Regulation of Leptin by Sexual Maturation and Energy Status in Male Atlantic Salmon (Salmo salar L.) Parr

SUSANNE TROMBLEY
Leptin is a peripheral adiposity signal and a key hormone in energy balance regulation in mammals, acting as a link between nutritional status and the endocrine reproductive axis. If this is also the role of leptin in fish is not fully understood. This thesis investigates how different components of the leptin system are affected by sexual maturation and seasonal changes in energy balance in male Atlantic salmon (Salmo salar L.) parr under fully fed and feed-restricted conditions. Moreover, the role of sex steroids as being one of the possible mechanisms by which sexual maturation interacts with leptin is explored.

The salmon leptin-a genes, lepa1 and lepa2, were expressed mainly in liver and the leptin receptor (lepr) in brain and ubiquitously in peripheral tissues. Seasonal characterization of the lepa genes and lepr during the growth and reproductive season in one-year old males showed that hepatic lepa1 and lepa2 mRNA levels and plasma leptin levels were down-regulated concomitantly with an increase in weight and body fat. Feed restriction up-regulated hepatic leptin, and pituitary lepr expression as well as plasma leptin levels. Correlation between leptin levels and body lipid stores were either lacking or negative. These findings show that leptin and lepr are sensitive to changes in energy balance, but that leptin might not reflect adiposity in juvenile salmon.

Hepatic lepa1 and lepa2, and testicular lepr expression increased during mid- to late spermatogenesis in early maturing males. This up-regulation was preceded by rapid gonadal growth and elevated pituitary follicle-stimulating hormone gene expression levels, whereas peak leptin levels coincided with peak pituitary luteinizing hormone expression and the presence of running milt in the testes. The sex steroids testosterone (T), 11-ketotestosterone and 17-β estradiol stimulated lepa1 and lepa2 gene expression in Atlantic salmon hepatocytes in vitro differentially depending on developmental stage. T was also able to stimulate hepatic lepa1 and pituitary lepa1 and lepr gene expression in immature male salmon in vivo. These results suggest that leptin plays a role in male fish reproduction during later stages of the maturational process and that the elevation of leptin expression during spermatogenesis could be caused by androgen stimulation.

Keywords: Leptin, leptin receptor, Atlantic salmon, Salmo salar, sexual maturation, puberty, energy balance, hepatocytes, sex steriods

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In Loving Memory of My Mother
Anne Hetty Arvidsson
LIST OF PAPERS

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


II  Trombley, S., Mustafa, A. and Schmitz, M. (2014) Regulation of the seasonal leptin and leptin receptor expression profile during early sexual maturation and feed restriction in male Atlantic salmon, Salmo salar L., parr. (Accepted to General and Comparative Endocrinology)


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Additional publications


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ABBREVIATIONS

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>11-KA</td>
<td>11-ketoandrostenedione</td>
</tr>
<tr>
<td>11-KT</td>
<td>11-ketotestosterone</td>
</tr>
<tr>
<td>ARα</td>
<td>androgen receptor-α</td>
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<td>ARβ</td>
<td>androgen receptor-β</td>
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<tr>
<td>BPG</td>
<td>brain-pituitary-gonad</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CF</td>
<td>condition factor</td>
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<tr>
<td>E2</td>
<td>17 β-estradiol</td>
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<td>ERα</td>
<td>estrogen receptor-α</td>
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<tr>
<td>ERβ</td>
<td>estrogen receptor-β</td>
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<td>FSH</td>
<td>follicle-stimulating hormone</td>
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<td>FSH-β</td>
<td>fsh β-subunit</td>
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<td>FSHR</td>
<td>FSH receptor</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
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<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
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<td>GPR54</td>
<td>kisspeptin receptor</td>
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<td>GSI</td>
<td>gonadosomatic index</td>
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<td>GTH</td>
<td>gonadotropin</td>
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<tr>
<td>Kiss</td>
<td>kisspeptin</td>
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<tr>
<td>lepa1</td>
<td>leptin-a1</td>
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<tr>
<td>lepb</td>
<td>leptin-b</td>
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<tr>
<td>lepr</td>
<td>leptin receptor</td>
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<td>LH</td>
<td>luteinizing hormone</td>
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<td>LHR</td>
<td>LH receptor</td>
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<td>NPY</td>
<td>neuropeptide Y</td>
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<tr>
<td>pomc-a1</td>
<td>pro-opiomeLANocortin a1</td>
</tr>
<tr>
<td>pomc-a2</td>
<td>pro-opiomeLANocortin a2</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>T</td>
<td>testosterone</td>
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INTRODUCTION

Atlantic salmon

Atlantic salmon (*Salmo salar* L.) belongs to the Salmonidae family (salmonids) of fishes which is divided into the subfamilies *Salmoniae* (salmons, trouts, charrs), *Coregoninae* (freshwater whitefishes) and *Thymallinae* (graylings) (Nelson 2006). The natural geographical distribution of Atlantic salmon extends along the west and the east coastlines of the northern part of the Atlantic Ocean and the Baltic Sea. Atlantic salmon is an anadromous species, which means that it spawns in freshwater but spends most of its life at sea. Spawning is seasonal and takes place during October-December at northern latitudes. The fertilized eggs hatch in spring and the fish remain in the freshwater habitat for one to several years, during which time they are called parr. They then go through the so called parr-smolt transformation (smoltification) and migrate to the sea to feed and only return to their native rivers as large adults to reproduce. The life cycle of salmon is highly plastic and, while females almost invariably mature as large anadromous fish returning from the sea, the males exhibit two alternative reproductive strategies: they can either mature at a large size after the sea migration or they can go through early maturation already as small one or two-year old parr without ever having left the freshwater habitat (Fleming 1996; Klemetsen et al. 2003). Anadromous large males will develop secondary sexual characters such as a hooked jaw, tough skin and changed color as they become sexually mature and they have adopted a fighting strategy to compete with other large males for access to females. Early maturing male parr, on the other hand, do not develop secondary sexual characters and remain small with similar cryptic coloration as the immature male and female parr. The strategy of the early maturing males is to rapidly sneak in by the adults during spawning and try to “steal” fertilizations from the large males. Anadromous males stop feeding as they begin their up-river spawning migration. They re-allocate energy resources by mobilizing lipids towards gonadal growth, spermiogenesis and other energetically demanding processes related to reproduction (Aksnes et al. 1986; Rowe et al. 1991). Maturing male parr invest a larger proportion of its body weight into gonadal development compared to the adult anadromous males (Fleming 1998). Mature male parr do not stop feeding entirely but the energetic cost of early maturation results in reduced growth (Rowe and Thorpe 1990a) and lower energy reserves compared to immature males of the same age (Saunders et al. 1982). The life history choice to mature early or not is dependent on growth performance and the size of energy stores at critical times of the year (Rowe et al. 1991; Silverstein et al. 1998; Thorpe et al. 1998; Shearer and Swanson 2000). The phenomenon of early sexual maturation is observed in wild populations as well as under farming conditions, where it often occurs in greater abundance due to favorable growth conditions (Taranger et al. 2010).
Sexual maturation

Reproduction in fish as in other vertebrates is controlled by the gonadotropic axis, also called the brain-pituitary-gonad axis (BPG axis) (Figure 1). The onset of sexual maturation, or puberty, is the developmental event by which a juvenile organism acquires the capacity to sexually reproduce for the first time. The BPG axis is quiescent during the juvenile stage and the onset of sexual maturation is characterized by the activation of this endocrine axis in fishes, as in other vertebrates (Ebling 2005). The activation of the BPG axis is controlled by a number of external (e.g. temperature and photoperiod) and internal factors (e.g. growth rates, adiposity). Several neural systems are involved in interpreting and integrating these signals, such as the kisspeptin (Kiss) and the kisspeptin receptor (GPR54) system in the brain which is considered the key gatekeeper of puberty and the activation of the BPG axis in vertebrates (Roa et al. 2008). This activation is marked by a surge in the synthesis and release of the neuropeptide gonadotropin releasing hormone (GnRH) from the hypothalamus. Most vertebrates express two to three types of GnRH (Zohar et al. 2010). The different GnRH types are expressed by distinct neuronal populations in the

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**Figure 1.** Schematic model of the brain-pituitary-gonad axis.
brain localized in separate brain areas. GnRH is the primary stimulating factor of the gonadotropins (GTHs) follicle stimulating hormone (FSH) and luteinizing hormone (LH), and GnRH receptors are found in the pituitary (Yaron et al. 2003). FSH and LH are released from the pituitary into the blood and act directly at the gonadal level to stimulate gonadal growth, gametogenesis and the synthesis and release of sex steroids. The sex steroids in turn exert both positive and negative feedback effects on the gonadotropins indirectly via the brain and also directly at the pituitary level (Levavi-Sivan et al. 2010).

Gonadotropins

The pituitary in fish, as in mammals, produces two gonadotropins: FSH and LH (Swanson et al. 1989). In contrast to mammals, the gonadotropins are produced by two distinct types of gonadotropes in the pituitary (FSH and LH cells) (Nozaki et al. 1990; Naito et al. 1991). The gonadotropins consist of two subunits; a common α-subunit and a β-subunit that is hormone specific and determines their separate biological function (Pierce and Parsons 1981). FSH and LH exert their biological action by binding to their cognate receptors, FSHR and LHR that are located in the fish gonads (Maugars and Schmitz 2006; Jeng et al. 2007; Rocha et al. 2007). The fsh-β and lh-β genes are differentially expressed in the pituitary throughout the reproductive cycle of salmonids (Gomez et al. 1999; Maugars and Schmitz 2008). The earliest sign indicating commitment to early maturation, prior to any observable gonadal growth, is an increase in fsh-β mRNA levels in spring (Maugars and Schmitz, 2008). In salmonids, FSH is present in the blood at low levels already in immature and levels increase gradually during spermatogenesis, while LH levels are very low or undetectable until early gametogenesis (for review see Swanson et al. 2003). FSH and LH potently stimulate the production of 11-ketotestosterone (11-KT) and testosterone (T) by the testes of salmon (Planas and Swanson 1995). The testes are more sensitive to stimulation of FSH during earlier stages of spermatogenesis while the steroidogenic effect of LH increases during late spermiogenesis and peaks at spermiation (Planas and Swanson 1995). FSH has therefore been suggested to play a crucial role in the onset of puberty and early stages of maturation while the main role of LH is during the final stages of maturation (Swanson et al. 1989; Loir 1999; Maugars and Schmitz 2008).

The testis and spermatogenesis

The two functions of the testes in all vertebrates are the production of male gametes (spermatogenesis) and sex steroids (steroidogenesis). The testes of most teleosts, including Atlantic salmon, are made up of a paired and, when immature, threadlike transparent structure extending along both sides of the dorsal part of the abdominal cavity (Schulz et al. 2010). They consist of the intertubular (or interstitial) and tubular (or germinal) compartments. In salmonids, the germinal compartment forms an
anastomosing tubular network which is lined with a germinal epithelium made up of somatic Sertoli cells and germ cells (Parenti and Grier 2004). Germ cells are dependent on Sertoli cells for their survival and development, and the two cell types form a tight bond, where Sertoli cells envelope the germ cells, creating units called spermatocysts (Schulz and Miura 2002). As spermatogonial proliferation within a spermatocyst starts, Sertoli cells increase in number and the spermatocyst increase in volume. The interstitial part of the testes contains Leydig cells as well as blood vessels, neural and connective tissue and immune system cells. The primary function of the Leydig cells is to produce sex steroids that are essential for the initiation and maintenance of spermatogenesis, i.e. the development of immature spermatogonial into fully functional spermatozoa and the completing event of spermiation.

Spermatogenesis is divided into the three major phases of 1) spermatogonial proliferation, 2) meiosis and 3) spermiogenesis (Schulz and Miura 2002). In short, the first developmental phase, that takes place when spermatogenesis is initiated, is the rapid proliferation of undifferentiated primary spermatogonia A cells through mitotic division. After a species specific number of cell cycles these start to differentiate into spermatogonia B. This next phase is the meiotic phase during which spermatogonia B cells go through DNA duplication, recombination and finally division to form haploid spermatids. During the last stage of spermiogenesis no further proliferation takes place as the spermatids develop into flagellated spermatozoa. The process of spermatogenesis is fully controlled by endocrine, paracrine as well as autocrine factors that coordinate and regulate all steps of the way.

A dramatic increase in gonad size occurs during spermatogenesis in early maturing male salmon. The immature testes make up less than 0.1 % of the total body weight while the mature testes can peak at over 12 % of the body weight at the stage of spermiogenesis during which time spermatozoa represents 70% the germ cell type (Maugars and Schmitz 2008). Once spermiation has been initiated testes mass is lost as milt and the size of the gonads start to decline.

**Sex steroids**

The teleost testis produces a number of sex steroids which are crucial for spermatogenesis, development of secondary sexual characters and reproductive behavior. The most important of these androgens in most fish species are T and 11-KT, in particular (Borg 1994). 11-KT is more effective than T in stimulating spermatogenesis and is able to induce all stages of spermatogenesis in Japanese eel, *Anguilla japonica* (Miura et al. 1991). In salmon, T stimulates testis growth (Berglund 1992) and is important for the feedback regulation of the GTHs in the pituitary (Antonopoulou et al. 1999a; Antonopoulou et al. 1999b).
The androgen levels in the blood change during the course of the reproductive season. Levels are low during the immature state and they start to rise as spermatogenesis is initiated (Mayer et al. 1990; Maugars and Schmitz 2008). T and 11-KT gradually increase in the blood as spermatogenesis proceeds, they peak during the pre-spawning stage and decline at spawning (Mayer et al. 1990). Another sex steroid produced by the testes is the progestogen 17α,20β-dihydroxi-4-pregnen-3-one, which peak at spermiation, and is important for final maturation and spawning (Planas and Swanson 1995). The estrogen 17 β-estadiol (E2) is also present in the blood of male salmonids (Mayer et al. 1990; Vizziano et al. 1996). The exact role E2 plays in male maturation in fish is not fully known, but it has been shown to be involved in steroidogenesis regulation and spermatogonial stem cell renewal (for review see Schulz et al. 2010).

The sex steroids exert their effects by binding to their specific nuclear receptors (Mangelsdorf et al. 1995), but also non-genomic receptors are important mediators of the effects of sex steroid in vertebrates (Boonyaratanakornkit and Edwards 2007). Ligand bound nuclear receptors directly regulate transcription by binding to specific response elements in the promoter region of a gene. Two genomic androgen receptors, ARα and AR β, have been identified in rainbow trout, but only the α-variant is activated by T and, to a lesser extent, 11-KT (Takeo and Yamashita 1999; Takeo and Yamashita 2000). An androgen receptor preferentially activated by 11-KT have been found in stickleback, Gasterosteus aculeatus (Olsson et al. 2005). Several nuclear estrogen receptors are present in teleosts (Hawkins et al. 2000; Filby and Tyler 2005), including Atlantic salmon (Rogers et al. 2000) and rainbow trout that possess two subtypes, ERα and ERβ (Nagler et al. 2007). Moreover, also novel non-genomic steroid receptors are found in fish (for review see Thomas et al. 2006).

Some androgens including T, but not 11-KT, can be converted (aromatized) to estrogens by the enzyme aromatase. The tissue-specific effects of T can therefore be esterogenic, through ER signaling, rather than anderogenic. Tissues that display high aromatase activity in salmon are brain and pituitary (Andersson et al. 1988) and aromatization of T has important reproductive functions e.g. the feedback effects of T on LH have been shown to be largely aromatase dependent (Crim et al. 1981; Antonopoulou et al. 2009).

**Interactions energy balance and reproduction**

The activation and regulation of the gonadotrope axis is influenced by a number of internal and external factors. Environmental cues such as photoperiod, water temperature and food availability influence timing of puberty as do internal peripheral metabolic factors conveying information of growth rates, nutritional status and the size of energy deposits to the reproductive centers in the brain. Internal assessment
of the size of energy stores allow for the proper timing of puberty onset, to ensure that the cost of attaining full reproductive capacity and production of offspring can be met. Growth and lipid accumulation is seasonal in salmon, as for other fish species in temperate regions that experience large variations in food availability and water temperatures over the year. In salmon, the size of lipid stores or the rate of lipid store accumulation during crucial times of the year strongly influences decision to mature or not (Rowe et al. 1991; Silverstein et al. 1998; Shearer and Swanson 2000). The proportion of early maturing salmon males in the fall can be reduced by restricted feeding during the growth season in spring, preceding the start of gonadal growth, demonstrating this to be a crucial time for the decision to mature or not (Rowe and Thorpe 1990b; Berglund 1992). In mammals, information regarding the size of adiposity stores is mediated to the brain mainly by the adipocyte-derived hormone leptin, which acts as a permissive signal for puberty onset (Barash et al. 1996) and modulates the BPG axis at all levels (Tena-Sempere 2007). If these are also the functions of this hormone in teleosts is not yet known. Considering the strong link between nutritional status and sexual maturation in Atlantic salmon, this species provides a useful model for studying not only the dynamics of metabolic factors, such as leptin, during rapid changes in nutritional status and growth, but also the role of these factors in sexual maturation.

The leptin system

Leptin is a 16-kD peptide hormone belonging to the cytokine family of proteins that was first discovered in mouse in 1994 as the product of the ob gene (Zhang et al., 1994). In mammals, leptin is produced primarily, although not exclusively, by adipose tissue. Circulating levels generally reflect the amount of stored body fat thereby acting as an adiposity signal to the brain (Maffei et al. 1995; Considine et al. 1996; Havel et al. 1996). Leptin is a crucial factor in energy homeostasis, body weight regulation and reproduction in mammals, but is also involved in a number of other physiological processes such as immunity, bone formation, thermogenesis and angiogenesis (Ahima and Flier 2000; Margetic et al. 2002).

In 2005, Kurokawa et al. identified a leptin gene for the first time in the teleost species pufferfish (Takifugu rubripes). Leptin genes have since been characterized in many different fishes (e.g. Huising et al. 2006; Murashita et al. 2008; Gorissen et al. 2009; Frøiland et al. 2010; Zhang et al. 2013) including Atlantic salmon (Rønnestad et al. 2010). Mammals express only a single leptin ortholog which is highly conserved across mammalian species (Zhang et al. 1994; Ahima and Osei 2004), while many fishes express two or more leptin genes (Gorissen et al. 2009; Kurokawa and Murashita 2009; Rønnestad et al. 2010; Angotzi et al. 2013). Two quite divergent variants of the leptin gene, lepa and lepb, are present in the teleost lineage, which is believed to be the consequence of a whole genome duplication event during the
early evolution of teleost fishes (Gorissen et al. 2009; Kurokawa and Murashita 2009). Two closely related paralogues of the *lepa* gene (*lepa1* and *lepa2*) have been identified in common carp, *Cyprinidae carpio* (Huising et al. 2006), Atlantic salmon (Rønnestad et al. 2010) and goldfish, *Carassius auratus* (Tinoco et al. 2012) which is thought to be due to a more recent genome/gene duplication event as indicated by their much higher sequence identity (Londraville et al. 2014). Few species have so far been shown to possess several paralogues of both leptin gene types, but Atlantic salmon express four leptin genes, *lepa1*, *lepa2*, *lepb1* and *lepb2* (Rønnestad et al. 2010; Angotzi et al. 2013). The nucleotide and amino acid sequence similarities between the mammalian leptins and the teleost leptins, as well as between species within the teleost group, is low; e.g. pufferfish leptin is only 13 % identical to human leptin (Kurokawa et al. 2005) and only 16 and 19 % similar to Atlantic salmon *lepa1* and *lepa2* respectively (Rønnestad et al. 2010). Despite the sequence divergence of the different leptins, the tertiary structure of the protein as well as the gene arrangement and gene synteny seems to be highly conserved in the different vertebrate lineages (Kurokawa et al. 2005; Gorissen et al. 2009; Won et al. 2012).

In contrast to mammals, leptin is predominantly expressed by the liver in many fishes (Kurokawa et al. 2005; Murashita et al. 2008; Won et al. 2012; Gong et al. 2013b). However, also other tissues, both centrally and peripherally (e.g. brain, pituitary and gonads), express leptin albeit at lower levels, and species differences are common (Gong et al. 2013b; He et al. 2013; Tang et al. 2013), and when two leptin paralogues are present, they often display a differential tissue expression pattern (Kurokawa and Murashita 2009; Angotzi et al. 2013; Zhang et al. 2013).

**The leptin receptor**

Leptin exerts its biological action through binding to the leptin receptor (lepR). LepR is a membrane spanning receptor belonging to the class-1 cytokine receptor family (Tartaglia 1997). There are six splice variants characterized in mammals of which only the long form contains the intracellular domain necessary for signal transduction. The long form of the receptor is expressed predominantly in the hypothalamus in mammals, while the shorter isoforms, lacking the intra- and transcellular segments, have a more ubiquitous expression pattern in central and peripheral tissues. The shorter isoforms, although not able to elicit intracellular signaling, also play important roles in leptin transportation, turnover, and serve as leptin reservoirs in the blood and tissues. The soluble isoforms of LepR act as leptin binding proteins and can bind up to 60-98 % of circulating leptin and are crucial modulators of the bioavailability of leptin to the tissues thereby regulating the actions of leptin (Sinha et al. 1996).

The long form of the leptin receptor (*lepr*) gene has been identified in a number of teleost species, e.g. pufferfish (Kurokawa et al. 2008), Japanese medaka (*Oryzias*
latipes) (Kurokawa and Murashita 2009), zebrafish, Danio rerio (Liu et al. 2010),
goldfish (Tinoco et al. 2012), yellow catfish, Pelteobagrus fulvidraco (Gong et al. 2013b) orange-spotted grouper, Epinephelus coioides (Zhang et al. 2013) and Atlantic salmon (Rønnestad et al. 2010). Several shorter isoforms of the receptor exist also in teleosts (Rønnestad et al. 2010; Cao et al. 2011) of which at least some act as leptin binding proteins in the blood (Gong et al. 2013a). Despite low leptin and leptin receptor sequence identity between mammals and teleost fish as well as between fish species, there is evidence for co-evolution of leptin and the leptin receptor which has preserved the binding efficiency of leptin to the receptor, although the binding efficiency varies between the different leptin paralogues (Prokop et al. 2012).

Role of leptin in energy homeostasis and metabolism
Leptin is a key regulator of body weight, appetite and metabolism. It signals the size of fat stores to the brain and thereby functions as an adipostat. This means that when fat stores are high, so is plasma leptin levels and vice versa; low fat stores lead to low plasma leptin levels. Therefore, under high adiposity levels leptin signaling increases and acts as an anorexigen i.e. leptin inhibits food intake centrally by affecting the appetite centers in the hypothalamus. It also regulates peripheral metabolism to increase energy expenditure in order to, in the long term, return adiposity levels to a previous set point. Mouse strains homozygote for the mutated version of the ob gene, the so called ob/ob mouse, are unable to produce bioactive leptin and are therefore in a perceived state of starvation, become overweight, hyperphagic and infertile (Chehab et al. 1997; Mounzih et al. 1997). Leptin administration to ob/ob mice restores reproductive ability, decreases food intake, increases metabolism and leads to weight loss (Zieba et al. 2005).

The initial belief was that the physiologically important effects of leptin were related to negative feedback effects in regulating body weight and hence being a crucial factor in e.g. obesity treatment (reviewed by Ahima and Osei 2004). However, the role for leptin in energy balance regulation has proven to be much more complicated than first envisioned. Obese conditions are often characterized by central leptin resistance, which do not respond to leptin treatment. Under normal conditions it has been suggested that leptin is far more important as a signal of short term starvation and long term body fat deficiency, rather than as a signal for excess body fat (Ahima 2000; Ahima and Osei 2004). This makes sense from an evolutionary perspective considering that animals more often experience periods of food deprivation rather than food excess. Circulating leptin levels decline during fasting and caloric restriction in mammals, and this decline is out of proportion to any loss in body fat (Boden et al. 1996). Decreased leptin signaling to the appetite centers of the brain stimulates orexigenic pathways while inhibiting anorexic pathways leading to an increase in
food intake and food searching behavior. Low leptin levels during fasting also cause a slowing down of metabolism to conserve energy, that are physiologically very important changes for endothermic animals that cannot normally survive extended periods without food. Leptin is thereby a crucial mediator of the adaptive response to fasting in mammals (Ahima 2000).

A less studied aspect of leptin is its role in seasonal mammals that go through periods of substantial fattening followed by a period of prolonged fasting, such as hibernating mammals and mammals with wither sleep. Although leptin levels reflect the size of adiposity store under normal conditions, an uncoupling of leptin levels and size of adiposity stores occur during the period of excessive food intake and body fat accumulation as seen in e.g. the raccoon dog, *Nyctereutes procyonoides* (Nieminen et al. 2002) and little brown bat, *Myotis lucifugus* (Kronfeld-Schor et al. 2000). This uncoupling is likely an adaptation to conditions where an increase in adiposity concomitant with an increase in leptin levels would cause an anorectic effect that would prevent the accumulation of energy stores necessary to survive the seasonal fasting period (Reidy and Weber 2000). During the winter sleep period in the raccoon dog, which can be considered a long starvation period, leptin levels are again elevated even as fat stores are being depleted (Nieminen et al. 2002). Fat is the main fuel during the winter fast and the proposed role for elevated leptin during this time was that leptin, probably in combination with other lipolytic factors such as growth hormone (GH), facilitates the mobilization of these fat stores. Even though the main effect of leptin on metabolism and adipose stores is considered to be indirect through its central effects on appetite and food intake (Prieur et al. 2008), leptin has also been shown to directly influences metabolic pathways in peripheral tissues to modulate fat metabolism (Mistry et al. 1997; Cohen and Friedman 2004) and high leptin concentrations enhance mobilization of fat (Reidy and Weber 2000).

**Leptin and energy balance in teleosts**

Numerous studies over the last decade show that the leptin system is sensitive to changes in energy state also in fish. However, the exact physiological functions of leptin in the regulation of energy balance in teleosts are still far from understood. Reports regarding fasting and restricted feeding experiments in different fishes do not paint a homogenous picture in the response of leptin to these conditions (see table 1). Considering the diversity of different components of the leptin that might have acquired species-specific functions, it has been difficult to compare results (Londraville et al. 2014). In long term fasting and restricted feeding experiments, leptin expression in the liver and leptin plasma levels has been shown to respond by increasing (Kling et al. 2009; Frøiland et al. 2012; Fuentes et al. 2012), decreasing (Johnson et al. 2000; Nieminen et al. 2003) or remaining unchanged (Huising et al. 2006; Kobayashi et al. 2011). In salmonids, feed deprivation and fasting in most
### Table 1. Effects of feeding, fasting and feed restriction, selected studies on teleosts

<table>
<thead>
<tr>
<th>Species</th>
<th>Experimental set-up</th>
<th>Expression level</th>
<th>Effect on leptin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burbot (<em>Lota lota</em>)</td>
<td>2 weeks fasting at 2 °C protein</td>
<td>protein</td>
<td>↓</td>
<td>Nieminen et al. 2003</td>
</tr>
<tr>
<td></td>
<td>2 weeks fasting at 10 °C protein</td>
<td>protein</td>
<td>→</td>
<td></td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>3 weeks fasting</td>
<td>protein</td>
<td>↑</td>
<td>Kling et al. 2009</td>
</tr>
<tr>
<td>Fine flounder</td>
<td>4 weeks fasting</td>
<td>protein</td>
<td>↑</td>
<td>Fuentes et al. 2012</td>
</tr>
<tr>
<td></td>
<td>Re-feeding</td>
<td>protein</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Common carp</td>
<td>4-6 h fasting mRNA lep1/lep2</td>
<td>→/→</td>
<td></td>
<td>Huising et al. 2007</td>
</tr>
<tr>
<td></td>
<td>6 weeks fasting mRNA lep1/lep2</td>
<td>→/→</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic salmon (adult)</td>
<td>10 months fasting protein</td>
<td>→</td>
<td></td>
<td>Rønnestad et al. 2010</td>
</tr>
<tr>
<td></td>
<td>mRNA lep1/lep2</td>
<td>→/↑</td>
<td></td>
<td></td>
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<tr>
<td>Arctic charr</td>
<td>10 weeks fasting mRNA lep1/lep2</td>
<td>→/↑</td>
<td></td>
<td>Frøiland et al. 2012</td>
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<tr>
<td></td>
<td>protein</td>
<td>→</td>
<td></td>
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</tr>
<tr>
<td>Zebrafish</td>
<td>1 week fasting mRNA lep1/lep2</td>
<td>→/↓</td>
<td></td>
<td>Gorissen et al. 2009</td>
</tr>
<tr>
<td>Goldfish</td>
<td>1 week fasting mRNA</td>
<td>→</td>
<td></td>
<td>Tinoco et al. 2012</td>
</tr>
<tr>
<td></td>
<td>2 weeks overfeeding mRNA</td>
<td>→</td>
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</tbody>
</table>

1. Protein indicates plasma levels and mRNA indicate hepatic expression
2. ↑ indicate up-regulation, ↓ indicate down-regulation and → indicate no changes
studies results in increasing leptin levels. Plasma leptin levels in rainbow trout, *Oncorhyncus mykiss*, increase during three weeks of fasting (Kling et al. 2009), hepatic *lepa1* gene expression levels in Arctic charr (*Salvelinus alpinus*) increase during feed restriction and are quickly returned to normal after re-feeding, while *lepa2* expression and plasma levels are unchanged (Frøiland et al. 2012) and a 10 month feed restriction in Atlantic salmon, up-regulates hepatic *lepa2* while *lepa1* and plasma leptin levels remain unchanged (Rønnestad et al. 2010). Leptin did not correlate positively with adiposity or condition factor in any of these studies and it has been argued that leptins role as an adiposity signal may not be conserved in fish (Frøiland et al. 2012; Londraville et al. 2014). A function of leptin that might be conserved between fish and mammals, however, is as the role of an anorexigen. Treatment with recombinant trout leptin decreased food intake and gene expression of the orexigenic factor neuropeptide Y (*npy*) while up-regulating the anorexic factors pro-opiomelanocortin a1 and a2 (*pomc-a1* and *pomc-a2*) in the brain of rainbow trout (Murashita et al. 2008), In Atlantic salmon, long term treatment with recombinant salmon leptin-A1 reduces growth and up-regulated the gene expression of *pomc-a1* while not having an effect on *pomc-a2* or *npy* (Murashita et al. 2011).

**Leptin in reproduction**

Reproduction is an energetically demanding event and the metabolic status of an organism is therefore important for puberty initiation as well as for sustained reproductive function. Leptin, being the principal peripheral adiposity signal in mammals, is therefore a crucial link between reproduction and energy status. Certain threshold levels of leptin are necessary for the onset of puberty and leptin thereby acts as a permissive signal, rather than a trigger, for this developmental event (Barash et al. 1996). The absence of functional leptin prevents pubertal development and impairs reproductive function as seen in the leptin deficient *ob/ob* mouse (Chehab et al. 1997). Failure of entering into puberty or loss of reproductive function due to low leptin levels, as is seen in cases of very low adipose stores, or non-functional leptin can be reversed by leptin administration, demonstrating the key role of this hormone in reproductive physiology (Chehab et al. 1996). Leptin modulates all levels of the BPG axis and plays multiple roles in reproduction in mammals including regulation of GnRH, gonadotropins, gonadal function, sustained fertility and pregnancy (Casabiell et al. 2001; Tena-Sempere 2007).

The leptin receptor is expressed at all levels of the reproductive neuroendocrine axis (Tartaglia et al. 1995; Zamorano et al. 1997). The primary target of leptin in mammals is the hypothalamus where the long form of the receptor is found in key areas in regard to reproductive and energy balance. One of the key effects of leptin on the BPG-axis is the activation of the GnRH-neurons (Donato et al. 2011). Experiments *in vitro* and *in vivo* have demonstrated that leptin acts at the level of the hypothala-
mus to stimulate GnRH secretion (Yu et al. 1997; Lebrethon et al. 2000; Parent et al. 2000; Watanobe 2002). But the GnRH-neurons do not express lepr and it is now understood that the stimulatory effect of leptin is mainly indirect by stimulating other hypothalamic circuits such as the kisspeptin system and by interacting with neuropeptides in the appetite control centers (for review see Tena-Sempere 2007; Hill et al. 2008).

In addition to the hypothalamic effects of leptin, the leptin receptor is expressed in the gonadotropes in the pituitary of mammals (Jin et al. 1999; Iqbal et al. 2000). Direct stimulatory effects of leptin on GnRH stimulated LH secretion at the pituitary level both in vivo and in vitro have been demonstrated in rats (Yu et al. 1997), although in another in vitro study this stimulatory effect was absent (Tena-Sempere et al. 2000). The importance of the role of leptin in regulating the BPG axis at the pituitary levels is still not fully known (Tena-Sempere 2007). In adult rat testes, leptin inhibits basal and stimulated T secretion in vitro (Tena-Sempere et al. 1999) and in rat Leydig cells in vitro (Caprio et al. 1999), an effect that was mediated by leptin down-regulation of key enzymes in the steroidogenic pathway. In addition, leptin inhibits also other gonadal factors and high leptin levels seems to impair gonadal function which has been proposed as an explanation for the gonadal reproductive dysfunction experienced by obese individuals (Agarwal et al. 1999; Merhi et al. 2013).

**Crosstalk between the leptin system and the gonadotropic axis**

Not only do leptin modulate all levels of the BPG-axis, but there is a crosstalk between the leptin system and the reproductive endocrine axis. Gonadal sex steroids in turn regulate the leptin system centrally as well as in peripheral tissues. Plasma leptin levels are sexual dimorphic in mammals which has been suggested to be due to gender differences in sex steroid levels (Demerath et al. 1999; Messinis et al. 1999). During puberty in humans, leptin levels increase concomitantly with the initial increase in gonadal estrogen in girls, while plasma leptin levels decline as testosterone production starts in boys (Garcia-Mayor et al. 1997; Mantzoros et al. 1997). In adults, women have substantially higher plasma leptin levels than men even after correcting for total body fat and body mass index, both of which are normally higher in females (Saad et al. 1997). E2 has been confirmed to be a potent stimulator of leptin gene expression and leptin secretion in adipose tissue in females both in vitro (Machinal-Quelin et al. 2002) and in vivo (Brann et al. 1999; Machinal et al. 1999). Studies have also shown that the stimulatory effect on leptin is specific to E2 and that other estrogens as well as progesterone fail to have any effect on leptin levels (Casabiell et al. 2001). The leptin gene promoter region in mammals contains an estrogen response element (ERE) and leptin transcription is activated directly by the E2-ER complex binding to the ERE’s (Machinal et al. 1999), but ligand bound ERs regulates transcription also at promoter sites other than the ERE’s.
as well as through non-genomic actions (Gambino et al. 2010). Evidence for the ability of estrogen to regulate also the leptin receptor in adipose tissue and hypothalamus have been reported (Meli et al. 2004).

**Leptin and reproduction in fish**

Few studies on leptin and its possible involvement in reproduction in fish have been published to date and there is a distinct gap of knowledge within this field. No functional studies have yet been performed to investigate any direct effects of leptin in the hypothalamus. There are, however, a few *in vitro* studies investigating the ability of leptin to regulate the gonadotropins directly at the levels of the pituitary, as is seen in mammals. Using European sea bass (*Dicentrarchus labrax*) primary pituitary cultures, Peyon et al. (2001) showed that mammalian recombinant leptin stimulates LH secretion *in vitro* with different potency depending on the developmental stage of the fish. Mammalian recombinant leptin was also able to increase LH as well as FSH secretion in primary culture of rainbow trout pituitary cells, but only at post-pubertal stages in both sexes (Weil et al. 2003). Although these studies used recombinant mammalian leptins at supraphysiological concentrations, they still provide some evidence to leptin being involved in the regulation of the gonadotropins directly at the level of the pituitary.

There are several studies indicating that leptin might be important during later stages of the maturational process in fish. Peak hepatic leptin gene expression coincide with attainment of sexual maturation in late fall in Arctic charr (Frøiland et al. 2010). This salmonid species, like Atlantic salmon, invest considerable energy into gonadal growth and peak leptin levels coincided with declining fat stores. Comparable results have also been found in other fishes such as ayu (*Plecoglossus altivelis*), where circulating leptin levels were elevated in both sexes during and after spawning compared to pre-spawning levels, coincided with loss of appetite and high sex steroid levels (Nagasaka et al. 2006). Leptin gene expression, leptin plasma levels as well as E2 and ER’s were increased in female chum salmon (*Oncorhynchus keta*) as a response to being transferred from seawater to freshwater, mimicking the conditions for upstream migration to reach the spawning grounds during which time they naturally reach sexual maturation (Choi et al. 2014). In the studies in ayu and chum salmon it was suggested that leptin might be stimulated by high sex steroid levels. The promoter region for the leptin gene (*lepa*-type) in grass carp contains a activator protein 1 (AP1 ) site (Li et al. 2010), and the promoter region of both grass carp leptin (Li et al. 2010) and Chinese perch leptin (He et al. 2013) contains a special protein 1 (SP1) site. ER’s do not directly bind to these sites, but ER enhances the activities of other transcription factors that do bind (Kushner et al. 2000). This provides evidence that both E2 and T, though its aromatization to E2, could potentially affect leptin transcription in fish.
AIMS OF THESIS

The overall aim of the thesis has been to investigate how different components of the leptin system are regulated by sexual maturation and changes in energy balance in fish, using male Atlantic salmon parr as a model. The specific aims were:

- To characterize the tissue distribution of the two leptin-a paralogues and the long form of the leptin receptor in juvenile Atlantic salmon parr (Paper I).

- To investigate the effect of feed restriction on the leptin system in one-year-old Atlantic salmon parr during rapid changes in growth and metabolism in spring (Paper I and II).

- To characterize the seasonal expression profile of the leptin system at different levels of the BPG axis during gonadal maturation in one-year old early maturing male Atlantic salmon parr under either an ad libitum or a restricted feeding regime (Paper II).

- To study sex steroid regulation of the leptin genes in the liver in vitro using primary hepatocyte cultures from Atlantic salmon (Paper III).

- To study the effects of the main teleost androgens on leptin and the leptin receptor in the liver and at different levels of the BPG axis in immature Atlantic salmon males (Paper IV).
MATERIALS AND METHODS

Experimental animals

The fish used in this thesis were one- or two-year old Atlantic salmon parr provided either by the Norrfors hatchery (63°N, 19°E) (Paper I) or the SLU Fishery Research Station in Älvdal (60°N, 17°E) (Paper II-IV) in Sweden. The fish were reared under natural photoperiod and through flowing water at ambient temperatures. Prior to sampling food was withheld for 24 h. Fish were anesthetized using metomidate hydrochloride (Aquacalm, Syndel Co., Vancouver, Canada). Tissue samples for gene expression analysis were always collected in RNAlater® solution except for brains in Paper I which were flash frozen in liquid nitrogen. Blood was collected via the caudal vein, kept on ice until centrifugation and centrifuged at 3000g for 5–10 min after which plasma was removed and stored at -80 °C until analysis. Fish were killed by decapitation and bodies were stored in -80° C until body lipid analysis. All animal experimentation was approved by the local committee of the Swedish Board for Laboratory Animals

Treatments

The experiments in Paper I and II were carried out at the Norrfors hatchery and SLU Fishery Research Station in Älvdal, respectively. Fish were fed either to satiation or a restricted diet for seven weeks (Paper I) or for five months (Paper II). Restricted feeding treatments were either 40 % of control or 50 % of fully fed control fish. Feed levels were calculated for optimized growth in the control groups and rations were adjusted throughout the trial period to correct for changes in fish number and size in both tanks. Commercial salmon feed was used for all experiments (Aller Aqua, Denmark or BioMar A/S Denmark). For experiments in Paper III and IV, fish were transported to a fish holding facility at Uppsala university were they were kept in 1 m³ tanks under simulated natural photoperiod, in 10 °C municipal water and left to acclimatize for at least 10 days before experiments were carried out.

For androgen treatments in vivo, one-year old fish were implanted in the abdominal cavity through a small e. 1.5 mm incision with a passive integrated transponder (PIT) tag, for individual identification, together with a Silastic capsule (5 mm length, 1.2 mm outer diameter, Silcrear™) filled with 11-ketoandrostenedione (11-KA) (4-androstene-3,11,17-trione; Sigma), T or empty capsules sealed with silicone glue at both ends.
**Body lipid content**

Total body lipid content including viscera (Paper I) or without viscera (Paper II) was measured using infrared transmission (MIT) spectroscopy technique (Elvingson and Sjaunja 1992). This was done using the Miris DMA instrument (Miris AB, Uppsala, Sweden) calibrated for fish tissue according to the manufacturer’s instructions.

**Relative gene quantification**

Tissue extraction of total RNA was performed using TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers’ protocol. Samples were DNase treated using TURBO DNase-free™ kit (Ambion) to remove genomic contamination. Total RNA was measured spectrophotometrically using Nanodrop (Thermo Fisher Scientific, Delaware, USA) and cDNA synthesis was performed by reverse transcription (SuperScript™ III Reverse Transcriptase, Invitrogen) and random primers. Relative levels of gene expression were measured by real-time quantitative polymerase chain reaction (qPCR) on the iCycler PCR detection system and a Sybr green I dye kit (Bio-Rad, Hercules, USA) or the Mx3000P real-time PCR machine (Stratagene, La Jolla, CA, USA) and the Brilliant II-III SYBR Green QPCR Master Mix (Agilent technologies, Stratagene). Gene specific primers were designed using the Primer3 software. Standard curves were generated by pooling cDNA from the RT reactions and spanned at least three orders of magnitude. $R^2$ values were consistently $>0.98$ and efficiencies $>93\%$. Samples were always run in duplicates and either whole standard curves or calibrator points in duplicate were included on each plate as well as a no temple control in duplicate containing water instead of sample. The specificity of the amplified product was verified by melting curve analysis after the amplification reactions. 18s rRNA or ef1a was selected as reference genes for relative gene expression data normalization.

**Radioimmunoassay**

Plasma leptin levels were measured according to a homologous salmonid radioimmunoassay (RIA) protocol established by Kling et al. (2009) (Paper I and IV). The assay is based on a 14 amino acid long sequence, identical between Atlantic salmon LepA1 (sLepA1) and rainbow trout leptin and polyclonal antibodies raised in rabbit against the leptin peptide fragment. The assay exhibits measuring parallelism for a range of fish species apart from rainbow trout and Atlantic salmon including Arctic charr, Atlantic cod and turbot. No reactivity between the sLep antibody and other antigens including GH, IGF-I, CCK8, ghrelin and GRP has been detected. Although designed to measure sLepA1, it is possible that the RIA detects both sLepA1 and sLepA2 due to high amino acid sequence identity between the two peptides. Because
of the limited amounts of plasma obtained from one-year old salmon, plasma samples were pooled in order to obtain the volumes needed for the RIA measurements.

**Primary hepatocyte protocol**

Hepatocytes were isolated following the procedure of Mommsen et al. (1994) with some modifications. Livers were dissected out and placed into a sterile petri dish filled with Dulbecco’s phosphate buffered saline without CaCl₂ and MgCl₂ (DPBS) (Gibco® Life Technologies, Carlsbad, CA, USA) supplemented with antibiotic/antimycotic solution (Gibco® Life Technologies, Carlsbad, CA, USA). The livers were cut into smaller pieces and washed repeatedly with DPBS until blood was cleared. Liver digestion was performed by incubation at 25 °C for 30 min using collagenase solution (type IV, Sigma, Saint Louis, MO, USA). Enzyme reaction was stopped by removing collagenase solution and washing digested tissue with DPBS. Cells were dispersed by repeated passages through a plastic transfer pipette. Cells were then filtered through a fine 70 µm mesh filter and subsequently collected by low speed centrifugation. DPBS was removed and cells were re-suspended in fresh DPBS, counted and viability was assessed by the Trypan blue exclusion test. Cell viability was >90 % and yields were typically in the order of 1 x 10⁸ cells/g liver. Cells were then collected by low speed centrifugation and re-suspended in L-15 medium (Leibowitz) (Sigma, Saint Louis, MO, USA) supplemented with antibiotic/antimycotic solution. Cells were cultured on 96-well plates (Costar) coated with poly-L-lysine (Sigma), for increased cell attachment, at 16 °C under plain air. A total volume of 125 µl cell suspension was added to each well and cell density was 1x 10⁵ cells/well. Cells were allowed to adhere for approximately 36 hours prior to treatment. At start of treatment medium was changed to fresh medium (final volume was 250 µl/well) with or without hormone treatment. Medium was changed on day three and treatments lasted for up to six days. Cultures were terminated by placing the plates on ice, removing the medium and RNA was extracted from the cells using TRIzol Reagent. Samples were then frozen in -80 °C until further analysis.
RESULTS

**Paper I**

Tissue expression profiles of both leptin-a genes and the long form of the leptin receptor were characterized in juvenile male Atlantic salmon parr. Both lepa1 and lepa2 were mainly, but not exclusively, expressed in the liver. The gene expression pattern differed between the two paralogues where lepa1 was also expressed in the brain, pituitary, gills, heart and muscle, and lepa2 expression was found in muscle and heart. No expression of either paralogue was detected in adipose tissue. Leptin receptor gene expression was localized to brain and pituitary as well as ubiquitously in all peripheral tissues investigated. The highest lepr expression levels were seen in testis, brain, kidney and heart.

Effects of 4 and 7 weeks of feed restriction (40 % of control) on the gene expression of lepa1 and lepa2 in the liver, the leptin receptor in the brain and the plasma leptin levels were investigated in juvenile male salmon during the rapid growth phase in spring (late April through mid-June). Feed restriction resulted in fish that were significantly shorter, weighed less and had less body fat compared to fully fed control fish at the end of the experiment. Lepa1 gene expression in the liver did not change during the study period in the fully fed control fish, while lepa2 decreased significantly by 16-fold compared to initial levels. Feed restriction resulted in a 20-fold increase in lepa1 expression levels after 7 weeks, and expression levels were at this time 10 times higher compared to control fish. Lepa2 expression decreased over the study period also in the feed restricted fish, but were still higher (7-fold) compared to controls after 7 weeks. Plasma leptin levels decreased slightly but not significantly from April through June in the control group. In contrast, plasma leptin levels in the feed-restricted group were 2.3 times higher compared to controls after 7 weeks. Plasma leptin levels significantly correlated with hepatic lepa1 mRNA expression in the restricted feed group ($r^2 = 0.77$), but not in the control group, in June. Neither lepa1 nor lepa2 was correlated to total body lipid content, and there was a negative correlation between plasma leptin levels and body lipid content in the control group in June ($r^2 = -0.57$). Lepr expression in the brain remained stable in both groups during the first four weeks of the study, but were significantly higher in the control group by 1.6 times compared to the feed restricted fish in June.

**Paper II**

Seasonal gene expression changes of lepa1, lepa2 and lepr were investigated during early sexual maturation in one-year old male Atlantic salmon parr under fully fed (control) and feed restricted conditions from April through September. Both lepa1 and lepa2 in the liver, and lepr in the brain, were significantly down-regulated in non-maturing control males during early spring. This period marked the seasonal
start of growth and fat accumulation. In the maturing control males, hepatic leptin expression increased during mid-spermatogenesis (July) compared to the non-maturing males, and at final maturation in September lepa1 and lepa2 mRNA levels were up-regulated by 7.7 and 49 times respectively. The increase of the two lepa genes in the maturing control males was preceded by a rapid increase in gonadal growth and high fsh-β expression levels in the pituitary, whereas peak lepa levels coincided with peak pituitary lh-β gene expression and the presence of running milt in the testis. In the testis of the maturing control males, lepr gene expression was significant up-regulation from mid- to late spermatogenesis compared to non-maturing fish as well as to initial levels in April. No differences in lepr gene expression in the brain or pituitary were seen between maturing and non-maturing fish.

Restricted feeding resulted in a dramatic increase in gene expression of both lepa genes in the liver and the leptin receptor in the pituitary from May through July and was independent of maturational stage. Feed restriction decreased the incidence of sexual maturation by 53 %. The maturational process was delayed in the feed restricted maturing males, as indicated by a delayed increase in lh-β expression levels in the pituitary and reduced gonadal growth and absence of running milt in September. Significantly higher lepa1 and lepa2 expression was seen in the maturing feed restricted males in August, one month later than for the maturing fish in the control group. The final lepa1 expression peak in September seen in the maturing control males was not observed in maturing feed restricted fish, although lepa2 levels remained higher. Also in the feed restricted group, lepr gene expression in the testes was up-regulated compared to feed restricted non-maturing males.

Lepa1 and lepa2 gene expression in the liver was highly correlated in both groups, and were also correlated with the lepr in the pituitary. In the feed restricted group, hepatic lepa1, lepa2 and pituitary lepr were all negatively correlated with body lipid content.

**Paper III**

This study investigated the direct effects of different sex steroids (T, 11-KT and E2) on the hepatic gene expression of lepa1 and lepa2 in vitro using primary cultures of Atlantic salmon hepatocytes. Testosterone (T) stimulated lepa1 and lepa2 in a dose-dependent manner after four days incubation. The non-aromatizable androgen 11-KT was generally equally potent as T in stimulating lepa1 and lepa2 gene expression. E2 treatment was more potent in stimulating the expression of both leptin genes and required lower doses. 10 nM E2 significantly up-regulated lepa1 by 1.9-fold and 1 nM up-regulated lepa2 by 1.5-fold compared to controls.

T, 11-KT and, more strongly, E2 stimulated lepa2 gene expression in a time-dependent manner over a 6 day period in hepatocytes from mature males. Lepa1 gene expression was on the other hand not stimulated by T and 11-KT, and was only
weakly stimulated by E2 over the treatment period. The two leptin-a paraloques responded differently to steroid treatment depending on developmental stage. T and 11-KT stimulated lepa1, but not lepa2, in both immature male and immature female parr, while E2 stimulated expression of both genes. In maturing males, the androgens and E2 stimulated lepa2 and not lepa1, while in immature males, the androgens and E2 stimulated lepa1, but only E2 stimulated lepa2. Co-incubation with the aromatase inhibitor fadrozole did not ablate the stimulatory effect of T on leptin expression.

**Paper IV**
The aim of this study was to explore the long term effects of 11-KA and T treatment on the leptin system in vivo in juvenile one-year old male Atlantic salmon parr. Treatment with 11-KA and T increased gonadosomatic index significantly. In the pituitary, 11-KA treatment down-regulated fsh-β and up-regulated lh-β mRNA levels, while T up-regulated both fsh-β and lh-β mRNA levels. T also induced a 5.5-fold lepa1 and a 4.2-fold lepr expression increase in the pituitary, while 11-KA did not have any effect. Lepr expression in the gonads was not effected by androgen treatment. In the liver, T treatment significantly stimulated lepa1 mRNA levels, but had no effect on lepa2, and 11-KA did not affect hepatic gene expression of either gene. Plasma leptin levels did not differ significantly between treatments.
DISCUSSION

Tissue expression profiles

The Atlantic salmon parr *lepa1* and *lepa2* genes were predominantly expressed in the liver of juvenile males (*Paper I*). These results are in agreement with previous reports in fish where liver seems to be the primary, although not the only, organ for leptin expression (pufferfish, Kurokawa et al. 2005; common carp, Huising et al. 2006; zebrafish, Gorissen et al. 2009; Japanese medaka, Kurokawa and Murashita 2009; yellow catfish, Gong et al. 2013b; Chinese perch, He et al. 2013). *Lepa1* and *lepa2* were differentially expressed at lower levels also in a few other tissues. Differential tissue expression patterns are commonly seen in species with two or more leptin paralogues (Huising et al. 2006; Rønnestad et al. 2010; Tinoco et al. 2012; Tang et al. 2013), which may indicate separate physiological functions for the two genes. The lack of expression in adipose tissue in the present study is notable, considering it being the main source of leptin in mammals (Zhang et al. 1994; Maffei et al. 1995). However, the absence of leptin expressed in adipose tissue is in agreement with many studies in teleosts (Murashita et al. 2008; Kobayashi et al. 2011; Won et al. 2012; Angotzi et al. 2013), although low transcript levels of leptin have been reported in visceral adipose tissue (Huising et al. 2006; Gong et al. 2013b; Zhang et al. 2013) and muscle (*Paper I; Huising et al. 2006; Rønnestad et al. 2010; Tang et al. 2013*), which can be a major fat storing site in fish. However, expression levels in the peripheral tissues are typically low compared to what is found in liver, making it likely that liver is a major source of circulating leptin, but does not exclude contribution from other tissues.

The gene expression of the long form of the leptin receptor was detected in the brain and pituitary as well as ubiquitously in peripheral tissues (*Paper I*), indicating a pleiotropic role for leptin in teleosts as has been suggested previously (Kurokawa et al. 2008; Liu et al. 2010; Rønnestad et al. 2010). Although four shorter isoforms (splice variants) of the leptin receptor has been identified in Atlantic salmon, it is only the long form that contains the intracellular domain necessary for full signal transduction (Rønnestad et al. 2010) in a similar manner as seen in mammals (Tartaglia 1997). Since many of the main effects of leptin in mammals (appetite regulation, body weight control and modulation of reproductive function) are mediated through binding to the long form of the receptor in the hypothalamus (Ahima and Osei 2004) the present results in salmon, along with other fish species, showing *lepr* expression in the brain indicate important central functions of leptin also in fish. Also pituitary and testes had high expression levels which is in line with what is seen in other teleosts (Kurokawa et al. 2008; Liu et al. 2010; Gong et al. 2013b) and...
are important sites for the actions of leptin in mammals (Tena-Sempere 2007; Ahima and Osei 2004).

Regulation of leptin by changes in energy balance

The seasonal decrease in hepatic leptin gene expression at the start of the growth season (April through June) coincided with increasing temperatures, weight gain and body fat accumulation in the male Atlantic salmon that were fed ad libitum (Paper I and II). The decrease in expression was also reflected by leptin plasma levels during spring/early summer (Paper I). Leptin expression levels remained low throughout the growth season until fall (Paper II). During winter, water temperatures are very low and juvenile salmon feed and grow very little if at all (Cunjak et al. 1998). If necessary, they live off energy reserves accumulated during the previous growth season, i.e. they are in a state of negative energy balance (Metcalfe and Thorpe 1992). When temperatures rise in the spring and food is available at sufficient levels, they quickly switch to an anabolic state (Paper I and II). This means that the decrease seen in leptin expression and plasma in male salmon fed ad libitum happen during a time when the fish go from a negative energy state to a positive energy state. Moreover, total body lipid content was inversely correlated with lepa2 in the liver, lepr in the brain (Paper II) and plasma leptin levels (Paper I). This is in contrast to the situation in mammals where circulating leptin levels generally correlate with the amount of stored body fat under normal conditions (Maffei et al. 1995; Havel et al. 1996). The absence of this positive association between leptin and adiposity in fish (Frøiland et al. 2012) indicates a different role for leptin other that as an adiposity signal.

Feed restriction resulted in an up-regulation of hepatic lepa1 and lepa2 gene expression and leptin plasma levels (Paper I and II), which is in agreement with a growing number of studies in teleosts. Fasting up-regulates leptin plasma levels in rainbow trout (Kling et al. 2009) and fine flounder, Paralichthys adspersus (Fuentes et al. 2012), and feed restriction up-regulates hepatic lepa1/mRNA levels, but not lepa2 mRNA levels or leptin plasma levels in the liver of Arctic charr (Frøiland et al. 2012). Reduced food intake due to long term exposure to high temperatures have also been shown to be accompanied by elevated sLepA1 plasma levels in Atlantic salmon (Kullgren et al. 2013). These are notable differences between fish and mammals since feed restriction and fasting in mammals causes leptin levels to decrease drastically (Ahima and Flier 2000). It should be kept in mind, however, that in studies in fish, plasma levels are not always investigated and it is presently not know if liver leptin expression is a good measure of plasma leptin levels. The RIA used in the present study (Paper I and IV) measures free plasma leptin levels and likely quantifies both leptin-a’s in salmon. In mammals, leptin binding proteins bind most of the circulating leptin, and the ratio of bound to free circulating leptin change
under different physiological conditions (Sinha et al. 1996). A recent study by Gong et al. (2013a) indicates that also in rainbow trout the presence of binding proteins is affected by changes in energy balance. This makes it difficult to estimate the amount of circulating leptin and in future studies it will be important to also consider the leptin binding proteins. That being said, it seems that, at least under some conditions, expression and plasma leptin levels are correlated and, considering the very low expression of leptin in peripheral tissues in salmon parr compared to liver expression levels, it appears that liver is an important source of leptin in this species (Paper I). It should also be noted that in some fish species, plasma leptin or leptin mRNA levels decrease during fasting/feed restriction similar to mammals (Nieminen et al. 2003; Gorissen et al. 2009; Won et al. 2012). These observed species differences in the response of leptin to fasting/feed restriction within the teleost group could be due to the great diversity environmental adaptations and life histories found between species which may have led to the evolution of novel functions for the different leptin paralogues that might not be the same in all species of fish (Won and Borski 2013).

The physiological significance of decreasing leptin levels during times of energy deposition and, conversely, increasing leptin expression and plasma leptin levels during feed restriction in teleosts is not fully understood. In mammals, leptin stimulates fatty acid oxidation and mobilize and deplete lipid stores (Reidy and Weber 2000; Cohen and Friedman 2004). Leptin has the ability of to affect peripheral metabolism also in fish (Londraville and Duvall 2002; Lu et al. 2012; Jørgensen et al. 2013). Moreover, leptin has been shown to act an anorexic signal in fish (Murashita et al. 2008; Li et al. 2010; Murashita et al. 2011). It has previously been hypothesized that increased leptin levels in fish species adapted to go through long periods of food deprivation, such as salmonids, might depress metabolism and appetite to avoid energy expenditure on food searching behavior when food availability is low while also facilitating the mobilization and use of stored energy as fuel (Frøiland et al. 2012; Fuentes et al. 2012; Fuentes et al. 2013; Jørgensen et al. 2013), which a mechanism for leptin also proposed in hibernating mammals (Kronfeld-Schor et al. 2000; Nieminen et al. 2002). This role for leptin would provide an interesting explanation for the present observations in the feed restricted fish (Paper I and II) and also for the maturing fish (see discussion below), but the effects of leptin on metabolism in teleosts are still far from understood and require further investigation.

The seasonal decrease in the brain expression of the leptin receptor in both fully fed and feed restricted fish indicates a decreased central sensitivity to the actions of leptin that is independent of the energy state of the fish (Paper II). This is in agreement with other studies in fish where lepr in brain was unaffected by feed restriction and fasting (Rønnestad et al. 2010; Tinoco et al. 2012). Lepr in the pituitary, on the other hand, was stimulated by feed restriction (Paper II), which is in line with what has been shown in common carp under feed restricted conditions (Bernier et al.
Moreover, the seasonal profile of lepr expression in the pituitary under feed restriction was similar to and correlated with both lepa1 and lepa2 expression in the liver. This suggests an increased sensitivity to leptin in this tissue and provides evidence for leptin regulation of pituitary function in regards to energy balance.

**Regulation of leptin by sexual maturation**

Leptin gene expression was up-regulated at mid- to late stages of spermatogenesis in early sexual maturing male salmon (Paper II). Hepatic lepa1 and lepa2 expression levels were significantly higher compared to immature males under fully fed conditions in July. This up-regulation coincides with highly elevated pituitary fsh-β mRNA levels, increasing GSI levels and decreasing lipid levels during mid-spermatogenesis when a re-allocation of stored lipids into gonadal growth and metabolism takes place. Peak levels of both leptin paralogues in the liver were measured in September, concomitant with peak pituitary lh-β expression and the presence of running milt in the testis, which is indicative of final maturation and spawning (Maugars and Schmitz 2008). These results are line with findings by Frøiland et al. (2010), where seasonal profiling of leptin expression in Arctic charr revealed peak leptin expression levels during late fall when fish had reached maturation, growth had ceased and adiposity levels had started to decline. Elevated plasma leptin levels were also reported in male and female ayu during and after spawning (Nagasaka et al. 2006). In this study, leptin was proposed to be responsible for the maturation-induced anorexia that occurs in this species during spawning, and that increasing levels of sex steroids might have stimulated the secretion of leptin. A recent study on female chum salmon also showed increased levels of hepatic leptin expression, leptin plasma levels, E2 and the estrogen receptors when transferred to freshwater, mimicking the spawning migration conditions and inducing maturation and anorexia (Choi et al. 2014). Fully fed maturing male parr had stunted growth and decreasing lipid stores (both muscle and visceral) during the later stages of spermatogenesis (Paper II) and many salmonids, such as Atlantic salmon, experience decreased appetite and anorexia during the maturational process (Kadri et al. 1997) which seems to be the case also for early maturing males, although this effect is not as pronounced as in adult maturation (Simpson et al. 1996). It is possible that the reduction in growth and adiposity in early maturing males is mediated through the anorectic effects of leptin that has been demonstrated in salmonids (Murashita et al. 2008; Murashita et al. 2011). However, no difference was seen between maturing and non-maturing males in leptin receptor expression in the brain (Paper II) indicating that any putative anorexic effect at this time would not be due to increased central sensitivity to leptin signaling in the maturing fish but rather to the increase in the signaling. Though, small changes in expression levels in specific brain areas might not be discovered when using whole brains for gene expression analysis, it cannot be ruled out that there were local changes in expression patterns that were not detected.
The major reproductive effects of leptin in mammals are exerted at the level of the hypothalamus (Elias and Purohit 2013), but modulation of the reproductive axis by leptin take place also in pituitary and gonads (Tena-Sempere 2007). Expression of lepr in the gonads have been found in a number of fish species (Paper I; Kurokawa et al. 2008; Liu et al. 2010; Gong et al. 2013b) and the up-regulation in total testicular content of lepr expression in early maturing salmon parr suggests a direct regulatory role of leptin in this tissue (Paper II), but it is not yet known which cells in the testis express lepr in teleosts. In rodents, expression of the long form of lepr as well as shorter isoforms have been detected in both Leydig cells and Sertoli cells (Tena-Sempere et al. 2001a) as well as germ cells (El-Hefnawy et al. 2000). Lepr expression in the testes of rats is inhibited by its ligand (Tena-Sempere et al. 2001b). Considering that the changes in testicular lepr expression levels occur around the same time as changes in hepatic leptin expression it could point to a regulatory mechanism of leptin on the receptor also in salmon. However, a negative regulation seems unlikely since lepr was elevated during this time, although this cannot be ruled out as the actual plasma leptin levels were not known.

Leptin directly affects steroidogenic pathways and inhibits basal and human chorionic gonadotropin-stimulated testosterone secretion in the testes of mammals (Caprio et al. 1999; Tena-Sempere et al. 1999; Tena-Sempere et al. 2001a). Indirect evidence of sex steroids being regulated by leptin has also been reported in fish. Zebrafish embryos exposed to hypoxic conditions had increased levels of leptin expression and were found to also have elevated levels of T and decreased levels of E2 (Yu et al. 2012). An important function of leptin in the testis could therefore be to modulate sex steroid production during spermatogenesis, although this will need further investigation.

**Regulation of leptin by sex steroids**

The increase of hepatic lepa1 and lepa2 gene expression levels in maturing male parr took place during mid- to late spermatogenesis (Paper II). It has previously been shown that androgen levels increase gradually during spermatogenesis of early maturing male salmon and peak just prior to spawning (Mayer et al. 1990; Maugars and Schmitz 2008). Since sex steroids are able to regulate leptin expression and leptin plasma levels in mammals (Mystkowski and Schwartz 2000), we hypothesized that the maturational effects observed on leptin expression in the liver could be, at least partly, caused by stimulation of sex steroids. In Paper III and IV we demonstrated that the T and 11-KT, the most important androgens in teleosts (Borg 1994), as well as E2 stimulates gene expression of both leptin genes in primary cultures of Atlantic salmon hepatocytes *in vitro* (Paper III) and that T also stimulated leptin and leptin receptor expression *in vivo* (Paper IV).
Both T and 11-KT increased *lepa1* and *lepa2* gene expression in salmon hepatocytes *in vitro* (Paper III). This is in contrast to many mammalian studies in males where androgens often either fail to alter leptin expression or act as inhibitors of leptin expression (Wabitsch et al. 1997; Pienceiro et al. 1999; Casabiell et al. 2001; Apostolova et al. 2005), and testosteron levels are inversely correlated with leptin plasma levels in males (Thomas et al. 2000; Söderberg et al. 2001). However, stimulatory effects of androgens on leptin have been reported in female adipose tissue *in vitro*, although these effects were not androgenic, but rather caused by the aromatization of T to E2 (Machinal-Quelin et al. 2002). In salmon, the T stimulation of leptin gene expression in hepatocytes seems not to be mediated by the aromatization of T to estrogen since co-incubation with the aromatase inhibitor fadrozole did not ablate the stimulatory effects (Paper III). Moreover, 11-KT which is non-aromatizable was either slightly less potent or equally potent in stimulating expression of the leptin gene. The observed effects are therefore likely mediated through the binding of T and 11-KT to an androgen receptor or receptors. No androgen receptor has yet been characterized in Atlantic salmon, but the closely related rainbow trout express two AR’s (ARα and ARβ) of which only ARα is activated by T and weakly also by 11-KT (Takeo and Yamashita 1999), and existence of a teleost AR preferentially activated by 11-KT has also been found (Olsson et al. 2005). AR transcripts were detected in the hepatocyte culture from Atlantic salmon, but further validation is needed (Paper III).

Interestingly, the androgens differed in their ability to stimulate *lepa1* or *lepa2* *in vitro* depending on developmental stage of the fish, while the effect of E2 was more consistent (Paper III). In immature males and females, both androgens stimulated *lepa1*, but not *lepa2*, while in mature males the opposite was seen and in previously mature males both genes were up-regulated by 11-KT, but T stimulated only *lepa1*. This indicates that the two genes could be regulated by different mechanisms and they also might have different function. The notion of different regulatory mechanisms is also supported by studies in salmonids *in vivo* where differences in the profiles of the hepatic expression of the two genes have been reported (Paper I; Paper II; Ronnestad et al. 2010; Frøiland et al. 2012).

*In vivo*, only T was able to stimulate hepatic leptin expression, and it was only the *lepa1*, and not the *lepa2* parologue that was affected (Paper IV) which is partly in line with the findings *in vitro*, where *lepa1* was generally more responsive during the immature stage (Paper III). However, both leptin paralogues were up-regulated during spermatogenesis in the early maturing males (Paper II), and if this up-regulation is caused by increased androgen stimulation it could indicate that the responsiveness of the two leptin genes to the androgens change during the maturational process, but also that sex steroids are likely not the only factors responsible...
for this effect since leptin gene expression is known to be affected directly by many factors (Margetic et al. 2002).

Although androgens, especially 11-KT, are the sex steroids that are the main regulators of reproductive function and behavior in male fish (Borg 1994), E2 is also present in the plasma of maturing Atlantic salmon males (Mayer et al. 1990). E2 plays a role testicular functions such as regulation of steroidogenesis and spermatogonial stem cell renewal, although the systemic effects of E2 in male reproduction in fish is less known (Schulz et al. 2010). Moreover, since T can be converted to E2 by the enzyme aromatase that is present in many tissues in fish it is important to understand the effects also of this sex steroid on leptin expression in regards to male reproduction.

*In vitro*, E2 was more potent than the androgens in stimulating leptin expression and the effect was seen already at physiological levels (1-10 μM) (Paper III). These results are in agreement with the effects of E2 on leptin in mammals. E2 potently stimulates leptin gene expression and secretion in human adipose tissue in females (Casabiell et al. 2001; Machinal-Quelin et al. 2002), both *in vitro* and *in vivo* in rat adipocytes (Kristensen et al. 1999; Machinal et al. 1999) and mouse adipocytes *in vitro* (Monjo et al. 2005). The estrogenic effect on leptin is mediated through E2 binding to the estrogen receptors and it acts directly at the transcriptional level (Machinal et al. 1999; O'Neil et al. 2001; Machinal-Quelin et al. 2002). The ERα was expressed in hepatocyte cultures from both males and females in the present study, and E2 treatment increased expression of ERα in both sexes suggesting that E2 may act via the same mechanism also in fish (Paper III). Response elements known to bind transcription factors that are modulated by ligand-bound ER’s have been found in the promoter region of the leptin gene in two fishes (Li et al. 2010; He et al. 2013), which suggests genomic effects of E2 on leptin expression. It further suggests that the effects of T in tissues with high aromatase activity, such as the salmonid pituitary (Andersson et al. 1988), might be, at least partly, estrogenic. This could explain why only T, and not 11-KA, stimulated *lepal* and *lepr* expression in the pituitary *in vivo* (Paper IV), or alternatively, T might interact with an androgen receptor that do not bind, or only weakly bind, 11-KT (Takeo and Yamashita 1999).
CONCLUSIONS AND FUTURE PERSPECTIVES

The work of this thesis aimed to increase the knowledge regarding the role of leptin in reproduction and energy balance in male Atlantic salmon parr. For this purpose we characterized the gene expression profiles of leptin and the leptin receptor in several key tissues under feed restriction and during sexual maturation, from the immature state to the spermiating state. Furthermore, we studied sex steroids as potential regulators of leptin gene expression both in vivo and in vitro.

Gene expression of both leptin paralogues in the liver decreased concomitantly with an increase in body weight and fat stores at the beginning of the growth season in spring, and they remained low until fall (Paper II). The decrease in hepatic leptin gene expression was also reflected in declining plasma levels between April and June (Paper I). Feed restriction, on the other hand, resulted in a considerable up-regulation in hepatic lepa1 and lepa2 gene expression (Paper I and II), plasma leptin levels (Paper I) and the leptin receptor in the pituitary (Paper II). This demonstrates a sensitivity of the leptin system to changes in energy balance. In addition, the association between leptin was generally negative or lacking, suggesting that leptin might not serve as an adiposity signal during this life stage in salmon parr. These results are opposite to what is generally seen in mammals under normal and fasted/feed restricted conditions and point to divergence in function between these animal groups.

In early maturing male parr, gene expression of lepa1 and lepa2 in the liver and lepr in the testes were up-regulated during mid-spermatogenesis when testes size increased rapidly and fsh-β expression levels in the pituitary were highly elevated (Paper II). Levels of lepa1 and lepa2 expression peaked during final stages of maturation coinciding with peak pituitary lh-β expression and the presence of running milt in the testes. The period of mid- to late spermatogenesis was also characterized by increased lipid depletion and stunted growth that take place due to the increased metabolic demand of maturation. This suggests that leptin is regulated by mechanisms related to sexual maturation and that it might have a role to play during the later stages of the maturational process.

Moreover, both leptin genes were sensitive to stimulation by T, 11-KT and E2 in hepatocyte cultures from Atlantic salmon in vitro (Paper III). The response of the different leptin gene paralogues to the androgen treatments depended on the developmental stage, where lepa2 was more responsive during the maturing stage and lepa1 during the immature stage, indicating separate regulatory mechanisms for the two genes. These findings fit well with the observation that lepa2 was more strongly up-regulated during final maturation than lepa1 (Paper II). In addition, T also ex-
erted a stimulatory effect of hepatic expression on lepal in immature males in vivo and, in addition, stimulated both lepal and lepr also in the pituitary (Paper IV). Overall, these studies provide evidence that the androgens have the ability to regulate leptin gene expression in the liver as well as leptin and the leptin receptor at different levels of the BPG axis. It also indicates different regulatory mechanisms for the two lepa genes. It is therefore possible that androgens might be the mechanism by which leptin is stimulated during sexual maturation, although these effects seem to differ depending on developmental stage. So far, it cannot be concluded what the physiological relevance of elevated leptin expression levels during the late stages of spermatogenesis is. Considering that males at this stage of development show similarities to the feed restricted fish (depletion of lipid stores, stunted growth) (Paper II), it is interesting to speculate that leptin might have a role to play in regulating metabolism during this time in order to facilitate a mobilization and reallocation of energy stores towards gonadal growth and the metabolic cost of reproduction, but this remains to be investigated.

In conclusion, this thesis has provided new insights on how sexual maturation and changes in energy balance affect parts of the leptin system, and by what mechanism the maturational effects could, at least partly, be mediated in male Atlantic salmon parr. A summary of the present findings in regards to maturation is illustrated in Figure 2. In order to gain a more complete understanding of what role the leptin system plays in reproduction and energy balance it will be important in future studies to: 1) investigate which cell types in the different tissues express the leptin genes and the receptor, 2) to conduct functional studies, using species specific recombinant leptins, in order to explore how the different leptin paralogues regulate key factors at all levels of the BPG axis, such as GnRH in the brain and the gonadotropins in the pituitary, as well as effects on metabolism, and 3) to take into account the two leptin-b genes and the shorter isoforms of the receptor found in salmon.
Figure 2. Putative model of the interactions between the two leptin-a genes, the leptin receptor gene and sexual maturation at different levels of the BPG-axis in Atlantic salmon. Known stimulatory effects are indicated by + and no effect indicated by X (thick lines). Possible endocrine and paracrine interactions that are not yet fully elucidated are indicted by dashed lines. *applies to pituitary lepr only.

Laxhanar kan mogna antingen som stora fiskar efter att ha spenderat ett till flera år i havet eller redan som små 1-åringar, s.k. tidig könsmognad, när de fortfarande befinner sig i det sötvatten där de kläcktes. I varje årskull av laxhanar kommer en viss andel att mogna tidigt medan andra inte gör det. Laxen börjar könsmognadssessensen under perioden vår-tidig sommar och blir lekmogen på senhösten. "Beslutet" att mogna tidigt eller inte är starkt kopplad till fiskens näringsstatus vid specifika tidpunkter på året, så som under tidig vår när tillväxtsäsongen börjar. Lax utgör därför en bra modell för att studera kopplingen mellan leptin, energistatus och könsmognad hos fisk.

För att undersöka hur leptin och dess receptor (lepr) påverkas av förändringar i energibalans och av könsmognad hos 1-åriga Atlantlax-hanar studerade vi hur dessa påverkas av sju veckors begränsad födottillgång under våren, samt genuttryckssprofilerna av leptin och lepr under tillväxt- och mognadssäsongen, april-september, både hos icke-mognande och tidigt mognande 1-åriga hanar som fick antingen en full födoration eller en begränsad födoration (50 % av full ration). Atlantlax har två leptin-a gener, lepa1 och lepa2, som båda var högst uttryckta i levern, i motsats till däggdjur, medan lepr-genen var uttryckt i hjärna, hypofys och allmänt i perifera vävnader, så som gonader, muskel och fett. Starten på tillväxtsäsongen hos fiskarna
med full ration ledde till en nedreglering av båda leptingenen i levern och lepr i hjärnan samt att plasma nivåerna av leptin sjönk. Detta skedde parallellt med att fiskarna ökade i vikt och längd samt fettinlagring. Begränsad födoration ledde istället till att genuttrycket av lepal och lepa2 i levern, samt lepr i hypofysen uppreglades över flera månaders tid. Dessa fiskar växte mycket lite och förödade fettvävnad under samma period. Sambandet mellan leptin-nivåer och fettmängd var antingen negativt eller saknades. Det verkar därmed som om leptin är känslig för förändringar av energistatus också hos fisk, men att reglering verkar vara motsatt till vad man ser hos många däggdjur och tyder på att leptin inte fungerar som en fettsignal hos Atlantlax.

Yttrycket av båda leptin-generna i levern och lepr i gonaderna hos de tidigt mognande fullt utfodrade hanar ökade då könsmognadsprocessen inlemts. Denna uppreglings sammanföll med den största gonadtillväxten (>14 % av kroppsvikten) och höga genuttrycksnivåer av follikelstimulerande hormon i hypofysen. De högsta uttrycksnivåerna för leptin uppmättes senare under hösten när fiskarna hade rinnande mjölke och höga nivåer av luteiniserande hormon (lh-β) i hypofysen dvs. när fiskarna var redo för lek. Andelen mognande hanar som gick på en begränsad diet var lägre än de på en full ration, vilket troligen beror på att färre fiskar i denna grupp uppnådde de nödvändig tröskelvärdena för fettlager som krävs för denna investering. De som faktiskt mognade i denna grupp investerade dock mindre i gonadtillväxt och uppräckades en fördröjd könsmognad vilket visade sig i att uppreglering av lh-β skedde senare samt att uppregleringen av leptingenerna också skedde senare.

Hos laxar har man sett att nivåerna av androgener ökar i blodet under könsmognadsprocessen och förblir förhöjda ända fram till leken. För att undersöka om ökningen av leptin som sågs under könsmognadsprocessens senare del hos de tidigt mognande hanarna kan ha orsakats av sexhormoner testades effekten av olika sexhormoner på leptinintrycket både genom in vitro och in vivo studier. Testosteron (T), 11-ketotestosteron och 17β-estradiol stimulerade genuttrycket av lepal och lepa2 i levercellskulturer från Atlantlax in vitro, fast vilken leptin-gen som stimulerades mest varierade beroende på fiskens utvecklingsstadium. Genom att implantera omogna 1-åriga lax-hanar med kapslar innehållande T eller 11-ketoandrostenedion (11-KA), kunde vi visa att T stimulerade genuttrycket av lepal, men inte lepa2, i levern också in vivo. T uppreglade även genuttrycket av lepal och lepr i hypofysen. Ingen effekt sågs av 11-KA behandlingen och ingen av androgenerna påverkade plasma nivåerna av leptin eller lepr i testes. Sammantaget tyder resultaten på att sexsteroider är en av de mekanismer som reglerar leptin under könsmognaden och att leptin i sin tur troligen har en funktion att fylla under den senare delen av denna process.
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