HORMONAL AND NEUROPEPTIDE MECHANISMS OF APPETITE CONTROL IN ATLANTIC SALMON

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I dedicate this dissertation to my beloved father, I miss you every day, and I know you are proud of me today. This was all for you and thank you for making me into the person I am today.

Anne-Grethe Gamst Moen

Bergen, June, 2010.

I believe everything happens for a reason.
People change so you can learn how to let go. Things go wrong, so that you appreciate when they're right, and sometimes good things fall apart so better things can fall together.

- Marilyn Monroe -

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Introduction

Background for studies

Studies of cellular mechanisms of appetite control in fishes are only just beginning. In 2006 when the work contained in this thesis commenced, only 2 papers were published with a specific focus of endocrine mechanisms of appetite control in fishes (Kurokawa et al., 2005; Huising et al., 2006a). Considerably more work has been conducted in mammals in association with the pathophysiological condition of obesity. Zhang and colleagues (1994) were the first to demonstrate a clear link between the absence of the hormone leptin (LEP) and severe obesity in mice. The subsequent discovery that both leptin and the appetite stimulating hormone ghrelin (GHRL) are also found in teleosts (Kurokawa et al., 2005; Kaiya et al., 2003a,b) provided the stimulus for the studies conducted in this thesis. Since considerable advancements have been made in the mammalian research field, a brief review is provided before discussing the status of research in fish.

Mammals

Central regulation of energy homeostasis

Discoveries in mammals concerning the endocrine control of appetite and growth have led to a new understanding of how the metabolic demand for energy is regulated in the central nervous system. In mammals, it has been suggested that signals arising from peripheral tissues can be systematically divided into two categories; those that are produced in proportion to the amount of fat in the body (adiposity signals), and those generated during meals to cause satiation (satiety signals) (Woods et al. 1998; Schwartz et al. 2000; Margetic et al. 2002; Klok et al. 2007). Central control of food intake and energy expenditure occurs in the hypothalamus (Woods et al., 1998; Schwartz et al., 2000). Amongst other nuclei, the

arcuate nucleus (ARC) is an important integrator of endocrine signals arising from peripheral tissues (Morton et al., 2005). ARC contains orexigenic neurons that co-express neuropeptide Y (NPY) and agouti-related protein (AgRP) (Ollmann et al., 1997; Baskin et al., 1999a) and anorexigenic neurons that co-express proopiomelanocortin (POMC) and cocaine- and amphetamine- regulated transcript (CART) (Boston et al., 1997; Ellacott and Cone, 2004). The opposing neuropeptide circuits promote feeding and inhibition of energy expenditure and food intake and increased catabolism, respectively (Wynne et al., 2005; Gao and Horvath, 2007; review: Sainsbury and Zhang, 2010). Sophisticated control is augmented through the antagonism of each opposing neuropeptide system (Fig. 1).

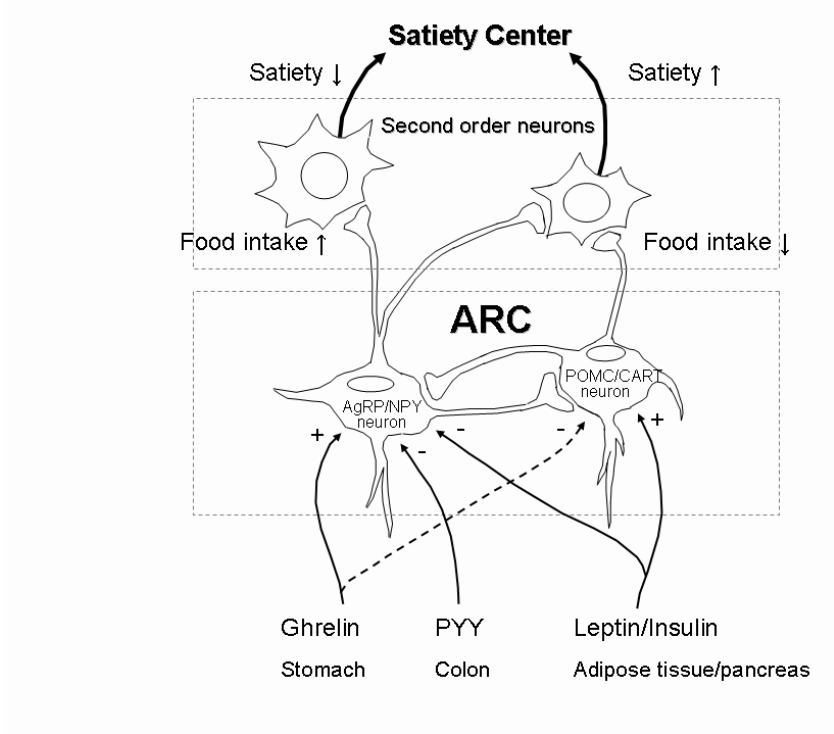

Figure 1. Schematic illustration of central nervous pathways involved in the regulation of food intake and satiety in mammals. + = agonistic; - = antagonistic; (redrawn after Sànchez-Lasheras et al., 2010 and Katharine Stuliff, 2003).

In mammalian adults, peripheral secretory peptides such as leptin (LEP) from the adipose tissue, or ghrelin (GHRL) from the stomach enter the circulation and act via their cognate receptors, which are expressed throughout the body tissues, but also in the brain (Mercer et al., 1996; Elmquist et al., 1998; Nakazato et al., 2001; Zigman et al., 2006). Signal integration occurs in the ARC to regulate nutritional status through the expression of the appetite and energy-regulating neuropeptides. Consequently, the LEP-POMC-CART axis is thought to control satiety, while the GHRL-NPY-AGRP axis controls appetite (Wynne et al., 2005; Gao and Horvath, 2007).

Actions of leptin and ghrelin

LEP is a hormonal product of the obese (*OB*) gene, which is a member of the cytokine family expressed primarily by adipose tissue (Friedrich et al., 1995; Ahima and Flier, 2000). LEP is also secreted in the brain and other peripheral tissues such as the stomach (Tartaglia et al., 1995; Bado et al., 1998). Early experiments revealed that the absence of LEP causes extreme obesity in mice (Campfield et al., 1995; Halaas et al., 1995; Pellymounter et al., 1995). The hypothesised cause was thought to be the ablation of the anorectic signalling cascade. For example, anorectic neuropeptides that are upregulated by LEP include POMC, CART and corticotrophin- releasing hormone (CRH) (Cowley et al., 2003; Dimeraki and Jatte, 2006; Abizaid and Horvath, 2008). The POMC-secreting neurons generate the cleavage product α-melanocyte-stimulating hormone (α –MSH), which is thought to be a key mediator of the anorectic LEP response (Schwartz, 2001; Gao et al., 2004). Simultaneously, the ablation of orexigenic signals occurs through the antagonistic actions of LEP on NPY, melanin concentrating hormone (MCH), orexins and AgRP. Consequently, the absence of LEP was argued to send the orexigenic pathways into overdrive (Zhang et al. 1994).

However, despite the early promise of these results, emerging evidence indicates that the link between adiposity and its hypothalamic regulation is more complex than originally envisaged (Margetic et al., 2002; Ahima and Osei, 2004).

There has been an increasing amount of evidence that leptin exerts a pleiotropic effect. Multiple peripheral effects of leptin have been recently described, including synthesis of sexual, thyroid and growth hormone, as well as regulation of blood pressure, reproduction, osteogenesis, hematopoesis and angiogenesis. LEP also plays a regulatory function in immunity and in the process of tumorigenesis (reviewed by Himms-Hagen, 1999; Margetic et al., 2002; Bjørbæk and Kahn, 2004).

Contrasting the anorectic effects of LEP, GHRL promotes growth as the endogenous ligand of the growth hormone secretagogue receptor (GHSR) (Fig. 2) (Bowers et al., 1984; Smith, 2005). In this respect, GHRL not only stimulates the growth hormone (GH) axis (Farhy et al., 2007), but also induces feeding and modifies body energy composition (Nakazato et al., 2001; Hosoda et al., 2006), as well as modulating the gonadotropic axis (Tena-Sempere, 2008). This occurs partly through induction of the NPY and AgRP circuits. However, stimulation of NPY or AgRP by GHRL is not an obligate requirement for the regulation of energy balance (Qian et al., 2002). Other growth-promoting effects of GHRL that are unrelated to appetite occur through stimulation of adipogenesis. This latter effect may be mediated through GHSR-independent pathways (Fig. 2) (Tschop et al., 2000; Thompson et al., 2004). The observation that *GHRL* is also expressed in the placenta (Gualillo et al., 2001) as well as in the fetal and/or neonatal pancreas (Chanoine and Wong, 2004; Prado et al. 2004; Wierup et al. 2002, 2004), pituitary (Kamegai et al. 2001) and hypothalamus (Torsello et al. 2003) has led some investigators to propose that GHRL developmentally regulates the onset of energy homeostatic mechanisms.

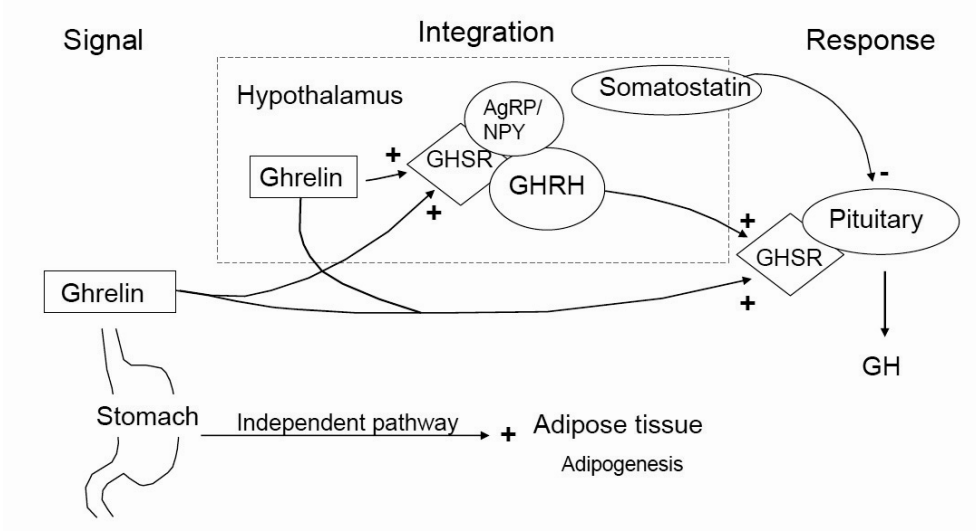

Figure 2. Schematic diagram of the effect of ghrelin on growth hormone (GH) and energy metabolism in adults. AgRP – agouti-related protein; GHRH – growth hormone releasing hormone; GHSR- growth hormone secretagogue receptor; NPY – neuropeptide Y; + = agonistic; - = antagonistic (redrawn after Chanoine 2005).

Leptin signalling transduction

LEP achieves most of its metabolic effects by interacting with specific receptors (LEPR) located in the central nervous system (CNS) (Bjørbæk et al., 1997) and ubiquitously distributed in peripheral tissues (Akerman et al., 2002; Buyse et al., 2001; Ebenbichler et al., 2002; Frank et al., 2000; Goiot et al., 2001; Kim et al., 2000; Lee et al., 2002; Morton et al., 1999). Although several splice variants of LEPR are known to exist (Chua et al., 1996), signal transduction only seems to occur when LEP binds to the long isoform of LEPRb (Ahima and Flier, 2000). The long-form receptor regulates multiple intracellular signalling cascades (Bjørbæk and Kahn, 2004), including the activation of the Janus kinase (JAK)-2/signal transducer and activator of transcription (STAT)-3, mitogen-activated protein kinase (MAPK3/1, formerly Erk1/2), and inositol 1,4,5-triphosphate (IP_3) pathways (Sahu, 2004b). JAK2/STAT3 is the main pathway activated by LEP, with phosphorylation of JAK2 and

STAT3 which subsequently translocates to the nucleus to initiate transcription of target genes (Fig 3).

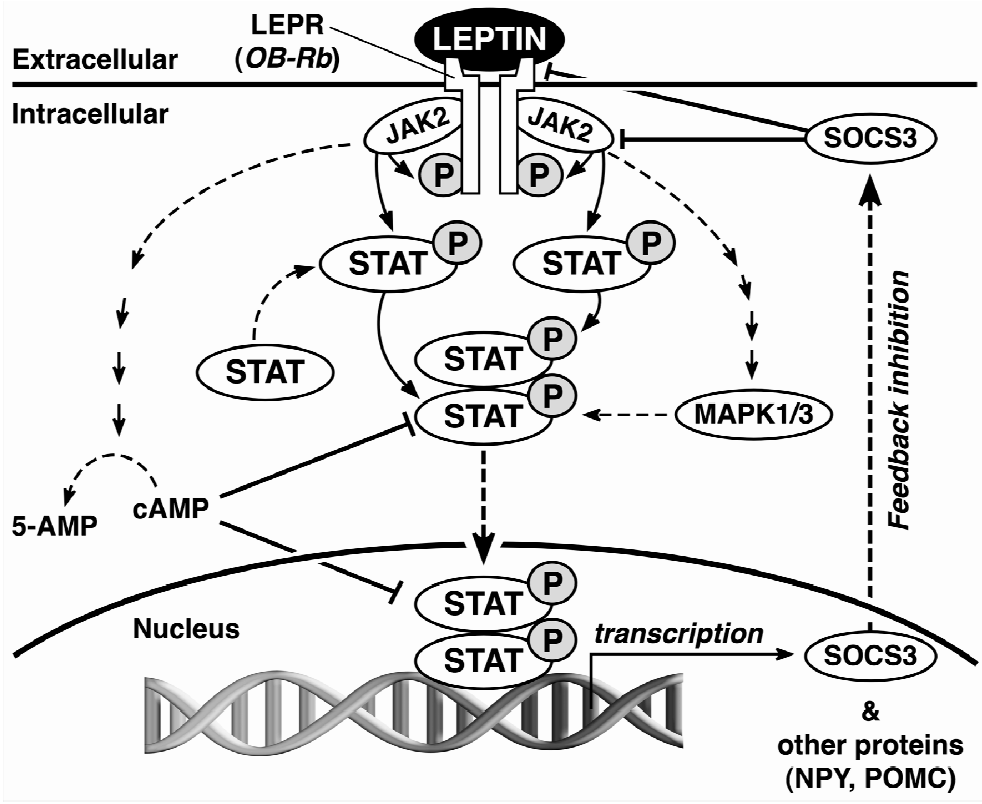

Figure 3. Schematic diagram of the signalling transduction pathways of leptin (adapted from Sahu, 2004b). Abbreviations: JAK2 – janus kinase 2; STAT3 – signal transducer and activator of transcription 3; MAPK - mitogen activated protein kinase; suppressor of cytokine signaling 3 protein - SOCS3.

Ghrelin signalling transduction

The actions of GHRL are mediated through G-protein coupled GHSRs (Howard et al., 1996; McKee et al., 1997). Ligand binding promotes the release of guanosine diphosphate (GDP) and binding of guanosine triphosphate (GTP) to the G protein α subunit, thus activating G protein subunits to initiate signaling cascades. The regulation of ionic currents is triggered by a Gαq/11 protein that activates phosphatidylinositol-specific phospholipase C

(PI-PLC) to generate IP_3 and diacylglycerol (DAG) from phosphatidylinositol 4,5-diphophate (PIP_2) (Howard et al., 1996; McKee et al. 1997). IP_3 mobilizes intracellular Ca^{2+}, which in conjunction with DAG activate PKC. This results in inhibition of the K^+ channels to cause cellular depolarization (Fig. 4) (Camina 2006).

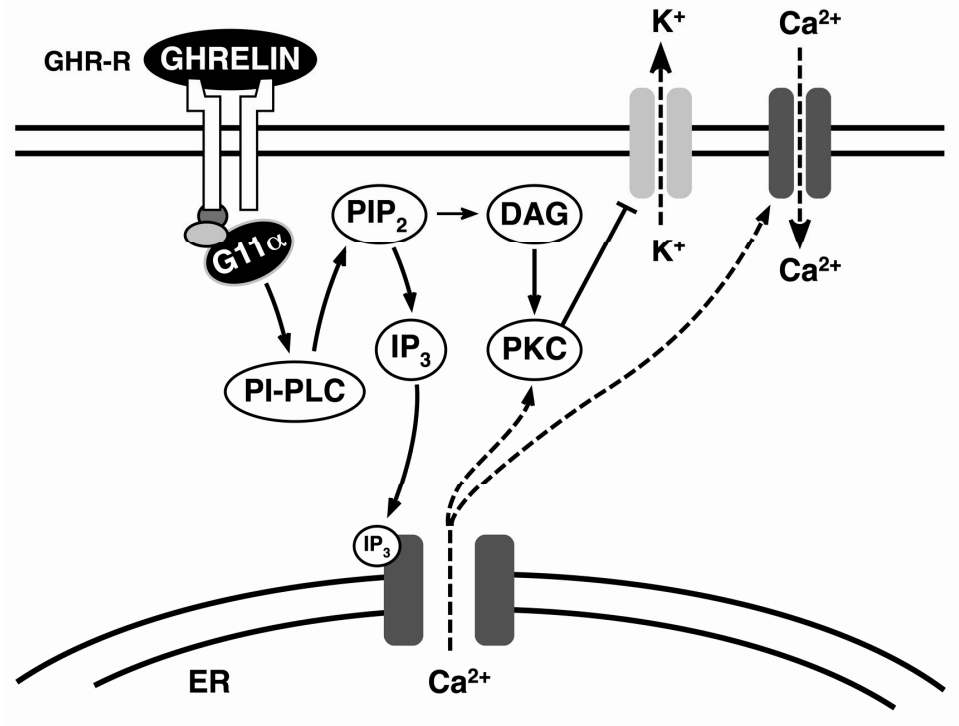

Figure 4. Schematic diagram of the signalling transduction pathways of ghrelin. (Redrawn from Gao et al., 2007). Abbrevations: Phosphatidylinositol-specific phospholipase C – PI-PLC; inositol 1,4,5-triphosphate – IP3; diaclyglicerol – DAG; phosphatidylinositol 4,5-diphosphate – PIP2; Protein Kinase C - PKC.

Fish

Leptin

The LEP hormone was long thought to be specific to Mammalia, but in the recent years the gene (*lep*) has been found in Amphibia (Boswell et al., 2006; Crespi and Denver 2006) and Teleostei. The discovery of *lep* in torafugu, *Takifugu rubripes,* by Kurokawa and colleages

(2005) revealed the ancient ancestry of this hormone. During the course of this thesis, several orthologues of lep have been identified in other teleosts. A single *lep* gene has been documented for green-spotted pufferfish, *Tetraodon nigroviridis*, (Kurokawa et al. 2005), rainbow trout, *Oncorhynchus mykiss* (Murashita et al. 2008), Arctic charr, *Salvelinus alpinus* (Frøiland et al. 2010), silver carp, *Hypophthalmichthys molitrix*, and grass carp, *Ctenopharyngodon idellus* (Li et al. 2009). In other species there are reports of two closely related *lep* paralogues, including common carp, *Cyprinus carpio* (Huising et al. 2006) and goldfish, *Carassius auratus* (unpubished GenBank sequences; ACL68083, ACG69476). More distantly related *lep* genes have been found in medaka, *Oryzias latipes*, (Kurokawa et al. 2009) and zebrafish, *Danio rerio* (Gorissen et al. 2009). The present work led to the discovery of two closely related *lep* genes in Atlantic salmon (*Salmo salar*) (Paper I).

The large differences in the primary structures between endothermic mammalian and ectothermic teleost leptins raised the question of whether the energy homeostatic functions of the teleost leptins are conserved. Initial phylogenetic analysis has revealed that amino acid conservation with other mammalia LEP orthologues is low, with only ~13% sequence identity between torafugu and human LEP (Kurokawa et al., 2005). Subsequent investigations have confirmed the low amino acid identity of teleost Leps compared to mammalian LEP (Huising et al., 2006; Kurokawa et al., 2008; Kurokawa et al., 2005; Li et al., 2010; Paper I). Despite the low conservation of the primary structure, available data indicate that the three-dimensional conformation is putatively conserved between teleosts and mammals (Kurokawa et al., 2005; Huising et al., 2006; Murashita et al., 2008; Gorissen et al., 2009; Kurokawa and Murashita, 2009; Li et al., 2010, Paper I). The study on torafugu (Kurokawa et al., 2005) indicated that *lep* mRNA is mainly expressed in the liver in contrast to the adipose secretion in mammals (Zhang et al., 1994; Montague et al., 1997). However recent studies have shown that *lep* is expressed in several peripheral tissues, including the intestine, kidney, ovary,

muscle and adipose tissues (Murashita et al., 2008; Gorissen et al., 2009; Kurokawa and Murashita, 2009; Frøiland et al, 2010; Paper I; Paper II). The multiplicity of *lep* genes and their low conservation in Teleostei (Huising et al., 2006; Gorissen et al., 2009; Kurokawa and Murashita, 2009; Li et al., 2010), suggest that their physiological roles may be more divergent than reported for mammals.

Ghrelin

Ghrelin (*ghrl*) has been indentified in a number of fish species, including rainbow trout (Kaiya et al., 2003a,b), European sea bass (*Diecentrarchus labrax*; Terova et al., 2008), Atlantic salmon (Murashita et al., 2009) and Artic charr (Frøiland et al., 2010). *ghrl* has been demonstrated to be essential for GH expression, growth and metabolism during embryonic development (Xi et al., 2009) and that Ghrl exerts both GH secretagogue (Kaiya et al., 2005) and appetite stimulating roles (Riley et al., 2005; Matsuda et al., 2006a,b; Miura et al., 2006, 2007) in fish. Recent studies have demonstrated that both short-term central or more long-term peripheral treatment with homologous Ghrl may decrease food intake in rainbow trout, suggesting that Ghrl is an anorexigenic hormone in this species (Jönsson et al., 2010). Nevertheless, injection of rainbow trout with human growth hormone releasing hormone (GHRH) did stimulate feed intake (Shepherd et al., 2007). The former Ghrl study by Jönsson et al. (2010) is in contrast with studies on goldfish (Matsuda et al., 2006a,b; Miura et al., 2006, 2007; Unniappan et al., 2002, 2004) and Mozambique tilapia (*Oreochromis mossambicus*) (Riley et al., 2005), where central and peripheral treatments of Ghrl increases food intake, similar to its action in mammals (Choi et al., 2003; Druce et al., 2005, 2006; Tschop et al., 2000; Wren et al., 2000, 2001a, b). Hence, the role of Ghrl in fish is still far from elucidated and more research in the putative role of Ghrl in short- and long-term regulation of appetite and energy homeostasis is needed.

Though LEP and GHRL are known to work in parallel at an equivalent level in mammals (Gao and Horvath, 2007; Cummings and Foster, 2003), most studies have focused on either one or the other. Studies in fish have lagged behind, with few studies yet demonstrating definitive physiological roles of either hormone. However Frøiland et al. (2010) suggested a possible role of these hormones in the long-term regulation of energy homeostasis during a seasonal feeding cycle.

Neuropeptides

In fish, a number of peptides homologuous to the mammalian appetite-regulating peptides have been identified, such as NPY (Blomquist et al., 1992; Cerdà-Reverter et al., 2000a; Murashita et al., 2009a), MCH (Baker et al., 1995; Logan et al., 2003a,b; Pissios and Maratos-Flier, 2003), POMC-derived peptides (Cerdà-Reverter et al., 2003a; Okuta et al., 1996; Herzog et al., 2003; Paper IV), CART (Volkoff and Peter, 2001a, Murashita et al., 2009a) and AgRP (Cerdà-Reverter and Peter 2003; Song et al., 2003; Klovins et al., 2004; Murashita et al., 2009a). Data on the role of these neuropeptides in the control of food intake and their mechanism of action in fish is growing but still limited. However it has been suggested that the same series of neuropeptides and their receptor systems are involved in the control of feeding behaviour throughout the vertebrate phylum, although major differences may occur in their mode of action between fish and mammals (Matsuda, 2009).

Atlantic salmon as a model species

Atlantic salmon is a commercially important species. It is a representative of the class Protacanthopterygii and inhabits both freshwater and marine cold water environments. Although principally anadromous this species shows a wide range of life histories, which are linked to body size and energy stores (Verspoor et al., 2007). Adult Atlantic salmon are

known to biologically adjust to long periods of reduced feed intake during their spawning migration, and it may therefore take a long time before energy stores are affected compared with what is seen in other species (Bertile et al., 2003; Huising et al., 2006a). It is therefore of interest to examine the long- and short-term effects of feed restriction and response to hormone dose. From the perspective of growth, it is important to know what effect these experimental treatments might have on the putative signalling pathways (Fig. 5) involved in the regulation of food intake and appetite control in fish.

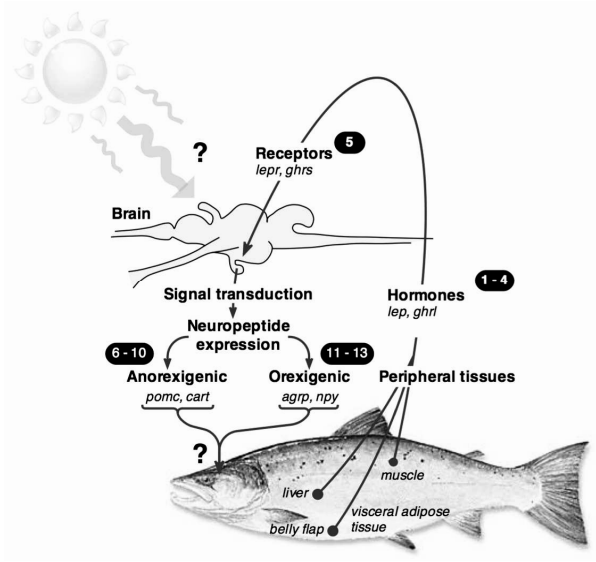

Figure 5. Illustration of possible signalling pathways involved in the regulation of food intake and appetite control in fish. Numbers indicate the number of genes that were studied during this thesis; 1-4: *lepa1*, *lepa2*, *ghrl1*, *ghrl2*; 5: *lepr*; 6-10: *pomca1*, *pomca2*, *poma2s*, *pomcb*, *cart*; 11-13: *agrp1*, *agrp2*, *npy*.

Salmon undergo their greatest feeding and growth in seawater, however they return as adults to spawn in freshwater streams where the eggs hatch and juveniles grow through several distinct phases. The embryos and larvae of Atlantic salmon are ideally suited for investigations involving maternal effects and the onset of energy homeostatic systems prior to

exogenous feed intake, because the long period of yolk-sac nutrition removes any variation in feeding behaviour between treatments or individual.

The work in this thesis therefore focused on:

1. Characterisation of *lep* paralogues in Atlantic salmon
2. The effects of feed restriction in adults
3. The effects of recombinant salmon leptin on growth and appetite related peptide hormones in the brain
4. The onset of energy homeostatic systems in embryos and larvae

Aims of the thesis

In light of the present lack of knowledge on energy homeostatic signalling pathways and leptin function in fish the aims of the thesis work were:

- To obtain full-length mRNA sequences for Atlantic salmon *leps* and the cognate receptor. The sequence information was then used to establish methods for quantifying mRNA levels, and to elucidate the pattern of tissue expression. (**Paper I**).

- To investigate the long- and short-term relationships between *lep* expression and feeding regime in adult Atlantic salmon (**Paper II**).

- To establish a production protocol for recombinant salmon *lepa1* and how administration of this affected growth and appetite related neuropeptide genes in the brain (**Paper III**).

- To elucidate the ontogeny of energy homeostatic pathways via neuroendocrine signalling in Atlantic salmon and how they relate to the present mammalian model. (**Paper IV**).

List of publications

Paper I Rønnestad, I., Nilsen, T.O., Murashita, K., Angotzi, A.R., Moen, A-G.G., Stefansson, S.O., Kling, P., Björnsson, B.Th., Kurokawa, T., 2010. "Leptin and leptin receptor genes in Atlantic salmon: cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status". General and Comparative Endocrinology 168, 55-70.

Paper II Moen, A-G.G., Kling, P., Jordal, A-E.O., Stefansson, S.O., Björnsson, B.T., Taranger, G.L., Finn, R.N., Rønnestad, I., 2010. "Short-term, but not long-term feed restriction causes differential expression of leptins in Atlantic salmon". General and Comparative Endocrinology. Submitted manuscript.

Paper III Murashita, K., Jordal, A-E.O., Nilsen, T.O., Stefansson, S.O., Kurokawa, T., Björnsson, B.Th., Moen, A-G.G., Rønnestad, I., 2010. "Recombinant salmon leptin A1 (sLepA1) affects growth and appetite related peptide hormones in the brain of Atlantic salmon (*Salmo salar*)". Manuscript.

Paper IV Moen, A-G.G., Murashita, K., Finn, R.N. 2010. "Ontogeny of energy homeostatic pathways via neuroendocrine signalling in Atlantic salmon". Developmental Neurobiology. Doi: 10.1002/dneu.20803

Synopsis of Papers

Paper I

In this study the cloning and sequencing of two *lep* paralogues (*lepa1* and *lepa2*) and the cognate receptor (*lepr*) in Atlantic salmon were reported. The deduced amino acids (aa) sequences of the two Leps showed 71.6% identity to each other, but only 22.4% (*lepa1*) and 24.1% (*lepa2*) identity to human LEP, respectively. The deduced amino acid sequences of the Atlantic salmon paralogues clustered phylogenetically with teleost type A Lep. Expression analyses revealed a differential distribution in peripheral tissues, with the highest *lepa1* levels in the brain, white muscle, liver and ovaries. Conversely, *lepa2* was mainly expressed in the stomach, midgut and kidney. Only one *lepr* orthologue was identified, which shared 24.2% aa sequence identity to human LEPR. However, several splice-variants were detected in Atlantic salmon that appear to match similar isoforms in human. The expression of *lepr* was ubiquitous in peripheral tissues, with the highest levels in the ovary. The initial test to verify if energy status affected *lep* levels showed that restricted feeding (60%) for 10 months resulted in lower *lepa1* mRNA levels in visceral adipose tissue, and higher mRNA levels of *lepa2* in the liver. For belly flap and white muscle no differences between treatments were observed. Interestingly *lepa2* levels were more highly expressed in adult males compared to *lepa1*, which contrasts our observations for mRNA tissue distribution in parr. Neither the circulating plasma Lep levels nor *lepr* mRNA levels in the brain appeared to be affected by long-term feed restriction.

The study partly supports anorectic signaling effects of *lepa1* in line with the mammalian model, while *lepa2* might have an unknown role in the digestive tract and liver. However, more specific studies on this hormone will be necessary to verify how leptin is involved in the regulation of energy homeostasis of adult Atlantic salmon.

Paper II

This study investigated whether dietary restriction affects the expression of leptin paralogues (*lepa1* and *lepa2*), in relation to plasma Lep, growth hormone (Gh) and insulin-like growth factor 1 (Igf1), as well as body weight and adiposity. We found that long-term feed restriction in Atlantic salmon did not significantly affect transcriptional regulation of either *lepa1* or *lepa2*. This trend was also reflected in the plasma levels of Lep, Gh and Igf1. Unexpectedly, however, we noted that transcriptional levels of *lepa1* in the visceral adipose tissue appeared to follow ambient day length. Only white muscle *lepa1* expression showed the positive correlation reported in mammals when comparing leptin expression to the muscle lipid content, while hepatic expression of *lepa1* showed a negative correlation to the organ lipid content. To further study the regulatory role of leptin, fish were trained to receive a single meal, and then the endocrine response in the presence or absence of a meal during a 24 hr period was examined. These studies showed that in the unfed fish, *lepa1* expression peaked initially in the white muscle at 6 hr, followed by other peripheral tissues at 9 hr. The one exception was *lepa2* in visceral adipose tissue which peaked in the fed fish at 9 hr. However, *lepa2* levels were expressed at an order of magnitude lower than that of *lepa1*, and likely do not reflect a physiological effect. However, no differences were noted between the treatments for plasma Lep. The absence of a clear leptin response to a single meal is consistent with data for humans but appears to contrast recent findings for other teleosts where injection of leptin has shown to reduce appetite. Taken together it can be suggested that leptins have a complex lipostatic function in Atlantic salmon, possibly under the influence of photoperiod as seen in some mammals.

Paper III

In order to investigate the effect of leptin on growth and the expression of appetite related peptide hormones (*agrp1*, *agrp2*, *npy*, *cart*, *pomca1*, *pomca2*, *pomca2s* and *pomcb*) in Atlantic salmon, *lepa1* was used to establish a production protocol for recombinant rsLepA1 in *Echerischia coli*. This protocol yielded 1.7 mg pure protein per culture. rsLepA1 was then administered via intraperitoneal osmotic pumps at four different delivery concentrations (estimated pumping rates were 0, 10, 100 and 1000 ng h^{-1}) to Atlantic salmon fed to satiation over 20 days. Weight gain and specific growth rate over this period were significantly reduced in the highest dosage groups (1000 ng h^{-1}). Measurements of the appetite-related neuropeptide mRNA levels via qPCR revealed that *pomca1* levels were higher than controls in this high dose group, while no differences were observed between treatments for *agrp1*, *agrp2*, *npy*, *cart*, *pomca2*, *pomca2s* and *pomcb*. These findings indicate that high concentrations of *lepa1* in Atlantic salmon can have an anorectic effect on the regulation of body weight in Atlantic salmon, however such dosages may represent a pharmacological effect, since they are an order of magnitude higher than concentrations measured in plasma. Nevertheless, the data demonstrate that *lepa1* can illicit an anorectic response as found in mammals.

Paper IV

In this study the expression of leptins (*lepa1*, *lepa2*), ghrelins (*ghrl1*, *ghrl2*), their target neuropeptides (*cart*, *pomca1*, *agrp*, *npy*) and several growth factors (*gh*, *igf1*, *igf2*, *igf1r*) were examined during embryonic and larval development of Atlantic salmon. During embryogenesis initial experiments revealed differential expression of *lep* paralogues and *ghrl* isoforms. Prior to exogenous feeding an upregulation in *ghrl1* and *ghrl2* was observed, while *lepa1* did not surge until one week after first-feeding. Subsequent dissection of the embryos and larvae showed that *lepa1*, *cart*, *pomca1*, *agrp* are supplied as maternal transcripts. At 320 day-degrees (dd) the first zygotic expression was detected for *lepa1* and *cart*, which at 400 dd was localized in the head and coincided with similar upregulation of *ghrl2* and *npy*. Over the hatching period growth factor signalling predominated. The observed ghrelin surge prior to first-feeding was mainly expressed in the internal organs and coincided with a surge of *npy* and *agrp* in the head and *agrp* in the trunk. Major peaks were detected in the head for *lepa1* and *pomca1*, and *lepa1* in the trunk at one week after exogenous feeding was established. This coincided with increasing levels of *cart*. By integrating these results into an ontogenetic model the data suggest that mediation of Atlantic salmon energy homeostatic pathways via endocrine and neuropeptide signaling retains putative features of the mammalian system.

General discussion

Teleost leptins and receptors

The present work demonstrates the presence of two closely related *lep* paralogues in Atlantic salmon (Fig. 6). Recent studies on teleosts have shown that at least two leptin genes (*lepa* and *lepb*) exist in the crown-clade (Kurokawa et al. 2005; Huising et al., 2006; Gorissen et al., 2009; Kurokawa and Murashita, 2009). Early findings have shown that *lepa* and *lepb* share low interspecific aa identity, and are argued to have arisen through whole genome duplication (Kurokawa et al. 2005), which occurred early in the teleost lineage (Jaillon et al., 2004). The duplicity of genes has been described for Atlantic salmon (Paper I), Japanese medaka, common carp and zebrafish (Gorissen et al., 2009; Kurokawa and Murashita, 2009; Huising et al., 2006). Both *lep* paralogues identified for Atlantic salmon in the present study cluster with *lepa*, and therefore suggest that at least one or more forms (*lepb*) may exist in this species, since it is tetraploid (Allendorf and Thorgaard, 1984). However, previous attempts using genomic synteny have only found the putative genomic duplicates in medaka and zebrafish paralogue (Gorissen et al., 2009; Kurokawa and Murashita, 2009). The presence of two *lepa* genes in other ostariophysan species such as goldfish and common carp are thus suggested to have arisen in a lineage-specific manner due to recent autotetraploidy in these species (Gorissen et al., 2009). Similarly the data for two *lepa* paralogues in Atlantic salmon likely represent a similar scenario, and it therefore remains unclear, whether *lepb* exists in other teleosts due to the degenerative nature of this paralogue.

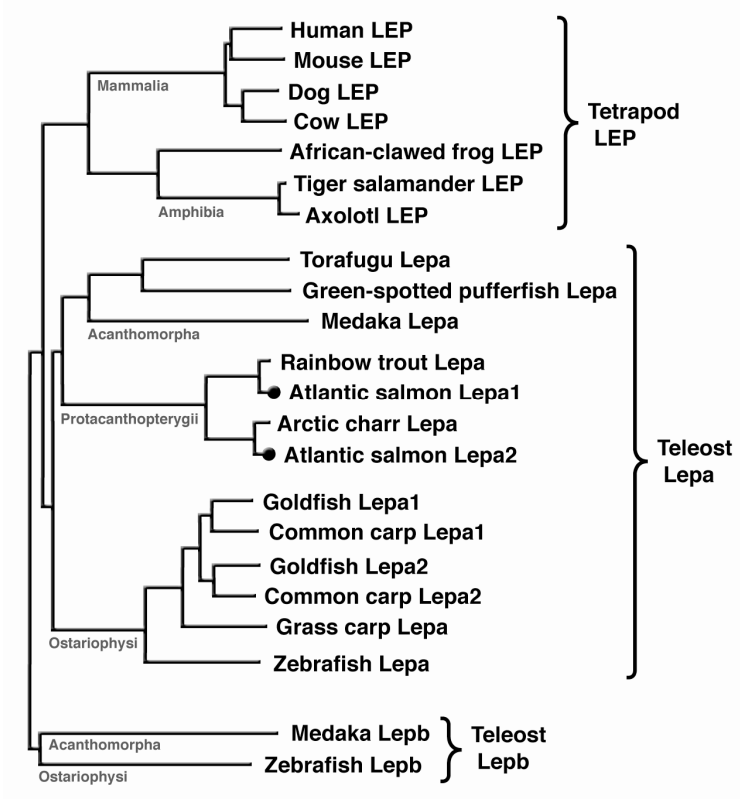

Figure 6. Phylogenetic tree of vertebrate leptins redrawn from Paper I.

Despite low amino acid identity between the different orthologues, three-dimensional modelling predicts conservation of the tertiary structure between Atlantic salmon and other teleost Leps compared to their mammalian orthologues (Fig. 7) (Paper I; Kurokawa et al. 2005; Murashita et al., 2008; Gorissen et al., 2009; Kurokawa and Murashita, 2009).

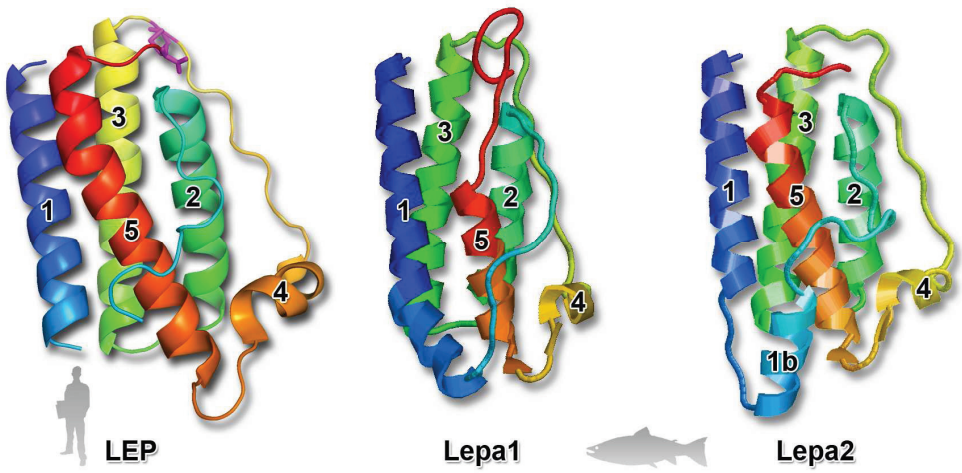

Figure 7. Models of Atlantic salmon leptins (*lepa1*, *lepa2*) compared to human leptin (LEP) based on ProModII (Paper I). The human molecule 1AX8.pdb shows the four anti-parallel α-helices (1, 2, 4, 5) with corresponding domains in the Atlantic salmon proteins. The C-terminal cysteine (magenta) covalently linked to loop 3-4 is shown as ball and stick in the human render.

Both *lepa1* and *lepa2* have two characteristic cysteine residues which are predicted to form disulfide bonds between the C-terminus and the loop between α-helics 3 and 4. This covalent linkage is considered a pre-equisite for the correct conformation and bioactivity of human LEP (Zhang et al., 1997; York et al., 1996).

The putative models suggest that the folding of *lepa2* might be different to *lepa1*. There are several differences between the 3D structures of *lepa1* and *lepa2; e.g.* α-helix 5 is considerably shorter in *lepa1* than *lepa2*. Furthermore α-helix 1 for *lepa2* appears to be split by a short-disordered region, and may therefore have a poorer affinity. However considering that it is a predicted model based upon the structure mask of human LEP, the significance of these putative conformational adjustments remains to be tested.

The importance of the conserved tertiary structure of Lep is most likely explained by requirements for specific LepR-binding affinity and is constrained by the structure of the

receptor-binding pocket (Crespi and Denver, 2006). This might also explain some of the results from studies on teleosts using heterologous mammalian LEP. For example, treatment with the mammalian hormone caused an anorectic effect in goldfish (Volkoff et al., 2003; De Pedro et al., 2006) and green sunfish (*Lepomis cyanellus*) (Johnson et al., 2000), but not in coho salmon (*Oncorhynchus kisutch*) (Baker et al., 2000), channel catfish *(Ictalurus punctatus*) (Silverstein and Plisetskaya 2000) or green sunfish (Londraville and Duvall 2002). These contradicting results have been explained by the relatively large differences in amino acid sequences observed between mammals and fish (Kurokawa et al., 2005; Crespi and Denver 2006; Huising et al., 2006). However future studies in this area should focus on verifying whether or not the observed differences using mammalian or species-specific hormones are valid or whether there is actually a pharmacological effect.

The present work identified five isoforms of *lepr* that have differences in the 3'-end of the mRNA sequence (Paper I). Of these, only the longest form retains each of the functionally important domains (such as three FN III domains, the Ig C2-like domain, a pair of WSXWS motifs, two JAK2-binding motif boxes, and a STAT-binding domain, Paper I), while the other four forms were predicted to only have the intracellular subdomains. The long form of mammalian LEPR has a function for full signal transduction through the JAK/STAT pathways, whereas the shorter isoforms exhibit partial or no signalling capabilities (Baumann et al., 1996; Tartaglia, 1997). The biological importance of the long form LEPR via the JAK/STAT pathway in maintaining body weight and energy homeostasis has been definitively demonstrated (Bates et al., 2003). Previous studies in teleosts have only identified a single *lepr* (Kurokawa and Murashita, 2009; Kurokoawa et al., 2008; Liu et al., 2010). Consequently, this work is the first identify multiple *lepr* transcripts in any ectothermic species. Comparison of the tertiary structures of *lepa1* and *lepa2* (Fig. 7) indicates that *lepa1* shows greater conformational similarity to human LEP, and it could therefore be inferred that

similar binding properties exist for this paralogue. However, further studies would be needed to test this hypothesis. Nevertheless, the relatively ubiquitous expression of *lepr* in Atlantic salmon tissues implies diverse roles of Lep in teleosts (Paper I).

The tissue expression pattern for the Atlantic salmon *lep* paralogues differs substantially (Paper I) and hence indicates a possible difference in function. With the exception of the results presented here, and those of zebrafish and Japanese medaka (Paper I, Gorissen et al., 2009; Kurokawa and Murashita; 2009), few studies have investigated the broad tissue distribution of *lep* in teleost fishes. Interestingly the more distantly related *lep* genes (*lepa* and *lepb*) showed distinct differences in in tissue distribution, as shown in medaka, where *lepa* is being expressed in liver and muscle, while *lepb* are more highly expressed in the brain and eye (Kurokawa and Murashita; 2009). However these differences are also seen in more closely related *lep* paralogues, such as *lepa1* in Atlantic salmon, being more highly expressed in the brain, liver and white muscle, while *lepa2* is mainly expressed in the stomach and midgut (Fig. 8).

Teleost ghrelins

Previous studies have reported the cloning of *ghrl1*, *ghrl2* in Atlantic salmon (Murashita et al., 2009b). Comparative analysis of the sequences obtained indicates that they represent splice variants of a single *ghrl* gene in Atlantic salmon. In the rainbow trout *ghrl* gene, an alternative splicing site has been found at the second intron, which results in the production of two types of ghrl (rtGhrl and des-VRQ-rtGhrl) (Kaiya et al., 2003). Furthermore, an exon–intron boundary site was found in Atlantic salmon at the same position as the trout *ghrl* second intron (Murashita,K., unpublished results). These data indicate that conserved splice variants exist in the closely related Salmonidae. However, very recently, two different salmon *ghrl* mRNA isoforms have been uploaded to GenBank (*S. salar* preproghrelin-1: EU513378

and *S. salar* preproghrelin-2: EU513379), which suggests that Atlantic salmon may have up to four different *ghrl* mRNA isoforms. In other vertebrates, two different *ghrl* mRNA isoforms have been identified in channel catfish (*Ictalurus punctatus*) (Kaiya et al., 2005) and the red-eared slider turtle (*Trachemys scripta elegans*) (Kaiya et al., 2004). Atlantic salmon is thus appears to be the first species in which more than two different *ghrl* mRNA isoforms have been identified.

		Tissues																		
Leptin	Species	Li	Br	Pi	Ey	Gi	Sk	He	St	Pc	Mg	Ad	Bf	Wm	Rm	Ki	Te	Ov	Panc	Sp	References
lepa1	Atlantic salmon	++	+++	+	+	+	+	+	(+)	+	+	+	+	++	+	+	+	+			Rønnestad et al., 2010
lepa2	Atlantic salmon	+	+	-	-	+	-	-	++	+	++	-	+	+	-	+	-	+			Rønnestad et al., 2010
lepa	Rainbow trout	+++	-		++		+	+	-		-*	-		+		-			-		Murashita et al., 2008
lepa	Common carp	++																			Husiing et al., 2006
lepa	Grass carp	++																			Li et al., 2010
lepa	Silver carp	++																			Li et al., 2010
lepa	Pufferfish	++																+			Kurokawa et al., 2005
lepa	Janpanese Medaka	++	+		+	+	+				+*			++ ***							Kurokawa and Murashita 2009
lepa	Zebrafish	+++		+++		+++		+++			+++*							++		+++	Gorissen et al., 2009
lepa	Artic charr	++												+ ***							Frøiland et al., 2010
lepb	Janpanese Medaka	-	+++		+++	-	+				+*			++ ***							Kurokawa and Murashita 2009
lepb	Zebrafish	+		+++		++		+++			++*							+++		++	Gorissen et al., 2009
Lepr	Atlantic salmon	+	++	++	++	++	++	+	+	+	+	++	++	++	++	++	++	+++			Rønnestad et al., 2010
	Marine medaka **	+/+	+/+		+/+	++/++	+/+				+/+*			++/++***		++/++	+	+			Wong et al., 2007
	Pufferfish	++	++	+++	+						+*					++	+	+++			Kurokawa et al., 2008
	Japanese Medaka	+	+		+	++	++				+*			+++ ***		+					Kurokawa and Murashita 2009
	Zebrafish	++	+			+++		+						+++ ***			+++	+			Liu et al., 2010

Li, liver; Br, brain; Pi, pituitary; Ey, eye; Gi, gill; Sk, skin; He, heart; St, stomach; Pc, pyloric caeca; Mg, mid-gut; Ad, adipose tissue; Bf, belly flap; Wm, white muscle; Rm, red muscle; Ki, kidney; Te, testis; Ov, ovary; Sp, spleen

* = intestine/ gut ** = female / male *** = muscle

Figure 8. Summary of the tissue distribution of the distantly related *lep* genes and more closely related *lep* paralogues in teleosts.

Effects of nutritional status

The observations that long-term feed restriction does not significantly affect *lep* expression in Atlantic salmon (Paper II) has also been noted in other teleosts. However it is likely that prolonged feed restriction can influence several endocrine parameters to adapt to the nutritional condition. For example, in common carp, a rapid response was observed in *lep* gene expression in hepatic tissues shortly after feeding, but no changes in expression in response to different long-term feeding regime were observed (Huising et al. 2006). These authors suggested that this effect could be explained by the fact that starved fish do not lose weight as rapidly as mammals, due to not having to deal with thermoregulation, and therefore can withstand longer periods of starvation. A similar study on grass carp showed that chronic injection of species-specific Lep did not affect long-term food intake and body weight, while acute injection decreased food intake (Li et al. 2009). Conversely, we observed increased *pomca1* levels following chronic injection of Lep in Atlantic salmon (Paper III), which suggests that chronic exposure to elevated Lep levels can decrease food intake through the Pomc pathway in this species, and suggests that *pomca1* could be a particular sensitive gene (Murashita 2008; Paper III). However such dosages may represent a pharmacological effect, since the effective dosage were an order of magnitude higher than concentrations measured in plasma. We did not observe any difference of feed restriction in circulating plasma levels in our study (Paper II), which contrast recent results in rainbow trout (Kling et al., 2009) and suggests that the relation between circulating Lep levels and energy status differs from that in mammals. However the RIA protocol for Atlantic salmon appears to allow interspecies assessment of plasma Lep levels. This only confirms that more comprehensive studies are needed for conclusive data interpretation. Studies on rainbow trout also implicated Lep as an anorectic hormone as in mammals. Injection of rainbow trout with recombinant trout leptin (rtLep) resulted in a significantly reduced appetite over two days that coincided with a

decrease in hypothalamic mRNA expression of *npy* and increase of *pomc* mRNAs, respectively (Murashita et al., 2008). Whether these observations are due to species-specific differences in the regulation of appetite or growth is not known, however, consensus data indicate that the effects of Lep on appetite regulation may be short-term in teleosts. Long-term effects of Lep may be tuned to season, rather than feeding status as suggested in Paper II. This latter notion is consistent with studies that found increased *lep* expression during summer months in teleosts and mammals entrained to photoperiods of northern latitudes (Concannon et al., 2001; Frøiland et al., 2010).

Daylength effect

In the long term-trials, the *lepa1* transcriptional expression in VAT seemed to be related to changing day-length. Leptin transcription could thus be associated with the pineal-melatonin axis, a finding that has also been proposed for mammals (Kus et al., 2004; Zieba et al., 2007; 2008; Klocek-Gorka et al., 2010). Melatonin is a common output signal of the vertebrate circadian clock which is produced at two major sites, the pineal organ and the retina (Falcòn 1999; Deguchi et al., 2005; Falcòn et al., 2007). Despite the fact that the photoperiodic and circadian controls of melatonin production have been profoundly modified during evolution, the melatonin signal released into the blood is the same from fish to mammals (Colin et al., 1989). Plasma melatonin content is higher at night than during the day, thus reflecting the prevailing dark cycle of the photoperiod. The shape of these oscillations changes with the season. In fish, they are of short duration and high amplitude in the summer and of long duration and low amplitude in the winter, with intermediate situations occurring in the spring and autumn. Thus, the plasma melatonin profile is an indicator of both day-length and season, and melatonin is considered to be a time-keeping molecule (Underwood, 1989; Arendt, 1997; Ekstrôm, 1997). Recent data have provided evidence in ewes that LEP upregulates

photoperiodic hormones, such as melatonin during short days, but downregulates melatonin during long-days (Zieba et al., 2008). Furthermore, seasonal sensitivity to LEP has been demonstrated in sheep and it appears to serve as a mechanism of adaption to feed availability (Zieba et al., 2007; Klocek-Gorka et al., 2010). In teleosts, a recent study on another salmonid, the Arctic charr (*Salvelinus alpinus*), also found that Lep may be seasonally regulated (Frøiland et al., 2010). Interestingly the seasonal dynamics of *lep* expression that putatively occur in Atlantic salmon have also been reported for woodchuck's photoentrained to a northern photoperiod (Concannon et al., 2001). Although the experiments in Paper II were not specifically designed as a photoperiod study, photoperiod was the only variable that seemed to influence *lep* expression during this period. It has also been suggested that melatonin might activate the phospholipase C (PLC) pathway through G_q proteins in mammals (Vanecek, 1998; Steffens et al., 2003), which is also implicated in the ghrelin signalling transduction pathway (Howard et al., 1996; McKee et al., 1997).

The plasma data for Lep and Igf1 further appeared to be more correlated to day-length than to long-term feed restriction as observed for *lep* mRNA. A similar finding has also been noted in gilthead seabream (*Sparus aurata*), in which dietary composition or ration size did not affect hepatic expression levels of *igf1* (Méton et al., 2000). Interestingly, however, this latter study found *igf1* expression levels to be diurnally regulated. Conversely, in rainbow trout, a strong relationship between season and levels of Igf1 in plasma has been reported (Taylor et al., 2008). Although the plasma Igf1 levels in the present study did not show a strong seasonal relationship, plasma Lep did reflect the mRNA expression levels in VAT of the FF group. Consequently, season may thus have influenced the observed variation. Further studies will be necessary to validate the role of photoperiod in the energy homeostasis of Atlantic salmon.

Short-term feed restriction

The short-term experiments revealed that *lepa1* expression specifically peaks in the peripheral tissues (white muscle, visceral adipose tissue, belly flap and liver) after 6 – 9 hr in the unfed fish. This suggests that the transcript-specific response could be associated with the absence of food. Conversely since the unfed fish had not received food for 33 hr (24 + 9 hr), the peaks could represent an unrelated effect. Since there were no significant differences ($p<0.05$) in plasma Lep levels between fed and unfed fish, and the temporal upregulation of *lepa1* occurred during a phase of falling plasma Lep, the increased expression does not in fact appear to be specifically related to the absence of food. The earliest peak of *lepa1* occurred in the white muscle, which represents an important lipid reservoir in Atlantic salmon (Aursand et al., 1994). Unlike pufferfish, which utilize the liver as a major lipid repository (Kurokawa et al., 2005), Atlantic salmon show that despite a high visceral lipid content (Table 2, Paper II), hepatocytes contain few lipid droplets compared to other species of teleost (Bruslé and Gonzàlez, 1996), yet are an important site for *lep* expression (Paper I, II). Both *lepa1* and *lepa2* peaked at 9 hr in the liver of unfed fish. By contrast, however, studies in common carp demonstrated a peak in *leptin-I* (*lepa1*) and *leptin–II* (*lepa2*; see Fig. 6) in the liver at 3 and 6 hr post feeding, respectively (Huising et al., 2006). The earlier expression response of *lep* in common carp likely reflects the higher temperature under which the experiments were conducted, but contrast the findings of upregulation of *lepa1* in Atlantic salmon due to the absence of food. Similarly, in mice, a postprandial increase in hepatic leptin expression has also been reported (Saladin et al., 1995). However, a recent study in grass carp has shown that intraperitoneal injection of recombinant Lep only alters the appetite on the first day, and does not influence food intake during the ensuing 12 days (Li et al., 2010). The present data for Atlantic salmon are therefore quite different and suggest that *lep* expression in this species may have a complex lipostatic function.

Acute administration of species specific lep (rs-lepa1)

To further elucidate the role of *leps* in energy homeostasis, a protocol for species-specific administration of *lep* was established (Paper III). Recombinant Lepa1 (rs-lepa1) was harvested from bacterial (*E. coli*) cultures and administered by intraperitoneal osmotic pumps to test the effects of the protein on appetite and growth of Atlantic salmon over 20 days. Weight gain and specific growth rate were significantly reduced at the highest dosage (1000 ng H-1). This high dosage also increased *pomca1* levels significantly in the brain. Lep treatment is known to reduce body weight and food intake in many mammalian species, such as rats, mice, pigs and monkeys (Seeley et al., 1996; Sahu 1998; 2004; Wetzler et al., 2004). However, these dosages may represent a pharmacological and not a physiological effect since when recalculated they are at least 10-fold higher than concentrations measured in Atlantic salmon plasma (Paper II). Furthermore we do not know how much leptin was absorbed into the blood, and it is a possibility that some of the rs-lepa1 was broken before absorption. Nevertheless, the data demonstrate that *lepa1* can illicit an anorectic response as found in mammals. The increased *pomca1* levels correspond to the role of α-MSH, as a derivative of pomc, which has a strong anorexigenic function, and is activated in a Lep downstream-pathway (Schwartz et al., 2000). This was also observed in acute short-term examination in rainbow trout (Murashita et al., 2008), and contrasts the findings of increased blood Lep concentration during long-term fasting in trout (Kling et al., 2009).

Ontogeny

In order to investigate the ontogeny of neuropeptide signalling, transcripts for several neuropeptides involved in the mammalian energy homeostatic circuitry were cloned from Atlantic salmon (Murashita et al., 2009a,b; Paper III). By studying the developmental expression levels, Paper IV is the first to show that *lepa1*, *cart*, *pomca1* and *agrp* transcripts

are maternally supplied in Atlantic salmon. The transcripts decreased significantly between 320-400 dd, suggesting that these mRNAs are latently degraded when zygotic expression of *lepa1* and *cart* are co-upregulated in the embryo. This suggests that the regulatory communication between Lep secretion and neuropeptide signalling may be established early in the developmental program. This notion is supported by related evidence demonstrating that Lep transactivates CART in mammalian ARC neurons (Schwartz et al., 1997; Ahima et al., 2000; Ahima and Osei, 2004; Bjørbæk and Kahn, 2004; Pinto et al., 2004; Sahu, 2004a, b; Gao and Horvath, 2007). By integrating the data observed here, and assuming that expression in the head is localised in the brain, a putative model is proposed (Paper IV) for the early endocrine and neuropeptide signalling of Atlantic salmon. While this model is consistent with known regulatory loops in mammals, it is recognised that other *lep* and *ghrl* paralogues may exist in Atlantic salmon, which is tetraploid (Allendorf and Thorgaard, 1984). Nevertheless, the current data for Atlantic salmon appear to support the putative neuropeptide circuitry induced by *lep* and *ghrl* as found for mammals.

The endocrine effects of mammalian LEP, however, have also been found to be pleiotropic. For example, *ob*$^{-}$/*ob*$^{-}$ mice with complete LEP deficiency show evidence of developmental defects, including the failure to undergo sexual maturation (Chehab et al., 1996; Barash et al., 1996), as well as structural neuronal abnormalities and impaired myelination in the brain (Bereiter, et al., 1979; 1980; Sena et al., 1985). This is further supported by the high levels of *lepr* in the brain and notochord of embryonic zebrafish (Liu et al., 2010). Taken together, these findings suggest that vertebrate Lep may be involved in various aspects of CNS development. Maturation of neuronal pathways by LEP might require basal leptin levels or an increase in LEP at some critical period, independent of its later role as a sensor of energy stores. LEP has also been implicated in promoting the development of mouse preimplantation embryos *in vitro* (Kawamura et al., 2002), which raises the possibility

of a developmentally regulated paracrine/autocrine LEP signalling system. Some of these functions appear to be conserved in amphibia, since studies in African clawed frog (*Xenopus laevis*) tadpoles, have shown that LEP stimulates food intake, limb and lung development (Crespi and Denver, 2006; Torday et al., 2009). Hence further studies are necessary to verify which role leptin might have during early development in fish.

Major Findings

- At least two *lep* genes (*lepa1* and *lepa2*) exist in Atlantic salmon
- Five receptor isoforms were found in Atlantic salmon
- Long-term feeding status and growth appear unaffected by *lep*
- Season may influence *lep* expression
- Short-term feed restriction induces latent (6-9 hr) peaks of *lep* in peripheral tissues
- High dosages of recombinant Lep can affect the appetite and growth of Atlantic salmon
- Ontogeny of energy homeostatic and neuropeptide signals reflect the mammalian appetite control circuitry

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