Leptin endocrinology and energy homeostasis in salmonids

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Dissertation abstract

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In salmonids, the physiological role of leptin is not completely elucidated. Nonetheless, the anorexigenic effect of leptin indicates a role in their energy homeostasis. This thesis focuses on advancing the understanding of the involvement of the leptin system in the regulation of energy homeostasis and food intake in teleost fish. Furthermore, it examines the question if adipose tissue is a leptin-producing tissue in fish.

By modulating food availability as well as studying rainbow trout breeding-selected for different muscle lipid content, the nutritional state was manipulated. By utilizing a range of homologous analytical tools, including radioimmunoassays for plasma leptin and leptin-binding protein levels, and quantitative expression analysis (qPCR) of leptin related genes *in vivo* and *in vitro*, the functional links between leptin endocrinology, food intake and nutritional state were studied.

The results presented in this thesis reveal that the leptin system in salmonids is highly complex, and that its regulatory response to periods of catabolism may depend on environmental or physiological conditions. The leptin-induced anorexic state is modulated during periods of food shortage or fast growth. When fish develop anorexia through high plasma leptin levels, the breaking of the anorexic state appears not to be due to a decrease in plasma leptin, but rather that consumption of food decreases plasma leptin.

A disparity between plasma leptin levels and both the gene expression of the leptin receptor isoforms and plasma leptin binding protein was observed. This indicates that more data on the protein level are needed to improve our understanding of leptin endocrinology in fish, and to compliment current knowledge which is mainly derived from gene studies.

The plasma leptin source has not yet been determined in salmonids, although the liver has been suggested as a main source due to high hepatic *lep* expression. This thesis demonstrates that visceral adipose tissue both secretes leptin and expresses the *lep* gene, supporting a leptin-secreting role.

Keywords: Leptin, Leptin binding-protein, Energy homeostasis, Oncorhynchus mykiss, Salvelinus alpinus, Adiposity, Food intake, Fasting, Refeeding, Catabolism, Anorexia.

List of papers

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

Paper I
Salmerón C, Johansson M, Angotzi AR, Rønnestad I, Jönsson E, Björnsson BTh, Gutiérrez J, Navarro I and Capilla E (2014) Effects of nutritional status on plasma leptin levels and *in vitro* regulation of adipocyte leptin expression and secretion on rainbow trout. *General and Comparative Endocrinology* **210**: 114-123.

Paper II
Árnason T, Gunnarsson S, Imsland AK, Thorarensen H, Sráradóttir H, Steinarsson A, Gústavsson A, Johansson M and Björnsson BTh (2014) Long-term rearing of Arctic charr (*Salvelinus alpinus*) under different salinity regimes at constant temperature. *Journal of Fish Biology* **85**: 1145-1162.

Paper III
Johansson M, and Björnsson BTh (2015) Elevated plasma leptin levels of fasted rainbow trout decrease rapidly in response to feed intake. *General and Comparative Endocrinology* **214**: 24-29.

Paper IV
Johansson M, Morgenroth D, Einarsdottir IE, Gong N, Björnsson BTh (2016) Energy stores, lipid mobilization and leptin endocrinology of rainbow trout. *Journal of Comparative Physiology B* (manuscript under revision).

Table of Contents

Introduction 1
Leptin and its diverse functionality 1
The origin of leptin and the lipostasis hypothesis 1
The molecular structure of leptin and the leptin receptor 1
The mammalian leptin model 2
Comparative aspects of mammalian leptin physiology 3
Leptin in non-mammalian vertebrates 4
Leptin in fish 4
Leptin receptor and leptin binding proteins 5
Leptin and nutrition status 6
Leptin and lipid metabolism 7
Leptin and sexual maturation 8
Leptin, stress and osmoregulation 8
Energy homeostasis 9
Lipid metabolism 10
Ghrelin 11
Growth hormone (GH) and insulin-like growth factor I (IGF-I) 12
Summary 13
Scientific aims 14
Methodological and terminological considerations 15
Manipulation of nutritional status 15
Experimental animal models 15
Radioimmunoassays 16
Quantitative PCR 16
Cell isolation 18
Fasting versus starvation 18
Findings and Discussion 19
Leptin endocrinology in relation to nutritional states 19
Leptin endocrinology during feeding conditions 19
Leptin endocrinology during voluntary fasting 21
Leptin endocrinology during fasting 22
Leptin and the initiation of feeding 26
Tissue sources of circulating leptin 27
Conclusions and future perspectives 30
Acknowledgments 32
References 33

Introduction

Leptin and its diverse functionality

The origin of leptin and the lipostasis hypothesis

Genetically induced obesity was first observed in the mouse, and this recessive obese mutation was designated as *ob* (Ingalls et al. 1950). The lipostasis hypothesis states that a peripheral signal is proportionally produced to the total amount of adipose tissue. The signal is then compared in the brain to a "set point" and any offset will either trigger energy intake or energy expenditure (Kennedy 1953). However, the set point could change with time and may be an integration of many signals, both peripheral and central (Rowland et al 1996). The *ob* gene in mouse and the human homologue were cloned and sequenced by Zhang and colleagues (1994) using the mutant obese C57BL/6J *ob*/*ob* mouse. Evidence indicated that an *ob* protein product was secreted mainly from adipose tissue in proportion to fat mass, supporting the lipostasis hypothesis and implying that the *ob* signal, later known as leptin, acted directly or indirectly on the CNS to regulate energy expenditure or inhibit food intake. Leptin was therefore suggested to be the explaining signal for the lipostasis hypothesis.

The molecular structure of leptin and the leptin receptor

The 16 kDa *ob* gene product was named leptin, which is derived from the Greek word leptós meaning thin, as early research indicated it as an anorexigenic hormone. In 1995, leptin was shown to be structurally related to the family of helical cytokines and to exert its effects in a similar manner as class I cytokines (Madej et al. 1995). Throughout the entire vertebrate series from fish to mammals, leptin has maintained both secondary and tertiary structures, with 4 conserved helixes and a disulphide bridge, while the primary amino acid sequence varies highly among species (Boswell et al. 2006; Crespi and Denver 2006; Denver et al. 2011; Gorissen et al. 2009; Huising et al. 2006; Kurokawa et al. 2005; Li et al. 2010; Morini et al. 2015; Murashita et al. 2008; Ohga et al. 2015; Rønnestad et al. 2010). The leptin receptor is closely related to the gp130 family of cytokine receptors and has a single membrane-spanning domain. The receptor has multiple isoforms (six in rodents, four in humans) derived from alternative splicing of mRNA (Lee et al. 1996; Tartaglia et al. 1995). Only the long form of the leptin

receptor (Ob-Rb) carries the long intracellular domain with the two protein motifs necessary for activation of the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, while Ob-Ra has a shorter intracellular domain and transduces signals through the mitogen-activated protein (MAP) kinase (Bjørbæk et al. 1997). In humans, the Ob-Re lacks the intracellular domain, but maintains the ligand-binding properties and has been demonstrated to be a leptin binding protein (LepBP) (Sinha et al. 1996).

The mammalian leptin model

The C57BL/6J *ob*/*ob* mice are obese and diabetic and have reduced metabolism, body temperature and physical activity. These symptoms can be alleviated by daily administrations of recombinant mouse leptin, suggesting that leptin regulates lipid deposition and body weight through effects on appetite and metabolism (Pelleymounter et al. 1995). Lean mice in the same study who received daily leptin injections lost less weight and had reduced food intake, while no changes in metabolic parameters were observed (Pelleymounter et al. 1995). In rodents and humans, plasma leptin correlates positively with the body mass index (BMI) (Maffei et al. 1995), and weight loss through food restriction lowers plasma leptin in humans and rodents (Ahima et al. 1996; Coppari and Bjørbæk 2012). The correlation between plasma leptin and BMI, together with the effects of administered recombinant leptin in mice indicates/strengthens the role of leptin as a peripheral/systemic lipostatic signal regulating adiposity and having a suppressive effect on appetite. Leptin crosses the blood brain barrier and exerts its central actions on both hypothalamic orexigenic and anorexigenic neurons to regulate energy balance and food intake (Coll et al. 2007; Harris 2014).

In mammals, leptin regulates lipid metabolism directly via stimulation of the lipolytic pathways. Leptin inhibits insulin-stimulated lipogenesis and promotes lipolysis (Cohen et al. 2002; Reidy and Weber 2000). Furthermore, leptin promotes the shift from carbohydrate oxidation to fat oxidation in *ob*/*ob* mice (Hwa et al. 1997). In adipocytes, leptin inhibits the expression of acetyl CoA carboxylase, an enzyme which converts carbohydrate to triacylglycerol (TG) (Bai et al. 1996). Fatty acid uptake inhibits leptin release from adipocytes *in vitro* (Cammisotto et al. 2006), and mice with diminished fatty acid uptake have an increased plasma leptin levels (Hajri et al. 2007) indicating a negative feedback loop.

Leptin is a highly pluripotent hormone with regulatory functions linked to energy homeostasis, obesity, reproduction, immunity, wound healing and bone formation (Ahima and Osei 2004). Even though leptin is mainly produced in adipose tissue (Zhang et al. 1994), it is also produced in placenta (Masuzaki et al. 1997), stomach (Bado et al. 1998) and skeletal muscle (Wang et al. 1998). Both sexes of the homozygous *ob*/*ob* mouse is infertile (Zhang et al. 1994), and administration of recombinant human leptin in these mice restores fertility and decrease weight in both sexes, whereas in female *ob*/*ob* mice, weight reduction alone does not restore fertility (Chehab et al. 1996; Mounzih et al. 1997) indicating that leptin has a direct function in reproduction and the infertility is not an effect of increased weight. Leptin is a permissive, but not stimulatory, signal in the control of puberty in mammals, both via and peripheral effects on the gonads and central effects (Vazquez et al. 2015).

Comparative aspects of mammalian leptin physiology

When comparing the biomedical model species, rodents and humans, with other mammalian species, a more complex picture of leptin and it functions emerge. Most mammals living in Arctic to temperate climates exhibit lifecycles with significant seasonal changes in adiposity from high to low lipid stores. These species show a disconnection between adiposity and leptin levels. In hibernating animals such as the Syrian hamster (*Mesocricetus auratus*), leptin appears more closely linked to short-term energy balance than changes in adiposity (Schneider et al. 2000), and in the little brown bat (*Myotis lucifugus*), plasma leptin levels increase before any increase in fat accumulation (Kronfeld-Schor et al. 2000). In other hibernating mammals such as the mink (*Mustela vison*), racoon dog (*Nyctereutes procyonoides*), woodchuck (*Marmota monax*) and the common shrew (*Sorex araneus*), the highest levels of plasma leptin are found in individuals with the lowest body adiposity (Concannon et al. 2001; Nieminen and Hyvarinen 2000; Nieminen et al. 2000; Nieminen et al. 2002).

Marine mammals have a thick, subcutaneous fat layer (blubber) as insulation from the cold, aquatic environment (Whittow 1987; Young 1976). In the bowhead (*Balaena mysticetus*) and beluga (*Delphinapterus leucas*) whales it has been shown that blubber expresses the *lep* gene, with higher leptin production in adipose than other tissues (Ball et al. 2013). In the Northern sea lion (*Mirounga angustirostris*) and the Antarctic fur seal (*Arctocephalus gazelle*), there is no correlation between plasma leptin and fat stores (Arnould et al. 2002; Ortiz et al. 2001), indicating that leptin is not

regulating these fat stores. Although, plasma leptin levels decrease during fasting in the subantarctic fur seal (*Arctocephalus tropicalis*; Verrier et al. 2012). Furthermore, while plasma leptin levels decrease during fasting in Antarctic fur seal pups and fasting, lactating females (Arnould et al. 2002), in Northern sea lion, plasma leptin levels are unaffected by fasting (Ortiz et al. 2001).

Leptin in non-mammalian vertebrates

Leptin was identified in South African clawed frog (*Xenopus laevis*), and found to have 35% primary amino acid similarity with human leptin and 13% to pufferfish leptin (Crespi and Denver 2006). Further, in the tiger salamander (*Ambystoma tigrinum*), the leptin gene has been identified with 29% similarity to human leptin primary amino acid sequence (Boswell et al. 2006). In 2011, two leptin genes were identified in the reptile, the green anole (*Anolis carolinensis*) with 36% similarity to human leptin (Denver et al. 2011). In spite of their relatively low sequence similarities with mammalian leptin, leptin in these amphibian and reptilian species have a similar tertiary helical structure as the mammalian leptin. In South African clawed frog, spadefoot toad (*Spea bombifrons*) and fence lizard (*Sceloporus undulates*) leptin reduces food intake (Crespi and Denver 2006; Garcia et al. 2015; Niewiarowski et al. 2000). Leptin also interacts with the immune system (Crespi et al. 2012; French et al. 2011; Hicks-Courant and Crespi 2006), in early development of the *Xenopus* tadpole (Crespi and Denver 2006; Love et al. 2011; Torday et al. 2009), and mating preferences (Garcia et al. 2015).

The early cloning of leptin in chicken (Taouis et al. 1998) was subsequently found to be incorrect due to contamination (Friedman-Einat et al. 1999), and the first true bird leptin was only recently sequenced in the peregrine falcon (Prokop et al. 2014).

Leptin in fish

In non-mammalian vertebrates, leptin was first identified and characterized in fish, when a homologue to mammalian leptin was identified in pufferfish (*Takifugu rubripes*; Kurokawa et al. 2005). Since then, leptin has been identified and characterized in common carp (*Cyprinus carpio*; Huising et al. 2006), rainbow trout (*Onchorhynchus mykiss*; Murashita et al. 2008), Japanese medaka (*Ortzias latipes;* Kurokawa and Murashita 2009),

zebrafish (*Dario rerio*; Gorissen et al. 2009), Atlantic salmon (*Salmo salar*; Rønnestad et al. 2010), Arctic charr (*Salvelinus alpinus*; Frøiland et al. 2010), grass carp (*Ctenopharyngodon idella*; Li et al. 2010), striped bass (*Morone saxatilis*; Won et al. 2012), orange-spotted grouper (*Epinephelus coioides*; Zhang et al. 2013), European and Japanese eel (*Anguilla anguilla, Anguilla japonica;* Morini et al. *2015)*, red-bellied piranha (*Pygnocentrus nattereri*; Volkoff 2015), and chub mackerel (*Scomber japonicas*; Ohga et al. 2015). The primary sequences differ highly among fish species and are only 6-26% identical with human leptin, although the teleost leptin structures all predict a similar class I helical cytokine tertiary structure as in mammals, (Gorissen et al. 2009; Huising et al. 2006; Kurokawa et al. 2005; Li et al. 2010; Morini et al. 2015; Murashita et al. 2008; Ohga et al. 2015; Rønnestad et al. 2010)

Two leptin paralogues (leptin A and B) have been identified in zebrafish, Japanese medaka, orange-spotted grouper, European and Japanese eel and chub mackerel (Gorissen et al. 2009; Kurokawa and Murashita 2009; Morini et al. 2015; Ogha et al. 2015; Zhang et al. 2013). These paralogues stem from ancient whole-genome duplication event (WGD; 3R) (Taylor et al. 2003; Volff 2005). In salmonids, a further lineage-specific WGD (Allendorf and Thorgaard 1984; Ohno 1970) has resulted in four paralogues; leptin-A1 and leptin-A2 as well as leptin-B1 and leptin–B2 (Angotzi et al. 2013; Rønnestad et al. 2010).

Based on gene expression analysis, the major leptin producing site in teleost fish appears to be the liver (Gorissen et al. 2009; Huising et al. 2006; Rønnestad et al. 2010; Tinoco et al. 2012; Won et al. 2012), this regardless of the relative importance of the liver as an energy-storing organ. Salmonids also express low levels of the *leptin* (*lep*) gene in adipose tissue (Murashita et al. 2008; Rønnestad et al. 2010; **Paper I**) and *in vitro* experiments demonstrate that leptin is secreted from adipocytes (**Paper I**).

Leptin receptor and leptin binding proteins

In several fish species, the functional (long form) leptin receptor ($LepR_L$, analogous to the mammalian Ob-Rb) gene has been cloned (Cao et al. 2011; Gong et al. 2013b; Kurokawa and Murashita 2009; Rønnestad et al. 2010; Shpilman et al. 2014; Tinoco et al. 2012; Zhang et al. 2013). In addition to the $LepR_L$, a truncated leptin receptor ($LepR_T$) has been characterized in rainbow trout (Gong et al 2013a). The affinity for leptin is similar for both receptor forms, although only the $LepR_L$ is capable of mediating the leptin signal through intracellular pathways, whereas the $LepR_T$ is suggested to be

linked to the central regulation of food intake and modulating the leptin signal at the tissue level (Gong and Björnsson 2014). Furthermore, in rainbow trout, there are three shorter leptin receptor isoforms ($LepR_{S1,}$ $LepR_{S2}$ and $LepR_{S3}$) which lack the transcellular domain, while maintaining the extracellular receptor domain and the ligand-binding properties similar to the $LepR_L$ (Gong et al. 2013a). These shorter isoforms are thought to act as plasma LepBPs when released into the circulation, as well as at the tissue level, and can thus modulate both the peripheral and central physiological leptin actions (Gong et al. 2013a).

Leptin and nutrition status

In teleost fish, leptin has an anorexigenic function (Aguilar et al. 2010; 2011; de Pedro et al. 2006; Gong et al. 2016a; Huising et al. 2006; Li et al. 2010; Murashita et al. 2008; Volkoff et al. 2003; Won et al. 2012) similar to mammals (Munzberg and Morrison 2015), even though leptin treatment has not always resulted in inhibition of food intake in teleosts (Baker et al. 2000; Londraville and Duvall 2002). In fish, changes in nutritional condition affect the leptin system, but there are large species differences in terms of the responses, both in tissue *lep* gene expression as well as in plasma leptin levels. Hepatic *lepb* expression in zebrafish (Gorissen et al. 2009) and hepatic *lepa* expression in orange-spotted grouper (Zhang et al. 2013) increases during fasting, whereas in striped bass liver (Won et al. 2012) and the proximal intestine of red-bellied piranha (Volkoff 2015), *lep* expression decreases during fasting. Further, fasting does not affect hepatic *lep* expression in the common carp (Huising et al. 2006), hepatic *lepa* expression in zebrafish (Gorissen et al. 2009), visceral adipose *lep* expression in zebrafish (Oka et al. 2010) and hypothalamic or hepatic *lep* expression in goldfish (Tinoco et al. 2012), for summary see table 1A. Likewise, changes in plasma leptin levels in response to food restriction or fasting vary among species, with decreased plasma leptin levels in the green sunfish (*Lepomis cyanellus*; Johnson et al. 2000) and burbot (*Lota lota*; Nieminen et al. 2003), whereas plasma leptin levels increase in rainbow trout (Kling et al. 2009; **Paper III**), Atlantic salmon (Johnsen et al. 2011; Trombley et al. 2012) and fine flounder (*Paralichthys adspersus*; Fuentes et al. 2012, 2013). In rainbow trout bred for high or low muscle lipid content for seven generations, plasma leptin levels decrease during fasting in individuals with low muscle lipid content, but not in individuals with high muscle lipid content (**Paper IV**), for summary see table 1B.

A	Common carp	Goldfish		Orange-spotted grouper	Red-bellied piranha	Striped bass	Zebrafish	
Tissue	Liver	Liver	Hypothalamus	Liver	Proximal Intestine	Liver	Liver	Visceral adipose
Leptin isoform	*lep*	*lep*	*lep*	*lep a*	*lep*	*lep*	*lep a* / *Le pb*	lep
Response to fasting	—	—	—	↑	↓	↓	— / ↑	—

B	Atlantic salmon	Burbot	Fine Flounder	Green sunfish	Rainbow trout	Rainbow Trout (lean line)	Rainbow Trout (fat line)
Plasma response to fasting	↑	↓	↑	↓	↑	↓	—

Table 1. Summary of the different responses to fasting in different species of fish. Changes in tissue *lep* gene expression in response to fasting (A), changes in plasma leptin levels in response to fasting (B)

Leptin and lipid metabolism

Given the known, metabolic effects of leptin in mammals, a major focus in non-mammalian research has been placed on elucidating the role of leptin in regulating energy balance, with emphasis on lipid metabolism. However, the role of leptin in the lipid metabolism in fish is still far from fully elucidated. Injections of mouse leptin increase carnitine palmitoyl transferase (CPT) and fatty acid binding proteins (FABP) in green sunfish (Londraville and Duvall 2002), and hepatic lipid content in goldfish decreases after ten days of human leptin injections (de Pedro et al. 2006). In grass carp, the effect of leptin on genes related to lipid metabolism are mostly short-term, such as reducing hepatic expression of stearoyl CoA deaturase-1, a critical enzyme for the biosynthesis of monounsaturated fatty acids (Li et al. 2010). In the study by li and colleagues, the only long-term effect after a 13 day long treatment period with repeated leptin injections was the reduction of hepatic lipoprotein lipase (Li et al. 2010). Leptin stimulates lipolysis via

JAK-STAT signaling and fatty acid β-oxidation gene expression in the fatty degenerated hepatocytes of grass carp (Lu et al. 2012). Treatment with 10nM of homologous leptin on isolated rainbow trout adipocytes increases glycerol without proportionate release of free fatty acids (FFA), and decreases fatty acid transporter-1 (FATP1) expression, indicating stimulation of lipolysis and a reduction of adipocyte FFA content *in vitro* (Salmeron et al. 2015).

Leptin and sexual maturation

Even though the greatest research focus so far has been on the involvement of leptin in feeding and energy metabolism, research into the involvement of the leptin system in other physiological processes is gaining momentum. One of these is sexual maturation, where leptin stimulates release of luteinizing hormone and somatolactin in sea bass (Peyon et al. 2001, 2003). The main androgens, testosterone and 11-ketotestosterone, as well as the 17β-estradiol directly affect the hepatic *lepa1* and *lepa2* in Atlantic salmon (Trombley et al. 2015) and during the spermatogenesis, hepatic *lepa1* and *lepa2* expression is elevated in maturing one-year old Atlantic salmon male (Trombley et al. 2014; Trombley and Schmitz 2013). An increase in *lep* expression has also been associated with sexual maturation in Arctic charr (Frøiland et al. 2010), and in ayu (*Plecoglossus altives*), high plasma levels of prolactin and 17β-estradiol coincide with increased plasma levels of leptin (Nagasaka et al. 2006). This suggests that leptin is involved in the process of sexual maturation, but it is unclear if this means that leptin is directly involved in the maturation processes or indirectly through regulation of energy metabolism, acting more in a permissive role as in mammals (Vazquez et al. 2015).

Leptin, stress and osmoregulation

Leptin attenuates the hypothalamo-pituitary-interrenal stress axis, as leptin decreases adrenocorticotropic hormone (ACTH) release from the pituitary, while decreasing cortisol release from the head kidney (Gorissen et al. 2012). Hypoxic stress increases *lep* expression in carp (Bernier et al. 2012) and zebrafish (Chu et al. 2010), and during acute hyperosmotic stress, leptin promotes glucose mobilization in tilapia (Baltzegar et al. 2014). Further indication of possible links between leptin and osmoregulation is its interaction with prolactin, a hormone known to be important for osmoregulation in euryhaline and freshwater fish (Sakamoto and McCormick

2006). In tilapia, leptin stimulates prolactin secretion *in vitro* from the rostral pars distalis (Tipsmark et al. 2008) and hepatic leptin gene expression as well as circulating plasma leptin A levels are inhibited by prolactin (Douros et al. 2014) indicating that leptin may be involved in responses to osmotic stress.

Energy homeostasis

Maintaining sufficient energy stores for both short- and long-term needs and the regulation of energy intake is defined as energy homeostasis whereas the process of converting energy from the stored or circulating nutrients to adenosine triphosphate (ATP) is defined as energy metabolism. The energy can be extracted from proteins, carbohydrates or lipids, with lipids as the most concentrated energy source as triacylglycerol (TG) with an average energy content of 9.5 kcal g^{-1} (Finn and Dice 2006). In most vertebrates, adipose tissue is the main storage of lipids as it is essentially (>90% by weight) made up of lipid droplets. However, many other tissues, including muscle and liver, also contain lipids as energy stores (Finn and Dice 2006; Sheridan 1994).

In mammals, the main long-term storage of energy is in the form of TG stored in adipose tissue, mostly located within the abdominal cavity and between muscle fibers (Deuel 1955). In fish, the principal storage tissues are visceral adipose tissue, liver and white and red muscle. Visceral adipose tissue is mainly composed of lipids (Sheridan 1988b). The liver can store considerable amount of lipids, often 10-20%, but up to 67% in some species such as the Atlantic cod. However, storage capacity varies among species as well as with developmental stage and/or season (Henderson and Tocher 1987; Sheridan 1988a, 1989). The liver also has a high capacity for *de novo* lipid synthesis, as shown in coho salmon (Lin et al. 1977). Both white and red muscles in fish contain lipids. White muscle contains adipocytes between the fibers, whereas in red muscle, most of the lipids are found within the fibers (Sheridan 1994). Salmonids have significant adipose stores in muscle and are regarded as a “fatty” fish species. In aquaculture production, filet adiposity content is usually 10-15% for salmonids. Salmonids also store lipids in visceral adipose tissue, and this is assumed to be the most important tissue for long-term storage of lipids, while muscle and liver lipid stores are regarded as more short-term storage (Sheridan 1994). During extreme energy needs, lipids can also been mobilized from the carcass, i.e. head, skeleton, fins and skin (Jobling et al. 1998; Jørgensen et al. 1997). The brain regions involved in the regulation of energy homeostasis

are mainly the hypothalamus and the brainstem, registering peripheral hormonal signals such as leptin and gut hormones such as ghrelin and cholecystokinin (CCK), as well as neural signals (Sherwood et al. 2005).

Lipid metabolism

The plasma lipoproteins in fish are similar to mammalian lipoproteins (Skinner and Rogie 1978). The lipoprotein containing the highest amount of TG is the very low-density lipoprotein (VLDL) and as TG is cleaved off and absorbed by the cells, VLDL changes to low-density lipoprotein (LDL). The liver can subsequently take up LDL and reassemble this to VLDL. Lipoprotein lipase (LPL) is responsible for the lipolysis of TG and for directing fatty acids (FA) into the cell. LPL is localized on the capillary endothelium outside the cell (Skinner and Youssef 1982). In rat, insulin has been suggested to affect LPL by stabilizing LPL mRNA (Raynolds et al. 1990). In gilthead seabream, insulin regulates both the gene expression and activity of LPL in adipose tissue, and during fasting, LPL activity in the adipose tissue is reduced and is elevated again when feeding is resumed (Albalat et al. 2007). LPL expression in gilthead sea bream appears to correlate with condition factor (CF) (Cruz-Garcia et al. 2009). In mammals, LPL also functions as an early marker for adipocyte differentiation (Ntambi et al. 2000), whereas in fish there is a gradual increase of LPL during adipocyte differentiation (Bouraoui et al. 2012; Todorčevič et al 2008).

During lipid mobilization, the TG molecule is hydrolyzed into one glycerol and three FA molecules. FA may be transported bound to albumin or other plasma proteins to various target tissues, or used for β-oxidation in cells. FAs may have a role in signaling or regulation of metabolic status as FAs exert negative feedback on LPL activity in rat adipocytes (Amri et al. 1996). Unsaturated FA reduces lipogenesis in rainbow trout (Alvarez et al. 2000) and reduces LPL expression by reducing the transcription factor liver X receptor (LXR) (Cruz-Garcia et al. 2011). The liver is important for the assembly and degradation of lipoproteins for lipid circulation. In fish, cholesterol and TG assemble into VLDL and are then released and transported to other tissues via the plasma (Babin and Vernier 1989).

Ghrelin

The peptide hormone ghrelin was first isolated from the mammalian stomach, but has since also been identified in other tissues including pancreas, the gastrointestinal tract ovary and adrenal cortex (Kojima et al. 1999; Date et al. 2000; Date et al. 2002; Gaytan et al. 2003; Tortorella et al 2003). In mammals, there is a preprandial increase and postprandial decrease in ghrelin secretion, modulated by the nutritional state (Ariyasu et al. 2001; Montague et al. 1997; Tschop et al. 2001a). In mammals, leptin and ghrelin are regarded as having opposite functions, although in obese patients, plasma ghrelin levels during fasting have a negative correlation to plasma leptin levels (Tschop et al. 2001b), whereas plasma levels of ghrelin and leptin during fasting in obese adolescents and children are not correlated (Ikezaki et al. 2002). It is therefore likely that ghrelin and leptin act to some extent independently in regulating energy homeostasis. Ghrelin mediates its effect on energy balance via an orexigenic effect in the hypothalamus in most species (Korbonits et al. 2004) and the preprandial increase in plasma ghrelin levels can initiate voluntary meals in the absence of food- and time-related cues (Cummings et al. 2004).

Ghrelin has been described and characterized in multiple species of fishes (Kaiya et al. 2011). The physiological roles of ghrelin have not been fully elucidated, and may be fairly species-specific. In orange-spotted grouper, goldfish and tilapia (Gao et al. 2012; Miura et al. 2006; Miura et al. 2007; Riley et al. 2005) ghrelin increases food intake. Changes in ghrelin gene expression indicates an involvement in long-term regulation of appetite and energy homeostasis in Arctic charr (Frøiland et al. 2010), whereas in Atlantic cod (*Gadus morhua*), ghrelin is only involved in meal initiation and not long-term feeding regulation (Xu and Volkoff 2009). In rainbow trout, the effect of ghrelin appears to vary depending on source, dose and route of administration. Both long-term intraperitoneal (ip) treatment and short-term intracerebroventricular (icv) with homologous ghrelin decreases food intake (Jönsson et al. 2010). Heterologous ip treatment stimulates food intake (Shepherd et al. 2007), whereas ip treatment with rainbow trout ghrelin has no short-term effect on food intake (Jönsson et al. 2007). In tilapia, a glucose load increases plasma ghrelin levels and gastric ghrelin gene expression (Riley et al. 2008). In line with this result plasma ghrelin increases after a meal in tilapia and rainbow trout (Pankhurst et al 2008; Peddu et al. 2009). Ghrelin appears to stimulate mobilization and synthesis of TG and increases lipolysis in rainbow trout adipocytes *in vitro* (Salmerón et al. 2015).

Growth hormone (GH) and insulin-like growth factor I (IGF-I)

Growth hormone (GH) is a pluripotent hormone that can stimulate both anabolic and catabolic processes. It improves growth in fish via an increase in feed conversion and elevated appetite (Johnsson and Björnsson 1994; Markert et al. 1977). Furthermore, GH is involved in lipid metabolism in both lipolytic and lipogenic processes by affecting multiple enzymes with effects being context- and tissue-dependent (Norrelund 2005). During highly energy-demanding processes where salmonids need to mobilize lipid stores such as during fasting, smoltification, and gonadal maturation, plasma GH levels increase (Farbridge and Leatherland 1992; Sheridan 1986; Björnsson et al 1994). GH increases TG lipase activity in adipose tissue and stimulates hepatic lipolysis (Albalat et al 2005; O'Connor et al. 1993) leading to increased plasma FFA levels (Leatherland and Nuti 1981) and reduced lipid stores in visceral adipose tissue and liver (Johnsson et al. 2000; Kling et al. 2012).

Leptin antiserum treatment decreases spontaneous GH secretion in rat, and leptin treatment of fasting rats reverses the inhibitory effect on GH secretion of fasting, while leptin treatment of fed rats does not modify the spontaneous GH secretion (Carro et al. 1997). During fasting, plasma GH increases coinciding with a decrease in insulin-like growth factor I (IGF-I) in both mammals and fish (Björnsson 1997; Björnsson et al. 2002; Fuentes et al. 2012; Imsland et al. 2008; Pierce et al. 2005; Reinecke et al. 2005; Shimizu et al. 2009; Wood et al. 2005). In fine flounder an increase in plasma GH coincides with the increase in plasma leptin (Fuentes et al. 2012). Plasma leptin is not affected by GH-treatment, but hepatic *lepa1* and lipid content decreases in a GH-dependent manner in rainbow trout (Kling et al. 2012). This indicates that both GH and leptin are affected by fasting and that there probably is a functional GH-leptin interaction in fish, although further studies are needed to clarify such relationship.

IGF-I is produced and released from liver by GH stimulation. Further, IGF-I is also expressed and produced in other tissues such as muscle, liver and skeleton, where GH may stimulate both local IGF-I and paracrine IGF-I signaling.

Summary

The background data presented here show a steady expansion of the research focusing on the physiological functions of leptin in fish, since its identification in pufferfish ten years ago.

The research has so far demonstrated that leptin is generally anorexigenic in fish as in mammals, but on the other hand, gene expression studies indicate that the liver is the main production site of leptin, rather than adipose tissue as is the case in mammals.

Recent research efforts have indicated the involvement of leptin in various physiological functions in various teleost species. While many interesting observations have been made, these have not allowed any generalizations in terms of other physiological functions of leptin in fish, such as the role of leptin in energy metabolism. Indeed, it may well be that such generalization for leptin function in fish is not possible, as the roles of leptin may differ significantly among teleost species, due to their enormous diversity in life-history strategies, environmental conditions and food sources.

Research efforts in fish as in other non-mammalian vertebrates are still hampered by scarcity of homologous leptin sources for treatment studies, as well as a lack of analytical methods for assessing circulating leptin levels.

Scientific aims

The over-all objective of this thesis has been to further clarify the involvement of the leptin system in regulating the energy homeostasis of teleost fish, by investigating functional links between leptin endocrinology, nutritional status and feed intake.
The specific aims have been to elucidate how changes in the lipid storing tissues; liver, muscle and adipose tissue, affect plasma levels of leptin and LepBPs, as well as expression levels of leptin and leptin receptor isoforms, in order to gain insights into if and how leptin is involved in the energy homeostasis, particularly in lipid mobilization and deposition. Further, another aim has been to investigate the possibility that other tissues than the liver are sources of leptin production, especially with focus on adipose tissue as a possible source of leptin, as well as the nutritional and/or hormonal regulation of secretion and leptin transcription on a cellular level.

Methodological and terminological considerations

Manipulation of nutritional status

In order to induce changes in energy stores and lipid content of the experimental animals, feed access was manipulated in **Papers I**, **III** and **IV** over a period of four to eight weeks. In **Papers III** and **IV**, groups of fish were fasted, whereas in **Paper I**, feed with high lipid content was used, and food restriction (25%) for eight weeks was utilized. Refeeding of fasted individuals was carried out in **Paper III**. In **Paper II**, food access was not manipulated, with the study focusing on rearing salinity. However, observations of feed intake were made and correlated to plasma leptin levels.

Experimental animal models

As outlined, leptin may have species-specific functions in different teleost species, depending on environmental conditions and life history strategies. The thesis work was thus limited to using the subfamily *Salmoninae* as model, as they are closely related with a lifecycle involving periods of food abundance and scarcity, as well as being important species in aquaculture.
The salmonids were also selected as model due to the availability of homologous analytical tools for analyzing leptin-related proteins and genes.
In this thesis, two species of salmonids were used as experimental animals. In **Paper II**, Arctic charr was used, specifically, a fourth generation from the Hólar strain, a crossbreed between an anadromous strain (Grenlækur, south Iceland) and a freshwater strain (Ölvesvatn, north-west Iceland). The rainbow trouts used in **Paper I** and **Paper III** were obtained from local fish farms in Spain and Sweden, respectively. In **Paper IV**, two breeding-selected rainbow trout strains were used, selected for either high or low muscle adiposity for seven generations (fig.1). The fish are being bred at the Pisciculture Expérimentale INRA des Monts d'Arrée (PEIMA-INRA) facility in Brittany, France, where the study was carried out.

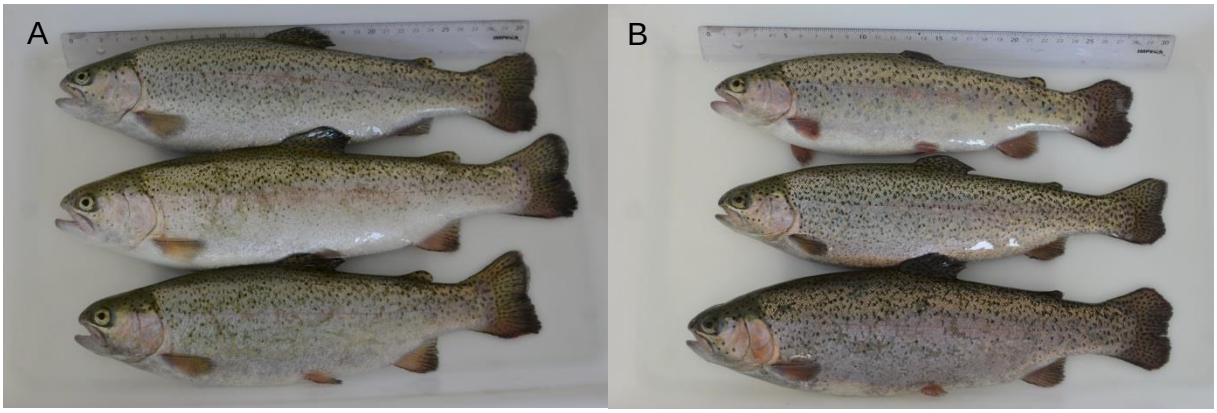

Figure 1. A sample of rainbow trout fasted for four weeks in the two lines bred for either (A) low or (B) high muscle lipid content. The ruler above goes from 0 to 30 cm.

Radioimmunoassays

In 1977, Rosalyn Yalow was awarded the Nobel Prize in Physiology or Medicine "for the development of radioimmunoassays of peptide hormones". The radioimmunoassay (RIA) still remains an assay method of unsurpassed sensitivity for measuring plasma or tissue hormone concentrations, essential when assessing levels of peptide hormones. RIAs are based on the use of a radioactive labeled hormone or hormone fragment, together with specific antibodies for the hormone examined. To evaluate the hormone concentration in a sample, the radioactive label and the native hormone are allowed to bind, either competitively or non-competitively to the specific antibody. The antibody-antigen complex is subsequently precipitated, the radioactivity of the pellet assessed, and the hormone concentration of the unknown samples derived from a standard curve of known hormone concentrations. In **Papers I-IV**, the homologous salmonid leptin RIA (Kling et al. 2009) was used, and in **Paper III**, a cortisol RIA established by Young (1986) and modified by Sundh et al. (2011) was utilized.

Quantitative PCR

Quantitative polymerase chain reactions (qPCR) are commonly used to quantify the transcriptions of genes. By quantifying the messenger RNA (mRNA) levels in cell cultures or tissues, a relative expression of gene levels can be assessed. Total mRNA is extracted from the sample and complementary DNA (cDNA) is synthesized from the mRNA. Then, with the use of targeted primers for specific nucleotide sequences in the mRNA of the gene of interest, the cDNA is amplified in a PCR reaction and mRNA

levels are presented as relative values compared with a reference gene. Relative gene transcription in isolated adipocytes (**Paper I**) and in muscle, liver and adipose tissue (**Paper IV**) was assessed using qPCR.

Protein vs gene analysis

The RIA and qPCR allow studies of endocrine systems such as the leptin system at the protein and the gene expression levels, respectively. The endocrine regulatory mechanisms, e.g. ligand-receptor interaction occur at the protein level and are directly dependent on circulating hormone concentrations and receptor protein densities and the target tissues. It can therefore be argued that endocrine systems are best studied at the protein level. However, partly due to the general lack of species-specific analytical tools such as specific antibodies and homologous hormone sources, and partly due to the relative easy by which gene expression can be quantified, research into leptin function in fish has so far been dominated by gene expression studies, and such studies are indeed included in this thesis (**Paper I** and **IV)**.

It must, however be kept in mind expression data on hormone and receptor gene expression cannot be interpreted to fully reflect hormone levels and receptor densities. Correlation between mRNA expression and protein levels is often only low, explaining only about 40% of the protein variation (Abreu et al. 2009; Vogel and Marcotte 2012). Correlation between mRNA expression and proteins varies among organisms with the squared Pearson's correlation coefficient (R^2) between 0.20-0.47 in bacteria, 0.34-0.87 in yeast and 0.09-0.46 in multi-cellular organisms (Abreu et al. 2009). In mustard weed (*Arabidopsis thaliana*) R^2 varies from 0.27-0.46 (Bärenfaller et al. 2008) while in humans, R^2 ranges from 0.09-0.40 (Abreu et al. 2009; Anderson and Seilhamer 1997; Guo et al. 2008; Pascal et al. 2008). Furthermore, the correlation between mRNA and protein may as well differ in different individuals (Guo et al. 2008). One of the major underlying causes for the dissociation between gene expression and protein levels is thought to be the differences in molecular half-life, which for of proteins can vary from minutes to days, whereas mRNA degradation rates fall within a much tighter range of hours (Vogel and Marcotte 2012).

Cell isolation

To study physiological responses in specific tissue/cell types to different treatment or agents, an *in vitro* approach may offer significant mechanistic details of directly or indirectly induced effects, not easily discerned through an *in vivo* approach. For studying acute effects at the cellular level, there are multiple methods available. Tissue slices or pieces can be incubated and studied for shorter periods of time (hours). Another approach is to create a single-cell suspension by treating a tissue with collagenase. Such primary cell cultures can be viable for longer studies up to a few days. Primary adipocyte cultures from the visceral adipose tissue were studied in **Paper I**.

Fasting versus starvation

When exploring physiological and endocrine mechanisms under catabolic conditions, experimental animals are often subjected to periods during which food is withheld. In the research literature, this is usually described as periods of "fasting" or "starvation", and these two terms are frequently used interchangeably as synonyms.

McCue (2010) argues that the two terms are not synonymous. Instead, he defines fasting as being a voluntary action, where the animal chooses not to feed. Accordingly, anorexia would be defined as fasting. Starvation, on the other hand, is defined a forced state, where the animal cannot eat in spite of having an appetite, i.e. a desire to feed, because there is no food available. Thus, starvation occurs under laboratory conditions when feed is withheld from experimental animals and under natural conditions if no food is available. While there is logic to this terminology, an alternative view is to relate "fasting" and "starvation" to the length of time the animal does not feed, irrespective of whether this is voluntary or forced, with fasting being shorter and starvation longer period of food deprivation. Although Garnett (1986) correctly points out that it is hard to define when fasting turns into starvation, in general terms, fasting can be said to be a mild catabolic state where neither the physiology nor the health of the animal is severely compromised, whereas starvation is a state of severe malnutrition which threatens both health and survival of the animal.

Based on the above considerations, in this thesis, the term fasting is used throughout the discussion, whether food has been withheld from the fish or they have voluntarily abstained from feeding. This relates to the fact that the species of salmonids studied are well adapted to relatively long periods

without access to food. When food is withheld for up to four weeks (**Papers III** and **IV**), the fish still appear in good physiological condition, with only moderate weight loss, as deduced from their relatively good condition factor (CF).

Findings and Discussion

Leptin endocrinology in relation to nutritional states

As stated, the main focus of this thesis work has been to explore if and how leptin has a role in regulating energy homeostasis in salmonids. The results are presented in four papers where both *in vivo* (**Papers I-IV**) and *in vitro* (**Paper I**) methods have been used, where fish have been studied under normal feeding conditions as well as during manipulation of food access, thus allowing observations under both anabolic and catabolic states.

Leptin endocrinology during feeding conditions

In all four studies (**Papers I-IV)**, plasma leptin levels in feeding salmonids were examined. In Arctic charr (**Paper II**), similar or slightly lower plasma leptin levels are noted as in previous studies (Gunnarsson et al. 2014; Jørgensen et al. 2016). For the rainbow trout in **Papers I** and **III**, the plasma leptin levels are similar in both experiments, being slightly lower than in earlier studies on rainbow trout (Kling et al. 2009, 2012). Interestingly, rainbow trout bred for a low muscle lipid content (lean line; LL), has higher plasma leptin levels than in rainbow trout bred for high muscle lipid content (fat line; FL) (**Paper IV)**, or other rainbow trout strains used earlier (Kling et al. 2009, 2012, **Paper I**, **Paper III**). The LL rainbow trout appear to compensate for low muscle lipid content with greater amount of visceral adipose tissue, likely in order to maintain necessary body energy stores (**Paper IV)**. The unusually high plasma leptin levels in the LL rainbow trout could be due to an elevated leptin secretion rate from leptin-producing tissues.

Given that the liver has been suggested to be the main production site of leptin in fish, due to the high level of hepatic *lep* gene expression, it could be assumed that the difference in plasma leptin levels between the LL and FL fish were due to differences in hepatic function between the strains. However, neither liver size not *lepa1* expression differs between the two lines

under normal feeding conditions. This indicates that the liver may not be the only source of plasma leptin. The greater amount of visceral adipose tissue in the LL fish makes it plausible that leptin secreted from this tissue explains the high plasma levels, similar as in mammals (Ahima et al. 1996; Coppari and Bjørbæk 2012; Maffei et al. 1995), and as indicated by the *in vitro* leptin production and secretion of rainbow trout adipocytes isolated from visceral tissue (**Paper I**). While the FL fish have higher muscle adiposity than the LL fish, it is important to note that not all adipose tissues are functionally the same. In mammals, there are metabolic and endocrine differences between visceral and subcutaneous lipid stores (Giralt and Villarroya 2013), and it may thus be that in the rainbow trout, leptin is secreted from visceral adipose tissue, but not from muscle adipocytes. Although the *lepa1* expression is lower in the adipose tissue than in the liver (Murashita et al. 2008; Ohga et al. 2015; Rønnestad et al. 2010; Won et al. 2012; Zhang et al. 2013), it is still unknown to what extent the liver secretes leptin into the circulation. Therefore, it is still unclear which tissues contribute, and to what extent, to plasma leptin levels in fish.

Alternatively, the higher plasma leptin levels in the LL fish could be the result of decreased leptin turn-over rate, due either to slower break-down, a mechanism which may be linked to higher LepBP levels, or lower LepR densities in target tissues. Such downregulation of receptor densities is often termed hormonal resistance. However, plasma LepBP levels as well as hepatic *leprl* expression are similar between the LL and FL fish. It is possible that the elevated plasma leptin levels in the LL fish are due to a reduced transport over the blood brain barrier, as an increase in plasma leptin levels does not lead to a proportional leptin uptake into the brain, indicating a saturable leptin transport mechanism (Banks 2004). During normal conditions, the greatest influx of leptin is to the hypothalamus (Ladyman and Grattan 2005), while reduced leptin transportation could be from altering *leprl*-associated signaling pathways caused from a reduction of *leprl* density on the cell surface in hypothalamus (Wilsey et al. 2003; Wilsey and Scarpace 2004). Tanycytes in median eminence promote leptin transport from the blood to the medio-basal hypothalamus in lean mice, while in diet-induced obese mice, leptin accumulates in median eminence, but does not reach the medio-basal hypothalamus; this transport being dependent on tanycytic LepR (Balland et al. 2014). In mice, intermediate leptin resistance is indicated by peripheral leptin insensitivity while central sensitivity is maintained (Lin et al. 2000b), together with increased plasma leptin levels and increased *lepr* expression in the hypothalamic arcuate nucleus (ARC) and choroid plexus, concurrent with a decreased *neuropeptide Y* (NPY) expression in ARC (Lin et al. 2000a). The plasma LepBP levels

and the expression of *lepa1*, *leprl leprs1* and *leprs3* in liver, muscle and adipose tissue is similar between the two rainbow trout strains (**Paper IV**), while in the hypothalamus, both *leprl* and *leprs3* is higher in the LL fish than FL fish (Gong et al. 2016b). If similar mechanisms are present in rainbow trout as in mice for peripheral leptin resistance (Balland and Cowley 2015), a reduction or modulation of the plasma leptin transport over the blood-brain barrier is a possible reason for the increased plasma leptin levels in the LL fish. However, as the peripheral expression (*lepa1*, *leprl*, *leprs1* and *lepr3*) and plasma LepBP levels remain similar between the two fish strains, it is clear that further research is needed to come to a clear conclusion.

Leptin endocrinology during voluntary fasting

In both **Papers II** and **III,** the observation was made that not all salmonids fed actively even though *ad lib* rations were provided. In the case of the Arctic charr (**Paper II**), 37% of the sampled individuals had empty gastro-intestinal tracts (GI) and these individuals had 13-30% higher plasma leptin levels than those with food in their GI tract. The feeding individuals also had higher CF. About one-third of the rainbow trout who were fed *ad lib* in **Paper III** had no food in their GI when sampled, and these fish had inter-mediate plasma leptin levels, higher than the actively feeding fish, and lower than the fasted fish.

The underlying reasons for why individual fish voluntarily abstained from feeding in the two experiments are unknown, but intriguing. The non-feeding Arctic charr were sampled from large production tanks with high fish density (**Paper II**). As the "non-feeding" fish still maintained an overall high CF (1.44), it is unlikely that these fish have abstained from feeding for long periods. If Arctic charr have similar evacuation time as Atlantic salmon at 7°C, the digestion and evacuation time for a single meal would be *c.* 48 h (Sveier et al. 1999). The conclusion would therefore be that the "non-feeding" Arctic charr had not fed for at least two days, but it's unlikely that they had not fed for significantly longer periods. Thus, it appears likely the under the commercial production conditions, the individual Arctic charr have an intermittent feeding pattern of abstaining and binging, in itself an interesting topic for research into mechanisms of appetite control. Conversely, the "non-feeding" rainbow trout (**Paper III**) had low CF, similar to fish that had fasted for four weeks. In that study, the rainbow trout were kept in smaller, experimental tanks at lower density. One reason could be that dominance hierarchies may have been established within the experi-mental tanks, resulting in a situation where subordinate individuals stopped feeding and entered anorexia early in the experimental study. If so,

the "non-feeding" fish would have progressively lost the ability to compete with the dominant fish, despite the hunger as a motivator of physical competitive ability (Johnsson et al. 1996).

It is hard to determine if the elevated plasma leptin levels are the cause or the result of the "non-feeding" behavior seen in **Papers II** and **III**. One hypothesis for why some of the Arctic charr have not eaten could be due to an intermittent feeding rhythm within the tanks, and that the fish have a few days digesting period between meals. The difference in plasma leptin levels between fed and non-feeding individuals could then be a result of a short-term postprandial decrease in plasma leptin levels, as seen in fine flounder two hours after re-feeding (Fuentes et al. 2012) and rainbow trout where high food content in the GI tract correlates with low plasma leptin levels (**Paper III**). However, it can also be hypothesized that the elevated plasma leptin levels are caused by a "non-feeding" behavior of individual Arctic charr, as fasting increases plasma leptin levels in rainbow trout (Kling et al. 2009; **Paper III**) and fine flounder (Fuentes et al. 2012), as well as during different food energy levels or food restricted diet (Johnsen et al. 2011; Trombley et al. 2012; **Paper I**). Furthermore, with leptin being an anorexigenic hormone in salmonids (Murashita et al. 2008, 2011) it can be speculated that the fish studied in **Paper II** which have higher plasma leptin levels, would also have less appetite and not feed as often, which in turn would lead to a lower CF and smaller body size than individuals with lower plasma leptin levels and higher appetite.

Irrespective of the underlying causal relationships, it appears unlikely that the Arctic charr and rainbow trout experienced comparable situations when they voluntarily abstained from eating. However, both cases indicate that leptin is involved in regulating processes related to the shift from feeding to fasting.

Leptin endocrinology during fasting

Many vertebrates, including salmonid fish, which live in Arctic, sub-Arctic and even in temperate climate zones, face seasonal challenges of limited or no food availability. In this thesis, manipulation of food access has been used in various ways to elucidate the role of leptin in energy and lipid metabolism during catabolic states, as prior studies have indicated that fasting or food restriction in salmonids increases plasma leptin levels (Johnsen et al. 2011; Kling et al. 2009; Trombley et al. 2012). In **Paper I** (food restriction for eight weeks) and **Paper III** (fasting for up to four weeks), similar changes in plasma leptin levels are noted. These can there-

fore be further examined together with concurrent changes in physiological parameters related to lipid/energy balance such as body and liver size, physical condition and plasma nutrient levels. Feed restriction and fasting result in similar physiological changes, indicating compromised energetic balance. Plasma glucose was, however, only reduced in fasting fish (**Paper III**), not during food restriction (**Paper I**), indicating that plasma glucose levels can be maintained during periods of low energy intake.

As in salmonids (**Papers I**, **II** and **III**), there appears to be possible link between a loss of appetite and increased plasma leptin levels during catabolic nutritional conditions in the fine flounder (Fuentes et al. 2012) and in hibernating mammals (Concannon et al. 2001; Nieminen et al. 2000; 2002). A hypothesis of why an anorexigenic hormone such as leptin would increase during periods of food shortage in fish has been put forth by Fuentes and colleagues (2012), suggesting that increased leptin levels might contribute to a passive survival strategy by reducing appetite and energy expenditure, and the results in **Papers I** and **III** support this hypothesis. In **Paper IV**, both the FL and LL lines of rainbow trout show fasting-induced changes in energy-related, physiological parameters after four weeks of fasting. However, while mobilization of energy from hepatic and visceral stores appears similar between the two fish lines, there was a striking difference in basal muscle lipid content and subsequent mobilization during fasting. While the FL fish rapidly mobilize muscle lipid reserves, no such mobilization is noted in the LL fish. This differentiated response results in both lines having similar energy stores in muscles, viscera and liver after four weeks of fasting (**Paper IV**). While the two fish lines also have similar plasma leptin and LepBP levels at that point in time, the leptin-related gene expression profiles reveal differences which may indicate different trajectories in terms of energy balance regulation (**Paper IV).** In retrospect, it would have been of interest to continue to observe the physiological and endocrine status of the two fish lines after an even longer period (six to eight) weeks of fasting.

In contrast to previous rainbow trout data (Kling et al 2009, **Papers I** and **III**), fasting did not elevate plasma leptin levels in the FL and LL fish (**Paper IV**) Furthermore, plasma LepBP levels in neither FL nor LL fish were affected by fasting (**Paper IV**), again in contrast with an earlier rainbow trout study where a three-week fasting caused a decrease in plasma LepBP levels (Gong et al. 2013a). This suggests that there is plasticity for the response of the leptin and the LepBP system to fasting, possibly related to environmental or physiological conditions. Indeed, the plasma leptin response observed in **Paper IV**, appears similar to that observed in Atlantic salmon after 4 weeks and 10 months of restricted feeding (Rønnestad et al.

2010; Trombley et al. 2012). The study on the high and low muscle adiposity rainbow trout (**Paper IV**) was carried out during a period of rapid spring-summer growth, in outdoor tanks under ambient increasing temperature and photoperiod, whereas previous rainbow trout studies (Kling et al 2009, **Papers I** and **III**) were carried out in laboratory environment with constant temperature and photoperiod. Seasonal changes in leptin endocrinology in salmonids have been indicated (Frøiland et al. 2010; Trombley et al. 2012). This could argue for the possibility that external cues can modulate the leptin-induced appetite-regulating response to fasting. However, the differences in leptin response to catabolic conditions could also be linked to endogenous factors related to the physiological state of the fish. The rainbow trout studied in **Paper IV** were in fine physiological condition with CF of 1.2-1.3, even after four weeks of fasting.

With not only adipose tissue, but also liver and muscle as important energy stores in salmonids, it is important to compare how the fasting affects all lipid stores. Both restricted feeding (**Paper I**) and fasting (**Papers III** and **IV**) reduces liver size relatively quickly during the first week of fasting and thereafter spared from further depletion indicating that hepatic energy stores are utilized during the initial week of fasting only (Jezerska et al. 1982; Milne et al. 1979; Takashima et al. 1971; **Paper IV**), probably in order to maintain other vital liver functions. After four weeks of fasting, hepatic gene expressions of *lepa1* and the two LepBPs *leprs1* and *leprs3* is elevated in the LL fish, with *leprs3* encoding for the major LepBP (Gong et al. 2013a). As neither plasma leptin nor LepBP levels are elevated, it indicates that either the mRNA is not being translated into protein, that the proteins are not being released into the circulation, or that they are acting in a paracrine manner within the hepatic tissue.

As noted earlier, the LL fish (**Paper IV**) maintain muscle lipid stores at ~4% during fasting while the FL fish, with the higher initial stores (>7% muscle lipids), continuously mobilize muscle lipids over the four-week fasting period. In comparison, during spawning migration, steelhead trout muscle lipids decrease from ~5% in in early stages to ~2-3% in later stages (Penney and Moffitt 2015), and small rainbow trout with 2.7% epaxial muscle lipid content primarily utilize visceral lipids, while only negligible amount of lipids are mobilized from muscle (Jezierska et al. 1982). Altogether, these data indicate that muscle lipids can be mobilized when needed, if the reserves are relative large from the onset, but if they are low, energy is mainly mobilized from visceral adipose tissue. One possible involvement of leptin in the mechanisms for maintaining the muscle lipid content could be linked to the elevated expression in the functional leptin receptor gene *leprl* and the LepBP genes *leprs1* and *leprs3*, which increase

after four weeks of fasting in the LL fish (**Paper IV**). Being the functional leptin receptor with the capacity to activate intracellular leptin signaling, increased $LepR_L$ density leads to tissue sensitization, resulting in elevated leptin stimulation of glucose and/or lipid metabolism within the muscle after four weeks of fasting (Bjørbæk and Kahn 2004) without elevation of plasma lipid levels. This is likely mediated through an activation of the AMP-activated protein kinase pathway and a reduction of target of rapamycin activation (Fuentes et al. 2013). Upregulation of the two LepBPs, *leprs1* and *leprs3*, might function locally as dominant negatives (Gong and Björnsson 2014) impeding the activation of the leptin signaling pathways. Why the leptin receptor upregulation only occurs after four week fasting and only in the LL fish can only be speculated on. Possibly, as the total energy stores have decreased significantly at that point, this response is linked to metabolic changes when the fish enter a state of long-term starvation. As the FL fish have greater, initial energy stores, they may not have reached the same metabolic state or negative energy balance as the LL fish (**Paper IV**) after four weeks. Again, if the study had included a longer period of fasting, these questions could have been addressed.

During fasting, there is a continuous mobilization of visceral lipid stores (**Papers III** and **IV**) whereas eight weeks of restricted (25% of satiation diet) feeding of a high energy diet is enough to maintain adiposity (**Paper I**). In fish, adipose tissue is the only tissue where leptin action has been studied at the cellular level and leptin has been found to suppress fatty acid transport-1 gene expression, indicating a reduced fatty acid uptake and storage (Salmerón et al 2015). Studies on the regulation of leptin itself in adipocytes from visceral lipid deposits show that food-restricted rainbow trout secrete more leptin and are more sensitive to insulin-induced leptin secretion, than adipocytes from fish on *ad lib* ration (**Paper I**). Insulin could promote leptin secretion by enhancing vesicle trafficking and/or leptin synthesis without any effects on gene transcription as seen in 3T3-L1 cells (Wang et al. 2014). Insulin may act in similar way as in mammals via increased leptin secretion from the endoplasmic reticulum (Barr et al. 1997) or by inducing mRNA stabilization (Moreno-Aliaga et al. 2003). Furthermore, leucine affects the adipocytes of the food-restricted fish only and not the adipocytes from *ad lib* fed fish, by reducing the secretion of leptin, while neither ghrelin nor eicosapentaenoic fatty acid (EPA) affected the leptin secretion. This indicates that leptin secretion may also be under the influence of leucine in rainbow trout, contrary to what is known in mammals (Lynch et al. 2006; Murata et al. 2000; Pérez-Matute et al. 2005; Roh et al. 2003), and that this effect is dependent on the energy status of the cells. However, *lepa1* expression in the adipose tissue was not affected by insulin, ghrelin, leucine or EPA (**Paper I**). This calls for further studies on

the effects of leptin on the cellular metabolism of lipids, not only in adipose tissue, but also in muscles and liver, where lipids are also deposited within myocytes and hepatocytes (Sheridan 1994), as well as the influence of specific nutrients in interaction with the animal's/cells' energy status on leptin secretion.

Leptin and the initiation of feeding

Plasma leptin levels increase in salmonids during periods of fasting (Johnsen et al. 2011; Kling et al. 2009; Trombley et al. 2014; **Paper III**), has led to the hypothesis that the anorexigenic effects of leptin may help fish to survive seasonal (winter) food shortage (Fuentes et al. 2012), during which it would be counterproductive to survival to spend energy on appetite-induced foraging behavior (Bull et al. 1996; Metcalfe and Thorpe 1992; Mrosovsky and Sherry 1980). This, however, leads to the important question of how the anorexic state is broken when feed becomes available again, *e.g.* when, over-wintering salmonids in freshwater either migrate to sea, or when pray items once again become available in their freshwater habitats. The question may even have wider implications, *e.g*. for hibernating mammals or even anorexic humans. It is known that salmonids that have entered an anorexic state, such as post-spawning Atlantic salmon kelts, do not instantly start feeding when presented with food (Crim et al. 1992; Jørgensen et al. 2013; Metcalfe and Thorpe 1992), and this also became apparent in the re-feeding phase of **Paper III**, when only a proportion of the four-week fasted fish commenced feeding again during the 72-hour refeeding period. Thus, after seven hours of refeeding, only five out of sixteen previously fasted fish had started feeding again, and only one of these ate more than one or two pellets. This indicates that the return of appetite, foraging behavior and feed intake is under complex control, both in terms of endocrine regulation and social interactions.

A plausible primary stimulus for breaking the anorexic state would be through sensory perception (sight, smell/taste) of food, followed by central mechanisms leading to decreased plasma leptin levels, which, in turn, would lead to the return of appetite. **Paper III** shows that while a few fish start feeding almost immediately when food is given to them after the fasting period, most fish have a much slower response, resuming feeding only after several hours or days. There is no indication that this delayed response is due to gradual leptin-related changes in regulation of appetite, as non-feeding fish have high plasma leptin levels, even if food is readily available. Instead, **Paper III** reveals a significant negative correlation

between the amount of food in the GI and plasma leptin levels. This indicates that the anorexic state of the rainbow trout is not broken by a reduction of leptin levels in the plasma, but rather, that plasma leptin levels are not reduced until the individuals have started feeding. Indeed, the data (**Paper III**) suggest a positive feed-back loop, where consumption of small amount of food leads to decrease in plasma leptin levels, which further increases appetite and food consumption. A prerequisite for such a mechanism to be plausible is that appetite is suppressed rather than completely absent, allowing the fish to respond to food/pray items by foraging, in spite of low appetite. This scenario is supported by the well-known fact that "anorexic" up-migrating salmonids can still be caught using bait or lure by sport fishermen. The data on the refeeding rainbow trout (**Paper III**) are also in line with data on fine flounder, where plasma leptin levels decrease rapidly two hours after refeeding (Fuentes et al. 2012).

When fish start feeding again after a period of fasting, they enter a state of temporal hyperphagia, a mechanism for compensatory growth which includes improved feed conversion efficiency and elevated specific growth rate (Won and Borski 2013). The rapid, food-intake related decrease in plasma leptin levels may be partly responsible for the onset of hyperphagia in rainbow trout, but as plasma leptin levels reach basal levels after 72 hours of refeeding, while food intake continues to be high (**Paper III**), regulatory mechanisms other thank leptin are likely to regulate the long-term compensatory growth process. Indeed, hepatic leptin expression in the European sea bass does not indicate that leptin is involved in compensatory growth in that species (Gambardella et al. 2012), and although long-term GH treatment improved feed conversion in rainbow trout, plasma leptin levels were not affected while hepatic *lepa1* decreased (Kling et al. 2012).

Tissue sources of circulating leptin

The tissue origin of plasma leptin in fish is still unclear. Although the relatively high hepatic *lep* expression makes the liver a likely source (Frøiland et al. 2010; Gong et al. 2013b; Gorissen et al. 2009; Huising et al. 2006; Kurokawa and Murashita 2009), direct evidence for this is still lacking. Even though Douros et al (2014) have demonstrated parallel changes of hepatic *lep* expression and plasma leptin levels in tilapia (under inhibitory influence of prolactin), it must be seen as an over-interpretation when the authors claim that they study hepatic leptin secretion. The direct proof of the hepatic origin of circulating leptin should come from *in vitro* studies on hepatocytes or liver slice cultures, or *in situ* studies of perfused

livers, where the release of leptin from hepatic tissue can be measured and demonstrated directly. Irrespective of the likely role of the liver as a leptin-secreting tissue, it is also of importance to recognize that this does not exclude other tissues as source(s) of plasma leptin. Indeed, **Paper I** demonstrate that rainbow trout visceral adipose tissue secretes leptin, despite relatively low *lep* expression.

Thus, in salmonids, both hepatic and adipose tissues are potential sources of plasma leptin. As both are energy stores, this indirectly links leptin to possible regulation of energy homeostasis, similar to the adipose source of leptin in mammals. Another potential source of plasma leptin is the GI tract. The *lep* gene is expressed in stomach (Rønnestad et al. 2010; Volkoff 2015) and midgut/intestine (Kurokawa and Murashita 2009; Gorissen et al. 2009; Rønnestad et al. 2010; Volkoff 2015) in various fish species, where the *lepr* gene is also expressed (Kurokawa et al. 2008; Kurokawa and Murashita 2009; Liu et al. 2010; Rønnestad et al. 2010; Wong et al. 2007). At the protein level, leptin has been immunohistochemically localized in the stomach of rainbow trout, sea bass, goldfish and red-bellied piranha (Ettore et al. 2012; Russo et al. 2011; Volkoff 2015; Johansson M and Björnsson BTh, unpublished data), with the leptin receptor found in stomach, proximal and distal intestine in rainbow trout (Johansson M and Björnsson BTh, unpublished data, fig. 2.). Together with the facts that plasma leptin levels are higher in individuals with empty GI tracts (**Papers II** and **III**) and plasma leptin levels do not decrease until food actually reaches the GI tract (**Paper III**), these data allow the speculation that leptin could be released from the GI tract and its secretion regulated by food intake. However, the regulation of leptin by food intake could also be indirect through *e.g.* cholecystokinin (CCK), a known appetite suppressor influencing digestion and feeding processes found both in the brain and GI tract in several fish species (Volkoff et al. 2005).

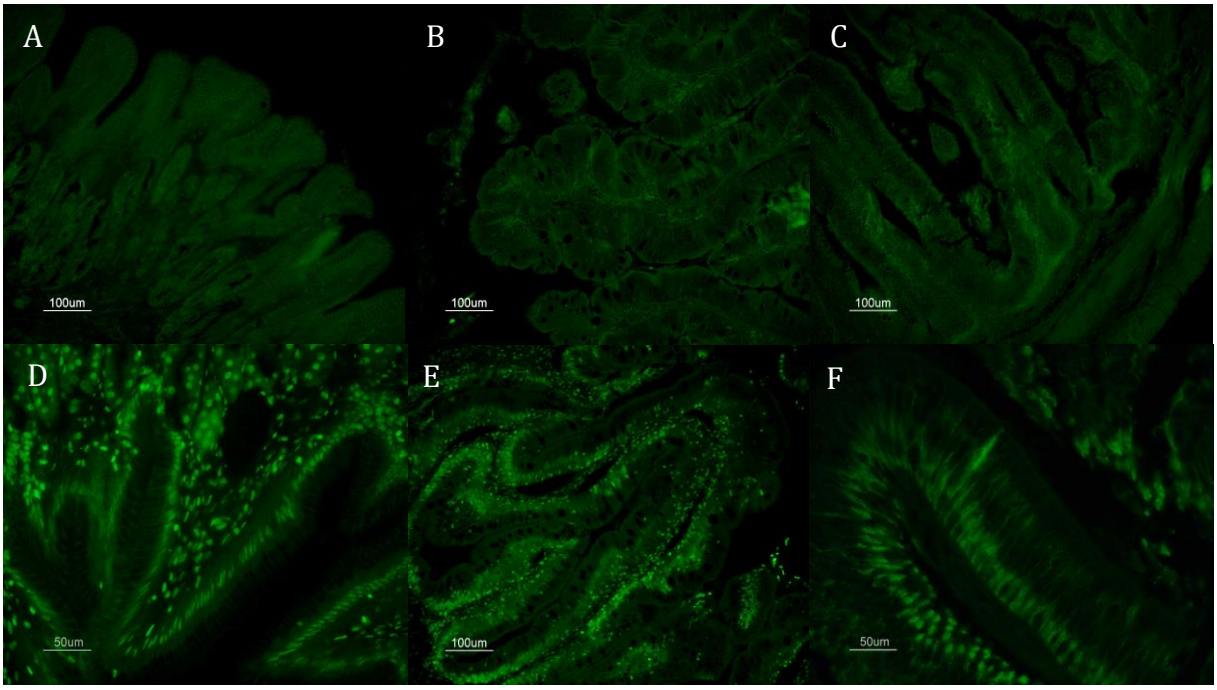

Figure 2. Immunofluorescence of the functional (long form) leptin receptor ($LepR_L$) in the gastrointestinal tract of rainbow trout. Panel A-C shows negative controls without the primary antibody in stomach (A), proximal intestine (B) and distal intestine (C). While panel D-F shows the presence of the LepRL in stomach (D), proximal intestine and distal intestine.

In mammals, it has been clearly demonstrated that leptin has both central and peripheral effects (Bjørbæk and Kahn 2004). In fish, the central, appetite-inhibiting effects have been fairly comprehensively demonstrated, while less data are available on the peripheral leptin action. Leptin does affect both glucose and lipid homeostasis in the liver, as leptin A mobilizes plasma glucose and reduces liver glycogen stores and glucose uptake as well as regulates some liver lipases (HSL, LPL) in tilapia (Baltzegar et al. 2014). Furthermore, heterologous leptin injection in green sunfish increases the lipid metabolism by acting on fatty acid binding proteins (FABP) in the heart enhancing the fatty acid oxidation, while only slightly (not significantly) increasing the 3-hydroxyacyl-CoA dehydrogenase (HOAD) in the heart and carnitine palmitoyl transferase (CPT) in liver. However, whether leptin specifically stimulates the fatty acid metabolism, or the metabolism of fatty acid and carbohydrates together, has not been determined (Londraville and Duvall 2002). Leptin has also a peripheral effect on adipose tissue; both leptin and ghrelin increases lipolysis in primary rainbow trout adipocytes *in vitro*, where leptin suppresses fatty acid uptake whereas ghrelin stimulates triglyceride mobilization and synthesis (Salmeron et al. 2015).

The central and peripheral action of leptin may be differentiated as fasting reduces intestinal leptin expression in red-bellied piranha (Volkoff 2015) and hepatic leptin expression in orange-spotted grouper (Zhang et al. 2013), while in both cases there is no effect on the leptin expression in the brain. Furthermore, leptin expression increases postprandially in liver, but not in the hypothalamus in goldfish (Tinoco et al. 2012). The peripheral leptin action may possibly be linked to the GI tract, but more research into the relation between the gastric leptin and GI function and/or feeding behavior is needed to elucidate this.

Conclusions and future perspectives

The leptin system is highly complex with multiple functions, some of which appear to have been conserved through evolution, while a functional diversity among species has arisen as well. The anorexigenic function of leptin has been conserved, as the effect is found both in fish and mammals.

However, for both fish and mammals, there are obviously species-specific differences in leptin function. In the biomedical literature, data from rodents and humans have been used to create a generalized "mammalian model" of leptin function (Park and Ahima 2015; Ahima and Flier 2000; Coppari and Bjørbæk 2012), while data on wild, non-model mammalian species have been largely ignored. Unfortunately, all too often, research into leptin function in non-mammalian vertebrates has been defined by this "mammalian model", a serious pitfall recently pointed out by Londraville and colleagues (2014). One of the greatest limitations of the biomedical mammalian model of leptin function is its basic supposition that animals strive to maintain constant body weight and lipid reserves. While laboratory rodents as well as humans in developed countries may do this, as their food access is constant, this is certainly not the case for most wild mammals, including rodents, nor is it true for most humans, as they too depend on seasonally fluctuating food availability.

Thus, in studying wild animals, including the salmonids studied in this thesis, a much more reasonable supposition is that the endocrine regulation of energy homeostasis is geared towards allowing major changes in appetite and adiposity linked to seasonal fluctuations in food availability. Thus animals need to be able to consume large quantities of food during seasons of high food availability, something which demands that appetite is not inhibited due to increased adiposity. Conversely, during seasons of little

or no food availability, a reasonable strategy may be to inhibit foraging to conserve energy, and to lower metabolic rate, as many hibernating mammals do.

Leptin appears to have such function in hibernating mammals and fish adapted to seasonal fasting. In salmonids, it is clear that during periods of fasting, but perhaps seasonally dependent, leptin levels increase and inhibit appetite. The anorexic state is broken when food is available again, and in the rainbow trout, this appears to be a complex process, the initial mechanism possibly linked to basic behavioral responses to the presence of food. However, when food enters the GI tract, leptin levels decrease rapidly, allowing appetite to return.

The research reported in this thesis has been carried out under laboratory conditions (**Papers I**, **III** and **IV**), or under commercial production conditions (**Paper II**). The studies (**Papers I**, **II** and **III**) have been carried out under constant temperature and light conditions, while in **Paper IV** rainbow trout were kept under ambient conditions. It is notable that the rainbow trout studied in **Paper IV**, had a different leptin response to fasting conditions that previously seen for rainbow trout constant environmental conditions (Kling et al. 2009; **Papers I** and **III**). This indicates plasticity in the leptin system dependent on the environment/season, and a need to further elucidate the function of leptin in fish under natural conditions.

The data presented in this thesis indicate that if rainbow trout enter a catabolic state indicated by a significant depletion of lipid stores, plasma leptin will increase and plasma LepBP will decrease, if the fish are primed for a period of food shortage a passive survival strategy is to reduce energy expenditure and appetite. On the other hand, if the fish are primed for a period of growth, plasma leptin will decrease, plasma LepBP will remain unchanged and expression of the short leptin receptor isoforms increased, indicating a strategy of elevated appetite while protecting the remaining lipid stores.

So far, no data on any aspects of leptin endocrinology of wild fish have been reported, but such information on seasonal profiles of leptin endocrinology and energy homeostasis would undoubtedly add significantly to the current understanding of leptin function in fish.

Much of the research and thus the basic understanding of the leptin endocrinology in fishes have been focused on gene expression studies, and while this provides important information, more data are still needed on leptin endocrinology at the protein level, as e.g. indicated by the generally low correlation between plasma leptin levels and *lep* gene expression has been

noted. Also, as it is seen in the rainbow trout bred for different muscle lipid content, neither plasma LepBP nor gene expression of the various LepR isoforms could explain the observed differences in plasma leptin levels.

In mammals, adipose tissue is the main plasma leptin source and also the main energy storage, but the gastric epithelium and in the glands of the gastric fundic mucosa in the stomach has also been identified to contribute to plasma leptin levels. The source of plasma leptin in fish has not been established yet, even if the liver has been suggested as the main source due to its relatively high *lep* expression. As leptin secretion appears to be linked to energy storing tissues as well as nutrient intake, both adipose and muscle tissue, as well as the GI tract could be tissue sources of plasma leptin. The leptin-secreting role of visceral adipose tissue in rainbow trout is supported by data showing both *lep* expression and leptin secretion from adipocytes *in vitro*, and it would be of future importance to carry out similar *in vitro* studies on hepatocytes, myocytes and cells from the GI tract.

For future research, it is important to take an integrated approach studying protein levels/density as well as gene expression, both *in vivo* and *in vitro*, using homologous analytical tools to obtain more complete picture of the dynamics and regulatory roles of the leptin system. It is also important to put obtained data in perspective of environmental conditions, physiological condition and genetic variation within a single species.

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