries had reached tertiary yolk (TY) stage. The ovarian sections were classified into four categories according to the most advanced stage of follicles, (Figure 8) according to the following morphological criteria:

1. Oil drop stage (OD). Previtellogenic oocytes with cortical alveoli and perinuclear oil droplets.
2. Primary yolk stage (PY). Oocytes in true vitellogenesis showing a limited number of small eosinophilic yolk globules in the periphery of the oocyte. When analyzing the resulting data, this stage was divided into early PY and late PY using February 11 as cutoff date as this was the first sampling date for the continuous light (LL) treatment. This separation took into account the effects of LL on the fish that were already in the PY stage before February 2 when the LL treatment began.

3. Secondary yolk stage (SY). Oocytes in true vitellogenesis with numerous but still small yolk globules distributed throughout the oocyte.
4. Tertiary yolk stage (TY). Oocytes in true vitellogenesis filled with large yolk globules.

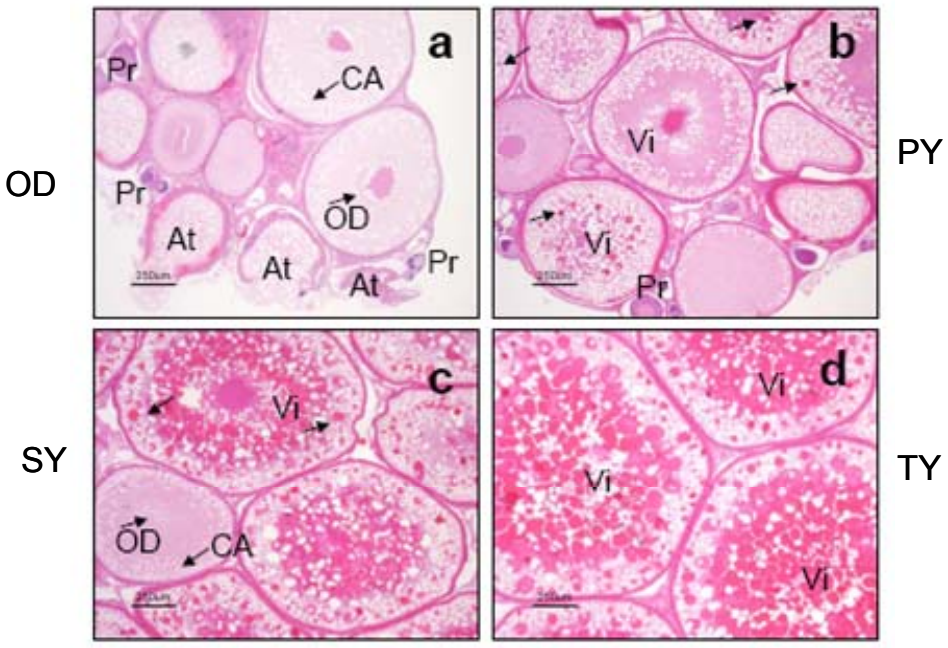

Figure 8. Classification of ovarian samples according to most advance follicle stage. a. OD (oil drop), b. PY (primary yolk), c. SY (secondary yolk), d. TY (tertiary yolk). Pr (primary growth phase oocyte), CA (cortical alveoli), At (atretic follicles), Vi (vitellogenic oocyte). Histological preparation and picture by Eva Andersson (Andersson et al. submitted).

Radioimmunoassays

Plasma and pituitary levels of GH (**Paper III** and **IV**) were measured by radioimmunoassay (RIA) as described in Bolton et al. 1986 and modified by Björnsson et al. 1994. Plasma IGF-I (**Paper IV**) was measured by homologous RIA as outlined by Moriyama et al. (1994). The GH and IGF-I RIAs used measure all the hormone present in the plasma, whether or not it is bound to binding proteins. As binding proteins can bind substantial amount of the circulating hormones, the assay results give a measure of the potentially available level of the hormone in the circulation.

Bioassay

The so called MTT bioassay (**Paper I**) for the characterization of the cloned GHR isoform 1 was carried out on CHO-K1 cells transfected with the Atlantic salmon GHR1. These epithelial cells from Chinese hamster ovary express little endogenous GHR (Emtner et al. 1990; Möller et al. 1992), and their use in PRLR transfection experiments suggests this is also the case for PRLR. The MTT assay is based on the reduction of the tetrazolium salt (MTT; 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product. By measuring this crystal, one has a measure of proliferation, which is one of the functional responses induced by binding of GH to transfected fish GHR in CHO-K1 cells (Lee et al. 2001; Tse et al. 2003). At the time when the bioassay for GHR1 was carried out, the question about the identity of teleost GHRs and SLRs had not surfaced and therefore, SL was not tested on the transfected cell line, but only recombinant coho salmon GH (GroPep Pty Ltd, North Adelaide, Australia) and native ovine PRL (Sigma-Aldrich, Stockholm, Sweden).

Phylogenetic analysis

Phylogenetic analysis of the deduced peptide sequences of Atlantic salmon GHR isoform 1 and isoform 2 (**Paper I**), as well as of SLα, SLβ and SLR (**Paper II**), were carried out using the freeware package PHYLIP 3.6a3 (Phylogeny Inference Package; Felsenstein 2005). Fish sequences that were relevant for each analysis were obtained from GenBank (NCBI, National Center for Biotechnology Information, Bethesda, MD, U.S.A.). After alignment with the general purpose multiple alignment program Clustal W 1.4 (Thompson et al. 1994), gaps were deleted to avoid any undue influence of incorrectly aligned gap regions on the resulting tree (Baldauf 2003). The resulting alignments consisted in around over 160 aa for the SLs and over 390 aa for the receptors, which is ample information to obtain a realistic phylogenetic tree. Four different methods for calculating phylogenetic trees were used in order to compare the consistency of the relationships. Two distance-matrix methods were used, neighbor-joining and UPGMA (unweighted pair group method arithmetic mean), and two discrete data methods, parsimony and maximum likelihood. One hundred bootstrap replicates were done for each type of tree (100 datasets) and bootstrap values are shown as % on the branches of the resulting consensus trees (**Paper I** and **Paper II**). The resulting consensus trees for each analysis (SLs and GHR-SLR) all had a similar typology. Because discrete data analyses are based on the original data and not on a calculated distance statistic, whenever the

bootstrap values were high enough (typically over 50%) these were the preferred methods (parsimony and maximum likelihood).

Cloning and sequencing of cDNA

The cDNA sequences of Atlantic salmon SLα, SLβ, SLR, GHR isoform 1 and GHR isoform 2 were obtained by a conventional PCR approach (**Paper I** and **Paper II**). The primers were designed based on homologous fish gene sequences but aiming for primer sequences that were divergent with respect to the other members of the GH family, so that unspecific binding would be minimal. The resulting bands on 1.2% agarose gel were purified and in some cases sequenced directly, in others subcloned into pGEM-T Easy Vector (Promega), purified and sequenced by an ABI capillary sequencer (MWG Biotech, Ebersberg, Germany). The partial sequences were compared with known cDNA sequences in GenBank using BLAST nucleotide search to confirm their identities. The corresponding 5' and 3' ends were obtained by rapid amplification of cDNA ends (RACE), using the SMART RACE cDNA Amplification Kit (Clontech) and gene-specific primers designed for minimal unspecific binding. All RACE products were gel purified, subcloned into the above mentioned sequencing vector and sequenced to yield the complete cDNA sequences with 3' untranslated region (UTR) of varying length and 5' UTRs which included the polyadenylation signal. Their identities were then reconfirmed by BLAST nucleotide search.

Real time quantitative PCR

Real time quantitative PCR (RTqPCR) has become a valuable and widely used methodology to quantify the expression of specific mRNA species in a precise manner. Elongation factor 1 alpha (Ef1α) was chosen as a housekeeping gene (**Papers II-IV**) after validating its stable temporal expression. There was no trend in the expression level of Ef1α throughout the 17-month study period and the few samples which deviated from the pooled mean ± 2 standard deviations were excluded from the analysis. The specificity of all primer pairs and corresponding probes was validated by PCR amplification, sequencing of products and melting curve analysis. The efficiency of the systems was validated by running dilution series that gave Δthreshold cycle (ΔCt)/log cDNA curves with slopes <0.1. Because of the stability of the system, only duplicates were run. Relative gene expression was calculated using the comparative method (ΔΔCt) where the target gene is normalized to the internal reference (Ef1α) and target amount given relative to the calibrator the August sample for the time based and the initial OD stage for the maturation analysis. This method is preferable to the stan-

dard curve method when there is a large number of experimental categories, this being 17 time points and 5 stages in **Papers II-IV**. Because gene expression is normalized to the housekeeping gene, small variations in mRNA extraction and cDNA synthesis efficiency are corrected for.

Conversion of relative gene expression data to total ovarian content

In order to obtain a measure in relative terms of how much mRNA of each gene studied was produced by the whole ovary in relation to body weight, one can use a conversion factor, based on Kusakabe et al. (2006), on the results obtained by the ΔΔCt method (relative expression per 500 ng total RNA) to express relative expression in ovary in relation to body weight (relative expression in the ovary) (**Papers II and IV**).

$$\text{Relative expression level} = \text{Relative expression per 500 ng total RNA} \times \frac{\text{Total RNA extracted (µg)}}{\text{Ovary used (mg)}} \times \frac{\text{Ovary weight (g)}}{\text{Body weight (kg)}}$$

This seems to work best when using the standard curve method and when analyzing male data. In females, the correction can be misleading, in particular when comparing early previtellogenic stages with vitellogenic fish, as the gonadal growth is so large that it masks any lack of variation detected in 500 ng total mRNA, while it exaggerates any significant differences found so that everything appears to follow the curve for mRNA content in the gonads. The comparative method already compensates for the decreasing amounts of RNA extracted and our own verification shows that variation in the RNA isolation efficiency was negligible.

Results and Discussion

Atlantic salmon SLα and SLβ

In **Paper II**, the complete cDNA sequences of Atlantic salmon SLα and SLβ were obtained. The main difference between the two forms is that mature SLα is 4 aa longer and has an extra cysteine residue. Both are believed to have two disulphide bonds. The phylogenetic tree of the SLs (**Paper II**) shows two well-differentiated clades, for SLα and SLβ, respectively. As mentioned in the introduction, Atlantic salmon SLα and SLβ are 54% similar in their peptide sequence, are produced in different cells of the pars intermedia (PI) (Jiang et al. 2008a), and are encoded by two paralogous genes (Zhu et al. 2004). The similarity of Atlantic salmon SLα and SLβ with Atlantic salmon GH1 is 26% and 23% respectively, and with Atlantic salmon PRL, 19% and 17%, respectively. SLα appears slightly more conserved among fish species (54-81% sequence similarity) than SLβ (42-53% sequence similarity). When studying the gene expression during sexual maturation of Atlantic salmon, SLα had generally much higher expression levels than SLβ (**Paper II**), although SLβ was subject to stronger up and down regulation. In grass carp, SLα mRNA levels are also much higher than SLβ levels, and the somatolactotropes occupied a larger area of the PI (Jiang et al. 2008a). The relatively high degree of dissimilarity between the two forms, and specific pituitary cell types would suggest differential evolution for the two forms.

GH is the ancestral GH-PRL-SL family hormone as it is the only one present in the lamprey, agnathans being the first vertebrates, emerging about 530 million years ago (Kawauchi et al. 2002) (Figure 9). Only ACTH, MSH, GTH and GH cells have been identified in the lamprey, and these are thought to be the original pituitary hormones, which have conserved functions (Kawauchi et al. 2002, Nozaki et al. 2008). The ancestral gene type for the GH family is thought to be the five-exon type, because in mammals, birds and teleosts (such as carp and catfish), elasmobranchs and lampreys, the GH gene has five exons and four introns (Moriyama et al. 2006), whereas in teleosts (all ray-fin fish after separation of the cypriniformes), such as the salmonids, tilapia, seabream or flounder, it has 6 exons and an additional intron insertion (Johansen et al. 1989; Male et al. 1992). In addition, all the PRL and SL genes have five exons (Moriyama et al. 2006). Non-teleostean SL has been cloned in the dogfish (Kawauchi and Sower 2006) and also in the sturgeon and lungfish. It is believed that genome duplication (2R) during early gnathostome evolution gave rise to PRL and/or SL (Kawauchi and Sower 2006). Therefore, SL existed in the ancestral

tetrapods, but was later lost in the tetrapod lineage (Fukamachi et al. 2007). The SLα sequence is better conserved among fish groups that those for GH and PRL (Amemiya et al. 1999).

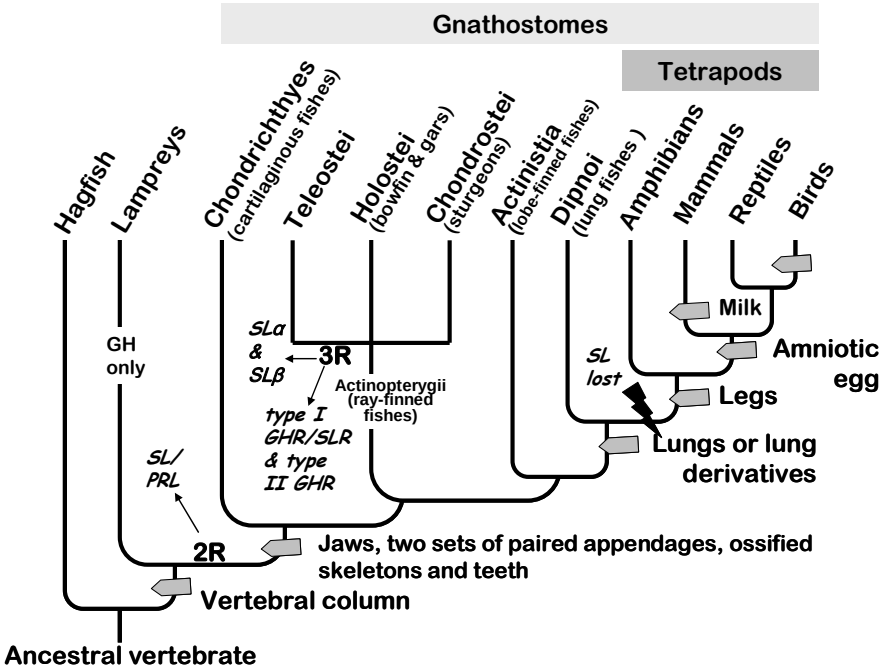

Figure 9. Vertebrate evolution and the appearance of SL and its receptor. 2R (second genome duplication event), 3R (third genome duplication event), GH (growth hormone), GHR (growth hormone receptor); SL (somatolactin), SLR (somatolactin receptor). Partly based on diagram © Benjamin Cummings, Pearson Education, Inc.

The two SL forms are believed to have originated in the teleost genome duplication event (3R), after the divergence of the sturgeons and prior to the divergence of cyprinids, salmonids and silurians. A genome search in *Fugu rubripes*, *Tetraodon nigroviridis* and medaka reveals only the SLα form, so that in some groups, the SLβ form is thought to have been lost (Zhu et al. 2004; Fukamachi et al. 2007). For the species studied, the within-species aa similarity between the two forms is relatively low, only 42-50%. The SLβ form lacks the third cysteine of the conserved seven found in the SLα form (Nguyen et al. 2006) which could reduce its dimerization potential. In non-salmonids, SLα and SLβ may be glycosylated as they have potential N-glycosylation sites and are sensitive to periodic acid Schiff staining used for

glycoprotein detection, while salmonids SLs are probably not glycosylated (Benedet et al. 2008; Jiang et al. 2008a). In gilthead sea bream only one SL gene was found but two forms of SL were secreted from the pituitary; a glycosylated and a non-glycosylated form at similar rates (Astola et al, 2005). This suggests that even if some teleosts groups might have lost one of the SL genes, they may still have retained the active use of two forms which differ in their glycosylation.

It would appear that SLα is slightly more conserved than SLβ. As SLβ has so far only been identified in a few species which spend all or part of their lifecycle in fresh water (Zhu et al. 2004; Cheng et al. 1997; Yang and Chen 2003), it has been proposed (Zhu et al. 2004) that SLβ could have a fresh-water adapting role, and its evolutionary origins could be linked to when marine teleosts first conquered freshwater habitats. However, in **Paper II**, where Atlantic salmon were transferred from seawater to fresh water in May, the gene expression did not change in a way indicating SLβ to have a stronger hypoosmoregulatory role than SLα. In **Paper II**, it appears that both SL forms are implicated in regulation of the same maturational events. The lower baseline level of expression of SLβ, but its stronger up- and down-regulation, suggests that SLβ could be recruited to enhance SL function at specific times, while SLα is the main signaling molecule. This would be in line with the fact that SLα has been found in all fish species studied and its structure is more conserved among fish groups than the structure of SLβ. This suggests a more central role of SLα than SLβ in endocrine regulation of physiological processes in teleost fish.

Using fish SL cDNA probes, corresponding sequences have been detected in mammalian and amphibians (Takayama et al. 1991), so it is possible that a SL-like gene exists in other vertebrate groups. However, to date, SL-encoding gene(s) homologous to those is fish, have not been found in tetrapods, and placental lactogens in mammals are thought to have arisen by subsequent duplication of GH genes (primates) and PRL genes (rodents) (Forsyth and Wallis 2002). Currently, the evolutionary question is thus what happened to the SL gene in tetrapods? Was it completely lost, or did it evolve so much that it is no longer recognizable? The recent characterization of leptin in fish, with only 13.2% sequence similarity between *Takifugu rubripes* and human, and no well-conserved regions (Kurokawa et al. 2005), illustrates how drastically peptide hormones can change during vertebrate evolution. For SL, perhaps the answer has to do with the fundamental difference between the aquatic habitat of fishes and terrestrial habitat of most current tetrapod lineages. In gnathostome fish, SL could have evolved important functions related to homeostasis in an aquatic environment. In this respect, SL has been implicated in homeostatic regulation of ion bal-

ance, including acid-base balance (Kakizawa et al. 1996), homeostasis of phosphate (Lu et al. 1995), sodium (Zhu et al. 1995) and calcium (Kakizawa et al. 1993; 1995; Abbink et al. 2006). However, considering that GH is the ancestral hormone and that it has conserved its role as main regulator of growth and metabolism throughout vertebrate evolution, perhaps the ancestral role for SL is also related to metabolism, and in particular, aspects of metabolism which are specific to fish.

SL and GH receptors

Atlantic salmon GHR1 and GHR2 isoforms were cloned in **Paper I** (identified as ssGHR1 and ssGHR2), and in **Paper II**, Atlantic salmon SLR was cloned. The Atlantic salmon SLR shares features and domains present in the GHRs: the typical FGEFS motif, and slightly different Box 1 (PPVPAPKIKGI) and Box 2 (DLYQDMPWVEFIELD) in the intracellular domain (ICD). It shares five of the cysteine residues present in the extracellular domain (ECD of most teleost GHRs and PRLRs, as well as two additional cysteine residues shared by most type II GHRs, making a total of seven. It has four, instead of five, potential N-linked glycosylation sites in the ECD and 11 intracellular tyrosine residues, whereas ssGHR1 and ssGHR2 only have seven. It has a cytokine receptor domain (aa 38-139) and a fibronectin type III domain (aa 128-245) in the ECD. The ssSLR is 61 and 65 aa longer than the ssGHR1 and ssGHR2, respectively, and the differences are mainly found in the intracellular signaling region.

The peptide sequence of the Atlantic salmon SLR has 44% and 43% aa similarity with Atlantic salmon GHR isoforms 1 and 2, respectively, and 17% similarity with rainbow trout PRLR. This shows that, in Atlantic salmon, the GHR-SLR receptors are more conserved than their ligands (43-44% for the receptors, compared with 23-26% for the hormones). Both the SLR and SLs have the same degree of similarity with respect to PRLR and PRL (17%). Further, the phylogenetic tree analysis (**Paper II**) shows that ssSLR protein shares greater similarity with so called type I GHRs (43-56% aa similarity with non salmonids) than with type II GHRs (35-43% aa similarity non salmonids). Similarity of ssSLR with the putative medaka SLR is 49%.

When putative SLRs and GHRs genes cloned from teleost are phylogenetically analyzed (**Paper II**), the salmonid SLRs for Masu salmon and Atlantic salmon form a clade amongst other non-salmonid GHRs, so-called type I GHRs (Tse et al. 2003; Saera-Vila et al. 2005). In contrast, the two isoforms of salmonid GHRs previously characterized (**Paper I**; Yang et al. 1997; Yang and Cheng 2003; Fukada et al. 2004; Very et al. 2005; NCBI accession numbers AF403539 and AF403540) form a distinct clade amongst

other recently cloned non-salmonid type II GHRs. Both type I and type II GHR/SLR have been found in several non-salmonid species. The degree of similarity between the salmonid SLRs and both the type I GHRs and type II GHRs including salmonid ones is quite low in both cases. In promoter activation studies using receptor transfected mammalian cells, both black sea bream type I and type II GHRs were characterized as being GHRs, not SLRs, when heterologous (salmon) PRL and SL was used (Jiao et al. 2006). Homologous hormones have only been used to obtain binding characteristics for the Masu salmon GHR and SLR. For non-salmonids, it is not yet been proved that type I GHR are not SLRs.

It appears that at least the salmonids, SLR can bind both GH and SL. The Masu salmon, SLR binds SL with greater affinity than GH (Fukada et al. 2005), whereas the Masu salmon type II GHR binds GH exclusively (Fukada et al. 2004). This has led some authors to postulate that type I GHRs can phylogenetically be regarded as SLRs, while type II GHRs should be defined as "true" GHRs (Fukamachi et al. 2005; Fukamachi and Meyer 2007).

A recent synteny analysis has established that SLR is a teleost specific paralogue of GHR, i.e. that it probably arose by duplication of GHR during the teleost 3R genome duplication event (Fukamachi and Meyer 2007). This is in contrast to SL which is thought to have originated in the 2R genome duplication event in the gnathostome line, but being lost later in tetrapod evolution. This would imply that non-teleostean gnathostome groups, including the elasmobranchs, lungfishes, coelacanths and chondrosteans, have a ligand (SLα), but not the receptor (SLR) (Fukamachi and Meyer 2007). In these groups, both SLα and GH would probably bind to the GHR. The GHR of the sturgeon (*Huso dauricus*) and the African lungfish (*Protopterus dolloi*) is indeed neither GHR type I or II, but something in between, more closely related to tetrapod GHRs (Fukamachi and Meyer 2007). The Salmonidae family underwent an additional whole-genome duplication event (4R) about 25-100 million years ago (Phillips and Ráb 2001). This gave rise to two closely related salmonid GHR type II isoforms. Only one type of salmonid SLR has been characterized so far, but another closely related isoform can exist, which perhaps differentially binds SLα and SLβ.

An *in vitro* transfection study of promoter stimulation in black sea bream (Jiao et al. 2006) revealed that type II GHR has reduced efficiency in receptor-mediated promoter activation when compared with GHR type I, i.e. the putative SLRs. This could reflect some of the structural differences between the two receptors. The ECD of type I has an extra pair of cysteines thought to help in ligand-binding by increasing the affinity for heterologous and closely related hormones, whereas in type II, fewer disulfide bonds could mean a more wobbly ligand-binding domain which reduces interaction with

ligand. The lack of the conserved site B in Masu salmon SLR involved in GH-binding in sheep and rat (Allan et al. 1999) could indicate lower affinity of SLR for GH (Fukada et al. 2005). Salmon SLR has been found to be less specific in differentiating between SL and GH than salmon GHR type II, and an eight-fold concentration of GH is needed to displace 50% on SL bound to SLR (GHR type I) (Fukada et al. 2005). This implies that in situations such as fasting, where GH plasma levels are often 10-fold higher than SL levels, GH could induce responses via the SLR.

Type I GHRs (putative SLR) are longer (49-109aa) than the type II GHRs in all species, mainly in the signal-transducing ICD which is less conserved. Type II GHRs have less Tyr residue sites for kinase phosphorylation, which could translate into reduced efficiency in signal translation. Differences in hormonal regulation of expression and tissue distribution of type I and II GHR have also found in black sea bream (Jiao et al, 2006). More *in vitro* and *in vivo* data, distinctly characterizing both types of receptors in their binding and signaling, are needed to resolve the true nature of type I and type II GHRs/SLRs.

Correlation between GH mRNA and protein levels

Paper II, III and **Paper IV** deal with complex set of data arising from a long-term (17-month) study during which Atlantic salmon females went through sexually maturation and spawning. **Paper III** describes a methodological innovation and focuses on the production dynamics of GH over time and the correlation between mRNA levels, pituitary protein levels and plasma levels of this pituitary protein. In **Paper IV**, the physiological role of the GH-IGF-I system in sexual maturation is explored, with specific focus on ovarian development and receptor expression.

In **Paper III**, a phenol-free, small-scale kit (PARIS^{TM} Kit, Ambion) was used in the processing of whole pituitary lysate for both total RNA extraction and protein content analysis. Therefore, for the first time, the expression level of pituitary GH mRNA as measured by RTqPCR, pituitary GH content and plasma GH as measured by RIA could be assessed for the same individual fish. One of the main findings of **Paper III** is that due to the complex and shifting relationships between pituitary GH mRNA expression, pituitary GH content and plasma GH levels there is no strong correlation among the three parameters, except between pituitary GH mRNA and plasma GH levels at specific time points. This low degree of correlation between pituitary GH mRNA and plasma GH levels has also been found to be the case in young rainbow trout (Moav and McKeown 1992). Since the popularization of RTqPCR, many investigations, including **Paper II**, have favored assessment of mRNA levels over the more tedious immunological

assessment of protein levels. Both measurements do, of course, give important information about the regulatory state of an endocrine system. The risk lies in the data interpretation, when gene expression data is assumed to give information about protein levels. Although many studies indicate that this can be acceptable, there are important exceptions. Studies in yeast (Futcher et al. 1999; Greenbaum et al. 2002) find quite good correlation between mRNA and protein levels. In mouse, mitochondrial genes from different tissues show a good correlation on a bulk level (Mootha et al. 2003), and in mice hematopoietic cell lines and livers, a moderate correlation has been found (Tian et al. 2004). In human liver, a positive correlation has been found between transcript levels and plasma proteins secreted by the liver (Kawamoto et al. 1996; Anderson et al. 1997). **Paper III** shows that in the case of putative pulsatile hormones such as pituitary hormones, inferring protein levels from measurements of mRNA levels would seldom give correct information. Pituitary hormone mRNA levels appear almost never reflect the levels of protein in circulation as these dynamic systems that controlled at various levels, and pulsatility makes any correlation analysis extremely difficult. Thus, great caution is needed when interpreting pituitary GH mRNA data (**Paper II**) and when extrapolating conclusions to plasma protein levels.

Paper III also highlights a problem related to correlation analysis of time series, which occurs when correlation between two variables is not stable over time (e.g. the correlation is positive at one time point, but negative at another). In such cases, pooling data from the various data points into a single correlation analysis, whether these are individual data or group means, is likely to lead to data misinterpretation, as physiologically relevant shifts in relationships are lost. This is e.g. relevant when analyzing the endocrine data of **Paper III**, where pulsatility in the system makes correlations among variables shift rapidly in strength and direction. This can also be seen in a recent study on maturing female rainbow trout (Taylor et al. 2008) which found that when 16 means were correlated (n=10 for plasma IGF-I vs specific growth rate in length) a strong correlation was obtained ($r=0.8$ $P<0.01$), but when the correlation analysis was carried out at the level of the individual at each time point, no consistent correlation was found.

Role of GH and IGF-I during sexual maturation of female Atlantic salmon

The aim of **Papers III** and **IV** was to analyze the role of the GH-IGF-I system during the sexual maturation of Atlantic salmon females from endogenous vitellogenesis to post-ovulation.

mRNA expression in different tissues of immature Atlantic salmon females (**Paper IV**) shows that both GHR and IGF-IR are highly expressed in the ovary, and local production of IGF-I has previously been observed (Gray et al. 1990; Sakamoto and Hirano 1991; Yao et al. 1991; Gutierrez et al. 1993; Gomez et al. 1999; Kajimura et al. 2004; Gioacchini et al. 2005). While IGF-IR expression is high in the ovary, ovarian IGF-I expression is proportionally lower, so that plasma IGF-I could be acting on the ovary in a significant way at the same time ovarian IGF-I acts in a paracrine fashion.

Starting in February, at the late PY stage of ovarian development (**Paper IV**), there is a downregulation of both plasma IGF-I and ovarian IGF-I mRNA levels by some unknown factor. One plausible candidate is E_2 because as it is known to downregulate plasma IGF-I, liver GHR, IGF-I and IGF-II mRNA levels during the transition from somatic to gonadal growth in tilapia (*O. mossambicus*) (Davis et al. 2007, 2008). There were also significant increases in FSH and LH in February that could be related to this IGF-I downregulation (data not shown). This initial inhibition of IGF-I is inhibited by the continuous light (LL) photoperiod treatment so that whatever factor is driving it probably is also inhibited by LL. In **Paper IV**, it is argued that the downregulation of plasma IGF-I and ovarian IGF-I mRNA levels could inhibit somatic cell proliferation (Duan 1997).

Paper III demonstrates that there is an activation of the GH system during late winter/early spring which is probably driven by changes in photoperiod. The initial IGF-I downregulation appears to be reversed by the ensuing increase in plasma GH observed during February and March (SY stage) which probably stimulates both the increase in plasma IGF-I observed in March and the increase ovarian IGF-I mRNA in March and April (SY stage). By May (TY stage), all females have significantly increased their gonadal growth.

Interestingly, the transfer from seawater cages to freshwater tanks after the May sampling did not produce any significant changes in mRNA, pituitary protein or plasma protein. Starting in August, there is a new upregulation of the GH system, but this does not translate into an increases plasma GH levels, probably due to an increase in the turnover rate. A pre-spawning peak in plasma GH levels which occurs in September and October (**Paper IV**) has previously been observed in maturing (but not immature) Atlantic salmon (Björnsson 1994) and rainbow trout (Sumpter et al. 1991; Le Gac et al. 1992). It is argued in **Paper III** that the peak in plasma GH appears to be related to decreased turn-over rate of plasma GH. One explanation for this decrease in turn-over rate could be related to the significant decrease in ovarian GHR levels detected at this time (**Paper IV**), which could translate into decreased GH internalization mediated by GHR. Considering the high

levels of GHR expressed by the ovary (**Paper IV**; Gray et al. 1990; Gomez et al. 1999) and that it represents 20% of body weight, the effect on the turn-over rate could be significant. In October, there is a significant decrease in ovarian IGF-I mRNA levels, concurrent to the decline in GHR mRNA level, but no decline in IGF-IR mRNA expression. This could imply a GHR-mediated regulation of local IGF-I expression at this time, but no effects on it receptor. In conclusion, both plasma GH via ovarian GHR, and locally produced IGF-I could have important roles in inducing ovulation. Peaks in GHR in vertebral bone have been associated with growth activation, while local IGF-I regulates matrix growth (Wargelius et al. 2005). It can be speculated that the downregulation of GHR in October could signal the end of oocyte growth, partly mediated by locally produced IGF-I, as seen by the elevated IGF-I transcripts from July to September. During ovulation in November, there is clearly a down-regulation of the GH system. In fish, IGF-I has been found to be important for final oocyte maturation which it can induce *in vitro* (Kagawa et al. 1994; Weber et al. 2007; Weber and Sullivan 2000, 2005), and IGF-I and IGF-II mRNA levels increase during competence acquisition and oocyte maturation (Bobe 2003, 2004). GH is also thought to have a role during final follicular maturation (Gomez et al. 1999) and appears to be necessary for final gonadal growth in *Fundulus* (Singh et al. 1988).

GH and IGF-I could also have a role in vitellogenesis, as pituitary hormones such as GH and PRL can stimulate vitellogenesis in fish (Kwon and Mugiya 1994; Mosconi et al. 2002; Peyon et al. 1996). IGF-I stimulates vitellogenesis in frogs, and perhaps the GH effect on vitellogenesis is mediated in part via IGF-I (Carnevali et al. 2005).

The GH-IGF-I-system seems to be activating for a role in the post-ovulation in December, with additional significant increase in pituitary GH mRNA expression and plasma GH. All this could point to a specific role of GH or IGF-I in gonadal resorption.

The effects of photoperiod on the GH-IGF-I system

In **Papers III** and **IV**, a sub-group of maturing Atlantic salmon females was placed on a continuous light (LL) regime in February, resulting in a sharp increase in daylength at that time. The fish were then kept on LL for about five months to study the effects long day on the GH-IGF-I system in relation to oocyte maturation.

Photoperiod is thought to be the determining environmental cue, timing the initiation of maturation in many teleosts, including salmonids. Photoperiod manipulation can be used to advance or delay ovulation and spermiation in salmonids (reviewed by Bromage 2001). Several studies have shown

that subjecting salmonids to a LL regime in the early spring brings on different results with respect to percentage of maturing fish, both male and female, depending on the exact moment the regime is implemented (Björnsson et al. 1994; Taranger et al. 1999). In Atlantic salmon, if the females mature, ovulation can be advanced or delayed depending on which photoperiod regime is imposed after the summer solstice, with short-day photoperiod advancing it while long-day or LL regime delaying it (Bromage et al. 1993; Björnsson et al. 1994; Taranger et al. 1999). Abrupt changes in photoperiod enhance this effect (Taranger et al. 1998). In Atlantic salmon it has been found that changing from NL to LL in January reduces maturation in the following autumn to 9% as compared with 91% in the NL group, but when the same photoperiod manipulation was carried out in March, maturation is only reduced to 67%.

One of the important results of **Paper III** is that the LL regime downregulates the GH production by the pituitary at all levels. LL also has other effects on the GH-IGF-I system (**Paper IV**). Thus, the histology-based analysis of ovarian development shows that LL abolishes all the significant changes in pituitary, plasma and ovarian GH and IGF-I system components and downregulates their receptors. The effect was to cancel the initial plasma IGF-I and ovarian IGF-I mRNA downregulation and the spring activation of the GH-IGF-I-ovarian system. This is the first time such a comprehensive inhibitory response of LL at the pituitary, plasma and peripheral organ level of the GH-IGF-I system is demonstrated, but earlier data on salmon smolts have also demonstrated the inhibitory effect of LL on plasma GH levels and the smoltification process (Björnsson et al. 2000).

Even though LL initially inhibited the GH-IGF-I-gonad system activation, by May and the TY stage, the effect was to accelerate the oocyte growth. So even if it took the fish longer to reach the TY stage under LL regime, gonadal growth appears to be faster from that point on. In this case it has been argued (**Paper IV**) that GH is stimulating gonadal growth of the LL fish more strongly than that of the NL fish. Therefore, it would appear that the initial IGF-I downregulation observed in the NL fish was inhibiting somatic cell proliferation and GH system activation was reversing this at the same time it restricted the energy allocation going into gonadal growth.

In a study on two sea-winter female Atlantic salmon, maturing for the first time (Taranger et al. 1999), arrested oocyte development was observed 4-6 weeks after LL treatment at the OD stage. For the fish in **Paper IV** which were the same age, but considerably larger, a few individuals had arrested ovarian development at the OD stage, four-eight weeks after onset of LL treatment. These females were excluded from the histology-based analysis of ovarian development, which therefore only included females with

maturing gonads (**Paper IV**). The females exposed to LL were initially somewhat inhibited in their development at the PY and SY stage, but later caught up in ovarian development at the TY stage (**Paper IV**). The Taranger et al. (1999) data also indicate that maturation was not arrested in the LL group by reduced growth performance. Long day photoperiods also diminish the percentage of maturing female rainbow trout, but advance spawning in the maturing ones (Taylor et al. 2008). Therefore, there seems to be a window of opportunity between January and March for the decision of Atlantic salmon at this latitude to mature.

The endocrine regulation probably involves signaling from one or several factors relating information on growth and metabolic status (one candidate could be leptin) to the reproductive control canters in the brain, to trigger amongst other processes, the initial gonadotropic cascade (E Andersson, J Bijl, R Male, D Patiña, S Benedet, B Norberg, GL Taranger and R Schulz, unpublished results) and subsequent GH-IGF-I system activation seen at this time. The timing of the LL regime is determinant in that if it precedes the triggering of the gonadotropic cascade, maturation is strongly inhibited, but if LL treatment is started after the initial gonadotropic cascade, something which is likely to inhibit the GH-IGF-I system at the level of the somatotropes (Falcon et al. 2003), then maturation is initially delayed, but then advanced.

Taranger et al. (1998) show that that de degree of advancement or delay in spawning in maturing females is dependent on LL treatment altering some response mechanisms in spring as well as in autumn, or short-day photoperiod alters responses in the autumn, and that the autumn changes have greater consequences. In **Paper IV**, the spring inhibition of the GH-IGF-I system by LL could have aided in the acceleration of gonadal development observed in May. It can be speculated that as LL inhibits GH mRNA synthesis in the spring, a short-day photoperiod would up-regulate GH mRNA synthesis. This, in fact, happens naturally in the autumn when there is an activation of the GH system with increased pituitary GH mRNA and GH secretion **(Paper III**). This, in turn, results in the pre-spawning peak of plasma GH in September and October coinciding with up-regulation of ovarian IGF-I mRNA at this time (**Paper IV**). It can be speculated that exposing fish to a short-day photoperiod from the summer solstice would activate the GH-IGF-I-gonad axis (amongst other things) and potentiate stimulatory effects on oocyte growth, helping accelerating maturation and eventually advancing spawning. On the other hand, exposing fish to LL regime during the autumn would inhibit the GH-IGF-I-gonad axis, cancelling out the effects of plasma GH and ovarian IGF-I at this time and delaying spawning. That autumn increase in plasma GH is related to spawning is

supported by the fact that GH levels, like E_2 and T, are also phase-shifted by photoperiod manipulation, in a similar manner as the spawning itself is shifted (Björnsson et al. 1994). Assessing each photoperiod manipulation separately (Taranger et al. 1998) it can be concluded that potentially stimulating the GH-IGF-I system by 8L in the autumn advances spawning four weeks, inhibiting the GH-IGF-I system in spring by LL advances the spawning three weeks, but inhibiting the GH-IGF-I system in the autumn by LL delays spawning five weeks.

The biological significance of this transient down- and upregulation of ovarian IGF-I mRNA levels could be related to the spring window of opportunity. E_2 is known to downregulate liver GHR, IGF-I and IGF-II in female tilapia, diverting resources from somatic to gonadal growth (Davis et al. 2007, 2008). An increase in E_2 seen in Atlantic salmon at this time could be stimulating the initial downregulation of ovarian and plasma IGF-I. However, the correlation results by date (**Paper IV**, data not shown) indicate that plasma IGF-I correlate significantly with T, but not E_2, in February and March. The fish were in a very good nutritional condition, and GH plasma could be giving a permissive signal, directly or indirectly. This is the first time that the ovarian GH-IGF-I system has been seen to be directly active during spring growth activation in a salmonid species. In salmonids, the GH-IGF-I system is considered to provide an integrated signal for growth and metabolic status, and IGF-I has also been proposed to be the metabolic trigger for puberty in fish (Shearer and Swanson 2000; Furukuma et al. 2008). The presence of GHR in somatic granulosa and theca cells has been confirmed by *in situ* hybridization (Kajimura et al. 2004) and is believed to have a potentially important role during oocyte recruitment into vitellogenesis and initiation of growth (Gomez et al. 1999). IGF-I is also thought to have a role in recruitment of oocytes into secondary oocyte growth (Campbell et al. 2006).

In males from the same study, GHR and IGF-I transcripts in the testis were significantly downregulated in January-February and late summer but up-regulated during spring, more so in fish on LL (U Nordgarden, E Andersson, BTh Björnsson, S Benedet and GL Taranger, unpublished results). This probably reflects differential endocrine regulation of male and female maturation, with differential roles and expression patterns for GH and IGF-I at the level of the gonads. It reinforces the implication of an activation of the GH-IGF-I system during early spring at the onset of sexual maturation.

This said, the effects produced by photoperiod manipulation on spawning time could also be mediated by other known and unknown factors, and the GH-IGF-I effects could be secondary to this. For example, LL treat-

ment could disrupt other key endocrine regulators such as LH, E_2, and T, which are known to be phases shifted in photoperiod manipulation studies (Davies et al. 1999), thyroid hormones or SL. As for a direct effect of LL regime on gonadotropes, little is known and with regard to reproduction, the link between photoperiod, melatonin production and reproduction in fish is disputable and experiments show equivocal results (Mayer et al. 1997; Bromage 2001). The effect of photoperiod on reproduction could be mediated by hormones such as GH or thyroid hormones, which have been found to interrelate with the pineal gland (Falcón et al. 2003).

Role of SL during sexual maturation of female Atlantic salmon

SL is thought to have a role in reproduction. It has been suggested that it might act during early oogenesis (Campbell et al. 2006), gonadal maturation (Rand-Weaver et al. 1992; Planas et al. 1992) and gonadal steroid biosynthesis (Planas et al. 1992). On the other hand, other studies found no correlation between plasma SL and final gonadal maturation (Kakizawa et al. 1995b). The specific actions of SL during salmonid reproduction are not known.

Paper II supports the notion that SL plays an indirect role during sexual maturation of the female Atlantic salmon. The high level of SLR expression in the ovary of immature Atlantic salmon (**Paper II**) and coho salmon (Fukada et al. 2005) points to a gonad-specific role for SL. The transient increase in SLα and SLβ mRNA levels observed in February appears to be related to the significant increase in SLβ in the late PY stage so that the activation of the GH system coincides with an upregulation in SL. The significant increase in both ssSLα and ssSLβ expression in the pituitary detected during the TY phase is concurrent with the significant increase detected in May and perhaps is related the increase in GSI detected at this time.

The data of **Paper II** show that the major SLα and SLβ upregulation takes place during late vitellogenesis and spawning (September, October and November) followed by downregulation in December. This could possibly indicate a role for SL during final follicle maturation, although there is no direct evidence for this. Another explanation could be a role in maturational changes in plasma calcium and phosphate metabolism during vitellogenesis and spawning (Johnson et al. 1997; Mousa and Mousa 2000). Further, **Paper II** demonstrates some degree of correlation between SL, E_2 and gonadal development (increased GSI), especially after the onset of rapid oocyte growth in August. Circumstantial evidence points to a role of SL in Ca

regulation during vitellogenesis: First, PTHrP a hypercalcemic factor in fish believed to mediate the hypercalcemic effect of E_2 in fish (Guerreiro et al. 2002) localizes with somatolactotropes in sea bream (Ingleton et al. 1998; Abbink et al. 2006). Second, SL may have a hypercalcemic action during gonadal maturation, as SL cells are activated by low environmental Ca levels (Kakizawa et al. 1993), and plasma SL levels correlate with increased plasma Ca levels in stressed and exercised fish (Kakizawa et al. 1995a; 1996). Likewise, changes in plasma SL could be related to inorganic phosphate metabolism during vitellogenesis as egg yolk proteins and Vg contain significant amounts of phosphate (Norberg, 1989). This is in agreement with the finding that SL stimulates inorganic phosphate reabsorption by the proximal tubule cells of the flounder kidney (Lu et al. 1995). However, there is no direct evidence for this and the role of SL during the sexual maturation of female Atlantic salmon could be limited to metabolic control.

Metabolic roles for GH and SL during sexual maturation of female Atlantic salmon

The analysis of mRNA levels in different tissues (albeit not in muscle or fat) of immature Atlantic salmon SLR in **Paper II** is in line with previous studies in salmonids (Fukada et al. 2005) and non-salmonid species (Jiao et al. 2006; Pierce et al. 2007) that find that SLR is highest either in the liver or visceral fat/fat in comparison to muscle. The results for GHR (**Paper III**) are a bit unusual in that the highest expression level was not found in the liver, but in the heart, followed by the ovary and the spleen, but somewhat similar pattern was also observed in mature tilapia (Pierce et al. 2007) where expression of GHR type II was highest in muscle and heart. Usually GHR expression is highest in the liver, followed by muscle, but in salmonids there is differential expression of the two GHR isoforms (Very et al. 2005). It is clear from tissue expression studies that both SL and GH mediate important action at the level of the liver, but that SL is associated with fat while GH is associated with muscle.

Both SL and GH enhance lipid catabolism and inhibit hepatic activity of acetyl–coA carboxylase (Vega-Rubín de Celis et al. 2003). SL appears to affect energy mobilization and stimulates lipolysis during and after the transition from growing to resting season of temperate fish (Mingarro et al. 2002). The profile of plasma SL levels is opposite of GH and it is thought that GH-IGF-I system could synchronize the summer growth spurt while the rise in SL could serve to prepare sea bass for the cold season (feeding inhibition) and perhaps the priming of reproductive processes or the onset of puberty. A circannual cycle in plasma SL levels has been detected in rain-

bow trout, with lower winter levels and higher summer levels which appear to correspond to the time of the year when fat stores are replenished (Rand-Weaver et al. 1995). The cycle was correlated with water temperature, but out of phase with daylight changes. Increase in plasma SL levels has been observed in gilthead seabream correlated to increased ration size (Company et al. 2001). On a daily basis, SL increases two hours after eating, thus acting as marker of energy surplus daily and annually. It would appear that under normal growing conditions, the role of SL might be regulation of energy resources into fat reserves and/or signaling the replenishment of these. Of course, there are probably great species differences in the regulation of metabolism, and in this respect salmonids are known to differ from temperate species (Company et al. 2001).

GH is known to play a role under fasting conditions, and this also seems to be the case for SL. In fasting rainbow trout, plasma SL levels increase significantly after 3 days, but decrease after 14 days, when GH levels increase, coinciding with a decrease in condition factor, hepatosomatic index and abdominal fat (Kakizawa et al. 1995). In rainbow trout fasted for 16 weeks, SL and GH correlate with most metabolic parameters, but in an opposing manner, as the starved fish had highly elevated GH while SL levels were suppressed (Rand-Weaver et al. 1995). In short-term fasted sparid fish, there is a transient increase of plasma SL which is thought to mediate the adaptation to fasting until the lipolytic action of GH is established (Company et al. 2001). In this respect, it would appear that SL maintains lipolytic tonus immediately after the onset of starvation, but that in the long term, GH plays this role and its plasma levels are elevated together with liver GHR and plasma IGF-I downregulation (Pérez-Sánchez et al. 2000). It is not clear where energy is mobilized from as starvation progresses, but it can be speculated that SL first taps into fat reserves, and perhaps after about two weeks, GH starts it own lipolytic action, at the same time its signals with high GH resistance levels that the mobilized energy should not go towards continued body growth.

The significant increase in plasma GH that precedes spawning (**Paper II** and **Paper IV**) could be related to metabolic regulation during the period of voluntary anorexia that starts in May. However, it appears just as likely that this GH peak is correlated to important gonadal events that are taking place. The downregulation of GHR in October appears to be signaling the end of GH-mediated action on the ovary, perhaps the end of rapid oocyte growth and the allocation of energy resources to it. There are also strong indications of a post-spawning role for plasma GH and the ovarian GH-IGF-I system, perhaps in the re-mobilization of energy resources from the spent tissue.

Regarding SL, even if the significant increase of both SLα and SLβ mRNA pituitary levels before and during spawning (September-November) could partly be related to Ca mobilization during vitellogenesis, the absence of any SLR up- and downregulation in the ovary during sexual maturation, and the fact that mRNA levels are still high during spawning, suggests an energy mobilizing role for SL during these final stages of oocyte maturation. If plasma GH is signaling for important events at the level of the gonads as it appears from **Papers III and IV** under the exceptional circumstances of anorexia and gonadal growth, SL could take over the somatic lipolytic control functions until feeding is resumed after spawning. In this scenario, the transient peak in SL in May which precedes the start of anorexia could be signaling this relay in GH-SL metabolic regulation. **Paper II** shows a transient peak in pituitary SL mRNA levels detected in February which was not commented before but that could actually be of real importance. It shows that SL could also be part of the GH-IGF-I mediated signaling of favorable conditions for reproduction during the late winter/early spring window of opportunity. If SL does indeed control visceral fat depositions, one of its roles could be to signal the level of visceral fat to the brain control areas while GH could be signaling on the level of muscular lipid content. The two could determine if maturation can proceed. There are indications that higher muscular lipid content positively influences sexual precocity and advances initiation of gametogenesis in juvenile rainbow trout (Weil 2007), while lower visceral fat content could be an indicator of lower maturation incidence (Páll et al. 2007).

Concluding remarks and future perspectives

This thesis work has been aimed at increasing our knowledge about the regulatory role(s) of the GH-IGF-I system during sexual maturation in female Atlantic salmon. Also, emphasis has been placed on advancing our knowledge about the SL system, as the hormone is closely related to GH, and both hormones may exert regulatory effects through closely related GH/SL receptors. For this purpose, the cDNA sequences of Atlantic salmon GHRs (two isoforms), SLR, as well as SLα and SLβ were obtained with the double goal of carrying out phylogenetic analysis and of developing molecular tools for analysis of mRNA levels using RTqPCR. The sequences have revealed the placement of Atlantic salmon GHR in the GHR type II clade and SLR in the controversial GHR type I clade (putative SLRs). A concurrent analyses of pituitary GH mRNA levels, GH protein and plasma GH protein on the same individual fish revealed part of the complex dynamics of the GH system and has demonstrated that a continuous light regime downregulates the system, something which could not have been deduced by studying only plasma hormone levels. Evidence has been found for a photoperiod-driven GH-system activation which is initiated in January with stimulation of GH secretion from pituitary somatotropes. The role of this activation of the GH system in late winter/early spring appears to be the reversal of a prior plasma IGF-I and ovarian IGF-I mRNA downregulation driven by an unknown factor(s). This downregulation in IGF-I is thought to inhibit somatic cell proliferation. The activation of the GH-IGF-I-gonadal system also appears to limit energy allocation to gonadal growth. These series of events involving the GH-IGF-I system appear to take place during the so-called spring window of opportunity and it is the first time this has been described. The GH-IGF-I system also appears to have an important role during final oocyte growth, spawning and post-spawning events. SLα and SLβ are both actively regulated during sexual maturation and could have several roles, such as signaling the status of visceral fat reserves during the spring window of opportunity, signaling lipid metabolic status before the onset of anorexia, involvement in Ca mobilization during vitellogenesis and/or control of lipid metabolism in lieu of GH during the final stages of oocyte growth.

Although the studies carried out in **Papers II** and **IV** are largely descriptive in nature, and data interpretation on physiological causal relationships thus largely speculative, these studies are an important and necessary step in generating hypotheses about how the hypophyseal-hepatic regulation of

growth is interrelated with the hypophyseal-gonadal regulation of maturation. In the future, it would be interesting to test some of the hypothesis generated by this thesis using *in vitro* and *in vivo* experimental approaches, including gonadal and pituitary tissue/cell cultures, transfected cell cultures and knock-out models, where hormonal-receptor interactions, as well as cellular effects of hormone action could be teased apart to discover detailed mechanisms of action and causal relationships. However, one of the most important experiments pending is the definite characterization of GHRs and SLRs in their specific binding properties. Until this issue is resolved, work on the so-called type I GHRs, which are the majority of non-salmonid GHRs sequenced so far, could be in fact be reflecting SLR actions.

After the initial discovery of SL (Ono et al. 1990; Rand-Weaver et al. 1991) there was much research into the role of this hormone which had to be of importance given its descent. However, the clarification of its physiological role(s) has proved frustratingly elusive to obtain. SL has been shown to have a role in control of body coloration of some species, but it is less clear if and how the hormone is involved in regulation of metabolism. However, considering that both SL and GH could share the type I GHR (SLR) and that it appears to have an important lipolytic action, SL should be included in future studies designed to elucidate the endocrine control of metabolism in fish.

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Swedish summary

Bakgrund och syfte: Det endokrina styrsystem som består av tillväxthormon (GH) och insulin-liknande tillväxtfaktor I (IGF-I), det såkallade GH-IGF-I systemet deltar i könsmognaden hos laxfiskar men dess exakta roll är inte känd. Somatolaktin (SL) är ett hypofyshormon, nära besläktad med GH, som endast förekommer hos fiskar. Hos vissa arter, däribland laxfiskar, finns två former av hormonet, SLα och SLβ. SL receptorn (SLR) har nyligen karakteriserats och fylogenetisk analys visar att den har stora strukturella likheter med de tidigare kända GH receptorerna (GHRs) från icke-laxfiskar. Hur hormonen GH och SL binder till dessa närbesläktade receptorer är okänt. Vilken roll SL spelar i reglering av könsmognad hos fiskar är också okänt.
Syftet med avhandlingsarbetet har varit att öka kunskapen om hur både GH-IGF-I systemet och SL deltar i regleringen av könsmognaden hos Atlantlaxhonan.
Metoder: cDNA sekvenser av Atlantlax GHRs (två isoformer), SLR, samt SLα och SLβ togs fram för att genomföra en fylogenetisk analys, samt att utveckla molekylära analysmetoder för att studera mRNA uttryck med hjälp av realtids kvantitativ PCR (RTqPCR). GH, IGF-I och SL systemen analyserades under en 17-månader lång studie av Atlantlaxhonor under könsmognad. mRNA uttryck av GH-IGF-I systemet och SLR mättes i ovarierna and i hypofysen mättes GH, SLα and SLβ mha RTqPCR. Plasmahalter av GH och IGF-I mättes, samt GH halterna i hypofysen, mha radioimmunoassay.
Resultat och Slutsatser: Den fylogenetiska analysen (**Paper I** och **II**) av de klonade sekvenserna placerar Atlantlax GHR i den sk GHR typ II kladen och SLR i den kontroversiella GHR typ I kladen (möjligtvis SLRs). Samtidig analys av hypofys GH mRNA halter, GH protein i hypofysen och plasma GH halter i samma fiskar visar mycket komplex dynamik i GH systemet, som hämmas om fiskarna placeras under kontinuerligt ljus. Paper **III** och **IV** fastställer att det finns en aktiv GH-IGF-I-ovarie axel hos Atlantlaxhonan som ser ut att ha en reglerande roll i början av den exogena vitellogenesen, oocyternas slutmognad, lek och möjligtvis också efter leken. Bevis har hittats för en fotoperiod-driven aktivering av GH-systemet som initieras i januari genom stimulering av GH sekretion från hypofysens somatotroper. Denna aktivering av GH systemet under vinter/tidig vår verkar ha som syfte att motverka tidigare nedreglering av plasma IGF-I och ovarie IGF-I mRNA. Denna nedreglering av IGF-I anses hämma cellutveckling. Aktiveringen av GH-IGF-I-ovarie systemet ser också ut att begränsa den energi som ovarierna får för tillväxt. GH-IGF-I systemets regleringsaktivitet ser ut att äga rum inom den sk "window of opportunity" som äger rum på våren, och det är första gången som detta har beskrivits. GH-IGF-I systemet ser också ut att ha en viktig reglerande roll under oocyternas slutmognad, lek och efterlek. SLα och SLβ regleras båda aktivt under könsmognaden och de kan ha flera olika funktioner såsom att signalera information om mängden bukfett och/eller fettmetabolism under våren, innan fiskarna slutar äta. SLs kan också vara involverad i Ca mobilisering under vitellogenesen och/eller regleringen av fettmetabolism tillsammans med GH under oocyternas slutliga tillväxt fas.