The effects of leptin and ghrelin on voluntary feed intake and appetite in Atlantic salmon *Salmo salar.*

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Abstract

Salmon farming has expanded dramatically in recent years, increasing the demand for both traditional feed ingredients and alternative proteins and oils. Developing new feed requires a deep understanding of appetite, feed intake, growth, and physiology in Atlantic salmon *Salmo salar*. Regulation of appetite involves interactions between peripheral signals and the brain that influence feed intake and metabolism. The brain and primarily the hypothalamus produce orexigenic or anorexigenic neuropeptides that inhibit or stimulate food intake. Feed intake can also be affected by peripheral hormones, such as leptin and ghrelin. In this study individual Atlantic salmon were placed in tanks and feed intake was monitored until feed intake stabilized, after which each individual was injected intraperitoneally with recombinant salmon *LEPA1* or rainbow trout *Oncorhynchus mykiss GHRL1* emulsified in vegetable oil. Feed intake was monitored for a further four days after which fish were removed 4 hours after feeding for sampling. Brain, liver and stomach samples were taken for QPCR analysis of appetite related genes. Plasma samples were analysed for ghrelin, triglycerides, glucose, free fatty acids, lactate and D-3-Hydroxybutyrate. This study reports that administered r*sLEPA1* causes a significant reduction in feed intake and SGR in Atlantic salmon. *POMCA1* and *POMCA2* expression in brain was only upregulated in fish with a significant reduction in feed intake and we suggest that *POMC* expression is linked to appetite reduction. Hepatic *LEPA1* and *LEPA2* mRNA expression was upregulated in *rsLEPA1* administered fish which also showed a reduction in feed intake and growth, therefore we suggest that hepatic expression of *LEP* isoforms are linked to metabolism. *PYY* mRNA expression was down-regulated in brain tissue in fish with a significant reduction in feed intake and could be involved in the leptin-signaling pathway at a central level. Significantly higher mRNA expression of *GHRL1A* and *GHRLIB* was found in the stomach of *rtGHRL* administered fish compared to control, and mRNA expression of both genes was only up-regulated when plasma ghrelin levels were found to be elevated. Rainbow trout *GHRL1* administered IP was not found to affect feed intake in Atlantic salmon in this study. *CCKL* and *CCKN* mRNA expression was downregulated in the brain tissue of *rtGHRL* administered fish which also showed elevated plasma ghrelin levels. Furthermore we have validated a method incorporating an individual based system and IP administration of hormones with minimal stress on fish which allows Atlantic salmon to resume feeding within hours of being returned to the tanks.

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AA: Amino acid
AGRP: Agouti-related peptide
ARC: Arcuate nucleus
BIO: Institute of biology at the University of Bergen
BW: Bodyweight
CART: Cocaine and amphetamine regulated transcript
CCK: Cholecystokinin
CCKL: Cholecystokinin L
CCKN: Cholecystokinin N
cDNA: complementary deoxyribonucleic acid
DFI: Daily feed intake
DNA: Deoxyribonucleic acid
EtOH: Ethanol
FFA: Free fatty acids
FSH: Follicle stimulating hormone
GH: Growth hormone
GHR1: Growth hormone receptor 1
GHR2: Growth hormone receptor 2
GHR2C: Growth hormone receptor 2c
GHRL: Ghrelin
GHRL1A: Ghrelin 1A
GHRL1B: Ghrelin 1B
GHSR1A: growth hormone secretagogue receptor 1A
GI: Gastrointestinal
ICV: Intracerebroventricular
IGF1: Insulin-like growth factor 1
IGF2: Insulin-like growth factor 2
IP: Intraperitoneal
JAK2: Janus Kinase 2
LEP: Leptin
LEPA1: Leptin A1
LEPA2: Leptin A2
LEPR1: Leptin receptor 1
LEPR2: Leptin receptor 2
LEPR: Leptin receptor
LHA: Lateral hypothalamic area
MBH: Mediobasal hypothalamic
MDB: Marine developmental biology
NaAC: Sodium acetate
NIFES: National Institute of Nutrition and seafood research
NPY: Neuropeptide-Y
OD: Optical density
POMC: Pro-opiomelanocortin
POMCA1: Pro-opiomelanocortin A1
POMCA2: Pro-opiomelanocortin A2
POMCA2s: Pro-opiomelanocortin A2s
POMCB: Pro-opiomelanocortin B
PYY: Peptide YY
qPCR: Quantitative polymerase chain reaction
rsLEP: recombinant salmon leptin
rtGHRL: rainbow trout ghrelin
SE: Standard error
TGC: Thermal growth coefficient
UIB: University of Bergen

1. Introduction

1.1 General background

The salmon farming industry has expanded dramatically during the past few decades, thus the demand for feed and major feed ingredients such as fishmeal and fish oil have risen (Waagbo *et al.,* 2001). Marine feed ingredients are a limited resource and have become increasingly expensive as supplies have diminished, which has resulted in the increased use of alternative proteins and oils, such as soybean meal or rapeseed oil in the fish feed industry (Carter and Hauler, 2000, Bell *et al.,* 2002). The use of alternative oils and proteins into feed requires that developers of new feed have a deep understanding of how plant based ingredients may interact with and affect the appetite, feed intake, growth and physiology of Atlantic salmon *Salmo salar* (Sissener et al. 2013; Hevrøy et al. 2008).

The regulation of appetite is a complex process involving a number of interactions between peripheral signals and the brain, these signals influence metabolism and determine growth in vertebrates, including Atlantic salmon (Volkoff *et al.,* 2005). The brain, primarily the hypothalamus produces orexigenic or anorexigenic neuropeptides that inhibit or stimulate food intake respectively (Volkoff *et al.,* 2005). Feed intake is also affected by external factors such as environment, season, time of day, availability of food, and stress, or internal factors such as circulating levels of glucose or hormones such as leptin and ghrelin (Hoskins and Volkoff, 2012). The nervous system, gastrointestinal (GI) tract, adipose tissue and external environment mediate afferent signals that are involved in maintaining energy homeostasis (Valen et al., 2011). A number of studies have dealt with the effects of external factors such as photoperiod, temperature and stress on the appetite and feed intake of Atlantic salmon, however the knowledge of neuropeptides and their role in appetite regulation is still limited (Volkoff *et al.,* 2005). In terms of optimizing and developing feed for farmed aquaculture species such as Atlantic salmon it is important to understand the processes that regulate feed intake, metabolism and growth. As knowledge of neuropeptides and their influence on the regulation of feed intake and metabolism in teleosts is limited, it is very important to focus on this area of research. In vertebrates hormones produced by the brain and peripheral organs that regulate feeding behaviour and food intake are defined as appetite regulating hormones (Hoskins and Volkoff,

2012). These include appetite stimulators or orexigenic factors such as ghrelin, neuropeptide Y (NPY), agouti-related peptide (AGRP) and appetite inhibitors or anorexigenic factors such as leptin, cocaine and amphetamine regulated transcript (CART), pro-opiomelanocortin (POMC) and cholecystokinin (CCK). Leptin and ghrelin specifically are peptide hormones with key regulatory effects on feed intake and energy homeostasis in mammals (Murashita *et al.,* 2008; Kaiya *et al.,* 2008). Further investigation of these hormones and their effects in teleosts will increase the understanding of how feed intake and appetite is regulated in an economically very important aquaculture species, such as the Atlantic salmon.

1.2 Leptin

Leptin is a 16-kDa protein hormone belonging to the class-1 helical cytokine family of proteins (Trombley *et al.,* 2012). Leptin was first discovered in the mouse *Mus musculus* by Zhang *et al.,* (1994) and has a central role in the regulation of appetite, energy metabolism, body composition, immune functions and reproduction in mammals (Trombley et al., 2012, Pelleymounter *et al.,* 1995, Barb *et al.,* 2001, Leininger *et al.,* 2009, Miller et al., 2002, Yu et al., 1997, Amstalden *et al.,* 2002). Leptin is primarily produced in adipose tissue and is secreted into the blood stream after cleavage of the 21 amino acid signal peptide (Barb et al., 2001), secretion occurs in response to changes in body fat levels or energy status (Zhang *et al.,* 1994, Barb *et al.,* 2001). Leptin acts as an anorexigenic signal through a negative feedback loop to the appetite centre in the hypothalamus causing long term and short-term effects on feed uptake and energy homeostasis (Trombley *et al.,* 2012). Elevated plasma leptin levels inhibit continued feeding and regulate body weight in the long term as well as promoting postprandial satiety (Trombley *et al.,* 2012). Low leptin levels are associated with low body fat levels and starvation (Kolaczynski *et al.,* 1996), signaling energy insufficiency and stimulating appetite in humans (Dardeno *et al.,* 2010) rats *Rattus spp.* (Shiraishi *et al.,* 2000) and pigs *Sur spp.* (Ramsay *et al.,* 2004). The leptin gene is highly conserved across mammalian species, and the mouse *Mus spp.* protein exhibits 83% homology with human *Homo sapiens* leptin (Zhang *et al.,* 1994). The gene contains three exons, separated by two introns (Green *et al.,* 1995).

In teleosts, leptin was first discovered by Kurokawa *et al.,* (2005), who identified a cDNA coding homologue to mammalian leptin in the pufferfish *Takifugu rubripes.*

Phylogenetic analysis revealed that the degree of amino acid (aa) conservation was low between fish and higher vertebrates, with only 13.2% sequence identity between pufferfish and the human sequence. Leptin has since been described in a number of teleost species such as; Atlantic salmon, rainbow trout *Oncorhynchus mykiss,* Striped bass *Morone saxatilis,* Orange-spotted grouper *Epinephelus coioides,* Grass carp *Ctenopharyngodon idellus* and Yellow catfish *Pelteobagrus fulvidraco.* (Ronnestad *et al.,* 2010; Murashita *et al*., 2008; Won *et al*., 2012; Zhang *et al*., 2013; Li *et al*., 2010; Gong *et al*., 2013). Leptin in teleost species generally shows low aa identity between species and with human leptin, *LEP* in striped bass for example is 52% homologous to *LEP* in Atlantic salmon *LEP* and 46% homologous to the human *LEP* sequence (Won et *al.,* 2012).

The first study on Atlantic salmon identified two paralogues for leptin, *LEPA1* and *LEPA2,* where the 171 aa sequence for *LEPA1* and 175 aa sequence for *LEPA2* shared 71,6% identity with each other and 22,4% and 24.1% identity to human leptin, respectively (Rønnestad *et al*., 2010). Recently, Angotzi et al., (2013) also identified *LEPB* duplicated leptin genes in Atlantic salmon, which have 98% aa identity between *LEPB* variants. As mentioned leptin belongs to the class-1 helical cytokine family, which possesses a distinctive α-helix bundle in the 3D structure, this tertiary structure is generally conserved throughout leptin orthologs across species. Leptin in pufferfish and Atlantic salmon both closely resemble mammalian leptin in structure based on *in silico* analysis (Kurokawa *et al.,* 2005; Rønnestad et al., 2010). Later Angotzi *et al.,* (2013) examined the structure of *LEPB* and found it too was a four helical cytokine and that the basic structural pattern of the protein was similar to that of human leptin and leptin orthologs in other teleost species. *LEPB* has also been found in japanese medaka *Oryzias latipes* (Kurokawa and Murashita, 2009), zebrafish *Danio rerio* (Gorissen et al., 2009) and Orange spotted grouper (Zhang *et al.,* 2013).

In teleosts leptin is expressed in a variety of tissues and there is much variation in areas of expression between species and variation in the expression of the different leptin orthologues. Unlike mammals the major site of leptin expression in Pufferfish, rainbow trout and Atlantic salmon is the liver (Kurokawa *et al.,* 2005, Murashita et al., 2008, Kling *et al.,* 2012, Trombley *et al.,* 2012). Won *et al.,* 2012 found that

mRNA expression of leptin occurred solely in the liver of Striped bass and suggest that the liver is the major site of leptin production in bass and other teleosts. Gong *et al.,* (2013) found that *LEP* was expressed in a wide range of tissues but the highest levels of expression were found in the liver of yellow catfish. Rønnestad *et al.,* (2010) found that *LEPA1* and *LEPA2* were expressed in a range of tissues and organs in Atlantic salmon although at very low levels. The highest mRNA levels of *LEPA1* were observed in the brain, however white muscle; liver and ovaries also had high expression. mRNA levels of *LEPA2* where generally found to be lower than *LEPA1* except in the stomach, midgut and kidney (Rønnestad *et al.,* 2010). The multiple expression sites for the different orthologues indicate that leptin may have multiple roles in Atlantic salmon. However the identification of high *LEPA2* expression in the stomach is concurrent with results in mammals, as leptin is also produced in the stomach and released into the gastric juice following a meal, and it is suggested that leptin may act in regulating the absorptive capacity for nutrients in the intestine, thereby indirectly affecting energy homeostasis (Rønnestad *et al.,* 2010). In Atlantic salmon specifically there are varying results in terms of the main site of expression, Trombley *et al.,* (2012) found that *LEPA1* and *LEPA2* genes were mainly expressed in the liver of juvenile Atlantic salmon. Furthermore Kling *et al.,* (2012) also found highest levels of *LEPA1* expression in the liver of juvenile rainbow trout. However life history stage may influence the level of different isoforms of *LEP* expression in a given region of the body.

In mammals, leptin informs the hypothalamus (Baskin *et al.,* 1998; Barb et al., 2001) about the amount of fat stored in the body through short and long forms of leptin receptor, *LEPR* (Roubos *et al.,* 2012). Six *LEPR* mRNA transcripts that produce various *LEPR* protein isoforms have been identified in mammals (Zabeau et al., 2003, Roubos et al., 2012). According to Robertson et al., (2008) the isoform *LEPRB* has no enzymatic activity but propagates downstream leptin signals through *LEPRB* associated tyrosine kinase *JAK2*. In mammals *LEPRB* expressing neurons mediate leptin action and different brain centres elicit different responses, the hindbrain is in control of satiety along with the hypothalamus, but the hypothalamus also controls glycemic, thyroid, reproductive functions by leptin signaling through *LEPRB* and *JAK2* pathway. In fish *LEPR* and the associated leptin receptor overlapping transcript (LEPROT) are found mostly in the pituitary rather than the hypothalamus as is

common in mammalian counterparts and physiological actions of leptin are mediated the membrane bound leptin receptors or *LEPR* (Rønnestad *et al.,* 2010, Gong *et al.,* 2013). The anorexigenic effects of leptin are mostly mediated through the neurons expressing the long isoform of *LEPR* (Robertson *et al.,* 2008). A leptin receptor ortholog has also been identified in Atlantic salmon (Rønnestad et al., 2010), with 24,2% amino acid sequence similarity to human *LEPR*, however Atlantic salmon is a tetraploid species as the genome has been duplicated at some point in its life history and it has two *LEPR* genes (Rønnestad *et al.,* 2010). In yellow catfish only one copy of the *LEPR* gene has been discovered (Gong *et al.,* 2013). Atlantic salmon *LEPR* is also highly expressed in tissues such as the brain, eye, gill and visceral adipose tissue (Rønnestad et al., 2010). By further examining the expression of *LEPA1*, *LEPA2*, *LEPR1* and *LEPR2* in stomach, liver and brain tissue, it may be possible to shed light on the signaling pathways of leptin and describe what effects this signaling pathway may have on the regulation of appetite and metabolism in Atlantic salmon.

In teleosts including Atlantic salmon it is unclear how the leptin systems are influenced by tissue-specific energy status as data on leptin in teleosts is to scarce to allow a generalization (Rønnestad *et al.,* 2010). One approach to exploring the physiological role of leptin has been to analyse plasma leptin levels during periods of feeding and fasting and in fish fed different amounts of feed. Trombley *et al.,* (2012) found an increase in *LEPA1* expression and higher levels of plasma leptin in feed restricted fish, while *LEPA2* expression decreased in feed restricted and normal fed fish, and suggest that *LEPA1* and leptin plasma levels behave in an opposite way to that observed in mammals. These findings are supported by Kling *et al.,* (2009) who found that plasma leptin was elevated in rainbow trout during fasting, and suggest that the relation between circulating leptin levels and energy status differs from mammals; in cows for example fasting causes a decrease in leptin plasma concentrations (Chelikani *et al.,* 2004). Kling *et al.,* (2012) found that feed restriction causes an upregulation of the LEPA1 gene expression as well as an increase of plasma leptin, and state that liver-derived leptin reflects plasma levels. Furthermore Kling *et al.,* (2009, 2012) proposed that leptin is linked to energy balance, but that it may not act as an adiposity signal in salmonids, which could point to a functional divergence between ectothermic and endothermic vertebrates. On the other hand Tinoco *et al.,* (2012) could find no correlation between nutritional status and the leptin system in

goldfish, *Carassius auratus,* and suggested that leptin may signal short term changes in food intake but seem to operate independently of fasting and overfeed conditions.

Rønnestad et al., (2010) reported that Atlantic salmon fed on a moderately restricted feeding regime (60%) resulted in lower growth and lower *LEPA1* expression in the main lipid storing tissues. Furthermore they found central effects of plasma leptin on energy homeostasis acting through *LEPR* in the brain, as Atlantic salmon fed on rationed diets showed lower *LEPR* expression in the brain than fully fed fish. These results suggest that regulation of *LEPR* at the level of the brain may form part of the regulatory system for leptin on energy homeostasis in line with the mammalian model. Re-evaluation of the method used for qPCR assay of LEPA1 in this study may however question the validity of this result and this should be reassessed in a new study (Rønnestad et al., unpublished data)

The large variability in the AA sequence between teleosts and humans leads to the question to what extent the function of leptin is conserved across species, although a conserved 3D structure indicates similarities. Leptin may play a different role in the regulation of physiological functions compared with their endothermic mammalian counterparts. It is important to further examine the possible multiple physiological functions of leptin in order to have a better understanding of the underlying mechanisms that control the metabolic physiology of teleosts

1.2.1 Leptin- interactions with neuropeptides, gut peptides and appetite regulatory effects

Neuropeptides are peptides utilised by neurons for communication, they are essentially neuronal signalling molecules. Neuropeptides are also referred to as peptide hormones. Neuropeptides are involved in many brain functions including appetite, food intake and metabolism. The hypothalamus produces neuropeptides that stimulate and inhibit feeding. These peptides include, *NPY*, *AGRP, POMC* and *CART* amongst others. Peripheral signals, meaning signals coming from outside of the brain include *CCK*, *PYY*, leptin and ghrelin (Volkoff, 2006). Peptides acting as satiety signals primarily originate from the GI tract, but are also synthesized in the brain, which is why they are designated “gut-brain peptides” (Volkoff, 2006). Peripheral signals such as leptin can influence the brains release of appetite-related

neuropeptides and therefore also control food intake (Volkoff, 2006). Gut/brain peptides interact with specific receptors on major nerves or reach the brain directly via the bloodstream (Volkoff, 2006).

Cholecystokinin (*CCK*) is secreted by the proximal intestine and is an anorexigenic GI peptide. The physiological role of *CCK* involves regulation of food intake, satiation and digestion; it has a key role in the regulation of the intestinal phase (Murashita *et al.,* 2009; Webb *et al.,* 2010). *CCK* stimulates the exocrine pancreas and discharge of bile from the gallbladder and also affects smooth muscle contraction in the jejunum and pyloric sphincter (Murashita *et al.,* 2009). *CCK-L* and *CCK-N* in Atlantic salmon were described by Murashita et al., (2009) and were found to be highly expressed in the brain. Peptide YY (*PYY*) and *NPY* are peptide hormones belonging to the *NPY* family. *NPY* is mainly expressed in the hypothalamus and has strong orexigenic functions, while *PYY* is mainly expressed in the brain and anterior part of the intestine, including pyloric caeca and has anorexigenic functions. In mammals leptin activates the long form of leptin receptor *LEPRB* on central nervous system (CNS) neurons to mediate most leptin action. The *LEPRB* expressing neurons lie in regions associated with the regulation energy balance, such as the mediobasal hypothalamic (MBH) arcuate nucleus satiety centre (ARC) and lateral hypothalamic area (LHA) feeding centre. In mammals it seems that leptin action in the MBH is regulated through the *LEPRB* pro-opiomelanocortin *POMC* expressing neurons and their opposing *LEPRB* agouti-regulated protein (AGRP) neuropeptide Y (*NPY*) in the ARC (Leininger *et al.,* 2009). These neurons are active in controlling satiety response and mediate the anorectic response to leptin, and also modulate energy expenditure and glucose homeostasis (Leininger *et al.,* 2009). This is supported by Valen *et al.,* (2011) who found that food intake and processing in Atlantic salmon causes a change in the mRNA expression of neuropeptides*, NPY*, cocain-amphetamine regulated transcript (*CART)* and *PYY*. *CART*, *CCKL*, *POMCA1* and *POMCB* were found to increase within 3 hours of feeding while feed was in the stomach suggesting that these neuropeptides play a central anorexogenic role, similar to higher vertebrates (Valen *et al.,* 2011). *NPY* and *AGRP* isoforms described as playing orexigenic roles in mammals were up regulated after feeding, which is opposite to the effect in mammals.

In mammals leptin reduces food intake through the hypothalamus orexigenic *NPY/AGRP* and anorexigenic *POMC/CART* neurons. Studies have indicated that similar mechanisms may to some extent also exist in salmon where leptin decreases hypothalamic *NPY/AGRP* mRNA and increases *POMC/CART* mRNA (Murashita *et al.,* 2011). Atlantic salmon administered leptin through intraperitoneal (IP) osmotic pumps, showed increased expression of *POMCA1* when compared to controls and also a significantly decreased growth rate (Murashita *et al.,* 2011). Based on these results the authors suggested that leptin decreases food intake through the *POMC* pathway, which means that *LEPA1* may have an anorexigenic role in the regulation of bodyweight in Atlantic salmon that compares with mammals. A direct effect of injected leptin on short-term feed intake still remains to be shown for Atlantic salmon.

1.3 Ghrelin

Ghrelin producing cells can be found in the oxyntic glands of the stomach in rats (Date *et al.,* 2000) and colocalize with chromogranin A-immunoreactive cells, which suggests that ghrelin is produced by endocrine cells in the stomach (Date and Kangawa, 2012). Four types of cells have been identified in the oxyntic gland, the X/A cell is one of these and because there are similarities in the ultra structural features of ghrelin cells and X/A cells, ghrelin cells are believed to be a type of X/A cell (Date and Kangawa, 2012). Ghrelin was first described as an endogenous ligand for the growth hormone secretagogue receptor 1a or *GHSR1A*, the 28 amino acid long peptide with a unique fatty acid modification at the N-terminal third amino acid that comprises ghrelin was first discovered by Kojima et al., (1999). Ghrelin stimulates growth hormone (*GH*) release *in vivo* and *in vitro,* which supports that the hormone acts as an orexigen factor (Kaiya et al., 2008, Kaiya *et al.,* 2013). Ghrelin is derived from pre-progrelin and undergoes a post-translational modification where a serine-3 residue is covalently linked to octanoic acid. This post-translational acylation is unique to ghrelin and is necessary for the ghrelin to bind to the *GHSR1A* and cross the blood brain barrier (Karra and Batterham, 2010). In mammals ghrelin is the only gastrointestinal hormone known to increase feeding (Date and Kangawa, 2012) and has therefore been coined the “hunger hormone” in humans. In non-mammalian vertebrates the amino acid sequence of ghrelin has been reported in reptiles, birds, amphibians and fish including goldfish and rainbow trout (Kaiya et al., 2008) and Atlantic salmon (Hevrøy et al. 2011, Murashita et al. 2009). The ghrelin gene in

Goldfish, catfish, and seabream *Sparidae spp*. has four exons and three introns as in humans, the sequence identity of the short non coding 1st exon found in human and rodents ghrelin gene is not evident in the mentioned fish genes (Kaiya et al., 2008). However the ghrelin gene in rainbow trout is comprised of five exons and four introns as in humans, salmonids and rodents (Kaiya et al., 2008).

Ghrelin producing cells have been found in the hypothalamic arcuate nucleus which is an appetite regulating centre in the brain (Kojima *et al.,* 1999). Ghrelin cells have also been found in the duodenum, pancreatic A-cells, kidney and pituitary (Kaiya et al., 2008). In teleosts ghrelin mRNA expression has mainly been found in the stomach or intestine, however ghrelin expression in other non-mammalian vertebrates has also been found in a variety of organs such as brain, hypothalamus, heart, pancreas, spleen, head kidney, trunk kidney, and gills of rainbow trout (Murashita et al. 2009, Kaiya et al., 2008). In tilapia *Oreochromis niloticus* ghrelin expression has been found in the brain, stomach, and gill and in the goldfish ghrelin expressions has been found in brain, hypothalamus, spleen, liver, head, gill and intestine (Kaiya et al., 2008)

The growth hormone secretagogue receptor (*GHSR*) which mediates the biological actions of growth hormone secretagogue (GHS) and ghrelin have been reported in teleosts such as rainbow trout, pufferfish and Atlantic salmon (Kaiya *et al.,* 2008; Hevrøy *et al.,* 2011). The GHS-R gene has two exons and one intron in seabream, pufferfish, tilapia, rainbow trout and Atlantic salmon. In rainbow trout the ghrelin receptor is called GHSR- like receptor, due to uncertainties in *GHSRLR* mRNA responses in a functional study in rainbow trout, and the same applies to Atlantic salmon because of the 99 % sequence similarities (Hevrøy et al. 2011).

1.3.1 Ghrelin- interactions with neuropeptides and appetite regulatory effects

Ghrelin is the only gastrointestinal hormone known to increase feeding (Date and Kangawa, 2012). In humans plasma ghrelin levels have been found to increase prior to meals and decrease after meals, indicating that ghrelin acts as a signal of expectations of meal (Shiiya *et al.,* 2000). The fibres that contain ghrelin innervate neurons that produce *NPY* and *AGRP*, neuropeptides that are associated with increasing feeding (Zhou *et al.,* 2013) and ghrelin administered to the brain activates

NPY and *AGRP* producing neurons in rats (Date and Kangawa, 2012). Ghrelin has also been shown as the only peripheral gut hormone to stimulate GH release in rats, indirectly through the vagal nerve afferent (Date *et al.,* 2002) and directly through its action on the pituitary gland (Date *et al.,* 2006). Ghrelin also stimulates GH release in fish, specifically in rainbow trout *in vivo* and *in vitro* (Kaiya *et al.,* 2003) and hybrid striped bass *Morone chrysops X saxatilis in vivo* and *in vitro* (Picha *et al.,* 2009).

Ghrelin has orexigenic effects in mammals and promotes feed intake, weight gain and adiposity. In higher vertebrates such as humans intravenous infusions of ghrelin cause an increase in feed intake (Wren *et al.,* 2001), and in rats *Rattus spp.* ghrelin intracerebroventricular (ICV) injections increase food intake and body weight (Locke *et al.,* 1995), also with orexigenic properties that are independent of GH releasing properties (Torsello *et al.,* 1998). In goldfish*,* Matsuda *et al.,* (2006) found that ICV and IP administration of ghrelin stimulated food intake and suggest circulating ghrelin derived from peripheral tissues acts via primary sensory afferent pathways on feeding centres in the brain. This is further supported by Murashita *et al.,* (2009) who found that a six day starvation period led to increased expression of *GHRL1* in the GI tract, suggesting an orexigenic role of ghrelin in Atlantic salmon. However the picture is not complete concerning the role of ghrelin in salmonids. In rainbow trout ghrelin injections and implants have been found to reduce food intake (Jönsson *et al.,* 2007; Kling *et al.,* 2012), while circulating plasma ghrelin levels seem to peak after short time starvation (Pankhurst *et al.,* 2008). In Atlantic salmon circulating ghrelin concentrations are higher after two days of food deprivation but no ghrelin peaks were shown after fourteen days of starvation (Hevrøy *et al.,* 2011). Hevrøy *et al.,* (2011) also found a down-regulation of stomach ghrelin1 mRNA in food deprived fish after two days but no effect after fourteen days and no effects on *GHSR1aLR* mRNA expression, however in a study on salmon kept at elevated temperatures fish developed low feed intake at nineteen degrees which was associated with a lower ghrelin plasma concentration and down-regulation of stomach *GHRL1* and hypothalamic *GHSR1aLR* mRNA expression (Hevrøy *et al.,* 2012). Murashita *et al.,* (2009) found a significant up-regulation of stomach ghrelin-1 mRNA after six days of food deprivation. The results of increased plasma ghrelin concentrations and stomach ghrelin mRNA responses may suggest that ghrelin is a short time energy regulator

during starvation in Atlantic salmon and ghrelin suppression may be related to energy homeostasis (Hevrøy *et al.,* 2012)

1.4 Plasma metabolites as an indicator of metabolic status

Plasma metabolites such as glucose, lactate, free fatty acids (FFA), triglycerides and D-3-Hydroxybutyrate are indicators of metabolic status and energy balance in teleosts. Glucose functions as a source of energy and is usually available through the diet. However when fish go through periods of starvation or intense activity such as burst swimming available glucose reserves may become depleted and further synthesis of glucose is maintained through gluconeogenesis. This is the process of synthesizing glucose from non-carbohydrate sources such as lactate or glucogenic amino acids (Morata *et al.,* 1982). When glucose is in limited supply evidence from teleosts suggest the organs of several fish use β-hydroxybutyrate and acetoacetate as fuels. β-hydroxybutyrate utilization has been measured in rainbow trout brain and occurs at 1% the rate of glucose and lactate metabolism, which is a contrast to mammals where oxidation rates of ketone bodies are comparable to those of glucose and lactate (Soengas and Aldegunde, 2002). The lypolitically-generated FFA are important sources of energy for cells and are substrates for lipid biosynthesis (Chung *et al.,* 1998). Fatty acids also play a significant role in glucose homoeostasis and triglycerides specifically enable the transfer of adipose fat and blood glucose from the liver (Menoyo *et al.,* 2006). Changes in the levels of triglycerides in plasma indicate a change in glucose metabolism, thus measuring triglyceride levels in blood can give an indication of metabolic status in vertebrates such as fish. Plasma free fatty acids are considered to be the most dynamic form of lipid transport from the lipid depots to the various utilising tissues. In fish, red muscle fibres are known to possess a high capacity for FFA catabolism. In contrast to mammals, which possess a specialised adipose tissue, the lipid reserves of fish may be located in several tissues including mesenteric fat, liver and red muscle (Van Rajj, 1994). Compared with higher vertebrates in which plasma FFA levels usually range between 0.4 and 0.6 μmol/ml, plasma levels of fish are much more variable. Levels ranging from 0.09 to 2.84 μmol/ml have been reported. In fish most reports are that plasma levels range between 0.3 and 1.0 μmol/ml. The replacement time of plasma FFA is within minutes and fatty acid metabolism is of major importance for the energy metabolism in fish, thus the

level and fatty acid composition of plasma FFA form a very dynamic reflection of lipid metabolism (Van Rajj, 1994). In fatty fish such as salmonids starvation stimulates lipid metabolism and white muscle protein catabolism (Echevarria *et al.,* 1997), so high levels of FFA and triglycerides in the plasma could indicate lack of feeding. When oxidation of carbohydrates and lipids are unbalanced acetoacetate and β-hydroxybutyrate serve as transportable units of fat for oxidation in peripheral tissues (Willmott *et al.,* 2005). Some metabolites also function as an indicator of stress, as glucose and lactate form part of the secondary stress response in salmonids (Iversen *et al.,* 2005). It is important to consider indicators of stress as changes in the levels of metabolites may not necessarily be due to experimental parameters and stress could produce confounding results, especially when examining metabolic functions.

1.5 An individual-based system as a method of studying peptide hormone effects in Atlantic salmon

Atlantic salmon are an important aquaculture species in Norway and globally, and serve as a model species for the potential cultivation of other teleosts. Leptin and ghrelin are peptide hormones with key regulatory effects on feed intake and energy homeostasis in salmonids (Murashita *et al.,* 2008; Kaiya *et al.,* 2008).Whether administration of leptin or ghrelin will affect feed intake in Atlantic salmon, remains unknown.

There are different routes of delivery when administrating peptide hormones, which are known to cause different methodological challenges. The methodological challenges may in turn yield varying physiological results. Hormones such as leptin and ghrelin can be administered ICV or IP, as hormone and gut-brain peptide injection studies have yielded results in mammals such as rats *Rattus spp.* (Rüter *et al.,* 2003). In non mammalian vertebrates ICV and IP studies have also been conducted on Nile Tilapia *Oreochromis niloticus,* hybrid striped bass, grass carp *Ctenopharyngodon idella*, Atlantic salmon, coho salmon *Oncorhynchus kisutch* with varying effects on appetite regulating systems (Shved *et al.,* 2011; Picha *et al.,* 2009; Zhou *et al.,* 2013; Einarsson *et al.,* 1997; Baker *et al.,* 2000). Baker *et al.,* (2000) found that recombinant human leptin administered to coho salmon did not affect growth or food intake, energy stores, gonad weight, pituitary content of FSH or

plasma levels of *IGF1*, GH or thyroxine. Therefore Murashita *et al.,* (2011) suggested that in order to obtain accurate results for leptin, species-specific peptides must be applied. The protocol for the production of recombinant leptin was established by Murashita et al., (2008) in rainbow trout, since then the technique has also been utilised for Atlantic salmon (Murashita *et al.,* 2011). The cDNA sequence of Atlantic salmon *LEPA1* was utilised in order to produce recombinant Atlantic salmon *LEPA1* in *Escherichia coli* (Murashita *et al.,* 2011). This method was also applied for the current study.

Ghrelin has been purified and characterized in a number of fish including, japanese eel *Anguilla japonica*, channel catfish *Ictalarus punctatus* and rainbow trout (Miura *et al.,* 2009). The protocol for purifying ghrelin from the stomach of rainbow trout was established by Kaiya et al., (2003) and was utilized for the production of ghrelin for this study. Two types of ghrelin have been found in rainbow trout, rtGHRL and des-VRQ-rtGHRL Kaiya *et al.,* (2003) and according to Murashita *et al.,* (2009) the GHRL1 and GHRL2 found in Atlantic salmon were similar to those in rainbow trout and the deduced mature peptide sequences were identical in both species. Due to the similarity of ghrelin found in rainbow trout and Atlantic salmon, purified rainbow trout ghrelin most likely have similar effects when injected IP in Atlantic salmon.

A good experimental setting for testing any effect on appetite must also permit accurate registrations of feed intake. A method for measuring the feed uptake of individual salmonids is virtually non-existent, because it is extremely difficult to get Atlantic salmon to feed individually in tanks. Fish are increasingly used in a range of laboratory experiments yet there is little data and information on how fish should be housed. Housing conditions can influence the behaviour and physiology of laboratory animal, thus enriching empty environments by providing structural complexity or the companionship of other individuals is considered beneficial as it could decrease abnormal behaviour. It is important to evaluate the housing environment as it may influence the validity of experimental data, especially if housing with an unsuitable environment produces abnormal behaviour (Brydges and Braithwaite, 2009). Roberts *et al.,* (2011) showed that changes in rearing conditions had rapid and marked effects on risk-taking behaviour in Atlantic salmon, which indicates it is possible to modify at least one component of behaviour known to have clear adaptive implications

through environmental enrichment. An individual based method was developed in association with the aquaculture research station at Matre, in a pilot study with IP injected leptin. In the wild, parr show marked territoriality, occupy fixed positions faced into current flows and can be highly aggressive. When the salmon smoltify these behaviours change and the fish display schooling behaviour with downstream orientation and also swim with the current. This behaviour can be observed in tanks on farms during smoltification, at the same time appetite is stimulated and fish begin to feed more intensively (Stead and Laird, 2002). Prior to being placed in tanks individually, three juvenile Atlantic salmon were kept together encouraging schooling and feeding behaviour. After feeding behaviour was attained one individual juvenile Atlantic salmon was placed in each tank and hand fed, allowing each fed pellet to be counted and excess uneaten feed to be collected.

Netting and handling in connection with experimentation for weighing and injections may impose severe stress to fish. Salmon is known to be particularly sensitive to such stress and it is well known that they may cease to eat for a long period after experimental handling. In the current experiment we used a sedation protocol including both AQUI-s and MS-222 that allowed for the careful removal of each fish from the tank causing minimum stress so as not to interfere with feeding. The fish could be removed, injected with a recombinant peptide hormone, in this case leptin or ghrelin, thereafter replaced in the tank for recovery. Thus feed intake prior to and after treatment was accurately recorded and measured for each individual fish, with minimum interference. The recent development of such a model for Atlantic salmon (Hevrøy et al., unpublished) is of great importance in order to accurately measure the hormone effects on feed intake and was used in the current work.

1.6 Aims and objectives

The main aim of this project was to examine the effects of administered leptin and ghrelin on appetite, feed intake and neuropeptides in Atlantic salmon. The research will help provide a better understanding of the mechanisms that regulate appetite and feed intake in salmonids.

Main objective

- Assess the appetite effect of recombinant hormones *LEPA1* and rainbow trout *GHRL1A* in Atlantic salmon in an individual based system, in order to better understand appetite regulation and feed intake in salmon.

Minor objectives

- Investigate the effects of recombinant leptin and ghrelin on feed intake in salmon
- Examine the underlying effects of observed changes in feed intake focusing on the neuropeptides involved in appetite regulation in the brain
- Identify to what extent energy metabolism is influenced by leptin and ghrelin in selected tissues and organs including liver, stomach and brain.

2. Materials and Methods

In order to examine the function of leptin and ghrelin in Atlantic salmon individual injection experiments were performed in order to evaluate feed intake response. The performance response was confirmed through the collection of organ and plasma samples The individual feed intake model (Figure 1) utilized for this experiment was developed in collaboration with Matre Aquaculture Research Station.

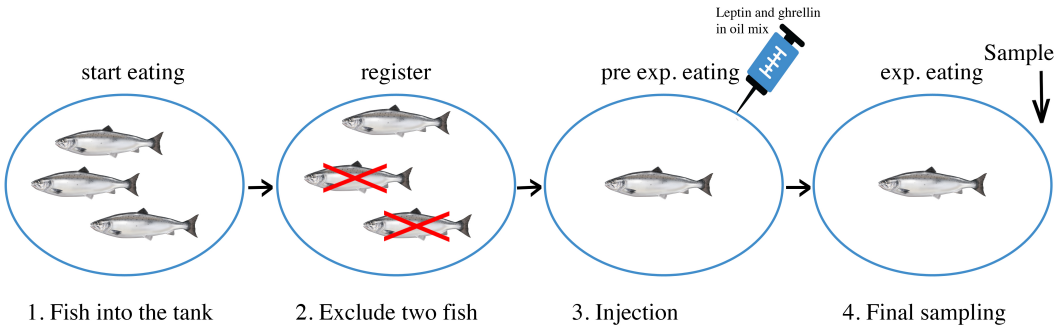

Figure 1 Overview of experimental setup (Hevrøy, unpublished)

Atlantic salmon were selected from stock and distributed throughout experimental tanks, three fish in each tank. After an acclimation period, during which most of the fish established a regular feed intake, individual fish were sampled during which weight and length was measured. One individual from each tank was selected for the next phase of the experiment. The selected fish had displayed feeding behaviour during the acclimation period and was within the mean size range of the group. The fish were fed individually and once the feeding had stabilized, the fish were sedated and anesthetized, weighed and measured before they were injected with hormone (IP) and returned to the tank. Feeding resumed rapidly and the fish were monitored for a further three to four days before they were removed and killed for final sampling.

2.1 Fish and experimental conditions

Two experiments were conducted. Both experiments were conducted at the IMR - Matre Aquaculture Research Station, Matre, Norway (60,87º N 5,58º E). The first

experiment was conducted during December 2011, and the second during January 2012.

2.1.1 Experiment one

On the 1st of December 2011, 48 post smolts (NLA strain, 0+) with an approximate body weight of 90-100g where randomly distributed in 16, 0.5 x 0.5 x 0.4 m indoor tanks with 85 L flow-through water volume, so that each tank contained three individuals. The water temperature was held constant at 12 °C and oxygen was added in order to keep the saturation above 90 %. Artificial light with natural day length was applied. The fish were kept on a 20/4 light regime prior to competition period. During the first 4 days of acclimation the light was kept on a 12/12 regime, however this interfered with the first feeding at 09:00 so the light regime was changed to 16/8. The light regime remained the same throughout the first and second experimental period.

The fish were handfed four times a day at 09:00, 12:00, 15:00 and 18:00. After 8 days all the individual fish had established a regular and high daily feed intake and consumed between 100 and 130 pellets per tank per day. Prior to removal of fish, pellets were hand fed into each tank and feeding behaviour was observed. Fish from tanks in which all three fish showed competitive feeding behaviour where selected for further experimentation. To prevent stress the fish were sedated in the tanks prior to netting as follows: Water flow was stopped and 1/3 of water was drained from the tank. 4 ml of AQUI-S (12 mg L^{-1}) (AQUI-S, New Zealand) dissolved in warm water was administered into each tank permitting all individuals to be sedated while they were in the tank. This normally took 3 to 5 min. After the fish had been removed from the tank, waterflow was returned to the original level to wash out remnants of sedating compound and volume to return to normal levels. After netting, the fish were anaesthetized with (FINQUEL) MS 222 (50 mg L^{-1}) before weight (OHAUS – Sterner Fishtech, Norway) and fork lengths were registered. Fish weighing more than 150 grams and less than 100 grams were discarded. The mean weight at the start of the experimental period was 130g±12.8g. One feeding individual was selected and returned to the tank. This procedure was repeated with all tanks, untill each of the 16 tanks contained one fish.

The experimental period started on the 8th of December and fish were handfed for another 7 days until the 14th of December at which point feeding had stabilized. At that point all fish were being fed 160 pellets a day (the uneaten pellets were removed and counted before the next meal) and the mean consumed pellets was 60.5±28.4. Fish were being fed until satiety. The fish were sedated and collected according to the method described previously, then they were injected with leptin, ghrelin or a sham injection for the control group. The fish were injected using a random design. The fish were handfed for another 3 days then netted, and quickly killed with a blow to the head on the 4th day (19th of December) 4 h post feeding. Blood was carefully withdrawn using a syringe. Aliquots of blood where stored in eppendorf tubes that were kept in ice before centrifugation and plasma was prepared for further analysis.
Samples of brain, liver and stomach were rapidly but carefully removed, collected and flash-frozen in liquid nitrogen stored at -80º and then transferred to a -80º freezer where they were kept until they were analyzed.

2.1.2 Experiment two

For Experiment two the start of the acclimation period was monitored by personnel at IMR - Matre Aquaculture Research Station. The average size and weight of fish entering the acclimation period was not registered. The feed consumption during the acclimation period was not registered. Weight and length measurements were recorded for each of the 3 individuals from the selected tank, fish weighing more than 210 grams and less than 150 grams were discarded. The mean at the start of the experimental period was 191.5g±16.4g. The experimental period was started on the 13th of January and fish were handfed for 13 days (26th January) until feeding had stabilized. At that point fish were being fed 190 pellets a day and the mean pellet consumption was 70.9±32.2. The fish were sedated, netted, injected and replaced according to the method described previously. Fish were handfed for 4 days post injection then netted, and quickly killed with a blow to the head on the 5th day (31st January) 4 h post feeding .

2.2 Feeding

The salmon where fed in excess four times a day at 09:00, 11:00, 13:00 and 15:00 using hand feeding and waste feed collectors. All fish were fed a commercial standard

diet, Nutra Parr LB 3 3.0 5mg 3.0mm (Skretting, Norway) with a mean dry weight of 0.0196 g.

2.3 Preparation for administration of leptin and ghrelin.

Recombinant leptin was prepared according to the published protocol (Murashita *et al.,* 2011) Crisco All Vegetable Shortening (solid at room temperature) was heated to 50C in a water bath and mixed 50:50 with Vegetable oil and temperature adjusted to 37C. Then, the appropriate amount of *rsLEPA1* was weighed and dissolved in 0.5 ml NaOH (0.01N) and HCl (0.1N) solution. The dissolved hormone was transferred to the shortening solutions to achieve doses of 1 and 5µg rsLEPA1/rtGHRL per gram body weight. Solutions were made in order to be able to inject 5µl per gram body weight. The protocol for preparing hormone in vegetable oil was also followed for *rtGHRL.*

2.4 Administration of leptin and ghrelin

The recombinant Atlantic salmon specific leptin dosage was set by a pilot trial (July 2011 – Data not shown). Based on previous experiments it was presumed there would be dose-dependent effects on feed intake as for ex. recombinant *LEP* injected ICV in chickens produced a dose-dependent reduction in feed intake, with the highest dose (10µg total injected recombinant human leptin per chicken) producing the most prominent reduction in feed intake (Denbow *et al.,* 2000). Individuals in treatment groups were given a specific dose, however in the present study there did not appear to be any clear dose-response relationship or trend (data not shown) so results are analysed and presented as the mean of a treatment.

2.4.1 Experiment one

Leptin (*rsLEPA1*; 150 amino acids; 16780 Da) and ghrelin (purified rainbow trout *GHRL*, 23 amino acids; 2082 Da) was administered at equal molar levels: 0.08, 0.16, 0.24, 0.32 and 0.40 nmol/g fish. This gave dosages of 1.34, 2.68, 4.03, 5.37 and 6.71 µg/g fish with leptin and 0.17, 0.33, 0.50, 0.67 and 0.83 µg/g fish with ghrelin.

2.4.2 Experiment two

Leptin doses were set and administered at the following molar levels: 0.02, 0.04, 0.16, 0.24, 0.32 and 0.40 nmol/g fish. This gave dosages of 0.34, 0.67, 2.68, 4.03, 5.37 and

6.71 µg/g fish. Ghrelin doses were set and administered at the following equal molar levels: 0.06, 0.13, 0.19, 0.26 and 0.32 nmol/g fish. This gave dosages of 0.17, 0.33, 0.50, 0.67 and 0.83 µg/g fish.

2.5 Sampling and anesthesia

All fish were sedated using ISO-eugenol (in tank) and anaesthetized with MS 222 before weight and length registration and sampling of tissue and organs. Fish that were used for blood and RNA samples were not anesthetized, but killed with a blow to the head.

2.6 Feed uptake and growth

Feed intake was recorded daily in a MS Excel spreadsheet (Appendix 1).

- Specific growth rate (SGR)
- Feed uptake, feed factor (feed efficiency), FCR feed conversion rate

2.7 Sampling: Energy partitioning and physiology

- Samples for gene expression analyses in whole brain, liver and stomach
- Blood plasma: nutrient metabolites (FFA, triglycerides, glucose, lactate and D-3-Hydroxybutyrate) and hormone (ghrelin)

Tissues for sampling of RNA (Liver, brain, stomach) were kept on liquid nitrogen before storage at – 80 °C.

2.8 Gene expression analysis: qPCR

Expression analysis for the selected genes was conducted as described below. Sample preparation (starting from RNA extraction) of the brain tissue was performed at the MDB laboratories at BIO, Bergen, Norway; sample preparation and analysis of liver and stomach was performed at the molecular lab at NIFES, Bergen, Norway.

2.8.1 Brain samples

RNA extraction from collected brain samples was performed using the Tri-Reagent protocol according to manufacturer's recommendations. Tri-Reagent (Sigma Aldrich, St luis, USA) is an improved version of the single-step total RNA isolation reagent developed by (Chomczynski and Sacchi, 1987). Homogenisation was done using a Fast Prep machine (Savant Instruments, Holbrook, NY, USA). The remaining pellet was reconstituted in nuclease free H_20, heated for 55-60ºC for 10-15 minutes and then the optical density (OD)value was assessed using the Nanodrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples had a concentration between 400 and 1000 ng/µl with A_{260}/A_{280} values between 1.9 and 2.1. For A_{260}/A_{230} a value of 2.3 for was considered optimal. For storage samples were precipitated with 3M NaAC pH 5.5 a 1/10 of sample volume and EtOH at 2.5 times the total volume of the sample. DNA removal was performed using an optimized protocol for the Turbo DNA-free kit (Life technologies, Carlsbad, California, USA). RNA integrity was assessed using the 6000 Nano Labchip kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All samples had RIN values above 8.80. cDNA synthesis was performed using the Invitrogen Superscript III Reverse transcriptase kit (Invitrogen Life Technologies, California, USA) according to the manufacturer's instructions. A minus reverse transcriptase (minus RT) control was created. The cDNA was incubated using the following reaction protocol, 60 minutes at 50ºC, 15 minutes at 70ºC.

Duplicates of each sample were run in 25 µl reactions, consisting of 1X Power SYBR Green Master Mix (Life technologies, UK), 400 nM primers and water. Sample and dilution curve dilutions are given in Table 1. For ghrelin like receptor assays in brain we used 200 nM of primers. A negative control of pooled RNA from all samples and no reverse transcriptase was included. A negative template control (NTC) was included on all plates. No signal was observed in the negative controls. A between plate control (BPC) was used on all plates existing of pooled cDNA from all samples from both experiments. 5 step dilution curves were constructed, and consisted of pooled cDNA from all samples from both experiments.

PCR parameters for all assays, except POMC-B were as follows; a first denaturation at 95 °C for 5 min then 45 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s.

POMC-B had an annealing temperature of 62 °C. A melting curve was performed for each assay, in order to verify the absence of primer dimers (60–95 °C read every 0.5 °C and held for 0,5 s). The melting curve analysis showed a single peak for each assay, confirming PCR specificity (data not shown).

Table 1 Criteria set for the construction of dilution curves, and sample dilution factors

Gene	Input RNA in to cDNA synthesis (ug)	dilution curve, initial dilution	dilution factor in dilution curve	sample dilution
POMCA1	4	1:4	10	1:5
POMCA2	4	1:4	10	1:5
AGRP1	4	1:4	10	1:5
NPY	4	1:10	2	1:20
CART	4	1:10	2	1:20
LEPR1	2	1:5	2	1:10
LEPR2	2	1:5	2	1:10
β-ACTIN	4	1:100	2	1:400
RPL13	4	1:100	2	1:400
EF1AB	4	1:100	2	1:400
POMCB	4	1:4	10	1:5
POMCA2S	4	1:4	10	1:5
AGRP2	4	1:4	10	1:5
CCKL	4	1:10	2	1:20
CCKN	4	1:10	2	1:20
PYY	4	1:5	2	1:10
GHSR1A-LR	4	1:10	2	1:20

2.8.2 Liver and stomach

RNA was extracted from samples using a Qiazol reagent and DNA removal was performed using the EZ1 cleaning robot. 750µl quiazol was added to precellys tubes containing 3-4 beads. For liver 50µg of tissue were added to tubes and for stomach 100µg tissue were added to the tubes. The samples were homogenized using the precellys. The blank supernatant was transferred to 2ml sample tubes for the EZ1 robot. DNase was added to tubes for the EZ1 robot. Elution tubes were also added to the EZ1 robot. Dnase removal program was run for 45 minutes on the EZ1 robot.

Prior to synthesizing cDNA sample RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) RNA Nano assay.

Only samples with RIN values above 7 were utilized for further analysis. All samples had RIN values above 7.

cDNA synthesis was performed using the Roche reverse transcriptase AMV kit according to the manufacturer's instructions (Roche Applied Science, Bavaria, Germany). Stomach and liver samples from each fish were run in duplicates or triplicates (250 ng) on 96 well plates for Reverse transcription. A minus reverse transcription control (–RT) was created. A No template control (NTC) and No amplification control (NAC) were also created. The PCR plate was placed in the PCR machine and the RT reaction was run at 50°C for 50 minutes. qPCR amplification and analysis was performed on a LightCycler 480 Real-time PCR system (Roche Applied Science, Bavaria, Germany). The LightCycler 480 SYBR Green master mix kit (Roche Applied Science, Bavaria, Germany) was utilized according to the manufacturer's instructions, the mastermix contained gene specific primers at a final concentration of 500 nM. Two μl of cDNA from each well were transferred to a realtime plate and ten μl of realtime master mix was added by a pippetting robot. The qPCR protocol in Table 2 was employed. Efficiency of qPCR was monitored using 2-fold dilution curves comprised of a pool of all RNA using a five point dilution range, 500ng – 31.25 ng/μl. PCR parameters for all assays were as follows; a first denaturation at 95 °C for 5 min, then 45 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s.

2.9 Primers

PCR primers for target genes insulin-like growth factor 1 (*IGF1*), insulin-like growth factor 2 (*IGF2*), growth hormone receptor 1 (*GHR1*), growth hormone receptor 2 (*GHR2*) were designed at NIFES with the Primer Express software and Biosoft software as previously described by Hevrøy *et al.,* (2008). Ghrelin1 (*GHRL1A*) and ghrelin2 (*GHRL1B*) were designed with the Primer Express software based on sequences of Atlantic salmon as described by Hevrøy *et al.,* (2011). Leptin A1 (*LEPA1*), leptin A2 (*LEPA2*), leptin receptor 1 (*LEPR1*), leptin receptor 2 (*LEPR2*) were designed and described at the MDB lab by Rønnestad *et al.,* (2010). Proopiomelanocortin a1 (*POMCA1*), proopiomelanocortin a2 (*POMCA2*), proopiomelanocortin a2s (*POMCA2S*), proopiomelanocortin b (*POMCB*), agouti-related peptide 1 (*AGRP1*), agouti-related peptide 2 (*AGRP2*), cocaine and amphetamine related transcript (*CART*), neuropeptide Y (*NPY*), peptide YY (*PYY*), cholecystokinin (*CCKL* and *CCKN*) as described previously by Murashita *et al.,* 2009a; 2009b.; 2011). Ribosomal protein L13 (*RPL-13*), beta actin (*β-actin*) and elongation factor 1 alpha beta (*EF1-αb*) were used as reference genes as described previously by Hevrøy *et al.,* (2011).

Table 2 Primer sequences of reference and target genes used for RT-PCR mRNA expression in brain, stomach and liver of Atlantic salmon. Reference genes; ribosomal protein L13 (*RPL-13*), beta actin (*β-ACTIN*), elongation factor 1 alpha beta (*EF1-αb*). Target genes; leptin A1 (LEPA1), leptin A2 (*LEPA2*), leptin receptor 1 (*LEPR1*), leptin receptor 2 (*LEPR2*), growth hormone receptor 1 (*GHR1*), growth hormone receptor 2 (*GHR2*), insulin-like growth factor 1 (*IGF1*), insulin-like growth factor 2 (*IGF2)*, ghrelin1a (*GHRL1A*), ghrelin1b (*GHRL1B*), proopopmelanocortin a1 (*POMCA1)*, proopopmelanocortin a2 (*POMCA2*), proopopmelanocortin a2s (*POMCA2S*), agouti-related peptide 1 (*AGRP1*), agouti-related peptide 2 (*AGRP2*), cocaine and amphetamine related transcript (*CART*), neuropeptide Y (*NPY)*, cholecystokinin-L (*CCK-L*).

Target	Sequence of primers	Primer efficiency
RPL-13	Forward 5′-CCAATGTACAGCGCCTGAAA	96%
	Reverse 5′-CGTGGCCATCTTGAGTTCCT	
β-ACTIN	Forward 5′-CCAAAGCCAACAGGGAGAA	94%
	Reverse 5′-AGGGACAACACTGCCTGGAT	
EF1AB	Forward 5′-TGCCCCTCCAGGATGTCTAC	101%
	Reverse 5′-CACGGCCCACAGGTACTG	
LEPA1	Forward 5′-TTGCTCAAACCATGGTGATTAGGA	91%
	Reverse 5′-GTCCATGCCCTCGATTAGGTTA	
LEPA2	Forward 5′-TGGGAATCAAAAAGCTCCCTTCCTCTT	106%

	Reverse 5′-GCCTCCTATAGGCTGGTCTCCTGCA	
LEPR1	Forward 5′-TAGAGGTAATTGAGGAGAAGGACCTCT	99% brain
	Reverse 5′-AACATAGAGTCCTGACTCCCGAGCAA	109% liver
LEPR2	Forward 5′-GGAGGAGAAGGACCTGGATTACCT	83%
	Reverse 5′-AACATAGAGTCCCGACACCCAAGTAG	104% liver
GHR1	Forward 5′-TGGACACCCAGTGCTTGATG	111%
	Reverse 5′-TCCCTGAAGCCAATGGTGAT	
GHR2c	Forward 5′-	106%
	Reverse 5′-	
IGF1	Forward 5′-TGACTTCGGCGGCAACA	186%
	Reverse 5′-GCCATAGCCCGTTGGTTTACT	
IGF2	Forward 5′-TGCCAAACCTGCCAAGTCA	96%
	Reverse 5′-GGCACCATGGGAATGATCTG	
GHRL1A	Forward 5′- CCCTCCCAGAAACCACAGGTA	84%
	Reverse 5′- TATTGTGTTTGTCTTCCTGGTGAAG	
GHRL1B	Forward 5′- TCCCAGAAACCACAGGGTAAA	85%
	Reverse 5′- GAGCCTTGATTGTATTGTGTTTGTCT	
POMCA1	Forward 5′-TGGAAGGGGGAGAGGGAGAG	114%
	Reverse 5′-CGTCCCAGCTCTTCATGAAC	
POMCA2	Forward 5′-CTGGAGGCTGGGACTGCGGA	94%
	Reverse 5′-CGTCCCAGCTCTTCATGAAC	
POMCA2S	Forward 5′-AGACGAGAGCTGGGGGGAGT	190%
	Reverse 5′-CGTCCCAGCTCTTCATGAAC	
POMCB	Forward 5′- GACTAAGGTAGTCCCCAGAACCCTCAC	84%
	Reverse 5′-GACAGCGGTTGGGCTACCCCAGCGG	
AGRP1	Forward 5′-GCGTTCTCCCCGTCGCTGTA	107%
	Reverse 5′-TGTTAGGGGCGCCTGTGAGC	
AGRP2	Forward 5′-GCGGTGTGGTCGTCTGATGG	95%
	Reverse 5′-GGGCCCAGTCTCCAGCAGTG	
CART	Forward 5′-AGCAACTGCTTGGAGCACTACATGAC	98%
	Reverse 5′-CAGTCGCACATTTTGCCGATTCTCGCGCCC	
NPY	Forward 5′-ACTGGCCAAGTATTACTCCGCTCTCA	89%
	Reverse 5′-CTGTGGGAGCGTGTCTGTGCTCTCCTTCAG	
PYY	Forward 5′-AGACCAGCGATTTGCTGCAAAGACACCAGT	96%
	Reverse 5′-AGACCAGCGATTTGCTGCAAAGACACC	
CCK-L	Forward 5′-CAGCCACAAGATAAAGGACAGAGA	86%
	Reverse 5′-GGTCCGTATGTTTCTATGAGGAGTACG	
CCK-N	Forward 5′- AGAAGTCCCTTCATCCCTCTCTCAAACACT	85%
	Reverse 5′- AGAAGTCCCTTCATCCCTCTCTCAAACACT	
GHRLR	Forward 5′-GCACACAGGGACAAGAGCAA	83%
	Reverse 5′-CCTCGGAGGAATGGGACATA	

2.10 Plasma Ghrelin

Ghrelin in plasma was measured with a heterologous assay using a ^{125}I-radioimmunoassay kit for human ghrelin (Linco Research Inc., St. Charles, Missouri) which is specfic for biologically active (octanoylated) ghrelin. The assay has been validated for use in Atlantic salmon (Pankhurst *et al.,* 2008; Hevrøy *et al.,* 2011)

2.11 Plasma metabolites

Triglycerides and glucose in plasma were measured with the MAXMAT immunoassay kit for human triglycerides and glucose (MAXMAT S.A. Zac du Millenaire 290, rue Alfred Nobel, 34000 Montpellier, France). FFA in plasma was measured with the DIALAB immunoassay kit for FFA (DIALAB, A-2351 Wiener Neudorf, Austria). Lactate in plasma was measured with the SPINREACT immunoassay kit for human lactate (SPINREACT , S.A.U, Ctra. Santa Coloma, 7 E-17176 Sant esteve de bas (GI), Spain). D-3-Hydroxybutyrate was measured with the RANDOX immunoassay kit for D-3-Hydroxybutyrate (Randox laboratories limited, 55 Diamond road, Crumlin, County Antrim, BT29 4QY, United Kingdom).

2.12 Calculations

2.12.1 TGC ratio

The somatic growth of the fish was measured as the thermal growth coefficient ratio:

$$TGC = 1000 \times \left\{ w2^{1/3} - w1^{1/3} \right\} \times \left(\sum T \right)^{-1}$$

w2 = final weight

w1 = initial weight

∑T = sum of daydegrees

$$\text{ratio} = \frac{postinjectionTGC}{preinjectionTGC}$$

postinjectionTGC = the thermal growth coefficient prior to injection

preinjectionTGC = the thermal growth coefficient post injection

2.12.2 Daily feed intake

Feed intake was measured as DFI % of BW with the following formula.

$$100 \times \frac{fu}{iw + (fu \times fcr)}$$

fu = feed uptake

iw = initial weight

fcr = feed conversion ratio

can also be calculated as:

$$100 \times \frac{(pf - pc) \times paw}{iw + ((pf - pc) \times paw) \times \frac{fi}{g})}$$

pf = pellets fed

pc = pellets collected

paw = pellet average weight

iw = initial weight of fish

fi = feed intake

g = growth

2.13 Statistical analysis

Statistical analysis was carried with the use of the software IBM SPSS statistics 19 (IBM corporation, New York, USA), graphs and tables where generated in Microsoft Excel 2003 (Microsoft, Redmond, Washington, USA). Each treatment group, leptin, ghrelin and a control, represents an independent statistical unit. In Experiment one, control n=6, leptin n=3, ghrelin n=5. In Experiment two, control n=4, leptin n=6, ghrelin n=5. A 95% confidence level was chosen for all tests, $p<0.05$ for statistical significance. All data was tested for a normal distribution, because parametric tests for a difference depend on a normally distributed dataset. In order to visually examine the distribution of the data a frequency distribution was created in SPSS, to determine whether the frequency distribution departed from normality the Shapiro-wilk was used. If the distribution did not differ significantly from a normal distribution ($p<0.05$), then the data set was considered normal and a general parametric one-way ANOVA was utilised to test for a difference between a treatment group and the control. If the data set differed significantly from a normal distribution ($p>0.05$), then the data set was considered not to conform to a normal distribution and the Mann-Witney U test (a general non-parametric test for two groups) was utilised to test for a difference between a treatment group and the control.

3. Results

3.1 Feed intake

Daily feed intake (DFI) expressed as % of bodyweight (BW) gradually increased and stabilized in leptin, ghrelin and control groups in experiments one and two (Figure 2). When feed intake stabilized at day 8 all fish where injected IP. Injection of leptin appears to lead to reduced feed intake in both experiments. However, a significant decrease in feed intake was only observed in the leptin group in Experiment two (Figure 2C; p=0.001 ANOVA). In Experiment two there was no significant difference in feed intake in the sham injected control group (Figure 2C: p=0.193 one way ANOVA). No significant difference in feed intake was found post injection in the leptin or ghrelin treated groups in Experiment one or in the ghrelin treated group in Experiment two (Figure 2A, B, D; p>0.05 ANOVA)

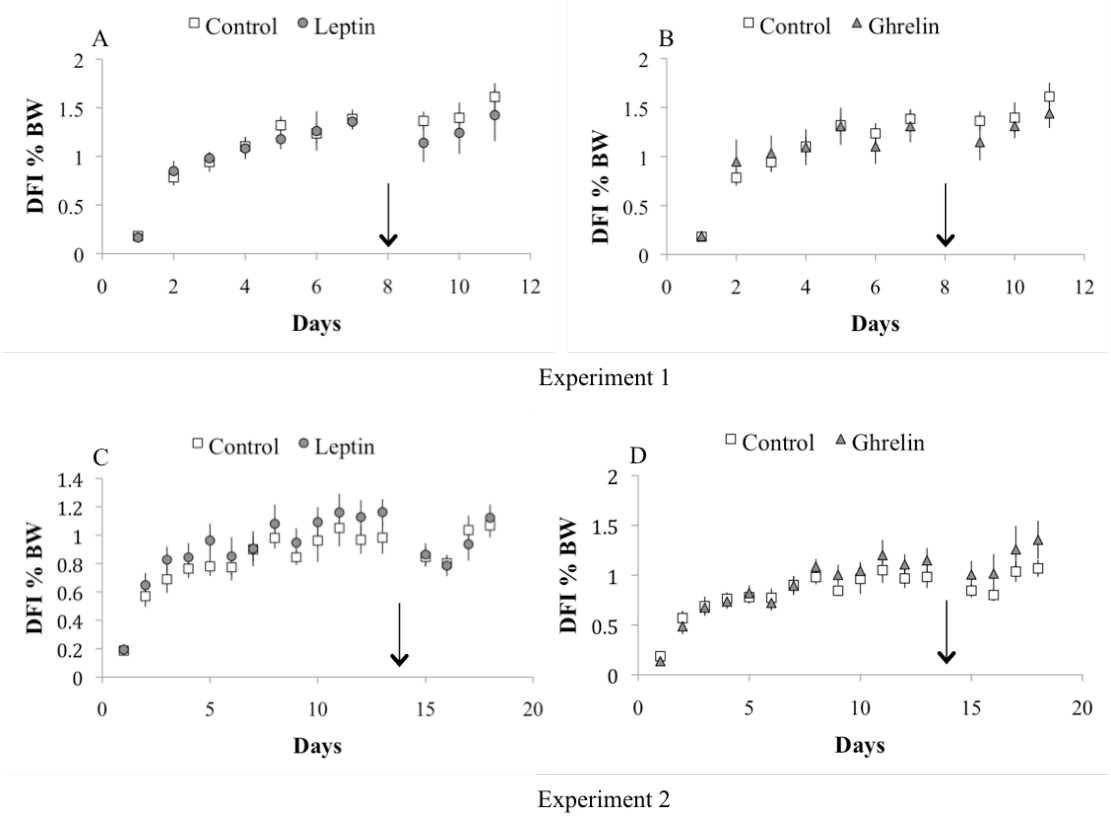

Figure 2 Daily feed intake of Atlantic salmon in % of bodyweight. Experiment one, leptin treated n=3, ghrelin treated n=5, sham treated control n=6. Experiment two, leptin treated n=6, sham treated control n=4. D Experiment two, ghrelin treated n=5, sham treated control n=4. Mean ± SE represented by bars, the mean is represented by DFI%BW each day of each fish in a treatment group. The comparison for a difference in feed intake is made between mean feed intake three days prior to injection and three days post injection. The arrow in each figure represents the point of injection. Significantly lower feed intake in leptin treated group, Experiment two.

3.2 Growth

Somatic growth of the fish was expressed as the thermal growth coefficient (TGC) ratio (Figure 3). In Experiment one (Figure 3A) no significant difference in growth was observed between treatments and control, (Figure 3A; $p>0.05$ ANOVA). In Experiment two a significantly decreased growth ratio was found in the leptin treated group (Figure 3B; $p=0.032$ ANOVA), however no significant difference in growth was found in the ghrelin treated group (Figure 3B; $p=0.971$ ANOVA).

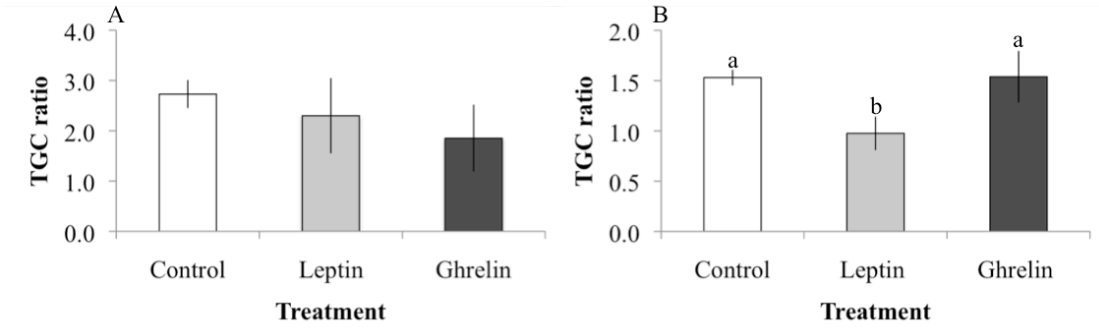

Figure 3 Mean TGC ratio of Atlantic salmon. A=Experiment one, control n=6, leptin n=3, ghrelin n=5. B=Experiment two, control n=4, leptin n=6, ghrelin n=5. Mean ± SE represented by bars.

3.3 Plasma Metabolites

There were no significant differences in plasma metabolites between treatments in Experiment one (Table 3; $p>0.05$ ANOVA). A significantly lower concentration of circulating D-3-Hydroxybutyrate was measured in the ghrelin treated group in Experiment two (Table 4; $p=0.024$ ANOVA). A similar trend in D-3-Hydroxybutyrate concentration was also observed in the ghrelin treated group in Experiment one and the difference could be considered significant (Table 3; $p=0.053$ ANOVA)

Table 3 Mean Plasma metabolites in mmol/L, Experiment one. Control n=6, Leptin n=3, Ghrelin n=5. Mean ± SE. No significant differences were observed.

Plasma metabolite concentrations Experiment one			
Metabolite	**Control**	**Leptin**	**Ghrelin**
FFA	0.21 ± 0.01	0.19 ± 0.03	0.17 ± 0.00
Triglycerides	1.46 ± 0.14	1.71 ± 0.06	1.61 ± 0.13
Glucose	5.14 ± 0.26	5.12 ± 0.26	5.62 ± 0.26
Lactate	1.79 ± 0.17	1.91 ± 0.14	1.68 ± 0.13
D-3-Hydroxybutyrate	0.065 ± 0.007	0.094 ± 0.012	0.053 ± 0.011

Table 4 Mean Plasma metabolites in mmol/L, Experiment two. Control n=4, Leptin n=6, Ghrelin n=5. Mean ± SE. Bold superscript denotes a significant difference in D-3-Hydroxybutyrate levels in the ghrelin treated group.

Plasma metabolite concentrations Experiment two			
Metabolite	**Control**	**Leptin**	**Ghrelin**
FFA	0.13 ± 0.01	0.12 ± 0.00	0.12 ± 0.01
Triglycerides	1.65 ± 0.28	1.52 ± 0.18	1.68 ± 0.28
Glucose	5.43 ± 0.44	5.47 ± 0.18	5.43 ± 0.31
Lactate	1.81 ± 0.19	2.12 ± 0.20	1.93 ± 0.19
D-3-Hydroxybutyrate	0.096 ± 0.015	0.101 ± 0.018	**0.054 ± 0.005**

3.4 Plasma ghrelin

A significantly higher concentration of circulating ghrelin was found in the ghrelin treated group in Experiment one (Figure 4A; p=0.013 ANOVA). No significant difference was found between leptin and control in Experiment one, or between injected groups and control in Experiment two (Figure 4B; p>0.05 ANOVA).

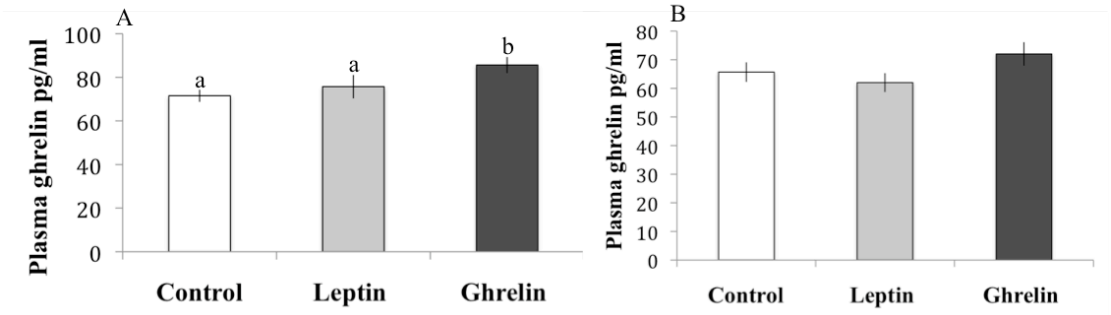

Figure 4 Mean plasma ghrelin in pg/ml. A=Experiment one. Control n=6, leptin n=3, ghrelin n=5. B=Experiment two. Control n=4, leptin n=6, ghrelin n=5. Mean ± SE represented by bars. The letter b denotes a significant difference from control marked a.

3.5 Gene expression

3.5.1 Leptin expression in liver and stomach

No significant difference in the expression of *LEP* in liver and *LEP* receptors in liver and stomach between treatment and control were found in Experiment one (Figure 5; p>0.05 ANOVA). *LEPR1* appeared to be highly expressed in liver in the leptin treated group (Figure 5C and Figure 6C), but the expression is not significantly different from the control. *LEPR2* appears to be highly expressed in the stomach tissue of the ghrelin treated group (Figure 5D and Figure 6D), however expression is not significantly higher than in the control group.

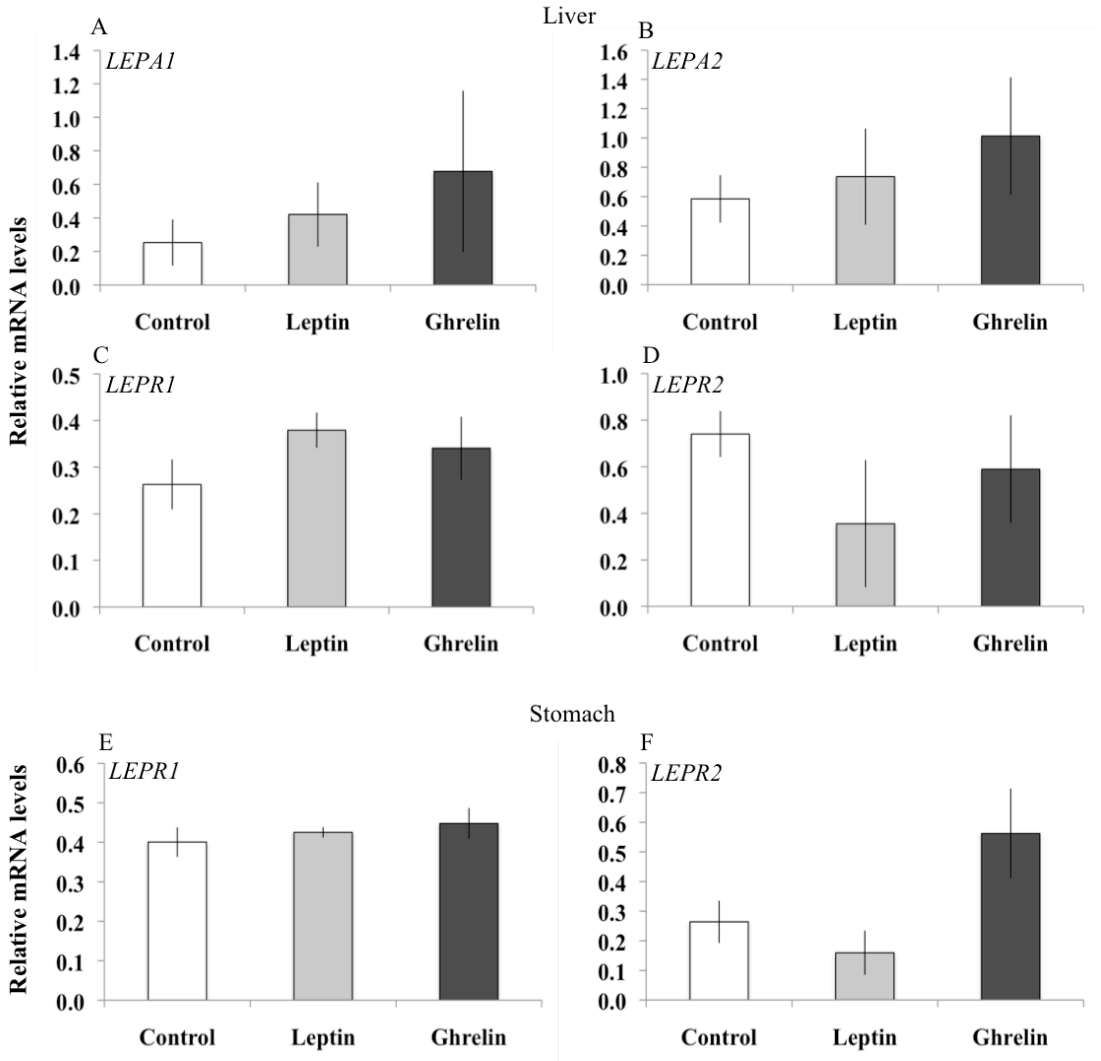

Figure 5 Mean relative expression of leptin and leptin receptors in liver and leptin receptors in stomach, Experiment one. Control n=6, leptin n=3, ghrelin n=5. Mean ± SE represented by bars. There were no significant differences between treatment groups and control group.

Expression of *LEPA1* was significantly highler in the liver of the leptin treated group compared to control (Figure 6A; p=0.021 ANOVA). Expression of *LEPA2* was also significantly higher in the liver of both the leptin and ghrelin treated group compared to control (Figure 6B; 0.005 ANOVA, p=0.019 ANOVA). No significant difference in the expression of *LEPR1* and *LEPR2* compared to control was found in liver or stomach in Experiment two (Figure 6; p>0.05 ANOVA)

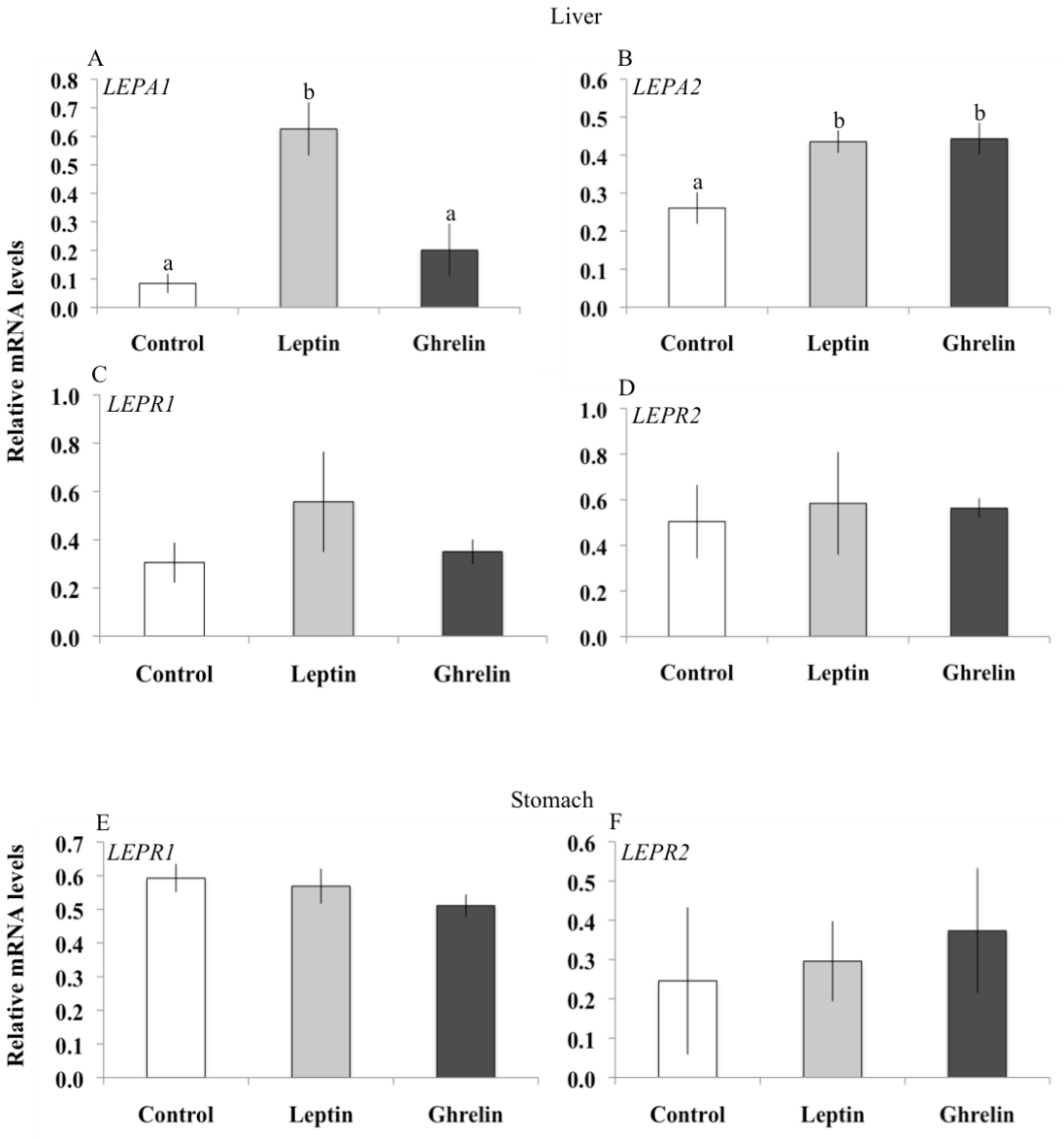

Figure 6 Mean relative expression of leptin and leptin receptors in liver and leptin receptors in stomach, Experiment two. Control n=4, leptin n=6, ghrelin n=5. Mean ± SE represented by bars. Letter b denotes a significant difference from the control marked a.

3.5.2 Ghrelin expression in liver and *GH* and *IGF* expression in stomach

The expression of *GHRL1A* and *GHRL1B* was significantly higher in the stomach tissue of the ghrelin treated group (Figure 7E; p=0.027 ANOVA and Figure 7F; p=0.016 ANOVA). No significant difference in the expression of *GHR1*, *GHR2*, *IGF1* and *IGF2* compared to control in liver or stomach tissue were found in Experiment one (Figure 7; p>0.05 ANOVA)

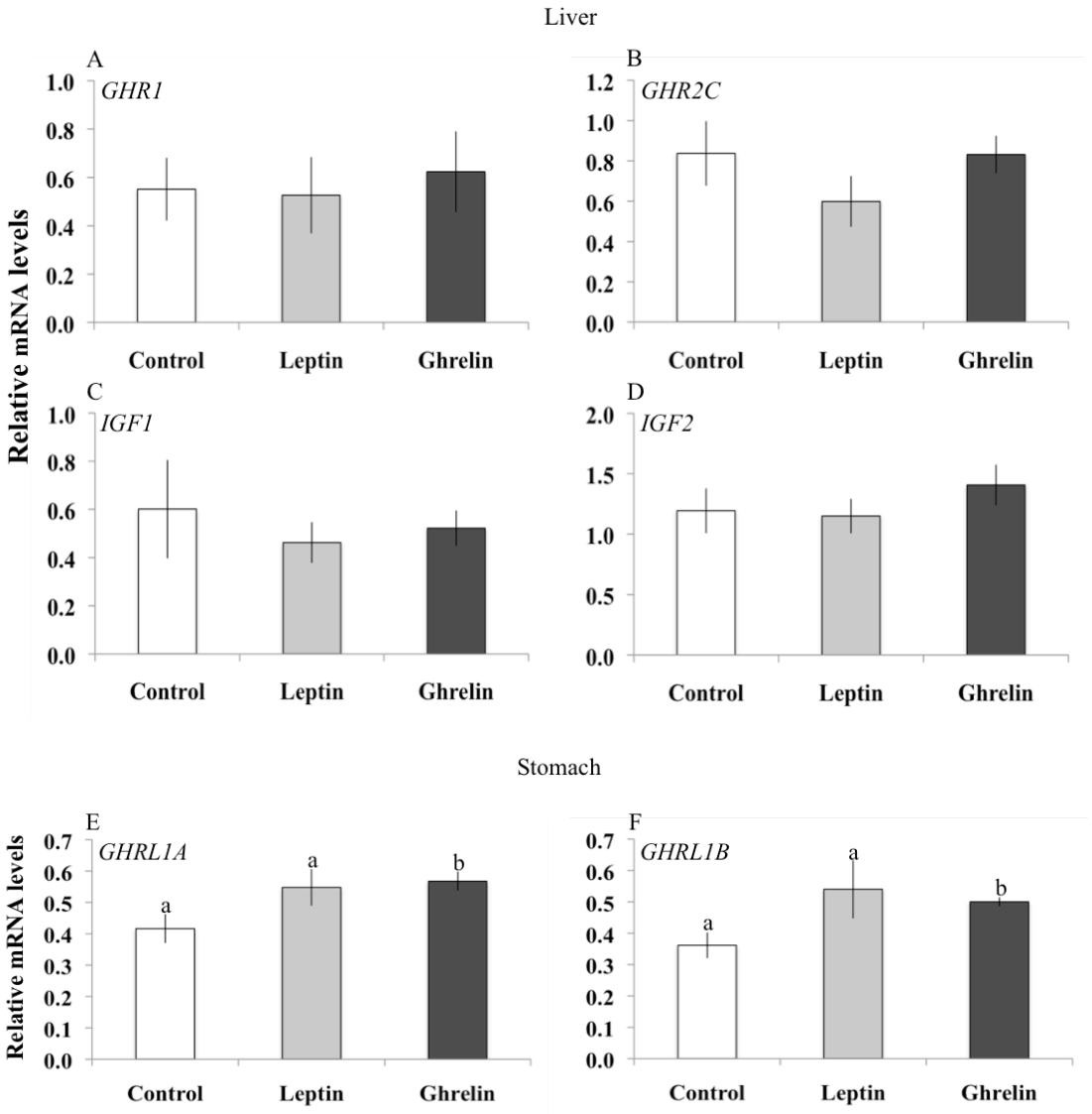

Figure 7 Mean relative expression of growth hormone and Insulin-like growth factor in liver and ghrelin in stomach, Experiment one. Control n=6, leptin n=3, ghrelin n=5. Mean ± SE represented by bars. The letter b denotes a significant difference from control marked a.

No significant difference in the expression of *GHR1*, *GHR2, IGF1,* and *IGF2* in liver and *GHRL1A* and *GHRL1B* in stomach were found in Experiment two (Figure 8; p>0.05 one way ANOVA).

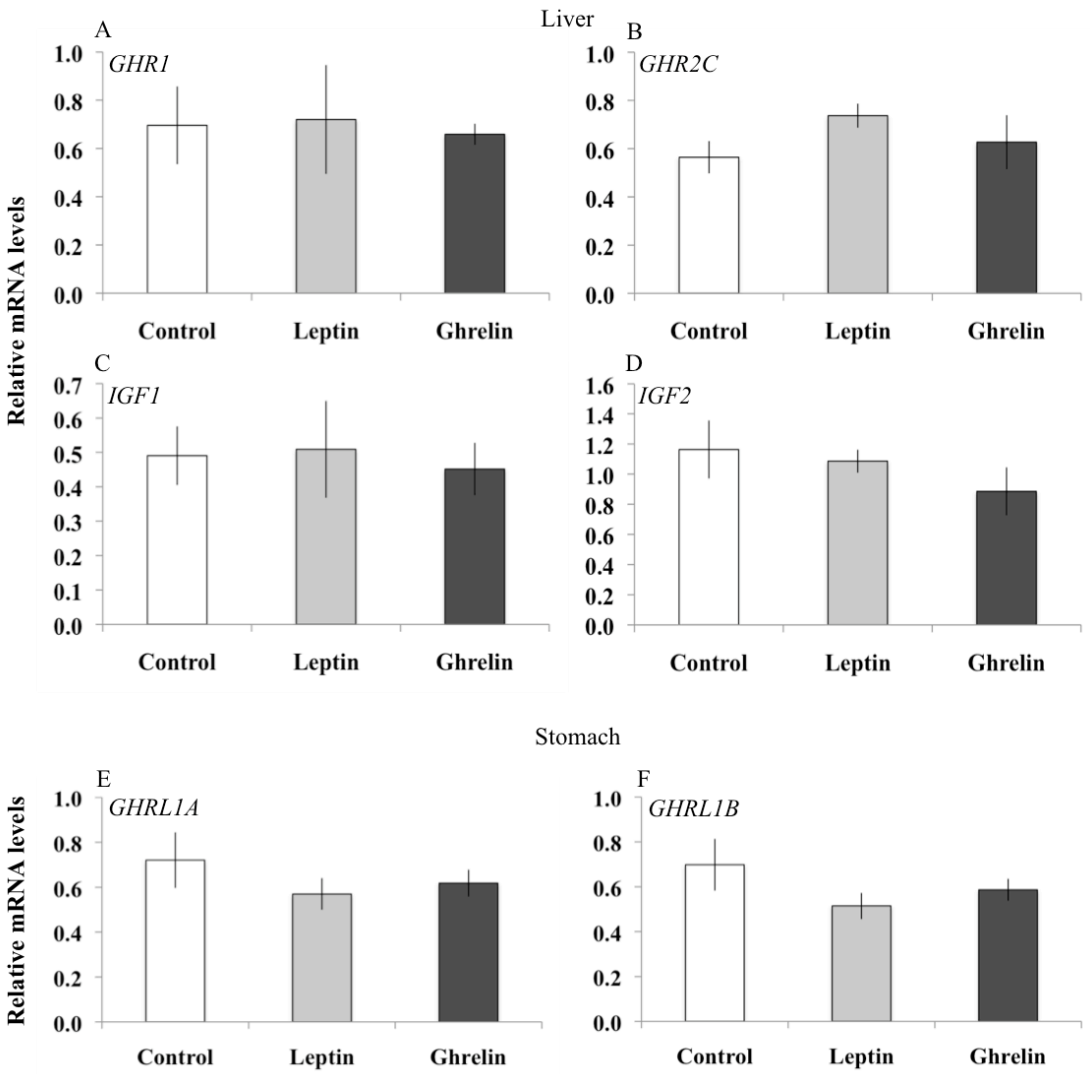

Figure 8 Mean relative gene expression of growth hormone and insulin –like growth factor in liver and ghrelin in stomach, Experiment two. Control n=4, leptin n=6, ghrelin n=5. Mean ± SE represented by bars. There were no significant differences between treatment groups and control group.

3.5.3 Neuropeptides

3.5.3.1 Anorexigenic Neuropeptides

No significant differences in the expression of neuropeptides; *POMCA1*, *POMCA2*, *POMCA2S*, *POMCB*, *CART*, *PYY*, *LEPR1*, *LEPR2* were found in Experiment one (Figure 9; $p>0.05$ ANOVA).

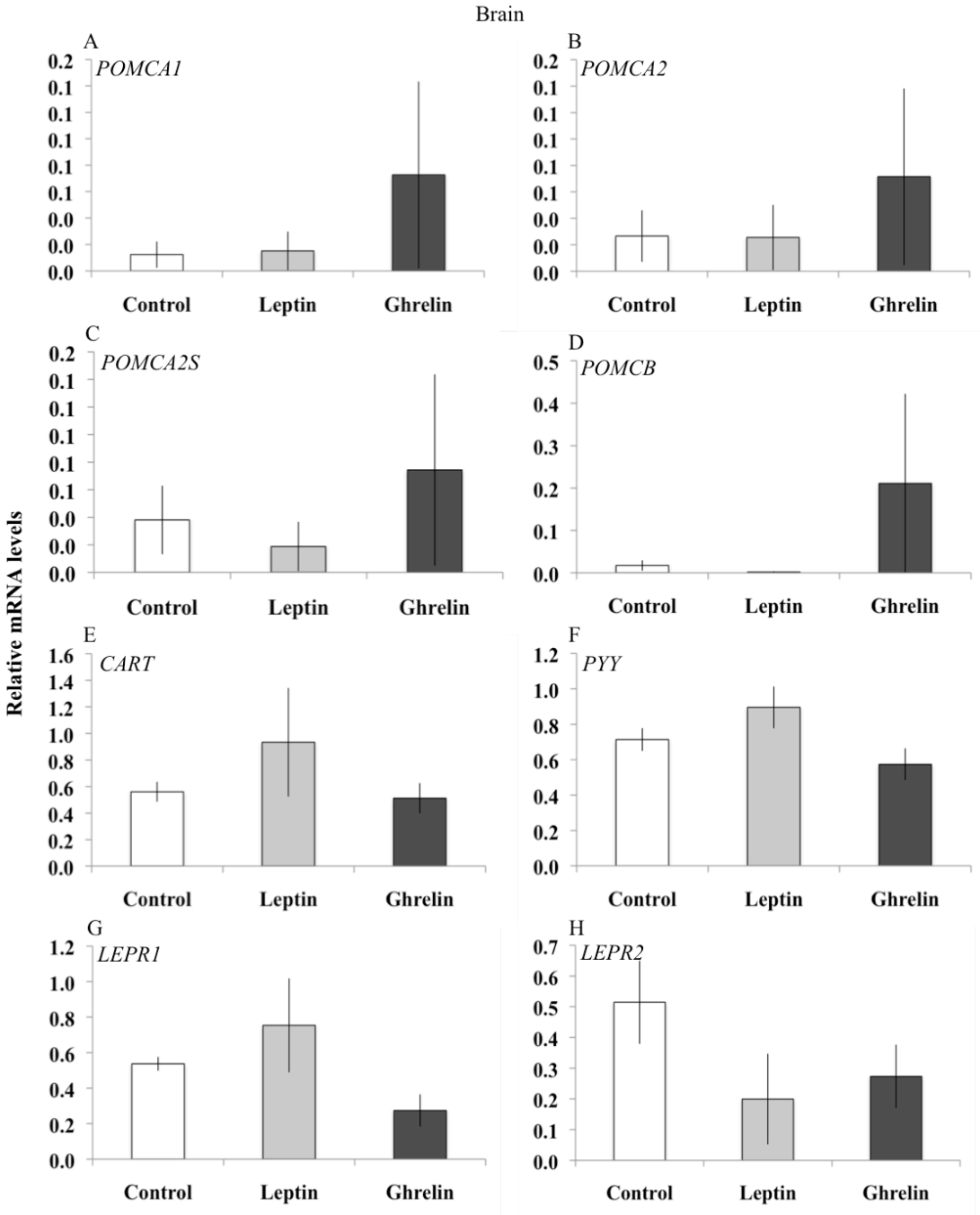

Figure 9 Mean relative gene expression of anorexigenic neuropeptides in brain, Experiment one. Control n=5, leptin n=3, ghrelin n=5. Mean ± SE represented by bars. There were no significant differences between treatment groups and control group.

POMCA1 and *POMCA2* appear to be up regulated in the leptin treated group compared to control (Figure 10A; p=0.067 Mann-Whitney U and Figure 10B; p=0.067 Mann-Whitney U). *PYY* was down regulated in the leptin treated group (Figure 10F; p=0.038 Mann-Whitney U) and the ghrelin treated group (Figure 10F; p=0.016 Mann-Whitney U). No significant differences in the expression of neuropeptides; *POMCA2S*, *POMCB*, *CART*, *LEPR1* and *LEPR2*, were found in Experiment two (Figure 10; p>0.05 ANOVA).

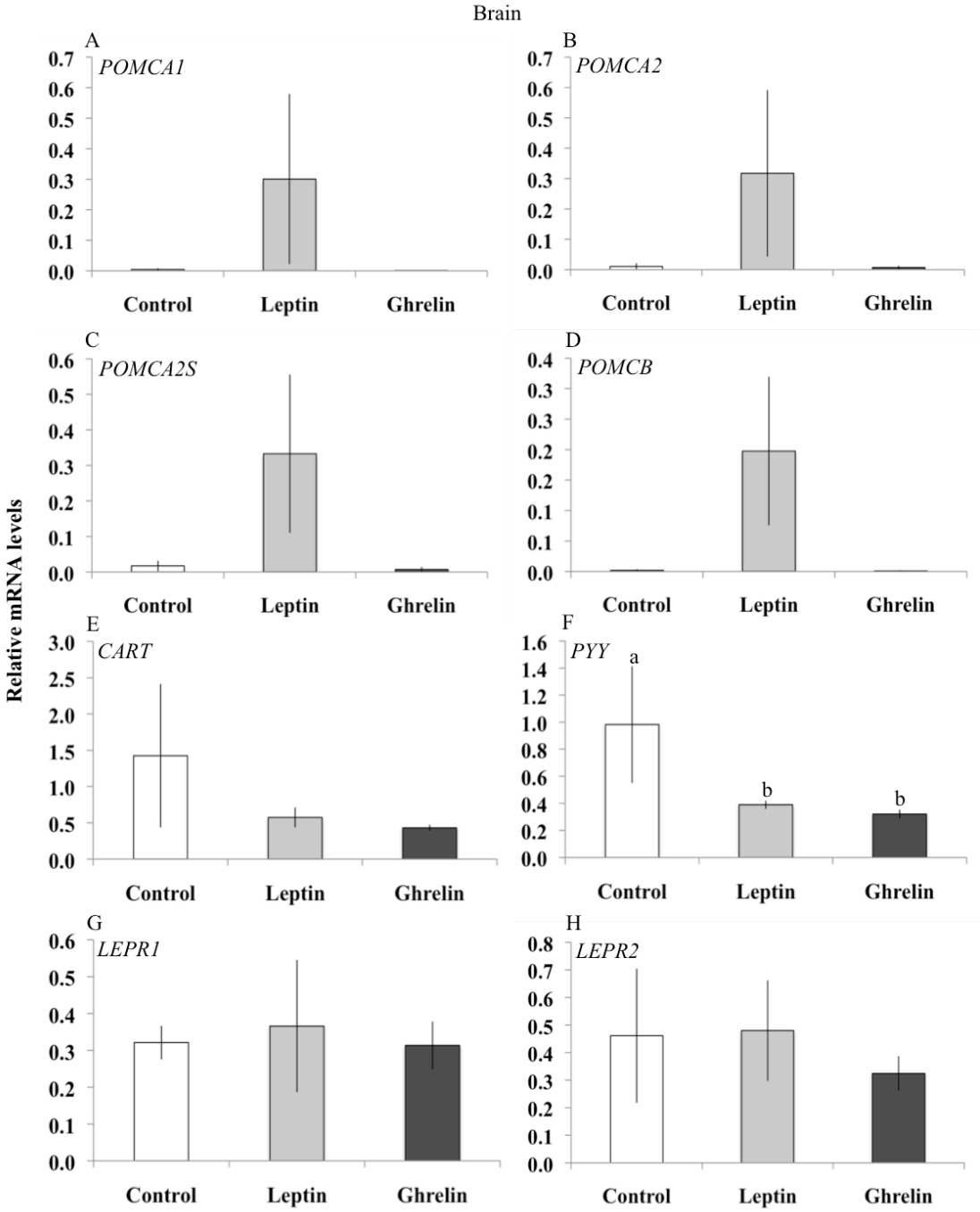

Figure 10 Mean relative gene expression of anorexigenic neuropeptides in the brain, Experiment two.

Control n=4, leptin n=6, ghrelin n=5. Mean ± SE represented by bars. The letter b denotes a significant difference from control marked a.

No significant difference in the expression of *CCKL* and *CCKN* expression were found in the leptin treated group compared to the control in Experiment one (Figure 11; p>0.05 Mann-Whitney U, p>0.05 ANOVA). Expression of *CCKL* and *CCKN* in the ghrelin treated group was significantly lower than in the control, indicating a down regulation of *CCKL* and *CCKN* in the ghrelin treated group in Experiment one (Figure 11; p=0.004 ANOVA, p=0.048 ANOVA).

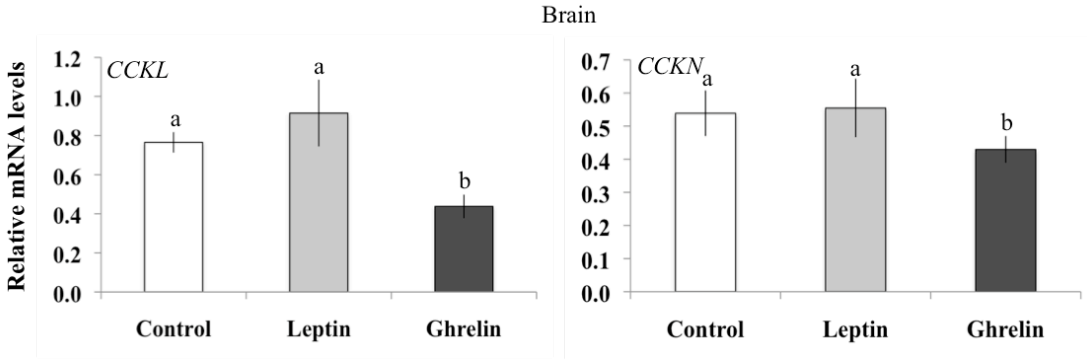

Figure 11 Mean relative expression of neuropeptides Cholecystokinin-L and Cholecystokinin-N in the brain, Experiment one. Control n=5, leptin n=3, ghrelin n=5. Mean ± SE represented by bars. Letter b denotes a significant difference from control marked a. Treatment group marked a denotes no significant difference from control also marked a.

No significant difference in the expression of *CCKL* and *CCKN* were found between treatment groups and control in Experiment two (Figure 12; p>0.05 Mann-Whitney U).

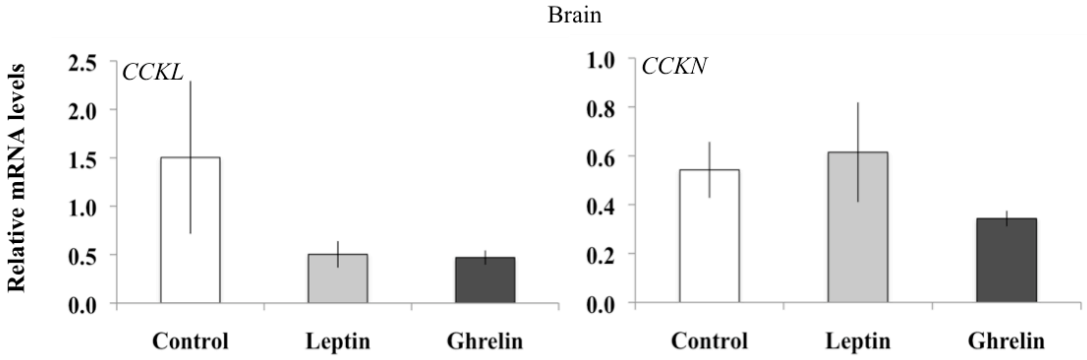

Figure 12 Mean relative expression of neuropeptides Cholecystokinin-L and Cholecystokinin-N in the brain, Experiment two. Control n=4, leptin n=6, ghrelin n=5. Mean ± SE represented by bars. No significant differences between treatment and control.

3.5.3.2 Orexigenic Neuropeptides

Expression of *AGRP1* was significantly higher in the leptin treated group than the control, indicating an upregulation of *AGRP1* (Figure 13A; p=0.047 ANOVA). No significant differences in the expression of *AGRP2*, *NPY* and *GHRLR* were found in the leptin or ghrelin treated groups compared to the control (Figure 13B, C, D p>0.05).

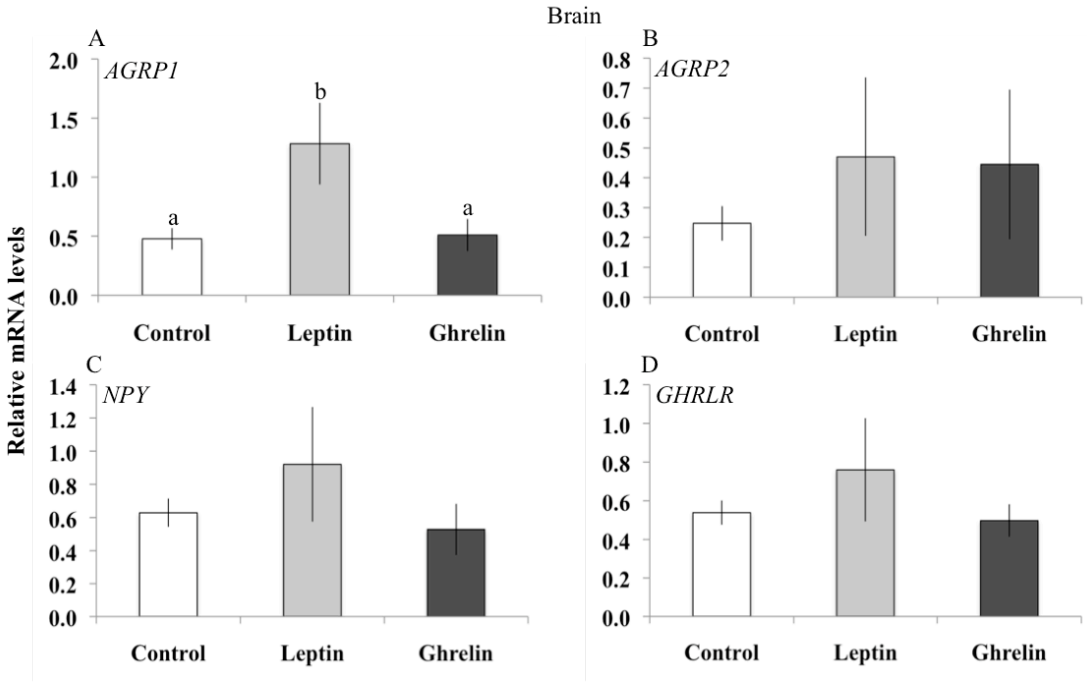

Figure 13 Mean relative gene expression of orexigenic neuropeptides in the brain. Control n=5, leptin n=3, ghrelin n=5. Mean ± SE represented by bars. The letter b denotes a significant difference from control marked a.

No significant differences in the expression of *AGRP1*, *AGRP2*, *NPY* or *GHRLR* where found in the leptin or ghrelin treated groups compared to the control in Experiment two (Figure 14A,B,C,D $p>0.05$ ANOVA).

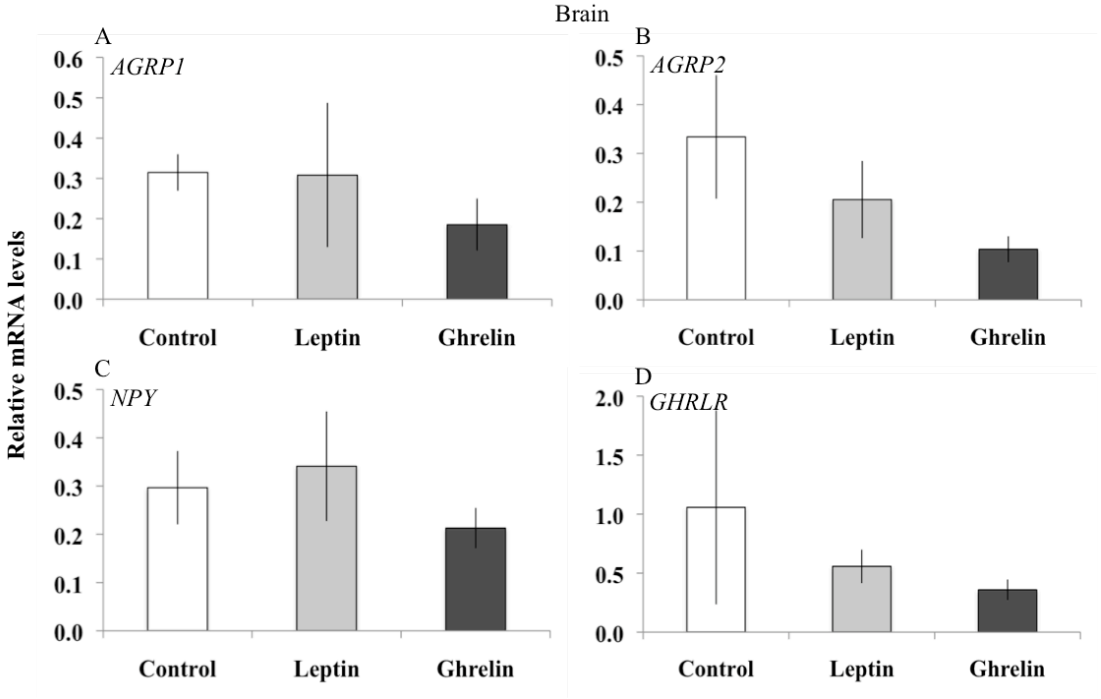

Figure 14 Mean relative gene expression of orexigenic neuropeptides in the brain. Control n=4, leptin n=6, ghrelin n=5. Mean ± SE represented by bars. No significant difference between treatment and control.

4. Discussion

4.1 Feed intake

This is the first report that demonstrates by direct measurement that *rsLEPA1* injected IP leads to a reduced feed-intake in Atlantic salmon. This was observed in Experiment two where there was a significant reduction in feed intake in *rsLEPA1* treated Atlantic salmon while the sham treated control had no change in feed intake. The data in Experiment one had trends suggesting similar results, but these were not significant. Murashita *et al.,* (2008) found a rapid suppression of feed intake post *rsLEPA1* injection indicating a strong short term anorexic effect, so one could expect to see the greatest reduction in feed intake the day after injection, which is not the case in Experiment one in this study, However Murashita *et al.,* (2008) injected recombinant rainbow trout leptin in phosphate buffered saline which may be released quicker than a hormone in vegetable oil. Although the results were not the same in Experiment one and two the results in Experiment two show that recombinant *rsLEPA1* injected IP causes a significant reduction in feed intake in Atlantic salmon (Appendix 1). These results support the findings that recombinant *LEP* treatment reduces feed intake in teleosts such as rainbow trout (Murashita *et al.,* 2008), goldfish (Vivas *et al.,* 2011) and Atlantic salmon (Murashita *et al.,* 2011) and suggest that *LEP* treatment in salmon produces a reduction in feed intake similar to that observed in higher vertebrates such as mammals; pigs *Sus spp*. (Barb *et al.,* 1998), rats *Rattus spp.* (Watzler *et al.,* 2004) and birds; chickens *Gallus spp.* (Denbow *et al.,* 2000), great tits *Parus major* (Lõhmus *et al.,* 2003).

Fish treated with ghrelin showed no alteration in feed intake compared to the control group (Appendix 1). These findings are contradictory to other studies in teleosts, as rainbow trout injections and implants have previously been found to both decrease food intake (Kling *et al.,* 2012) and increase feed intake in goldfish (Matsuda *et al.,* 2006). Ghrelin has also been found to increase feed intake in higher vertebrates such as humans (Wren *et al.,* 2001) and rats (Locke *et al.,* 1995). There does not seem to be such a clear relationship between the injection of purified rainbow trout and an increase in feed intake in Atlantic salmon. This may be due to the fact that rainbow trout ghrelin was administered to Atlantic salmon in this study, thus species specific ghrelin may be required to produce any effects on appetite. Ghrelin injected IP in

goldfish at 10 pmol/g BW produced an increase in feed intake (Matsuda *et al.,* 2006) and rainbow trout ghrelin injected with 0.05 or 5 nmol/kg^{-1} IP (Jönsson *et al.,* 2007) produced no increase in feed intake. It is unlikely that the dose set in this study was too low as Jönsson *et al.,* (2007) achieved similar results with lower dosages. It is possible that the effect of ghrelin on food intake varies with the route of administration, source of hormone, species and dose (Jönsson *et al.,* 2007)

4.2 Growth

The somatic growth of fish was significantly reduced in the *LEP* injected group in Experiment two. The growth rate appeared to be reduced in Experiment one but the reduction was not significantly compared to the control. The reduced growth rate of *LEP* treated fish in Experiment two is likely a consequence of the reduced feed intake that was also observed in Experiment two, which further supports that *ssLEPA1* causes a reduction in appetite and consequently reduces growth in Atlantic salmon. These results are supported by Murashita *et al.,* (2011) who also found that recombinant *ssLEPA1* administered IP significantly reduced the growth rate of Atlantic salmon. The reduction in growth observed in this study suggests that the role of *LEP* is comparable to that in other teleosts such as rainbow trout (Murashita *et al.,* 2008) and fine flounder *Paralichthys adspersus* and in higher vertebrates, such as humans (Dardeno *et al.,* 2010, Lee *et al.,* 2002), pigs (Barb *et al.,* 2001, Ramsay *et al.,* 2004) and mice (Leinninger et al., 2009).

Fish in the ghrelin injected group showed no significant difference in growth compared to the control, this could be expected as ghrelin treated fish showed no alteration in feed intake. These results are in contrast to other studies as Jönsson *et al.,* (2007) found that long-term peripheral ghrelin treatment of rainbow trout reduced feed intake, which was reflected in a ghrelin-inudced drecrease in weight growth rate. However Jönsson *et al.,* (2007) utilized native rainbow trout ghrelin in their study and ghrelin may well have species specific effects. Ghrelin is generally considered to cause growth through the stimulation of GH secretion from the pituitary in mammals, birds, amphibians and teleosts (Kaiya *et al.,* 2013). Ghrelin in Atlantic salmon may not function in a manner comparable to other species, but the evidence drawn from this study is not enough to make any conclusions.

4.3. IP injection – time and release

Leptin and ghrelin both form part of a feedback regulated loops, so one could expect that injecting these hormones would increase the level of the hormone in blood plasma. The levels of these hormones in plasma will in turn feedback to central systems possibly altering the regulation of appetite and affecting other pathways involved in appetite regulation. Previously, the method of administering peptide or steroid hormones emulsified in vegetable oil has worked well in terms of release and uptake in circulation in Atlantic salmon (McCormick, 1996; McCormick *et al.,* 2008; Specker *et al.,* 1994). This method of administrating hormones causes extended elevation of the hormone in plasma, for approx. 1-2 weeks depending on water temperature and can be considered the best method for administrating hormones in Atlantic salmon (Nilsen, T.O personal communication). Injecting hormones in an oil emulsion makes endogenous time of release difficult to determine. However it has been shown that by administering a certain volume of fat emulsion per gram of fish one can obtain a pellet of relatively the same size in the abdomen of the injected fish, therefore one can argue that the time of release should be the same in any given individual (Nilsen, T.O. personal communication). Variation can always occur but by measuring plasma levels of the given hormone one can find out if the hormone has been released at equally, in this study the different doses were set for each individual so one should expect to see different levels of hormone in plasma.

4.4 Plasma metabolites

The concentration of plasma metabolites FFA, Triglycerides, Glucose, Lactate and D-3-Hydroxybutyrate were similar in the *LEP* treated group and control. These results are comparable to those found in mammals as Tanida *et al.,* (2000) found that *LEP* injected into white adipose tissue (WAT) did not cause any change in the concentration of insulin, glucose and lactate in in *Rattus spp. LEP* may not influence neuroendocrine regulation through this route, but rather directly through sensors in afferent nerves (Tanida *et al.,* 2000). In the *GHRL* treated group no difference in the concentration of FFA, triglycerides, glucose or lactate where found compared to the control. However the concentration of D-3-hydroxybutyrate in plasma was significantly lower in the *GHRL* treated group in Experiment one and two.

Hydroxybutyrate is a ketone body, which is produced in the liver mainly from the oxidation of fatty acids and is exported to surrounding tissues as a source of energy (Guthrie and Jordan, 1972). Normal ketosis can indicate that lipid metabolism has been activated and the pathway of lipid degradation is intact, and is often found during fasting, prolonged exercise or a high fat diet (Galan *et al.,* 2001). As the concentration of D-3-hydroxybutyrate was significantly lower in the *GHRL* treated group, in both experiments it is likely that purified rainbow trout ghrelin somehow influences ketone metabolism in Atlantic salmon. As D-3-hydroxybutyrate is synthesized under nutritional states where carbohydrates and lipid oxidation are unbalanced (Willmott *et al.,* 2005) these results suggest that the *GHRL* treated fish do not have a reduced glucose or lipid metabolism, but a reduced lipid catabolism. In humans ghrelin has an effect on fatty acid release and ketone body formation and ghrelin infusions have been coupled with a significant increase in 3-hydroxybutyrate ketone bodies in plasma (Huda *et al.,* 2011), these findings could suggest that ghrelin has an alternative role in the stimulation of ketosis in teleosts.

4.5 Metabolites and Stress

Handling and physical disturbances can cause stress in fish (Barton and Iwama, 1991; Evans and Clairborne, 2006). In response to the stressor the fish will undergo biochemical and physiological changes in order to cope with the stress factor. The initial response includes a neuroendocrine response that includes a release of catecholamines and cortisol into circulation (Evans and Clairborne, 2006). A rise in cortisol following chronic stress has been linked to higher plasma glucose and energy mobilisation (McCormick *et al.,* 1998), thus changes in plasma glucose concentration have been used as an indicator of a metabolic response to stress in fish. Elevated cortisol levels that occur due to stress may reduce growth and administration of cortisol reduces growth in *Orynchus mykiss* and channel catfish *Ictalurus punctatus*. Unstressed sockeye salmon *Oncorhynchus nerka* have plasma glucose concentrations of 110 mg/ml in males and 103 mg/ml in females, when stressed the concentrations gradually increase to 150 mg/ml and 142 mg/ml after 15-30 minutes of stress (Kubokawa *et al.,* 1999). In the current study glucose concentrations in the *LEP* injected group were not elevated compared to the control in Experiment one or two. Throughout treatment and control groups plasma glucose levels were below 1 mg/ml in both Experiment one and Experiment two. The low and steady glucose

concentrations found in this study in control, rs*LEPA1* and *GHRL1* treated groups may indicate that the fish were not stressed by the experimental method and as such validate that the method used for sedating the fish in the tank before anesthesia and injection is not stressful to salmon. This is also verified by the fact that the salmon started to eat within a few hours after they were returned to the tanks post injection. Normal recovery back to pre-handling feeding levels takes days in Atlantic salmon. As a decreased growth rate was observed in the *LEP* treated group in Experiment two but the plasma glucose concentrations did not differ between treatment group and control and were not elevated it is likely that decreased growth was not caused by stress and elevated glucose levels, but by the recombinant *rsLEPA1* injection.

4.6 Plasma Ghrelin

Elevated levels of plasma ghrelin where found in the ghrelin injected fish in Experiment one, but not Experiment two. Fish that have been injected with ghrelin could be expected to show elevated levels of plasma ghrelin. In Experiment one *GHRL1A* and *GHRL1B* were upregulated in the stomach tissue of ghrelin injected fish, however these genes were not upregulated in Experiment two. As ghrelin is primarily produced in endocrine cells in the gastric glands or the mucosoal folds in the stomach the elevated concentration of ghrelin in blood plasma is likely related to elevated levels of ghrelin mRNA found at the main site of production. As *GHRL1A* and *GHRL1B* were not upregulated in the stomach tissue of ghrelin injected fish in Experiment two and no change in ghrelin plasma concentration levels where found further suggests that expression of ghrelin in the stomach and plasma ghrelin levels may be related. Matsuda *et al.,* (2006) suggest that the ghrelin signal that leads to increased food intake in goldfish is mediated through the afferent vagus nerve rather than the bloodstream, however we found no difference in feed intake between ghrelin injected fish and the control. The results in this study show no link between expression of ghrelin in stomach, elevated levels of plasma ghrelin and any short-term change in feed intake. However Hevrøy *et al.,* (2012) found reduced plasma ghrelin and stomach ghrelin mRNA levels in Atlantic salmon exhibiting voluntary fasting at elevated temperatures. Murashita et al., (2009) also found that mRNA levels of *GHRL1* but not *GHRL2* increased after 6 days fasting in Atlantic salmon. It is possible that elevated levels of ghrelin in plasma may be related to feed intake on a

longer time scale rather than short term. However the increased levels of ghrelin in plasma observed in this study may be due to the injection of *rtGHRL*.

4.7 Gene expression

4.7.1 Recombinant IP administered leptin and neuropeptide expression

This study reports significantly higher expression of *LEPA1* and *LEPA2* in the liver of *rsLEPA1*-injected fish compared to control. In mammals leptin is expressed mainly by adipose tissue which is reflected by the fact that mammals deposit and store energy in adipose tissue. Adipose tissue and plasma leptin concentrations are dependent on the amount of energy stored as fat as well as the energy balance, this means that leptin levels are higher in obese individuals and increase with overfeeding (Ahima and Flier, 2000; Ahima and Osei, 2004), thus leptin functions as an endocrine indicator of adipose energy reserves (Won *et al.,* 2013) Many teleost fish utilize the liver as a major lipid storage site and the liver is also considered a major site of leptin production as *LEP* genes have been found to be highly expressed in the livers of pufferfish (Kurokawa *et al.,* 2005), striped bass (Won *et al.,* 2012) and yellow catfish Gong *et al.,* (2013). On the other hand fatty fish such as Atlantic salmon mainly deposit and store energy in muscle tissue and visceral adipose tissue, which is to some extent reflected by a high expression of *LEPA1* in muscle tissue. In contrast to other teleosts *LEP* expression is lower in tissues containing more adipocytes, visceral adipose tissue for example consists almost entirely of adipocytes but displays a much lower expression of *LEP* than muscle and liver tissue (Rønnestad *et al.,* 2010). Whether leptin is produced or secreted in relation to the hepatic lipid content or adipose content of fish is still unknown (Won *et al.,* 2012). Although Atlantic salmon utilise the liver to minor extent for energy storage, hepatic expression of *LEPA1* is significant and comparable to the expression found in white muscle (Rønnestad et al., 2010). Previous research has shown that pufferfish hepatocytes contain abundant oil droplets and LEP has been detected in hepatocytes (Kurokawa *et al.,* 2005), indicating that liver expression of *LEP* is linked to energy metabolism of the hepatocytes. Our findings further support the hypothesis that expression of *LEP* in the liver is linked to metabolism.

Two experiments were performed in this study; in experiment one no significant reductions in feed intake or growth rate were found and *LEPA1* and *LEPA2* expression in liver were not affected. Plasma levels of leptin were not measured as part of this study so it is difficult to speculate on how leptin plasma levels were affected by exogenous administration of *LEPA1*, however we can assume that the levels were altered as *POMC* isoforms were upregulated at a central level in Experiment two and that these possibly form part of an appetite regulating pathway as feed intake was reduced. One might expect that *LEPA1* mRNA levels would be reduced at the sites of production such as in the liver due to external administration of *LEPA1* however in Experiment two a significant upregulation of *LEPA1* and *LEPA2* in liver was found, along with a reduction in feed intake and lower growth rate. These results suggest that *LEP* expression in the liver could be linked to the expression of *POMC* at a central level and short term changes in feed intake and metabolism. Previously Ronnestad et al., (2010) found that *LEPA2* mRNA levels in the liver were higher in a group of Atlantic salmon with reduced rations over an extended period of time, this study shows that *LEPA2* expression in liver may also be involved in short term changes in appetite in a manner comparable to that found in higher vertebrates. Our findings are also supported by Kullgren *et al.,* (2013) who found that leptin acted as a signaling factor contributing to reduced food intake in Atlantic salmon.

We found that administration of *rsLEPA1* did not affect mRNA expression of *NPY*, *AGRP* isoforms, or *CART* in brain tissue in Experiment two, which is supported by Murashita et al., (2011) who also found that administration of *rsLEPA1* did not affect *NPY*, *AGRP* and *CART* mRNA expression in the brain. However in Experiment one we found that *rsLEPA1* injected fish had upregulated mRNA expression of *AGRP1* in the brain. Leptin inhibits the *NPY*/*AGRP1* neuron from releasing orexigenic peptides, the fact that *AGRP1* is upregulated in Experiment one suggests that injected leptin may not be in circulation which is supported by the fact that feed intake is neither decreased, as one could expect in fish with increased leptin levels, or increased as one could expect with increased expression of *AGRP1*. Plasma leptin levels were not measured as part of this study, so no conclusions can be made based on plasma levels of leptin.

POMC may play a more important role in the short-term leptin-signaling pathway and could function independently of *AGRP/NPY* expressing neurons in Atlantic salmon as when the expression of *POMCA1* and *POMCA2* is upregulated a significant reduction in feed intake is also observed. The results presented in this study supports those of Murashita *et al.,* (2011), who also found increased expression of *POMCA1* in Atlantic salmon administered *rsLEPA1*. Previously IP injections of *rsLEPA1* reduced short-term food intake with increased expression of *POMCA1* and *POMCA2* and decreased expression of *NPY* in rainbow trout, meanwhile Atlantic salmon fasted for 6 days showed no significant change in the expression of NPY (Murashita *et al.,* 2011; Murashita *et al.,* 2009). Valen *et al.,* (2011) also found that *POMCA1* and *POMCB* were upregulated within 3 hours of feeding, which supports that these neuropeptides are involved in a short-term appetite regulating pathway. In relation to the upregulation of *POMCA1* and *POMCA2* it is important to note that the fish were fed one meal in the morning at the 09:00 and slaughtered at within 4 hours of the meal in both experiments, we found that *POMC*-type genes were only upregulated in Experiment two in which *LEP*-type genes were also upregulated and feed intake was reduced.

We found that *PYY* expression in the brain was downregulated in leptin injected fish in Experiment two. *PYY* is associated with a reduction of feed intake in mammals (Cummings and Overduin, 2007). Previous studies of Atlantic salmon show that fasting and feeding have minimal effects on *PYY* mRNA expression in the brain (Murashita et al., 2011; Valen et al., 2011). However Valen et al., (2011) did find postprandial changes in *PYY* expression in the GI tract, and suggest that *PYY* responds differently to feed intake depending on peripheral or central expression, but that *PYY* plays a minor role in the central control of short-term food intake in Atlantic salmon. These results are in contrast to the findings in this study where *PYY* is downregulated in fish with a significant reduction in feed intake and upregulation of hepatic *LEPA1* and *LEPA2* and *POMC* isoforms. *PYY* concentration in plasma increases after feeding and decreases during fasting in mammals (Murphy and Bloom, 2006), our findings are to some extent in line with the mammalian model as the apparent *rsLEPA1* induced reduction in feed intake could be considered involuntary fasting and thus may be involved in the reduced levels of *PYY* mRNA observed in the brain. It is possible that leptin-signaling pathway affects the expression of *PYY* at a central level.

4.7.2 Purified IP administered ghrelin and neuropeptide expression

This study reports significantly higher mRNA expression of *GHRL1A* and *GHRLIB* in the stomach of *rtGHRL*-injected fish compared to control. Two experiments were performed and the expression of *GHRL1A* and *GHRLIB* was only upregulated in the experiment in which plasma ghrelin levels were found to be elevated. Significant changes in the mRNA expression of neuropeptides associated with orexigenic functions *AGRP1*, *AGRP2*, *NPY* and receptor *GHRL1R* were not found in *rtGHRL* treated fish. However mRNA expression of the anorexigenic peptides *CCK* and *CCKL* was significantly lower than the control in Experiment one and also appeared to be lower in Experiment two, although not significantly.

Previously Gao *et al.,* 2012 found that ghrelin supplemented in the diet of grouper *Epinephelus coioides* caused an increase in the expression of hypothalamic NPY mRNA and also an increase in feed intake and weight. Furthermore Terova *et al.,* (2008) found mRNA levels of ghrelin in stomach were upregulated during negative energy balance, such as starvation, and downregulated during positive energy balance, such as refeeding and suggest that ghrelin has an orexigenic role in the regulation of food intake in sea bass. In salmonids the possible orexigenic function of ghrelin is not so clear. mRNA levels of *GHRL1* but not *GHRL2* have been found to increase during fasting in Atlantic salmon (Murashita *et al.,* 2009) indicating a possible orexigenic role in Atlantic salmon (Kaiya *et al.,* 2011). However injections of *rtGHRL* have decreased feed intake in rainbow trout and long-term peripheral IP implants of rt*GHRL* have also reduced daily feed intake compared to controls (Jönsson *et al.,* 2010). Furthermore Jönsson *et al.,* (2010) suggest that ghrelin may act on the GHS-R in the CNS in rainbow trout as an anorexigenic hormone and that elevated periheral ghrelin leads to decreased feed intake in the long term (14 days). The inhibitory mechanism of ghrelin on food intake described in rainbow trout is similar to that demonstrated in the chicken (Kaiya *et al.,* 2011).

As mentioned previously ghrelin is produced primarily in the stomach in response to hunger and circulates in the blood in mammals. Plasma ghrelin levels increase during fasting and decrease after ingesting glucose and lipid, but not protein. The efferent vagus nerve contributes to the fasting-induced increase in ghrelin secretion. Ghrelin secreted by the stomach stimulates the afferent vagus nerve and promotes feed intake.

The vagal circuit between the central nervous system and stomach has a crucial role in regulating plasma plasma ghrelin levels (Nonogaki, 2007). Thus it could be expected that the injected *rtGHRL* would cause an elevation of ghrelin plasma levels as the fish assimilate the cholesterol pellet formed by an IP injection. Based on this feedback mechanism one might expect to see a reduction in the fishes own production of ghrelin in the stomach and a downregulation of *GHRL1A* and *GHRLIB* in stomach tissue assuming that the fish did not have a hungersignal four hours after feeding at the point of sampling. Hevrøy *et al.,* (2012) found a reduced plasma ghrelin concentration and reduced *GHRL1* expression in stomach of Atlantic salmon kept at 19° C after 21 days and suggest this may be due to long-term neuropeptide signaling. It is possible that the potential orexigenic or anorexigenic effects of elevated plasma ghrelin only become evident after a longer period of time, bearing in mind that fish in this study were sampled after four days in Experiment one and five days in Experiment two.

Peripheral ghrelin can interfere with the *CCK* related mechanism in the regulation of satiety and food intake but also with other neuropeptides involved in homeostatic regulation of energy intake and expenditure (Kobelt *et al.,* 2005). Gastrointestinal peptides such as CCK can suppress ghrelin secretion, however the effects may be indirect and/or have been difficult to reproduce and the physiological relevance remains unclear (Engelstoft *et al.,* 2013). Known signals inhibiting ghrelin secretion are mainly endocrine and paracrine, known stimulatory signals are mainly neuronal (Engelstoft *et al.,* 2013). Sympathetic nerve and vagal stimulation increase ghrelin secretion. Sympathetic stimulation most likely occurs directly at the ghrelin cell via beta1 adrenergic receptors. Vagal stimulation depends on colinergic muscarinic mechanisms, but whether this effect is direct or indirect is unclear (Engelstoft *et al.,* 2013). Inhibition of these pathways prevents fasting-induced elevation of plasma ghrelin (Engelstoft *et al.,* 2013)

CCK exists in the endocrine cells of the GI tract and within the central and peripheral nervous system (Lin et al., 2000). CCK acts as a satiation factor at the levels of the gut and centrally in specific brain regions in mammals (Lin et al., 2000). In goldfish IP and ICV injections of CCK8 acutely suppresses food intake, supporting that CCK acts as a satiety factor in fish. Furthermore Peyon et al., (1999) had previously found

an acute increase in CCK mRNA levels in the olfactory bulbs, telencephalon-preoptic region, hypothalamus, and posterior brain 120 minutes after a meal. This evidence supports that *CCK* synthesis and release occur following a meal (Lin *et al.,* 2000). However Nguyen *et al.,* 2013 did not find any data that could support brain CCK acting as a satiety signal in Cobia *Rachycentron canadum* but these fish were sampled only once 15 minutes after a meal and changes in *CCK* expression may take longer to occur. There is evidence that CCK has a similar anorexigenic function in salmonids; Jönsson *et al.,* (2006) found a postprandial elevation of plasma CCK levels most evident after 4 and 6 hours in rainbow trout. Their results indicated that the endocrine release of gastrointestinal *CCK* was increased during feeding. CCK regulation also occurs at a central level postprandially, Valen *et al.,* (2011) found that *CCKL* expression in the brain was higher than in unfed controls 0.5, 1.5, 3, 6, 9 and 12 hours post feeding in Atlantic salmon. However Murashita *et al.,* (2009) found no change in the expression of *CCK* isoforms in the GI tract and brain of Atlantic salmon, but these fish were sampled after 6 days of fasting, and according to Valen *et al.,* (2011) this is due to *CCK* acting within a shorter timeframe. In immature rainbow trout oral administration of *CCK* antagonists increases feed intake in immature rainbow trout (Gelineau and Boujard, 2001)

In mammals the satiation effect of CCK depends on signaling via the vagus nerve and the vagal afferent-dependent mechanisms may be involved in peripheral ghrelin-induced increase in food intake (Kobelt *et al.,* 2005). Furthermore Kobelt *et al.,* 2005 claim there could be an antagonistic interaction between ghrelin and CCK to regulate food intake. Kobelt *et al.,* 2005 found that ghrelin injected IP exerted an orexigenic effect in freely fed rats. However, in rats injected with both ghrelin and CCK feed intake was reduced. They conclude that the stimulation of food intake and neuronal activity in the ARC induced by ghrelin administered intraperitoneally is abolished by intraperitoneal CCK.

In this study *CCKL* and *CCKN* were downregulated in brain tissue of *rtGHRL* treated fish in Experiment one. All the fish in this study were sampled four hours post feeding and according to the mentioned studies one might expect to see an upregulation of *CCK,* however no changes in mRNA expression of *CCKL* and *CCKN* were found in control or treatment groups except for in the ghrelin treated fish which

also had elevated plasma ghrelin levels and upregulated expression *GHRL1A* and *GHRLIB* in stomach tissue. It is possible that there could be a similar antagonistic relationship between ghrelin and *CCK* as described in rats by Kobelt *et al.,* 2005. However the downregulation of *CCK* isoforms in brain, upregulation of *GHRL1A* and *GHRLIB* in stomach and elevated plasma ghrelin levels did not coincide with any short term change in feed intake in this experiment. The data found in this study indicates that plasma ghrelin could have an effect on postprandial changes in *CCK* expression at a central level, which in turn could influence feeding regulation as *CCK* is generally found to increase postprandially and have inhibitory effects on further food intake in fish, although changes in appetite were not found in ghrelin injected fish in this experiment.

5. Conclusion

In summary the present study reports that administered r*sLEPA1* causes a significant reduction in feed intake and SGR in Atlantic salmon. *POMC* isoforms seem to be involved in the leptin-signaling pathway as mRNA expression of *POMCA1* and *POMCA2* is only upregulated when a significant reduction in feed intake is also observed in *rsLEPA1* administered Atlantic salmon. Hepatic *LEPA1* and *LEPA2* mRNA expression was upregulated compared to control in *rsLEPA1* administered fish which also showed a reduction in feed intake and growth linking hepatic expression of *LEP* isoforms to metabolism. *PYY* mRNA expression was downregulated in brain tissue in fish with a significant reduction in feed intake and could be involved in the leptin-signaling pathway at a central level.

Significantly higher mRNA expression of *GHRL1A* and *GHRLIB* was found in the stomach of *rtGHRL*-injected fish compared to control, and mRNA expression was only upregulated when plasma ghrelin levels were found to be elevated. Rainbow trout *GHRL1* administered IP was not found to affect feed intake in Atlantic salmon in this study. *CCKL* and *CCKN* mRNA expression was downregulated in the brain tissue of *rtGHRL* treated fish which also showed elevated plasma ghrelin levels. D-3-hydroxybutyrate levels in plasma were elevated in *GHRL1* administered fish suggesting that ghrelin has a role in the regulation of ketosis in Atlantic salmon.

The method incorporating an individual based system and IP administration of hormones was validated. Low and steady glucose and lactate concentrations were found in both control and hormone administered fish, indicating that the fish were not stressed. Furthermore salmon started to eat within hours of being returned to the tanks.

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Appendix 1

Table 1 Feed (g) eaten per fish per day Experiment 1 pre and post injection

Day	Treatment	Control	Control	Control	Control	Control	Control	Leptin	Leptin	Leptin	Ghrelin	Ghrelin	Ghrelin	Ghrelin	Ghrelin
	Dose (mol)	Sham	Sham	Sham	Sham	Sham	Sham	0.4	0.24	0.16	0.4	0.32	0.24	0.16	0.08
	Tank No.	2	5	9	11	13	16	1	15	12	3	6	8	10	14
1		0.22	0.18	0.18	0.20	0.29	0.27	0.14	0.25	0.24	0.29	0.25	0.00	0.25	0.37
2		1.10	0.96	0.55	1.20	0.88	1.20	0.82	1.14	1.27	1.22	0.86	0.51	1.57	1.92
3		1.27	1.33	0.92	0.84	1.14	1.59	1.10	1.18	1.45	1.20	1.25	0.82	1.53	1.92
4		1.51	1.37	1.10	1.20	1.37	1.88	1.31	1.10	1.65	1.47	1.25	0.86	1.61	2.02
5		1.57	1.71	1.45	1.53	1.65	2.41	1.47	1.29	1.55	1.65	1.61	1.22	1.92	2.35
6		1.53	1.67	1.43	1.35	1.84	1.82	1.55	1.14	1.82	1.45	1.06	1.10	1.78	2.06
7		1.57	1.86	1.55	1.69	1.98	2.43	1.31	1.59	1.98	1.67	1.67	1.29	2.08	2.25
8	injection														
10		1.59	1.69	1.65	1.63	1.82	2.72	1.33	1.02	1.69	1.39	1.39	0.98	2.02	2.12
11		1.45	1.74	1.59	1.69	2.47	2.39	1.43	1.10	1.94	2.04	1.59	1.43	2.08	2.10
12		1.78	2.04	1.94	2.20	2.70	2.59	1.84	1.37	1.88	2.18	1.80	1.55	2.35	2.37
13	one meal	0.67	0.57	0.63	0.69	0.71	0.74	0.69	0.63	0.78	0.63	0.51	0.53	0.74	0.61

Table 2 Feed (g) eaten per fish per day Experiment 2 pre and post injection

Day	Treatment	Control	Control	Control	Control	Leptin	Leptin	Leptin	Leptin	Leptin	Leptin	Ghrelin	Ghrelin	ghrelin	Ghrelin	Ghrelin
	Dose (mol)	Sham	Sham	Sham	Sham	0.4	0.32	0.24	0.16	0.04	0.02	0.32	0.26	0.19	0.13	0.06
	Tank No.	9	10	12	16	3	1	14	4	2	13	7	8	15	6	5
1		0.45	0.25	0.35	0.37	0.29	0.24	0.55	0.49	0.31	0.25	0.49	0.02	0.31	0.27	0.22
2		1.29	0.76	0.94	1.43	1.16	0.98	1.43	1.78	0.88	1.06	1.57	0.63	0.86	0.94	0.67
3		1.65	0.88	1.18	1.67	1.55	1.20	1.67	2.27	1.33	1.37	1.72	0.80	1.23	1.25	1.49
4		1.63	1.16	1.47	1.72	1.53	1.29	1.63	2.51	1.14	1.59	1.82	0.84	1.55	1.31	1.57
5		1.78	1.69	1.29	1.37	1.72	1.29	1.96	2.80	1.63	1.65	2.00	1.10	1.84	1.23	1.74
6		1.47	1.12	1.86	1.65	1.59	1.08	1.98	2.57	1.37	1.16	1.94	0.92	1.51	1.00	1.67
7		1.88	1.29	1.92	2.08	1.94	0.98	2.12	2.27	1.47	1.63	2.27	0.96	1.98	1.59	1.90
8		2.08	1.59	2.06	2.14	2.21	1.37	2.37	2.94	2.00	1.65	2.69	1.39	2.08	1.80	2.67
9		1.65	1.43	1.71	2.06	2.00	1.33	2.02	2.47	1.76	1.51	2.45	1.18	2.39	1.61	2.18
10		2.08	1.08	2.33	2.31	2.14	1.61	2.39	2.76	1.86	2.12	2.47	1.22	2.31	1.69	2.57
11		2.20	1.45	2.53	2.41	2.21	1.71	2.63	3.23	1.88	2.10	3.27	1.23	2.84	1.65	2.94
12		2.18	1.43	2.14	2.23	2.53	1.78	2.74	2.31	1.96	2.06	2.80	1.27	2.53	1.69	2.69
13		2.18	1.37	2.06	2.59	2.41	1.88	2.45	2.94	1.92	2.43	3.14	1.31	2.78	1.76	2.39
14	injection															
15		2.02	1.55	1.45	2.08	2.14	1.49	1.76	2.20	1.31	1.59	2.78	0.98	2.65	1.45	2.25
16		1.94	1.45	1.45	1.90	1.69	1.16	1.84	1.67	1.65	1.53	3.08	0.80	3.04	1.37	2.06
17		2.55	1.67	1.96	2.61	2.29	1.57	2.25	2.27	1.86	1.16	4.00	1.14	3.72	1.67	2.37
18		2.57	1.84	2.06	2.65	2.49	1.84	2.35	2.82	2.49	1.90	3.49	1.47	3.98	2.06	2.88
19	one meal	1.14	0.04	0.33	1.18	1.00	0.98	1.25	1.37	1.18	0.86	1.33	0.39	1.63	0.78	1.22