MASTER THESIS

University of Bergen, Norway

Spatial and temporal mRNA expression of key gastrointestinal peptide hormones in Atlantic salmon during processing of a single meal- towards an understanding of their roles in regulation of digestion and appetite

For the fulfillment of Master of Science in Cellular- and Developmental Biology

By

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Abstract

Ghrelin (GRLN), Cholecystokinin (CCK) and the Neuropeptide Y (NPY) family member; PYY are gastrointestinal (GI) tract peptide hormones involved in the optimization of the digestive process and regulation of appetite in mammals. Although the functions of these hormones have been extensively studied in higher vertebrates, less is known for fish. The high diversity between fish species, suggest a species specific approach when studying the role of GI tract peptide hormones. Two isoformes of GRLN (GRLN-1, GRLN-2) and CCK (CCK-L, CCK-N), in addition to PYY were recently cloned and characterized in Atlantic salmon. However, no information is available on the postprandial involvement of these peptide hormones in Atlantic salmon GI tract. Therefore an experiment was initiated where the aim was to increase the knowledge of regulation of the digestive process and appetite by these key GI tract peptide hormones in Atlantic salmon.

This study describes the spatial distribution and postprandial changes in GRLN, CCK and PYY mRNA expression in the GI tract during the complete processing of a single meal in Atlantic salmon (average body weight: 44.7 ± 2.1 g). The mRNA expression values were obtained by quantitative PCR from four GI tract segments covering stomach, pyloric cecae, midgut and hindgut. In order to assess the GI tract transit, the content of feed and chyme in each GI tract segment, and gallbladder content were assessed. Unfed fish were used as control, and these fish were also used to assess potential diurnal patterns of GRLN-1, GRLN-2, CCK-L, CCK-N and PYY mRNA expression in Atlantic salmon.

With the exception of CCK-N which was not expressed in midgut and hindgut, the genes for all peptides were expressed in all GI tract segments analyzed in Atlantic salmon, although at different levels. Both CCK-L and CCK-N mRNA expression increased within 1.5 hours past feeding (hpf) when the stomach was still ca 80 % full, the gallbladder was nearly empty and chyme had started to be transferred into the midgut. The CCK isoforms had different spatial and temporal mRNA expression patterns during the 24 h processing of the meal. PYY mRNA expression increased at 1.5 hpf and then decreased after 3 hpf. No differences were detected between GRLN isoforms and both forms increased after processing of a single meal, indicating minor effect of GRLN on regulation of digestion. Taken together this suggests that CCK and PYY have in part similar roles in regulating digestive processes to that observed in higher vertebrates. The two isoforms of CCK appear to have different functions. The results did not identify a role for GRLN in regulating digestion and appetite comparable to mammals.

Table of contents

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1. Introduction

1.1 Background

Atlantic salmon (*Salmo salar*) is an important species in aquaculture worldwide. The global production of Atlantic salmon was estimated to 1 433 708 tons in 2007 and is expected to increase in the future (FAO 2007). It is thus of uttermost interest to fish farmers to optimize fish growth while at the same time maintaining aquaculture as a sustainable industry. Increased knowledge of factors underlying growth, digestion, digestion efficiency, control systems of digestion and the integrated aspect of appetite and energy homeostasis is needed to ensure continued production of high quality feed for farmed Atlantic salmon when there is a gradual reduction of available fish meal and alternative feed ingredients are becoming increasingly important.

1.2 Digestion and absorption

The main function of the digestive process is to hydrolyze macro nutrients from feed in such a way that it can be easily absorbed by enterocytes in the gut and used in catabolic and anabolic reactions in the body. The process of digestion is complex and finely orchestrated by hormones and neurons culminating in responses such as secretion, motility, absorption and evacuation. Factors affecting and modifying digestion includes feed composition, changes in habitat characteristics, fish demand of nutrients, and the interplay with additional functions of the organism (Krogdahl 2001). The gastrointestinal (GI) tract of fish is a multifunctional organ involved not only in digestion, but also in regulation of water and electrolyte balance, immunity and endocrine regulation of digestion and metabolism (Buddington et al. 1997). An optimal digestive function requires proper coordination between GI tract and associated organs such as liver, gallbladder and pancreas.

Food processing can be divided into three phases; a cephalic phase, a gastric phase and an intestinal phase (Silverthorn 2004). The cephalic phase is provoked by the thought, sight, smell, taste and chewing of food (Feldman and Richardson 1986). The central nervous system (CNS) and enteric nervous system (ENS) are both involved in the cephalic phase. Responses elicited are stimulation of secretion and motility in GI tract and accessory organs. The gastric phase involves feed entering the stomach. The stomach digests and stores feed, and regulate the transit of feed into intestine. During the gastric phase several enzymes, paracrines and

hormones are secreted in addition to hydrochloric acid (HCl), mucus and tissue protective bicarbonate. Digestion is carried out by both mechanical and chemical breakdown of feed creating chyme. The presence of feed in the stomach creates distension of stomach walls which activates neuronal and hormonal pathways involved in controlling gastric motility and evacuation. Finally, the intestinal phase of digestion involves digestion of chyme entering the small intestine. In the intestine these is additional secretions both from pancreas, and the gallbladder but also by cells in the GI tract.

The cephalic phase overlaps and interacts with the gastric and intestinal phase contributing to overall GI response to a meal (Katschinski 2000). A wide range of mediators are involved in initiating the different phases and responses such as regulating secretion, gastric evacuation and gut transit time. The ultimate step of digestion is absorption of nutrients and evacuation of undigested material, thus enabling maintenance of body functions and growth. The dynamic process of digestion consequently involves multiple steps tightly regulated by both hormones and the nervous system. It is thus important to gain increased knowledge of the dynamics of meal digestion in relation to GI tract peptide hormones. This knowledge may then be useful in understanding the effects of alternative feed sources on digestion and growth.

Feed composition has been shown to influence the efficiency of the different phases of digestion. As Atlantic salmon is a carnivorous fish species, the structure and function of the digestive system is optimized for processing dietary proteins while it has reduced capacity to digest carbohydrates (Hemre et al. 1995; Buddington et al. 1997). The feed composition may not only affect the digestive process itself and digestibility, but also the level of feed intake, and control of appetite.

1.3 Challenges of new feed ingredients

Animal-based protein sources used in production of fish feeds constitutes a major cost of fish farming and is also becoming increasingly scarce as a feed resource (Naylor et al. 2009). This has led to an increase in use of vegetable ingredients in feed as a substitute for fish meal. These plant based proteins sources have been shown to contain variable amounts of a range of bioactive compounds many of which cause problems to fish. Feeding soybean meal

to salmonids causes enteritis[1] in the distal intestine, characterized by shortening of mucosal folds, widening of lamina propria and infiltration of inflammatory cells (Baeverfjord and Krogdahl 1996). In addition to enteritis, reduced activity of digestive enzymes have been found in salmonids fed only minor replacements with soybean (Krogdahl et al. 2003). A reduced nutrient uptake by the gut caused by decreased carrier-mediated transport of amino acids has also been found as a consequence of feeding a diet with 30 % of fish meal protein replaced with soybean meal (Nordrum et al. 2000). This poorer utilization of vegetable protein has been shown to affect the immune system and ultimately growth in rainbow trout (*Oncorhynchus mykiss*) (Rumsey et al. 1994).

Reduction in protein utilization and digestion in fish may be caused by the presence of antinutritional factors in vegetable derived feed ingredients such as protease inhibitors, tannins and lectins (Francis et al. 2001). Studies in Atlantic salmon show that high levels of trypsin inhibitors derived from soybean reduce trypsin activity and digestibility of protein and fat, and ultimately weight gain (Olli et al. 1994). At low levels of trypsin inhibitor, pyloric cecae compensated for trypsin inhibition by producing elevated levels of trypsin (Olli et al. 1994). In addition to plant derived proteins, carbohydrates are also used in different ratios in fish feed. Carbohydrates are cheap feed ingredients which are used for their pellet-binding properties and as energy sources (Krogdahl et al. 2005). As a consequence excess amount of carbohydrates may be added to the feed, thus above the levels which efficiently can be utilized (Krogdahl et al. 2005).

Studies in Atlantic salmon explain reduction in growth partially by a reduction in feed intake when 80-95 % of fish meal is replaced with vegetable protein from wheat, gluten and extracted soybean meal (Espe et al. 2006; Torstensen et al. 2008). Plant based ingredients may thus inhibit fish growth when added to feed by reducing appetite of fish. It may also be possible that the effect of enteritis and enzyme inhibitors partly is mediated through GI tract peptide hormones involved in appetite regulation. Studies in farmed rainbow trout and farmed turbot (*Scophthalmus maximus*) show an increased number of cholecystokinin(CCK)-8 immunoreactive (IR) cells in fish possibly caused by enteritis due to parasite infection (Bosi et al. 2005; Bermúdez et al. 2007). GI tract peptide hormones are key signals linking the regulation of digestion to changes in appetite. A reduction in appetite may thus serve as a

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[1] Enteritis is characterized as inflammation of the intestine (Baeverfjord and Krogdahl 1996)

protective mechanism in response to negative effects of vegetable feed ingredients or parasites.

In contrast to protein and carbohydrates, the effect of substituting fish oil with vegetable oil in fish feed appear to have a somewhat less detrimental effect on fish growth in terms of weight and feed conversion ratio, however certain types of lipids may affect muscle composition (Bell et al. 2001; Torstensen et al. 2008; Bahuaud et al. 2009). Whether different sources of lipids in fish feed result in different responses in appetite, remains to be elucidated.

Little is known regarding the basic regulation of digestion and absorption of nutrients in Atlantic salmon. Knowledge of the regulation of digestion and absorption will aid in understanding the physiological and nutritional effects of new feed ingredients and aid in optimizing feed composition and feeding regimes in aquaculture. As the digestive process is associated with appetite, the regulation of these processes in response to meal processing should be studied in Atlantic salmon. GI tract peptide hormones have been shown to be mediators of this regulation in mammals and fish (Buddington and Krogdahl 2004; Chaudhri et al. 2006).

1.4 Hormonal regulation of digestion

The GI tract is the largest endocrine organ in the body and while the endocrine regulation of digestion has been extensively studied in mammals, there are only a few studies describing these endocrine control systems in fish (see reviews by; (Holst et al. 1996; Buddington and Krogdahl 2004)). It has been suggested that the evolutionary signaling pathways and molecules involved are conserved among vertebrates, but some available data suggest that there are specific differences in fish (Buddington and Krogdahl 2004). These authors classify endocrine signaling as three operational networks based on distance of communication; including local paracrine, intra-GI tract and intra organ signaling networks. The digestive process involves the GI tract and accessory organs which collectively combine all of these signaling networks. An example of intra-GI tract signaling is the role of Cholecystokinin (CCK) in digestion. When hydrolyzed feed stuff enters the proximal intestine in mammals, CCK producing "I" cells in the intestine is stimulated and secretes CCK into the circulation (Liddle 1997). CCK then binds to receptors on the gallbladder and pancreas to induce secretion of bile and pancreatic enzymes respectively (Liddle 1997).

There are various types of GI tract signaling molecules, including hormones, neurotransmitters and neuromodulators. Most of the signaling molecules are peptides. Many of the regulatory peptide hormones in the GI tract are also involved in the regulation of feed intake and appetite control. The close interaction between endocrine and nervous regulation of GI tract is confounded by the sharing of similar signaling molecules. Gut hormones influence the rate at which feed is transferred to the different compartments of the GI tract, and also the level of appetite. Thus, the gut hormones play a dual role in regulation of digestion and nutrient uptake, both by optimizing the gut itself for nutrient processing, and by controlling the intake of nutrients. Both CNS classical neural reflexes and ENS short reflexes regulate GI function (Clements and Raubenheimer 2006). Motility and secretion can thus be altered by both hormones and the nervous system. The effect observed include variations in gastric emptying, gall bladder contraction and absorption.

The gut-brain axis provides a higher level of optimizing digestion and absorption, where gut hormones can alter food intake by providing a signal from the gut to brain centers involved in regulation of feed intake (Chaudhri et al. 2006). The appetite regulating hormones described in fish have been reviewed by several authors (Lin et al. 2000b; Volkoff et al. 2005; Volkoff 2006). The GI tract peptide hormones Ghrelin, CCK and neuropeptide Y (NPY) family of peptides have all been shown to be involved in the regulation of appetite as these peptides undergo periprandial variation in mRNA expression and plasma levels (Peyon et al. 1999; Narnaware and Peter 2001; Unniappan et al. 2004). Ghrelin (GRLN), CCK and PYY may thus regulate both the digestive process and appetite in Atlantic salmon.

1.4.1 GRLN

GRLN is an acylated peptide initially discovered in rat stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al. 1999). The name is derived from "ghre" meaning "grow" in Proto-Indo-European languages (Kojima et al. 1999). Although GRLN was initially shown to regulate growth hormone release from the pituitary, later studies showed that GRLN is a multifunctional peptide hormone involved in energy homeostasis in mammals (Tschop et al. 2000; Wren et al. 2000; Nakazato et al. 2001). GRLNs ranging from 12- to 23- amino acids have been identified in goldfish (*Carassius auratus*) (Unniappan et al. 2002), rainbow trout (Kaiya et al. 2003a), Japanese eel (*Anguilla japonica*) (Kaiya et al. 2003b), Mozambique tilapia (*Oreochromis mossambicus*) (Kaiya et al.

2003c), Nile tilapia (*Oreochromis niloticus*) (Parhar et al. 2003), channel catfish (*Ictalurus punctatus*) (Kaiya et al. 2005), seabream (*Acanthopagrus schlegeli*) (Yeung et al. 2006), European seabass (*Dicentrarchus labrax*) (Terova et al. 2008), zebrafish (*Danio rerio*) (Amole and Unniappan 2009) and Atlantic salmon (Murashita et al. 2009). While extensive studies in humans and other mammals have described multiple roles of GRLN, the role of GRLN in fish is less certain (Unniappan et al. 2004; Jönsson et al. 2007).

In rats and humans, the mature 28-amino acid GRLN peptide is produced by cleavage of a 117 amino acid precursor (Kojima et al. 1999; Kojima et al. 2001). In fish, species to species variation exists in the length of the precursor protein, but all teleosts have an amide structure on the C-terminus of the mature peptide, (reviewed by; (Kaiya et al. 2008)). A unique feature of GRLN is its post translational modification in which Ser-3 is covalently linked to octanoic acid. This acyl modification is required for binding to its receptor, GHS-R1a (Kojima et al. 1999) and thus for exhibiting hormonal effects. GRLN lacking acyl modification; des-acyl GRLN, has been identified in rats (Hosoda et al. 2000) and goldfish (Matsuda et al. 2006a). In goldfish des-acyl GRLN can inhibit GRLN-induced food intake (Matsuda et al. 2006a). GRLN modified by decanoic acid is the major form in Mozambique tilapia (Kaiya et al. 2003c). The "active core" of the peptide in mammals consists of the first four N-terminal amino acids including the acyl modification (Bednarek et al. 2000). This is required for GHS-R1a binding. Comparison between the N-terminal seven amino acids display high sequence homology among non-mammalian vertebrates such as; reptiles, amphibians, birds and teleost species (Kaiya et al. 2008).

In the non-mammalian vertebrates mentioned above the principal site of GRLN production was stomach and GI tract, but with species specific differences in tissue distribution (Kaiya et al. 2008). Recently Murashita et al. (2009) cloned two GRLN isoforms[2], GRLN-1 (Gene bank acc. no AB443431) and GRLN-2 (Gene bank acc. no AB443432) in Atlantic salmon. Both isoforms exhibit highest expression in stomach tissue, with decreasing expression levels in pyloric cecae and midgut, adipose tissue and testis. In addition to GI tract, GRLN mRNA is expressed in CNS in the hypothalamic arcuate nucleus and in pituitary of rats and human, though in lesser extent than the GI tract (Korbonits et al. 2001; Lu et al. 2002;

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[2] Murashita et al. (2009) suggest that GRLN-1 and GRLN-2 are alternatively spliced variants of the same gene. However, it is uncertain whether CCK-L and CCK-N are separate genes or alternatively spliced mRNA. Even though, the term isoforms will be used for both CCKs and GRLNs in this thesis.

Mondal et al. 2005). RT-PCR have also detected GRLN mRNA expression in fish brain for goldfish (Unniappan et al. 2002), rainbow trout (Kaiya et al. 2003a), Mozambique tilapia (Kaiya et al. 2003c), Japanese eel (Kaiya et al. 2003b) and hammerhead shark (Kawakoshi et al. 2007).

In mammals, the gastric fundus is the principal site of GRLN synthesis (Kojima et al. 1999; Ariyasu et al. 2001; Kojima et al. 2001; Taniaka-Shintani and Watanabe 2005), but GRLN can also be found in various other GI tract tissues (Gnanapavan et al. 2002). GRLN is produced from X/A like enteroendocrine cells in mucosal epithelium of the stomach, which recently have been renamed into "ghrelin cells" (Date et al. 2000; Cummings 2006). GRLN has been shown to promote gastric and gut motility in mammals (Peeters 2005). GRLN is further secreted into the bloodstream and circulates in the body (Kojima et al. 2001). Immunological studies in rats and human found GRLN IR cells in duodenum with a gradual decrease in distal intestines from ileum to colon (Date et al. 2000). For a schematic presentation of GRLN and its main known effects in mammals, see Figure 1.1.

GRLN is the endogenous ligand of GHS-R1a receptor and stimulates GH release (Kojima et al. 1999). Studies show that GRLN acts directly on the pituitary gland to induce GH release in a specific, dose dependent manner in both human and rats (Arvat et al. 2000; Seoane et al. 2000; Takaya et al. 2000). GRLN also stimulates GH release in several fish species. *In vitro* studies on Mozambique tilapia organ cultured pituitary cells show that GRLN stimulates release of both GH and prolactin in this species (Kaiya et al. 2003c). Though later studies failed to reproduce the effect of GRLN induced prolactin release in this species (Fox et al. 2007). In addition to studies in tilapia, reports on GRLN induced GH release exist for; seabream (Chan and Cheng 2004), goldfish (Unniappan and Peter 2004), orange spotted grouper (*Epinephelus coioides*) (Ran et al. 2004) and rainbow trout (Kaiya et al. 2003a).

GRLN has also been demonstrated to have a regulatory function on appetite in mammals. It is the only orexigenic GI peptide isolated to date, and stimulates food intake in mammals (Tschop et al. 2000; Wren et al. 2000; Nakazato et al. 2001). Whether the appetite regulatory mechanism is the same for non-mammals is uncertain. Both ICV and IP injection of GRLN in goldfish, stimulate food intake (Unniappan et al. 2002; Matsuda et al. 2006a). The effect of GRLN has been studied in rainbow trout by measuring periprandial changes in GRLN plasma levels and IP injection of GRLN (Jönsson et al. 2007). The result of this study

showed no postprandial changes in plasma GRLN levels and no effect on appetite, thus concluding no effect of GRLN on appetite regulation in this species. These opposing results suggest a species difference in GRLN function. GRLN may have additional roles in fish including reproduction, GI motility, water intake, immune function and behavior (Mustonen et al. 2002; Kozaka et al. 2003; Matsuda et al. 2006b; Yada et al. 2006; Olsson et al. 2008). The role of GRLN in Atlantic salmon is uncertain as very limited information exists for this species. The changes of GRLN in response to a meal should thus be studied in order to increase the understanding of the regulation of appetite and digestion, and thus verify whether GRLN has a potential orexigenic role in Atlantic salmon, similar to mammals.

1.4.2 CCK

Cholecystokinin (CCK) was originally identified by Ivy and Oldberg (1928). The name refers to "that which excite or moves the gallbladder which was consistent with the reported ability to induce gallbladder contraction (Ivy and Oldberg 1928). Since then numerous studies in mammals have demonstrated additional CCK regulating effects on digestion like stimulation of pancreatic enzyme secretion, inhibition of gastric acid production, regulation of gastric emptying (delaying), gut motility (peristalsis) and control of appetite (satiety) (Figure 1.1) (Liddle 1997).

In mammals CCK is secreted by enteroendocrine "I" cells located in the mucosa of the proximal intestine, and in neurons in the myenteric plexuses and brain (Chandra and Liddle 2007). CCK secreting cells have also been identified in proximal GI tract of herring (Kamisaka et al. 2005). The I cells have a triangular shape with their apical surface facing the intestinal lumen, and the secretory granules with CCK located towards the basolateral base (Buchan et al. 1978). CCK is secreted into the blood in response to a meal and binds to CCK-1 or CCK-2 receptors on target organs, where cellular signal transduction pathways are initiated (Chandra and Liddle 2007).

CCK is also well known as a potent satiety signal in mammals (Moran and Kinzig 2004). CCK has been identified in a number of teleost species; cod (*Gadus morhua*) (Jonsson et al. 1987), goldfish (Himick and Peter 1994), rainbow trout (Jensen et al. 2001), spotted green puffer (*Tetraodon nigroviridis*) and Japanese flounder (*Paralichtys olivaceus*) (Kurokawa et al. 2003), Atlantic herring (*Clupea harengus*) (Kamisaka et al. 2005) and Atlantic salmon (Murashita et al. 2009). In fish as in mammals, CCK has been shown to

regulate digestive function, although less knowledge exist on the endocrine effect of CCK on controlling feed intake.

By measuring gallbladder pressure, Aldman and Holmgren (1995) suggested a mechanism for CCK in gallbladder contraction in rainbow trout. In this study it was for the first time demonstrated that luminal fat and amino acids entering the proximal intestine induced gallbladder contraction by stimulating CCK release (Aldman and Holmgren 1995). Subsequent infusion of sulphated CCK octapetide mimicked this effect, which lead to the hypothesis that fat and amino acids in the proximal intestine stimulate CCK to induce gallbladder contraction. In addition to this effect, CCK is also suggested to have a role in the delaying of stomach emptying in the same species (Olsson et al. 1999). In Atlantic salmon, infusion of porcine CCK induced pancreatic cell secretion of trypsin and chymotrypsin *in vitro*. The same CCK also induced gallbladder discharge in a dose dependent manner *in vivo* (Einarsson et al. 1997).

In goldfish, CCK immunoreactivity was found in gut and brain and both centrally and peripheral injection of sulfated CCK-8 suppressed food intake (Himick and Peter 1994). High levels of CCK IR neurons and mRNA were found in the hypothalamic inferior lobe and posterior ventrolateral regions, suggesting that CCK also have a role as a neuroendocrine (and neurotransmitter) satiety signal in fish (Himick and Peter 1994; Peyon et al. 1998; Peyon et al. 1999). Peyon et al. (1999) further demonstrated that CCK mRNA increased 2 hours after a meal (postprandial) in goldfish brain. The mechanism CCK induces satiety in fish may be both direct by CCK acting as a neurotransmitter in the brain, or indirect through the gut by CCK delaying gastric emptying and thus maintaining stomach distension which further activates vagal afferents and induces satiety, as seen in rainbow trout (Olsson et al. 1999). In both ways vagal pathways are involved, though there might exist non-vagal pathways of hormonal relay systems (Bail and Ruf 1997). One such "relay system" may involve leptin, which in goldfish potentiates the satiety actions of CCK in hypothalamus and further blocking of CCK receptors in the brain attenuates leptin mediated inhibition of NPY and orexin (Volkoff et al. 2003b).

Studies on rainbow trout demonstrated for the first time the identification of three different CCK cDNAs in the same species, with the mRNA encoding three CCK preprohormones (Jensen et al. 2001). CCK-8 is well conserved in vertebrates within the

amino acid sequence; DYMGWMDF (·NH$_2$) with methionine as the sixth amino acid from the carboxy terminal. Amidation of the carboxy terminal is implicated in receptor binding and thus biological activity (Johnsen 1998). The three different CCK preprohormones identified in rainbow trout, is suggested based on processing signals to give rise to CCK-N (Aspargine), CCK-L (Leucine) and CCK-T (Threonine) named according to amino acid variations in C-terminal position six (Methionine) (Jensen et al. 2001). The three different CCK mRNA showed differences in tissue distribution. All CCK forms were expressed in brain and intestine. CCK-N showed highest expression in brain and pyloric cecae, while CCK-L was expressed more evenly throughout the gut including stomach, small intestine and large intestine, altogether potentially suggesting differences in biological functions.

Recently, Murashita et al (2009) cloned two isoforms of CCK in Atlantic salmon based on rainbow trout CCK sequences; CCK-L (GenBank acc.# AB443433) and CCK-N (GenBank acc.# AB443434) encoding the precursor peptides prepro-CCK-L and prepro-CCK-N, respectively (Murashita et al. 2009). When compared within a phylogenetic tree, salmonids CCKs separate as CCK-1 and CCK-2, with rainbow trout/Atlantic salmon CCK-N and rainbow trout CCK-T falling into CCK-1 group, and rainbow trout/Atlantic salmon CCK-L in fish CCK-2 group (Murashita et al. 2009). Both CCK-L and CCK-N were mainly expressed in Atlantic salmon brain tissue with lower expression in eye (with CCK-L expressing the highest level), pyloric cecae and midgut among others (Murashita et al. 2009)

Recent research has shown an additional role for CCK in mediating a protective effect against inflammatory responses in rats fed a high-fat diet (Luyer et al. 2005). Whether this effect is the same in fish remains to be elucidated. It is thus important to study the effect of CCK in regulating the digestive process and appetite in Atlantic salmon, and also the potential involvement in GI tract inflammatory responses. This knowledge may further be useful in understanding the growth inhibitory effect of increased inclusions of vegetable derived nutrients in fish feed.

1.4.3 <u>PYY</u>

PYY is a GI peptide hormone originally discovered in porcine intestine (Tatemoto and Mutt 1980). In mammals PYY is synthesized and secreted from intestinal "L" cells located in the distal intestinal mucosa, with the apical membrane facing towards the lumen sensing its content (Ueno et al. 2008) similar to the CCK "I" cells. In humans PYY release is particularly

high when a high-protein meal is ingested (Batterham et al. 2006). PYY immunoreactivity has also been located in neuronal cells in the stomach and proximal intestine (Bottcher et al. 1993). In response to luminal lipids, PYY mediates inhibition of intestinal motility partly by the "ileal brake mechanism" where lipids in the distal intestine inhibit motor activity in the proximal intestine (Spiller et al. 1984). PYY further inhibits gastric acid secretion and the cephalic phase of gallbladder secretion in mammals (Guo et al. 1987; Hoentjen et al. 2001). In mammals PYY also inhibits both pancreatic secretion stimulated by secretin and CCK and pancreatic secretion stimulated by the vagus nerve and luminal nutrients (Tatemoto 1982; Lluis et al. 1987). Thus, PYY act as an endogenous antagonist to CCK's stimulation of intestinal activity and gallbladder and pancreatic secretion (Figure 1.1). The role of PYY as a vasoconstrictor regulating blood flow to splanchnic vasculature may partly mediate these inhibitory effects (Adrian et al. 1986; Ueno et al. 2008). The hormone has been shown to have additional regulatory roles in mammals including regulation of feeding behavior and body weight (see Ueno et al. (2008) for review).

PYY belongs to the NPY family of peptides which includes; NPY, PYY, PP and PY where most are 36 amino acid long peptides including three proline and two tyrosine residues constructing the so-called PP-fold (Larhammar 1996). The NPY peptides mediate their effects through Y-receptors (Y1, Y2, Y4; Y5, Y6) which differ in their distribution and affinity for PYY (Ueno et al. 2008). The enzyme dipetidyl peptidase IV cleaves the N-terminal tyrosine and proline residues generating Y2 receptor specific PYY_{3-36} which is the major form in circulation as well as gut endocrine cells (Mentlein et al. 1993; Grandt et al. 1994). Y2 receptors can be found in the hypothalamus (including ARC), intestine and on vagal afferent nerves in mammals (Koda et al. 2005). In mammals, the role of PYY as a satiety factor is believed to be mediated through Y2 receptors on vagal afferents signaling to hypothalamic ARC via NTS in the brainstem (Ueno et al. 2008).

In fish, PYY sequences have been detected in lamprey (Soderberg et al. 1994), zebrafish (Larhammar et al. 1995), seabass (Cerda-Reverter et al. 2000), Japanese flounder (Kurokawa and Suzuki 2002), pufferfish, three-spined stickleback, medaka and torafagu (Sundstrom et al. 2008). Much of the research on PYY in fish has focused on the evolution of the neuropeptide Y family of peptides (Larhammar 1996; Sundstrom et al. 2008). Studies indicate that NPY and PYY evolved from a gene duplication event from a common ancestral gene, and a second duplication of PYY gave rise to PY (renamed PYYb) (Sundstrom et al.

2008). There appear to be a difference in distribution of PYY in fish compared to mammals, with fish PYY being more widespread in the nervous system and endocrine cells (Cerda-Reverter and Larhammar 2000; Soderberg et al. 2000; Kurokawa and Suzuki 2002). Recently Murashita et al. (2009) cloned the PYY gene in Atlantic salmon. Atlantic salmon PYY were found to be highly expressed in midgut, pyloric cecae and brain, with lower expression in eyes, stomach, adipose tissue and gonads (Murashita et al. 2009). In fish, PYY may thus be expressed in more proximal parts of the digestive system in contrast to mammals. The role of PYY in fish is unknown.
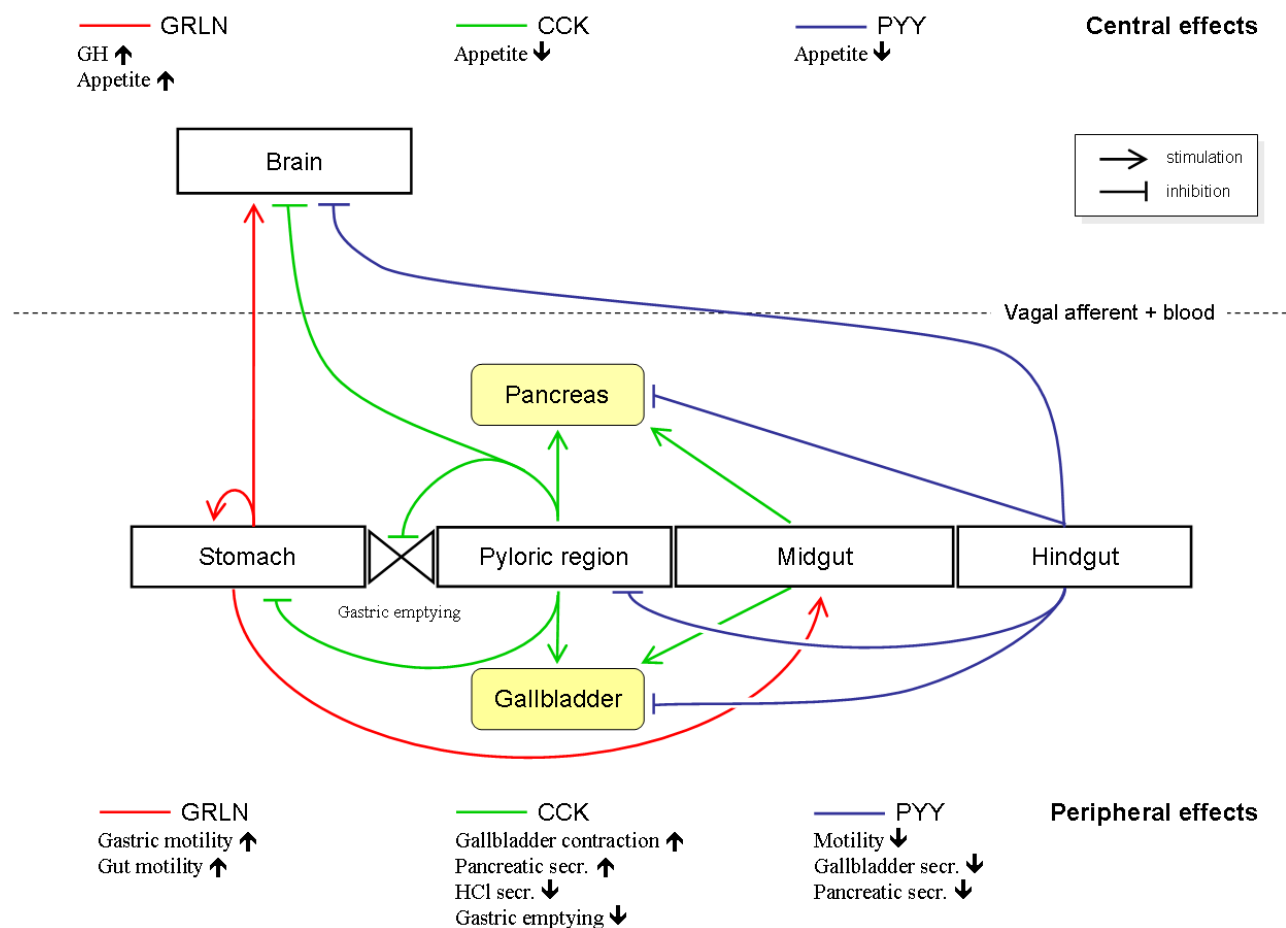
Figure 1.1. Central and peripheral effects of key gastrointestinal (GI) tract peptide hormones in mammals. *See next page for legend (page 19).*

Figure 1.1. Central and peripheral effects of key gastrointestinal (GI) tract peptide hormones in mammals. *The figure shows a schematic overview of the brain and GI tract compartments covering; stomach, pyloric region, midgut and hindgut. The area between the stomach and pyloric region illustrates the pyloric sphincter, while the pyloric region includes the proximal intestine. The origin of the lines indicates known site of peptide hormone secreting cells, while the end of the line indicates a stimulatory or an inhibitory effect on the target. A line originating and ending from/at the same target indicates a local, paracrine effect. The effect of Ghrelin (GRLN), cholecystokinin (CCK) and peptide-YY (PYY) are thought to be mediated both through the circulation and by activation of the nervous system, where they ultimately bind to receptors on their target. The dotted line illustrates GI tract peptide hormones mediating a signal from GI tract to brain areas. The central effects of each peptide hormone are listed below each peptide hormone on the top of the figure, while the peripheral effect is listed on the bottom of the figure. Arrows pointing upwards next to a described effect indicate a stimulatory effect, while arrows pointing downwards indicate inhibitory effect. GH: Growth hormone, HCl: Hydrochloric acid, Secr: Secretion. The scheme is compiled from available literature (see text for details)*

1.5 Summary

In summary, studies in fish indicate that GRLN, CCK and PYY are involved in the optimization of the digestive process and may also affect appetite (Figure 1.1). The dynamic process of digestion includes several phases initiated by higher levels in brain areas and the presence of feed in the GI tract, and the integrated response between digestion and appetite. The GI tract peptide hormones provide a finely tuned network of signaling molecules which mediates information from the gut and brain. The effects initiated by GI tract hormones in mammals involve regulation of gastric evacuation, gut transit of chyme, gallbladder contraction, pancreatic secretion and appetite (feed intake) (Figure 1.1).

In fish, less is known about the regulatory role of GI tract hormones. The large differences in environmental conditions and feed sources between mammals and fish could potentially result in a different response to luminal factors, hence alternative modes of digestive regulation. As fish constitutes a highly diverse group of species, species specific differences in regulation of digestion may exist. Species specific differences may include alternative isoforms, differential spatial distribution in the GI tract and different functions of the same GI tract peptide hormones. Consequently, when studying the role of GI tract peptide hormones in fish, a species specific approach needs to be chosen.

GRLN, CCK and PYY were recently cloned and characterized in Atlantic salmon (Murashita et al. 2009). However, no information was available on the short term postprandial effects on mRNA expression of these peptide hormones in the GI tract. Therefore an experiment was initiated with the aim of increasing the knowledge of postprandial regulation of the digestive process and appetite by GI tract peptide hormones in Atlantic salmon. Very limited information exists concerning changes in GRLN, CCK and PYY in relation to meal processing in Atlantic salmon, and studies on these genes in relation to meal digestion have only recently been described in goldfish and tilapia (Unniappan et al. 2004; Fox et al. 2009).

1.6 Hypothesis and aim of thesis

The hypothesis is that GRLN-1, GRLN-2, PYY, CCK-L and CCK-N are involved in the regulation of digestion and appetite in Atlantic salmon similar to mammals. If this is the case, these peptide hormones are expected to change in mRNA expression according to the dynamic process of digestion and appetite including stomach filling and evacuation, release of bile and pancreatic secretion, and the transit of feed through the gut. The regional distribution pattern of each peptide hormone is expected to reflect its role in the GI tract. If GRLN-1, GRLN-2, PYY, CCK-L and CCK-N are involved in regulation of digestion and appetite, potential postprandial changes in gene expression is expected to occur at specific time points related to the phase of digestion.

In order to test this hypothesis an experiment was conducted were Atlantic salmon were fed a single meal and the gene expression was analyzed during the postprandial processing until the GI tract was empty. Unfed fish were used as control and to determine potential diurnal patterns in mRNA expression. The main aim of the experiment was to:

1) Describe the regional tissue distribution of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N mRNA in stomach, pyloric cecae, midgut and hindgut.
2) Describe the processing of a single meal by looking at; changes in stomach filling and emptying, gut transit assessed as filling of pyloric cecae, midgut and hindgut.
3) Describe the release of bile and subsequent refilling of the gallbladder during processing of a meal.
4) Describe the regulation of meal digestion by analyzing postprandial changes in mRNA expression of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N during complete digestion of a meal.

5) Describe the potential involvement of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N in regulation of appetite by analyzing postprandial changes in mRNA expression during complete digestion of a meal.

6) Describe the diurnal variation in GRLN-1, GRLN-2, PYY, CCK-L and CCK-N by analyzing mRNA expression of these peptide hormones in unfed Atlantic salmon.

In this thesis GRLN, CCK and NPY are discussed as peptide hormones and thus refer to a group of peptides with described roles of hormones. GRLN, CCK and PYY have all been shown to act both in an endocrine and in a local paracrine manner. We therefore emphasize that we are not excluding additional modes of action.

2. Material and methods

2.1 Animals and samples

Atlantic salmon average body weight 44.7 ± 2.1 g, were reared at the Bergen High-Technology centre in indoor tanks supplied with a continuous flow of fresh water at 8° C and with a light regime of 12:12. The fish were fed a commercial pellet diet (EWOS, Bergen, Norway); see Appendix IV (Table AIV1). Two weeks prior to the postprandial sampling fish were divided in to two tanks, and hand fed *ad libitum* at 0900 every morning (7 days/week). At the first day of sampling, 6 fish in the first tank (unfed group) were sampled at each selected time point (0, 0.5, 1.5, 3, 6, 9, 12 and 24 hours), whereas fish in the second tank received food (fed group) as usual. Before sampling the second day, fish were fed as described above.

At sampling fish were anaesthetized using 50 mg/l MS-222 and killed with a blow to the head. The weight and length of each fish was recorded before the whole GI tract was removed. All dissecting work was done using dry-ice. The GI tract was selected for q-PCR, and the whole gallbladder and GI tract content were selected for weight assessment. The GI tract was divided into four segments as indicated in Figure 2.1, consisting of stomach, pyloric cecae, midgut and hindgut. The intestine was emptied of any leftover food and chyme by gently striking the content out in to pre-weighed aluminium foils. The stomach, midgut and hindgut segments were then each divided into three segments. Segments were then flashfrozen using liquid nitrogen, and stored at –80 ℃ until further use. The gall bladder was collected in pre-weighed eppendorf tubes.
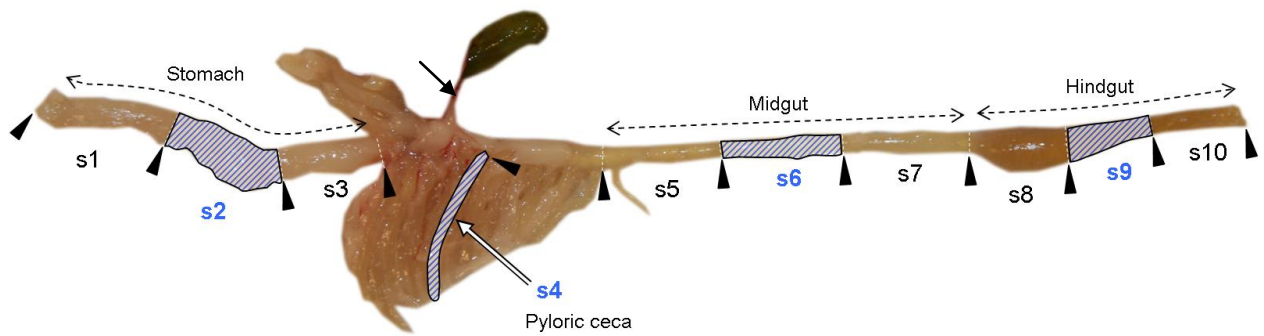
Figure 2.1. The Atlantic salmon gastrointestinal (GI) tract. *The picture shows an unfolded GI tract representative for Atlantic salmon sampled in this experiment (average weight 44.7 ± 2.1 g). During sampling, the GI tract were first cut into four main segments consisting of stomach, pyloric cecae region, midgut and hindgut segments indicated by the dotted line and the area between. The triangles below the end of each dotted line indicate cutting points. Food and chyme were emptied from stomach, pyloric cecae intestinal region, midgut and hindgut segments. Each segment excluding pyloric cecae region were then cut into three segments indicated by the arrows. Pyloric cecae used in gene expression analyses were cut at the indicated arrow at the base of the shaded area. In the current study S2, S4, S6 and S9 (indicated by the shaded areas and blue letters) were analyzed by q-PCR as these are representative for their respective GI regions. The gallbladder was cut off from the intestine and weighed with content (cutting point indicated by an arrow).*

2.2 RNA isolation

Total RNA was isolated according to (Chomczynski 1993) from Atlantic salmon GI tract segments including; stomach (S2), pyloric cecae (S4), midgut (S6) and hindgut (S9), using TRI Reagent® (Sigma, USA) for phenol-chloroform extraction.

Tissue samples were brought directly from -80 °C freezer and cut on dry ice to avoid thawing, then weighed. Each sample was kept in the weight range of 40-45 mg ± 10%. The tissue samples were quickly transferred to 2 ml FastPrep homogenization vials for tissue disruption, each vial containing 1 mL cold TRI Reagent® and kept on ice for at least 5 min. Tissue homogenization was executed using FastPrep™ FP120, BIO101 ThermoSavant (Q-BIOgene, USA), with the following settings: speed 4 for 20 seconds. The samples were then kept at room temperature (RT), for 5 minutes. Two hundred µl Chlorofrom (Sigma, USA) was added and samples thoroughly vortexed for 1 minute until appearance of a milky-white color. To

achieve phase separation, the samples were centrifuged for 15 minutes, 12000 x g on 4 °C (Eppendorf Centrifuge, 5415R, Heracus Instruments, Germany). The upper phase clear supernatant (approximately 360 µl), was transferred to a clean 1.5 mL microcentrifuge tube (Axygen, USA) leaving a small volume left to avoid DNA and protein residues. Five hundred µl of Isopropanol (Sigma) was added to the supernatant, sample inverted five times for mixing then left for precipitation of RNA for 10 minutes at RT. RNA was precipitated by centrifugation for 15 minutes, 12000 x g and 4 °C. To ensure a sample with pure RNA, the pellet was washed with 500 ul 80 % Ethanol (Arcus, Norway). The samples were centrifuged for 5 minutes, 7600 x g on 4 °C and the supernatant wash removed. Since ethanol may disturb further enzymatic analysis, all traces of ethanol were removed thoroughly. The samples were subsequently air dried for 5-10 minutes and reconstituted in 100 µl Nuclease-Free Water (Ambion, USA). Samples were placed on a heating block (Techne DRI-BLOCK, DB-3D, Techne, USA) for 5 min at 55 °C, with casual flicking of the vials to ensure a completely dissolved pellet.

2.3 RNA concentration and quality

RNA concentration was measured using NanoDrop ND-1000 absorbance technology (Thermo Scientific, USA), where 1.5 µl of total RNA was added to NanoDrop ND-1000 and quantitative/qualitative measurements were recorded. The 260/280 and 260/230 absorbance ratio were used as indicators of sample quality in terms of sample pureness. Concentration measurements for all samples were used as basis for calculations in downstream enzymatic reactions.

2.4 RNA integrity

RNA integrity was tested by the use of a RIN algorithm ('RNA Integrity Number' (RIN)) on the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) in conjunction with RNA 6000 Nano and RNA 6000 Pico LabChip kit (Agilent Technologies) to test sample RNA integrity. The procedure was followed according to manufacturer's guidelines.

2.5 DNase treatment of RNA

To remove traces of genomic DNA, TURBO DNA-*free* Kit™ (Ambion) was used on total isolated RNA, following manufacturer's instructions for Routine DNase treatment, with one

modification: instead of using 10 µg of total RNA into reaction, 12 µg was used. One sample was split into two parallels in DNase treatment. To minimize loss of RNA during precipitation and storage these samples were pooled and co-precipitated[3].

2.6 cDNA synthesis

cDNA was synthesized using Superscript III First-Strand Synthesis System (Invitrogen, USA) on 5 µg total RNA template. Oligo(dT) (Sigma, USA) was chosen as primer as it maximizes the number of mRNA molecules available for analysis in a small sample of RNA. Each individual sample was split into two parallels and kept as parallels during q-PCR. All reagents used were provided with the Superscript III kit. Eight µl of RNA sample and 1 µl of dNTPs and 1 µl of oligo(dT) was heat denatured for 5 min at 65 °C in Gene Amp PCR 2700 machine (ABI, USA) to limit secondary structures, then chilled on ice for at least 1 min for the primer to anneal properly. A mastermix containing RT buffer, $MgCl_2$, DTT, and SuperScript™ III RT was made to a total volume of 10 µl and added to the RNA/primer mixture. Samples were then heated at 50 °C for 50 min which is the optimum temperature for SuperScript™ III RT, 85 °C for 5 min for reaction termination, then chilled on ice. To remove RNA in the sample, one µl RNase H was added to each tube an incubated for 20 min at 37 °C. The cDNA reactions were stored in -20 °C freezer. Two negative controls were made by using RNA sample leftovers from all samples during each run, one excluding the SuperScript™ III RT, and one excluding the oligo(dT) primer. All reactions were run on Gene Amp PCR 2700 machine (ABI, USA).

2.7 Real-time quantitative PCR: quantification of gene expression

Real-time quantitative PCR (q-PCR) was used to measure mRNA expression in Atlantic salmon stomach, pyloric cecae, midgut and hindgut. Q-PCR amplification and analysis were performed using Chromo4 Continuous Fluorescence Detector (Bio-Rad) and MJ Opticom Monitor Analysis software (Version 3.1, Bio-Rad). For GRLN-1, GRLN-2 and Atlantic salmon elongation factor 1α (EF1α) 100 ng cDNA was used. Atlantic salmon EF1α was used as an internal standard reference gene. For PYY, CCK-N and CCK-L 500 ng cDNA was used.

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[3] All samples were precipitated with 1/10 of total sample volume with 3 M Sodium Acetate (pH 5.5) (Ambion) and 2.5 sample volume with 80 % Ethanol, then kept in -80 °C freezer for storage.

The quantities selected were within the range of the standard curve for the respective gene. Total q-PCR reaction volume of 25 µl was prepared using; 2.5 µl diluted cDNA, 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.75 µl forward primer (10 µM), 0.75 µl reverse primer (10 µM) and 8.5 µl Nuclease-Free Water (Ambion). See Table 2.1 for Genebank accession number and primer sequences. The enzymatic amplification reactions were carried out in 96-Well Skirted PCR plates (Bio-Rad) with thermal cycling protocol: A first degeneration at 94 °C for 3 min then 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. A melting curve was performed for each assay to verify the absence of primer dimers (60-95 °C, read every 0.2 °C and hold for 1 sec).

Each sample was run in duplicate reactions kept from the cDNA synthesis. Standards were also run in duplicates. Controls included in each plate were; non template negative control, a between plate control and minus RT. Cycle threshold was set manually to 0.010 which was within the exponential phase and above background noise for all assays. Q-PCR data were analyzed in RJ Opticom 3.2.32 (Bio-Rad) software and exported to Excel® and Statistica 8.0 (StatSoft, Inc., USA) for further analysis.

Table 2.1: *Primer sequences used in q-PCR*

Gene	Genebank Acc. No.	Primer name	Sequence (5'-3')
GRLN-1	AB443431	Ghr Fw3	CCAGAAACCACAGGTAAGACAGGGTA
		Ghr Rv3	CTCCTGAAACTCCTCCTCACTCATGG
GRLN-2	AB443432	Ghr Fw4	GCCCCTCCCAGAAACCACAGGGTAAA
		Ghr Rv3	CTCCTGAAACTCCTCCTCACTCATGG
PYY	AB443435	PYY Fw5	ACTACACCGCGCTCAGACACTACATC
		PYY Rv5	TCTCTGGTCTCTCTCTGCATTGTTGCCG
CCK-L	AB443433	CCK-L Fw3	GCGCGAACTACTGGCAAGATTGATA
		CCK-L Rv3	TGTCCTTTATCTTGTGGCTGGGACCCG
CCK-N	AB443434	CCK-N Fw3	CCTCTGAAGCACGTCTTGAAGCCTAC
		CCK-N Rv3	GAGATGAGTCTAGCCAACAGTTCACTGA
EF1α	AF321836	Ss EF1 SYBR Fw	GAGAACCATTGAGAAGTTCGAGAAG
		Ss EF1 SYBR Rv	GCACCCAGGCATACTTGAAAG

2.8 Calculation of copy number

The resulting cycle threshold (Ct) values from the two parallels of each sample were averaged and the average deviation between sample Ct values calculated. Samples with average deviation between parallels exceeding 20 % were rerun in q-PCR.

A dilution series of plasmid recDNA standards prepared was analyzed in duplicate reactions by q-PCR for each assay. The plasmid recDNA standards was made by Dr. Koji Murashita, (Murashita et al. 2009) according to protocols from Applied Biosystems (AppliedBiosystems 2003).

Standard curves were prepared by plotting the Ct values against the logarithm of the copy numbers in the standards, and linear regression was performed (Bustin 2000). The initial copy number was calculated using the following equation:

$$Ct = - slope \cdot (C_i) + intercept \qquad \text{(Equation 2.1)}$$

Where Ct is the sample Ct value from q-PCR and C_i is the unknown initial copy number in the sample. Rewriting equation (2.1):

$$C_i = (Ct - intercept) / (- slope) \qquad \text{(Equation 2.2)}$$

Based on the constructed dilution curves of the standard, the R^2 was calculated using least square linear regression, resulting in values > 0.99 for all assays. The obtained copy numbers for all samples were normalized against Atlantic salmon elongation factor 1 α by dividing sample copy number on EF1a copy number (target gene/reference gene).

2.9 Statistical analysis

All statistical analysis was performed in Statistica 8.0 (StatSoft, Inc., USA). Both unfed and fed fish groups were tested for normality within all time groups in separate segments (S2, S4, S6 and S9), using Shapiro-Wilk W-test. Homogeneity of variance was tested on the same

groups with Levene's F-test. Significant differences in mRNA copy number expression was determined with main effects ANOVA using fish group (unfed, fed) and time as effects. For both unfed and fed fish, a one way ANOVA was included to determine changes caused by time alone within each group. In addition an independent between-variable t-test was performed to determine differences between unfed and fed fish group means at each time point. For differences in mRNA expression between segments within each gene, factorial ANOVA was used with fish group and segment as variables.

The null hypothesis was rejected at significance level $\alpha < 0.05$, and a Tukey HSD post-hoc test was then followed up. Log transformation of data was performed to better meet the assumptions of ANOVA for normal distribution and homogeneity of variance. Some groups still failed to meet these demands (see Appendix III).

3. Results

3.1 GI tract compartment content and gallbladder weight

3.1.1 GI tract compartment content

Four compartments of the GI tract were assessed through the weighing of their contents to assess the amount of contents of feed and chyme. The average feed intake was estimated to 3.9 % (GI tract content (g) / average fish weight (g) at time 0.5). At the first sampling point, 30 min after termination of the meal, most of the ingested food (ca 87 %) was still in the stomach with only a small fraction transferred into the pyloric segment (Figure 3.1.1, a). Subsequently, there was a rapid emptying of stomach content and ca 50 % had been transferred from stomach at 4.5 hpf. Most of the stomach content (ca 83%) was emptied at 9-12 hpf, and at 24 hpf the stomach was empty. The unfed fish had an empty stomach throughout the experiment confirmed by visual inspection and content weight (Figure 3.1.1, b). The graph presenting measurements of pyloric cecae (S4) contents in fed fish (Figure 3.1.1, a) displayed a more gradual content increase, lacking a distinct peak but showed a decreasing trend from 9 hpf towards 24 hpf where it was empty. The midgut and hindgut compartments in fed fish displayed a weak gradual increase in chyme towards 12 h past feeding followed by minimal content at 24 hpf. In unfed fish, all gut compartments showed minimal and stable chyme content within the time of the experiment (Figure 3.1.1, b).

3.1.2 Gallbladder content

The gallbladder showed a rapid release of content 0.5 h past feeding shown by a decrease in gallbladder weight (g) (Figure 3.1.2, a). At 1.5 hpf the gallbladder was empty. From 6 and 9 hpf bile there was an increase in gallbladder weight (Figure 3.1.2, a). At 24 hpf the gallbladder shows high content levels equal to time 0 h (prefeeding levels). There are no differences in gallbladder weight throughout the whole sampling period for the unfed fish group (Figure 3.1.2, b).
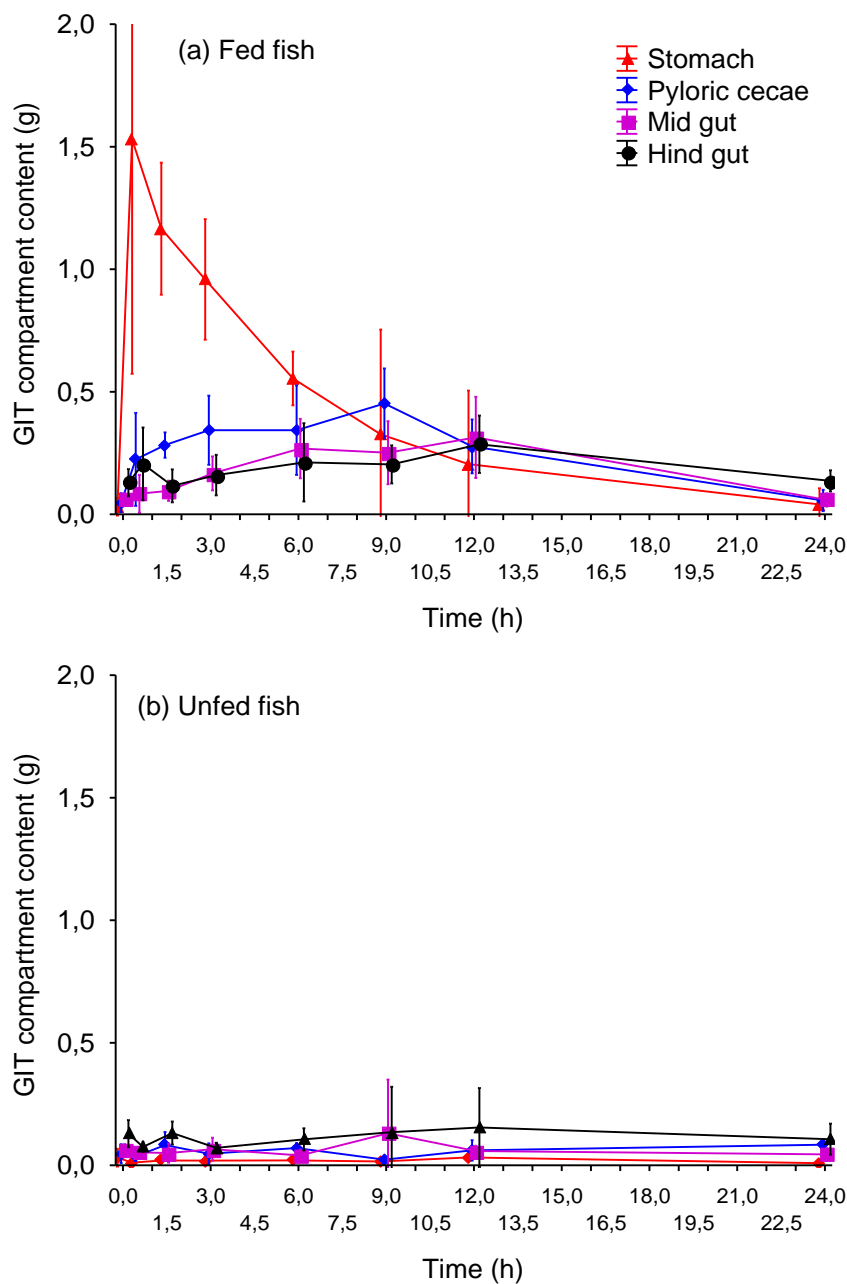
Figure 3.1.1 Measurements of GIT compartment content (g). *The data in graph (a) are represented as the average weight (g) of feed and chyme of each GIT compartment (stomach, pyloric cecae, midgut and hindgut) for six fish sampled at time (h): 0 (prefeeding), and 0.5, 1.5, 3, 6, 9, 12 and 24 after feeding ± SD. In (b) the data are represented as in (a), but for fish fasted for 24 h prior to sampling (Time (h): 0) and throughout the sampling period (unfed for 48 h in total). The data point for each segment at each time point is shifted relative to each other to better distinguish the segments. The fish in (a) and (b) were kept in separate tanks during the experiment.*
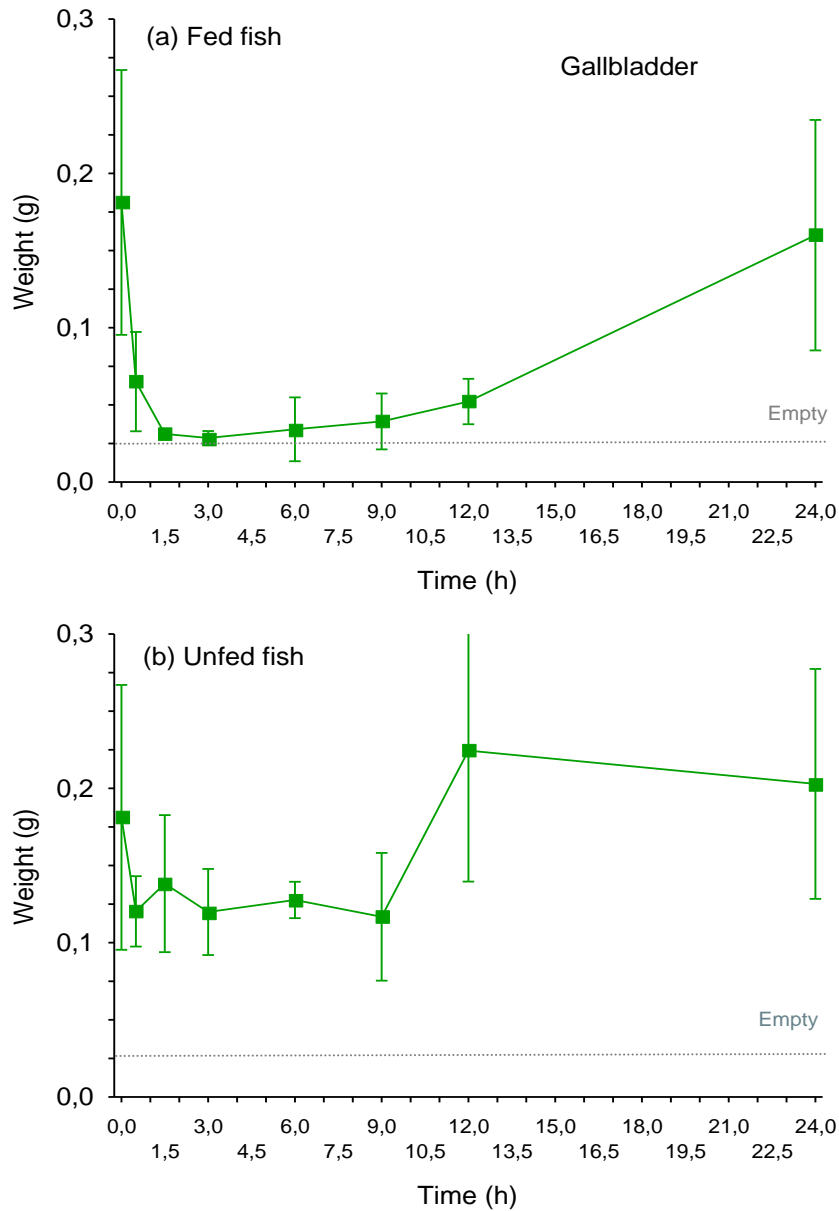
Figure 3.1.2 Measurements of gallbladder weight (g). *Graph (a) and (b) each represents the average weight of the gallbladder (g) for six fish sampled at time (h): 0 (prefeeding), 0.5, 1.5, 3, 6, 9, 12 and 24 after feeding in (a) = fed fish (with the exception of time (h): 0 which is fish sampled prior to feeding), and 24 h since last meal prior to sampling in (b) = unfed fish. The dotted line represents the weight (g) of an empty gallbladder (a gallbladder with minimal content).*

3.2 Spatial distribution of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N mRNA in Atlantic salmon GIT

3.2.1 GRLN-1

GRLN-1 mRNA copy numbers was highest in stomach tissue (S2) of Atlantic salmon. Figure 3.2.1 shows the GRLN-1 average copy numbers at all selected time points for each fish group (fed/unfed). GRLN-1 exhibited significantly lower copy numbers in pyloric cecae (S4), midgut (S6) and hindgut (S9) tissue (p<0.05) compared to stomach (S2), using factorial ANOVA. The hindgut (S9) exhibited lowest GRLN-1 mRNA copy numbers (p<0.01), (Figure 3.2.1, Segment 9). No significant difference in GRLN-1 mRNA copy numbers were found between pyloric cecae (S4) and midgut (S6) tissue for any of the fish. GRLN-1 mRNA copy numbers were not significant different between unfed and fed fish for any of the segments.

3.2.2 GRLN-2

GRLN-2 average mRNA copy numbers were highest in stomach tissue (S2) (Figure 3.2.2). GRLN-2 appeared to have the same spatial mRNA expression pattern as GRLN-1 in Atlantic salmon. The factorial ANOVA and post-hoc Tukey HSD test, showed statistical significant lower GRLN-2 copy numbers in pyloric cecae (S4), midgut (S6) and hindgut (S9) tissue (p<0.05) compared to stomach (S2) (p<0.001). GRLN-2 copy numbers in hindgut (S9) showed significant lower copy numbers than all other tissues studied (p<0.001, Tukey HSD). No significant difference in mRNA expression of GRLN-2 could be seen between fed and unfed fish for any of the tissues.

3.2.3 PYY

PYY mRNA copy numbers were higher in pyloric cecae (S4) and midgut (S6) in Atlantic salmon compared to stomach (S2) and hindgut (S9) tissue (p<0.001) (Figure 3.2.3). Fed fish exhibited higher PYY copy numbers than unfed fish in the stomach (S2) (p<0.05). The hindgut (S9) of Atlantic salmon was the third most expressing tissue of PYY mRNA, and exhibited a significant higher PYY copy number than stomach tissue (S2) (p<0.001). The largest variation in PYY copy numbers within fish groups were found in stomach tissue (S2) (unfed fish SD=0.02 and fed fish SD=0.03). The lowest variation within fish groups was found in midgut (S6) tissue for unfed and fed fish (SD=0.005 for both groups). No significant

differences were found in PYY mRNA copy numbers between pyloric cecae (S4) or midgut (S6) tissues for fed and unfed fish.

3.2.4 CCK-L

CCK-L mRNA copy numbers were found to be significant higher ($p<0.01$) in hindgut (S9) tissue of Atlantic salmon, compared with those in stomach (S2), pyloric cecae (S4) and midgut tissue (S6) (Figure 3.2.4). CCK-L copy numbers were expressed in moderate levels in pyloric cecae (S4) and midgut (S6). Stomach (S2) exhibited the lowest level of CCK-L copy numbers ($p<0.001$) compared to the other segments. Pyloric cecae showed the largest variation within each fish group compared with the other tissues, indicated by the error bars (±SD) in Figure 3.2.4. No significant differences were found in CCK-L mRNA expression between unfed and fed fish in any of the segments analyzed.

3.2.5 CCK-N

CCK-N mRNA copy numbers were found in stomach (S2) and pyloric cecae (S4) of Atlantic salmon (Figure 3.2.5). No significant differences in CCK-N copy numbers were detected between stomach (S2) and pyloric cecae (S4), neither between fish groups (fed/unfed) within each tissue (S2 and S4). Atlantic salmon only expressed low levels of CCK-N mRNA in midgut (S6) and hindgut (S9). Several fish sampled in these compartments were below the detectable limit for q-PCR. No statistical analyses could thus be performed on these two tissues.
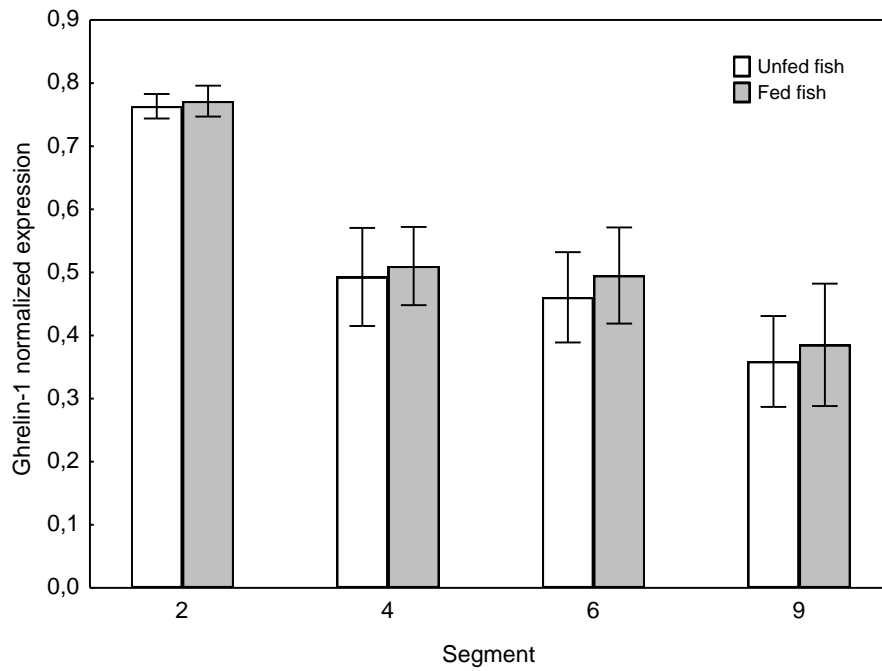
Figur 3.2.1. GRLN-1 mRNA distribution in Atlantic salmon GI tract. *Data are represented as mean calculated copy number for unfed fish (n= 18), and fed fish (n= 15) normalized against Atlantic salmon EF1α copy numbers ((copy numbers of target gene/copy numbers of EF1a reference gene) ± SD), for GI tract segments; stomach (2), pyloric cecae (4), midgut (6) and hindgut (9).*
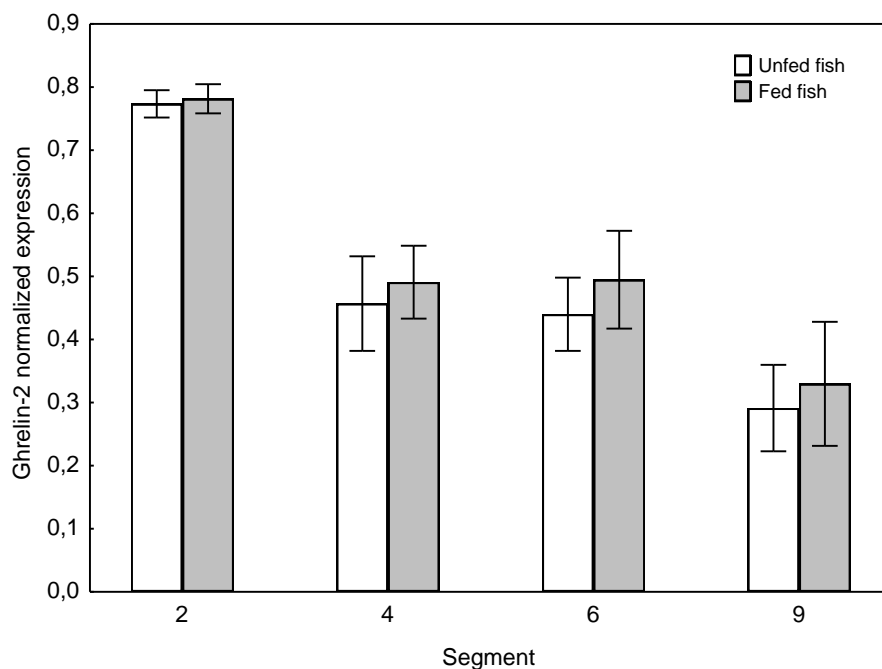
Figur 3.2.2. GRLN-2 mRNA distribution in Atlantic salmon GI tract. *See caption in Figure 3.2.1 for information on data representation.*
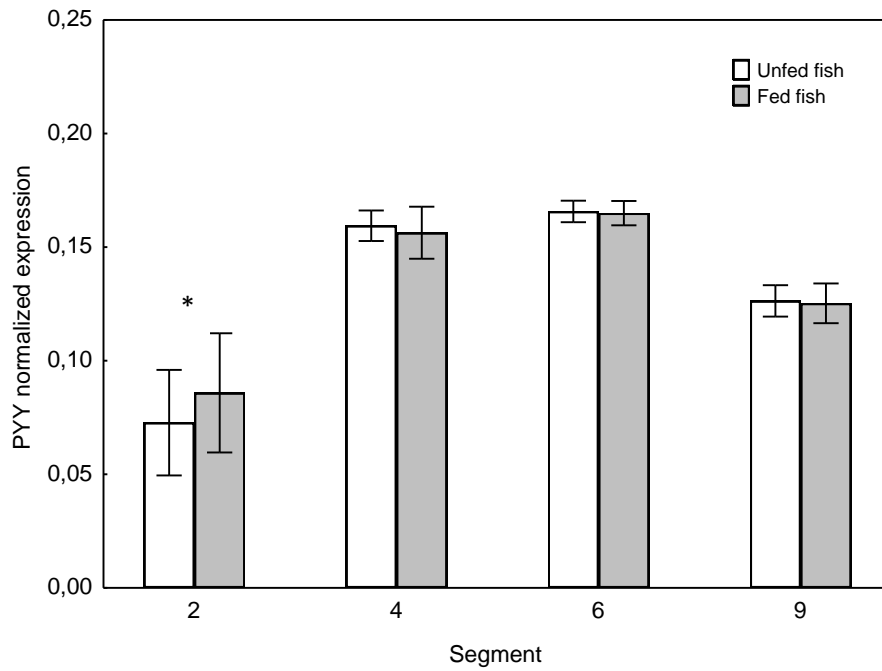
Figur 3.2.3. PYY mRNA distribution in Atlantic salmon GI tract. *See caption in Figure 3.2.1 for information on data representation. An asterisk above bars represents significant differences between unfed and fed fish within a segment.*
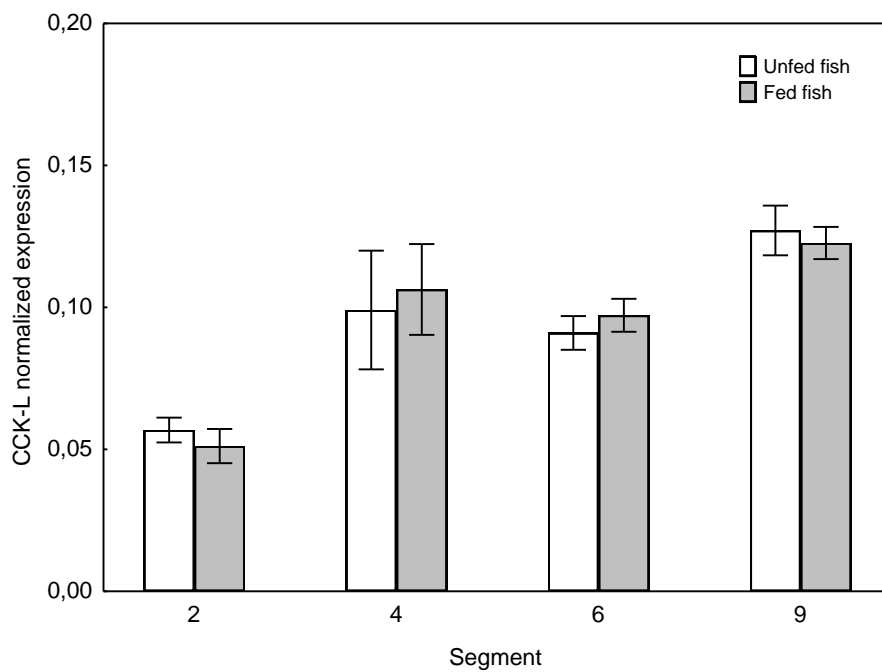
Figur 3.2.4. CCK-L mRNA distribution in Atlantic salmon GI tract. *See caption in Figure 3.2.1 for information on data representation.*
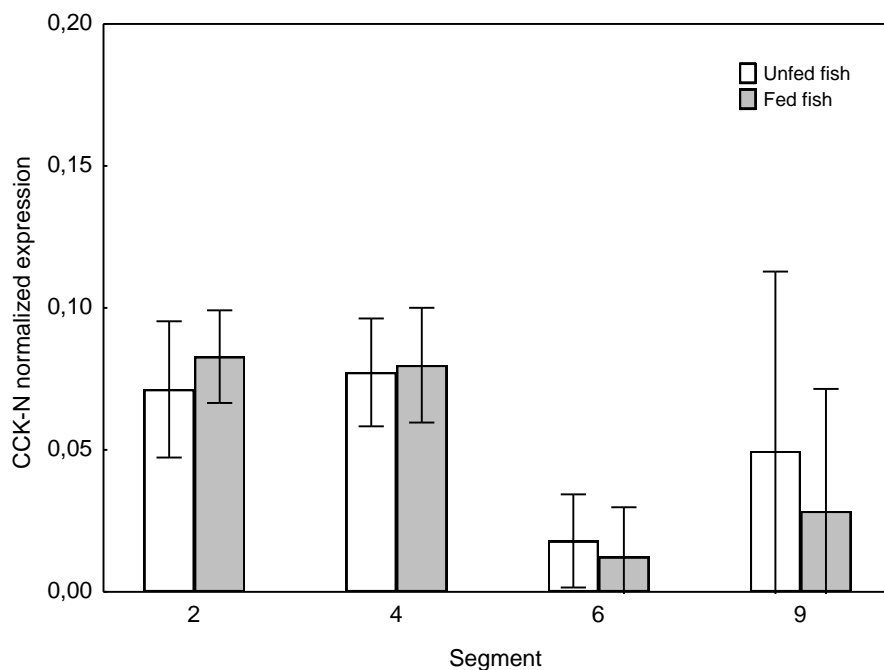
Figur 3.2.5. CCK-N mRNA distribution in Atlantic salmon GI tract. *See caption in Figure 3.2.1 for information on data representation. Several fish in midgut and hindgut tissue (Segment 6 and 9) showed CCK-N mRNA expression below the detectable limit for q-PCR. The bars representing midgut and hindgut should thus be interpreted with some caution as these bars include only the fish which showed CCK-N mRNA levels above the detectable limit in q-PCR.*

3.3 *Postprandial effect on mRNA copy numbers of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N in Atlantic salmon GI tract*

3.3.1 <u>GRLN-1</u>

Significant differences in mRNA copy numbers of GRLN-1 were found between unfed and fed fish in midgut (S6) ($p<0.05$), (Figure 3.3.1; c) and in hindgut (S9) ($p<0.05$), (Figure 3.3.1; d) at time 24 h. No significant differences were found in GRLN-1 mRNA expression between fed and unfed fish in stomach (S2), (Figure 3.3.1; a) or pyloric cecae (S4), (Figure 3.3.1; b). The mRNA expression pattern of GRLN-1 in stomach (S2) is the same for fed and unfed fish.

ANOVA analysis of main effects using time (h) and fish groups (fed, unfed) as effects, and one-way ANOVA to identify the influence of time within each fish group, did not show significant values at $p<0.05$.

Figure 3.3.1. GRLN-1 copy numbers in Atlantic salmon GI tract. *GRLN-1 mRNA copy numbers are shown for stomach (a), pyloric cecae (b), midgut (c) and hindgut (d). Data are presented as mean (n=3) calculated copy number (±S.D) normalized against EF1α reference gene (copy numbers), for unfed fish (black square) and fed fish (red triangle) at time (h); 0.5, 1.5, 3, 6 and 24. Time 0 is represented for unfed fish only. Mean values with an asterisk above show significant differences between fish groups at a given time point (p<0.5).*

3.3.2 GRLN-2

Significant higher GRLN-2 copy numbers ($p < 0.05$) were found in fed fish than unfed fish at time 24 h in midgut (S6) (Figure 3.3.2; c) and hindgut (S9) (Figure 3.3.2; d) of Atlantic salmon. No significant differences were found between fish groups at time; 0.5, 1.5, 3 and 6 hour (t-test) in any of the gut tissues studied (Figure 3.3.2; a, b, c, d). No significant differences were found between fish groups in stomach (S2) tissue (Figure 3.3.2; a) or pyloric cecae (Figure 3.3.2; b). GRLN-2 copy numbers in stomach (S2) exhibited the lowest variation in copy numbers both between and within fish groups (Figure 3.3.2; a).

ANOVA analysis of main effects using time (h) and fish groups (fed, unfed) as effects showed significant values at $p < 0.05$ for fish group as an effect. No significant differences were found in one-way ANOVA using time as an independent factor for each fish group.

Figure 3.3.2. GRLN-2 copy numbers in Atlantic salmon GI tract. *GRLN-2 mRNA copy numbers are shown for stomach (a), pyloric cecae (b), midgut (c) and hindgut (d). See caption in Figure 3.3.1 for information on data representation.*

3.3.3 PYY

Significant differences in PYY mRNA copy numbers were found between unfed and fed fish in hindgut (S9) of Atlantic salmon at time 1.5 h, 3 h and 24 h ($p<0.05$), (Figure 3.3.3; d). In hindgut (S9) at time 1.5 h, fed fish exhibited higher PYY copy numbers than unfed fish. At time 3 h and 24 h unfed fish exhibited the highest PYY copy numbers in hindgut (S9). No significant differences were found in PYY mRNA copy numbers between unfed or fed fish in; stomach (S2), pyloric cecae (S4) or midgut (S6) (Figure 3.3.3; a, b and c, respectively). Stomach (S2) tissue showed more variation within the time groups than pyloric cecae (S4), midgut (S6) and hindgut (S9), indicated by the standard deviation (SD) in Figure 3.3.3; a.

ANOVA analysis of main effects using time (h) and fish groups (fed, fasted) as effects showed significant values at $p<0.05$ for time on PYY mRNA expression in midgut, the Tukey HSD post-hoc test found this significant difference in PYY copy numbers between time 0 h and 1.5 h in midgut. However the one-way ANOVA using time as independent factor for each fish group did not show any significant effect of time alone.
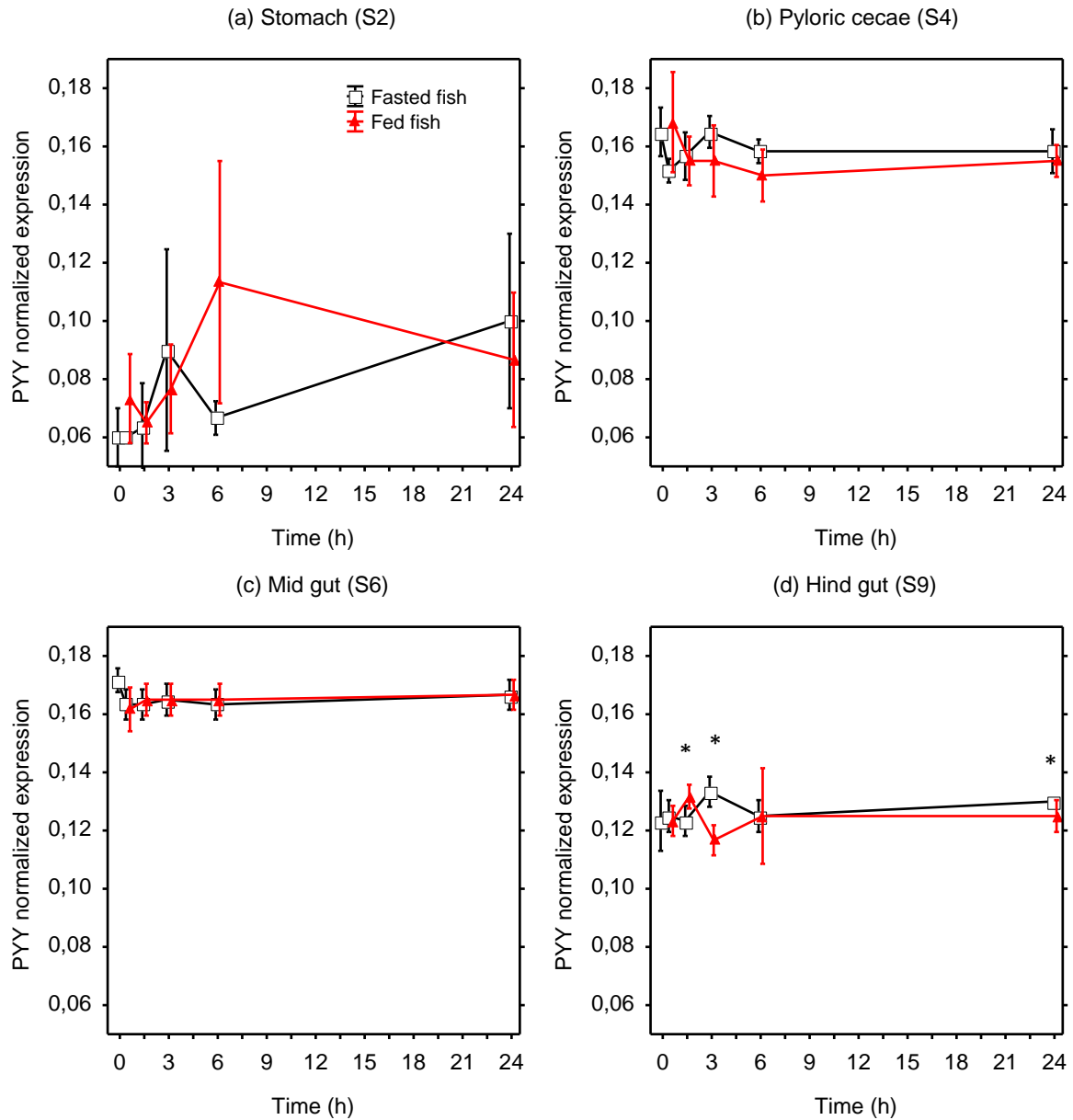
Figure 3.3.3. PYY copy numbers in Atlantic salmon GI tract. *PYY mRNA copy numbers are shown for stomach (a), pyloric cecae (b), midgut (c) and hindgut (d). See caption in Figure 3.3.1 for information on data representation.*

3.3.4 CCK-L

Significant differences in CCK-L mRNA copy numbers ($p < 0.05$) were found between fed and unfed fish in stomach (S2) of Atlantic salmon at time 3 h, where unfed fish exhibited higher CCK-L copy numbers than fed fish (Figure 3.3.4; a). Significant higher CCK-L copy numbers were found in the midgut (S6) of fed fish at time 0.5 h and time 24 h ($p < 0.05$), than in unfed fish at the same time points (Figure 3.3.4; c). CCK-L copy numbers were significantly higher in unfed fish than fed fish at time 1.5 h postprandial ($p < 0.05$) in hindgut (S9) (Figure 3.3.4; d). No significant differences between fish groups were found in pyloric cecae (S4) (Figure 3.3.4; b).

Results from main effect ANOVA using time (h) and fish groups (unfed, fed) as effects showed significant effect of time at $p < 0.05$ on CCK-L copy numbers in pyloric cecae. The followed up Tukey HSD post hoc test, found this significant effect between time 0 h and 0.5 h ($p < 0.05$). Main effect ANOVA detected additional significant effects of fish group on CCK-L mRNA copy numbers in stomach (S2) ($p < 0.01$) and midgut (S6) ($p < 0.01$). No significant effect of time was detected using one-way ANOVA with time as independent factor on CCK-L copy numbers within each fish group (fed/unfed).
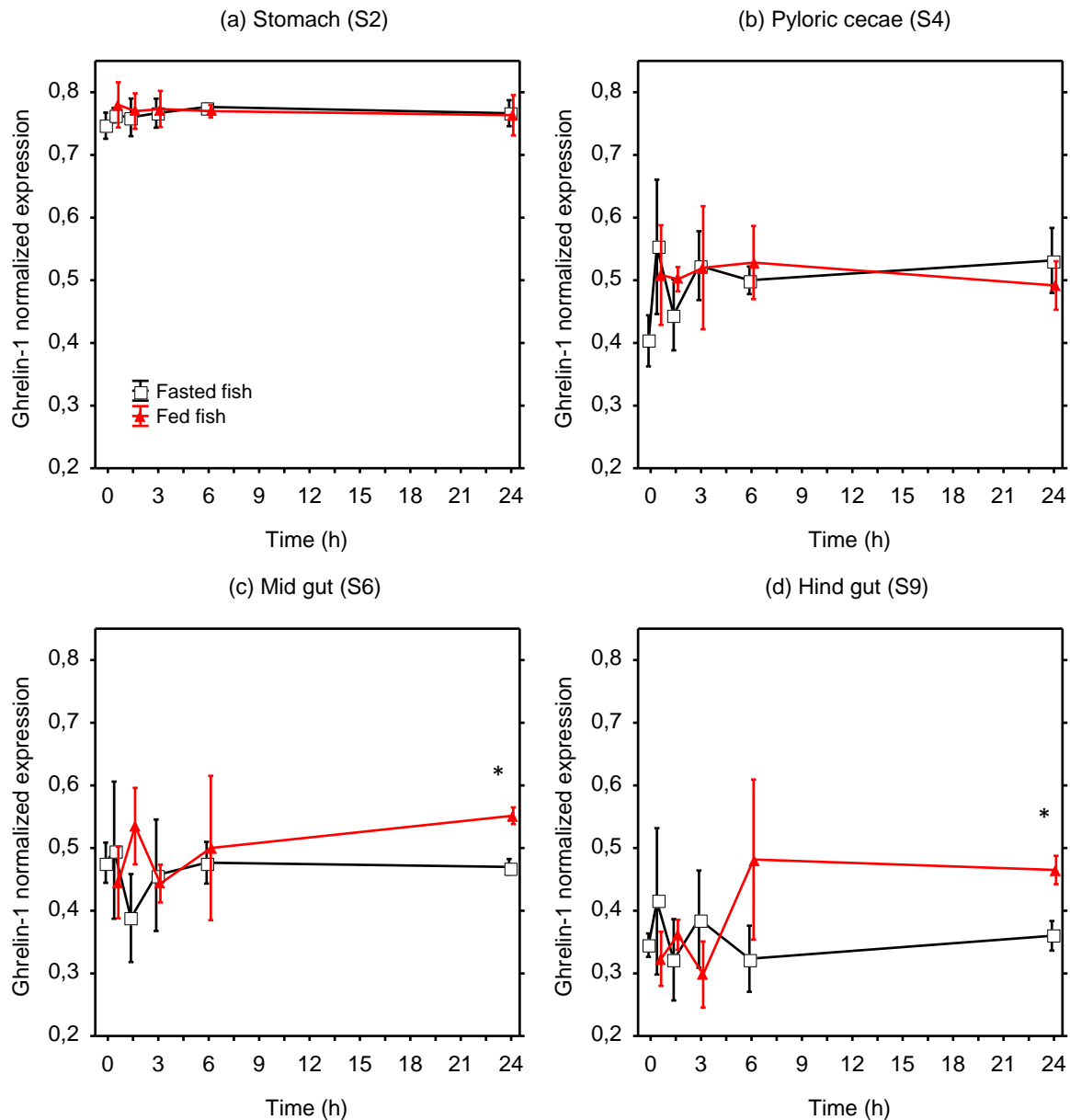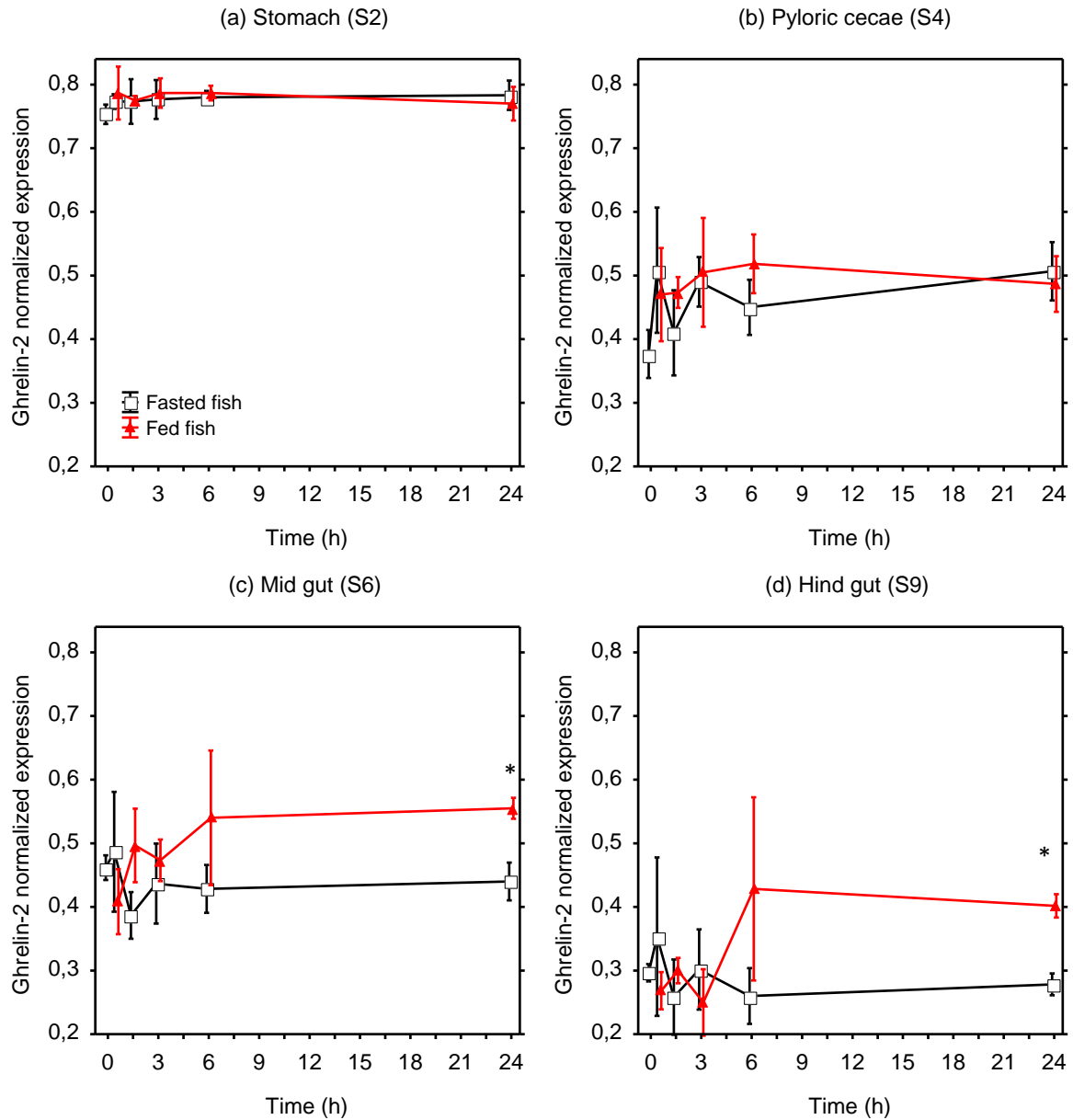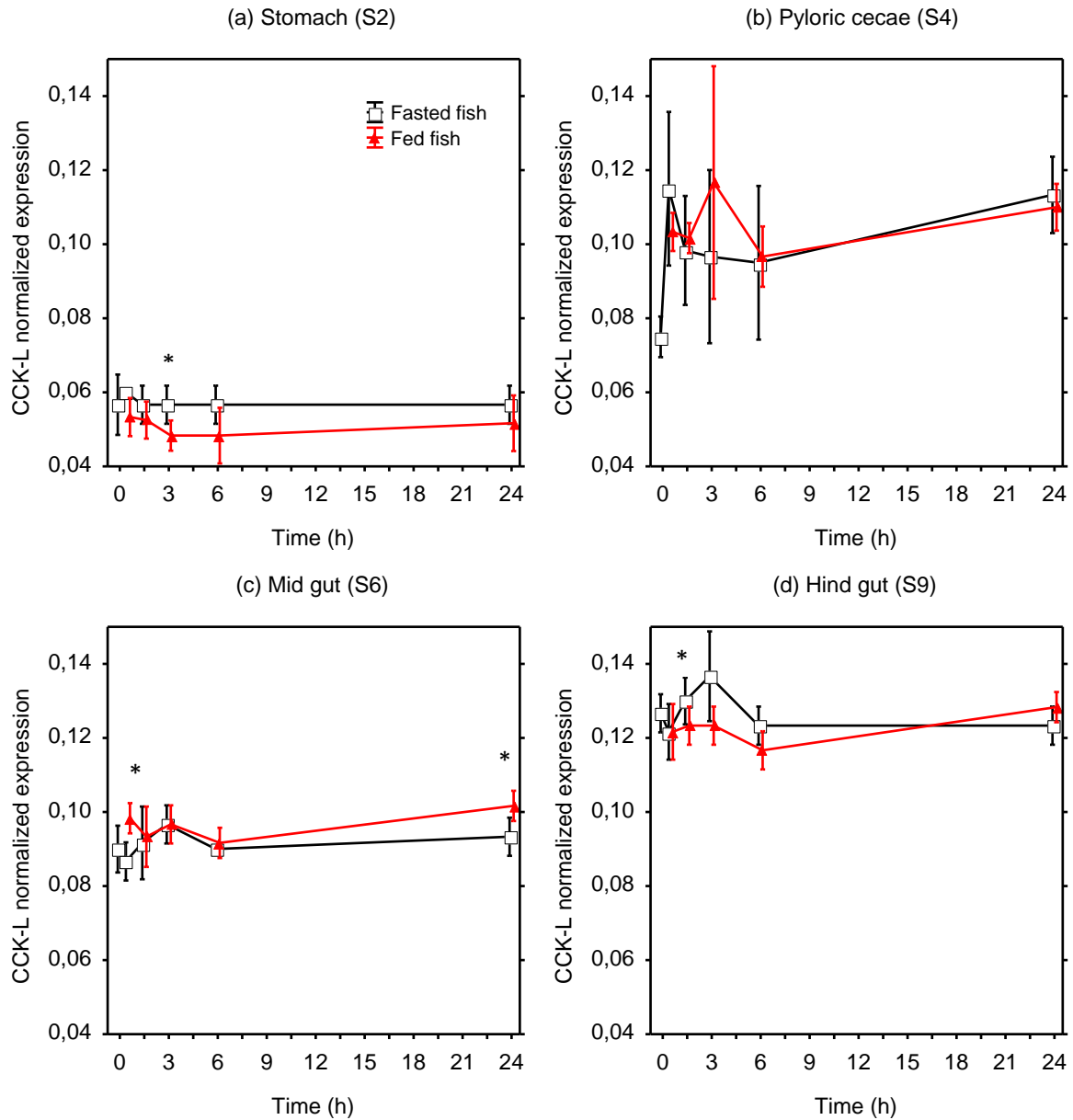
Figure 3.3.4. **CCK-L copy numbers in Atlantic salmon GI tract.** *CCK-L mRNA copy numbers are shown for stomach (a), pyloric cecae (b), midgut (c) and hindgut (d). See caption in Figure 3.3.1 for information on data representation.*

3.3.5 <u>CCK-N</u>

CCK-N mRNA copy numbers in Atlantic salmon stomach (S2) exhibited significant differences ($p < 0.05$) between unfed and fed fish at time 1.5 h, where fed fish show a higher CCK-N mRNA copy numbers than unfed fish (Figure 3.3.5; a). No significant differences were found in CCK-N mRNA expression in pyloric cecae (S4) (Figure 3.3.5; b). The midgut (S6) and hindgut (S9) are not presented for CCK-N as q-PCR results of CCK-N mRNA copy numbers were below the detectable limit for q-PCR. Results from main effect ANOVA using time (h) and fish groups (fed, unfed) as effects failed to show significant values at $p < 0.05$. This was also the case for one-way ANOVA using time as independent factor for each fish group.
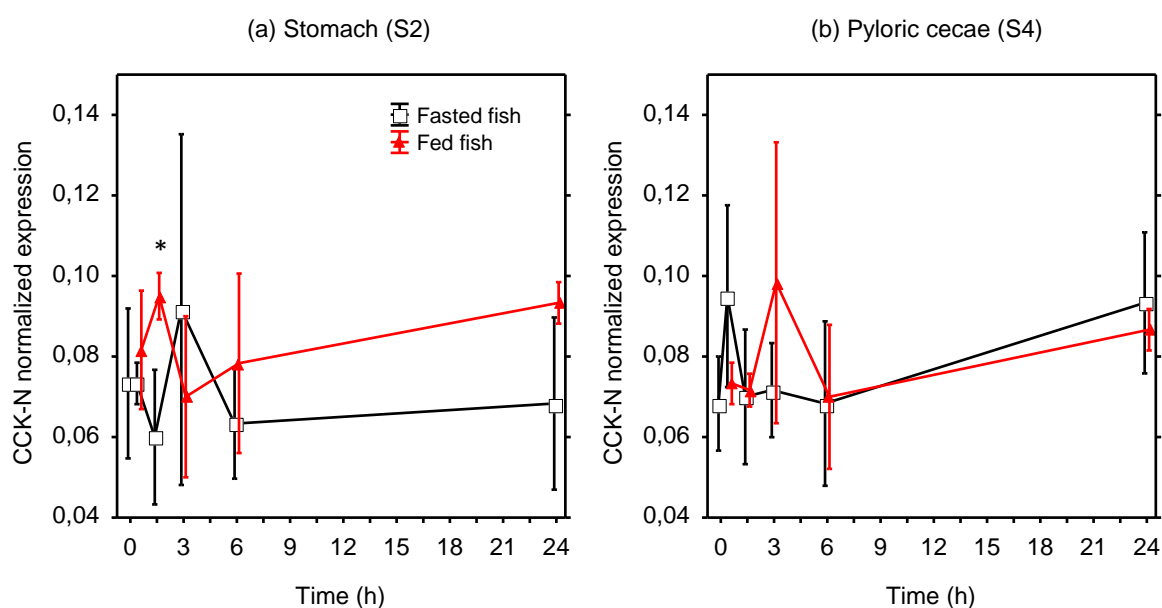
Figure 3.3.5 CCK-N copy numbers in Atlantic salmon GI tract. *CCK-N mRNA copy numbers are shown for stomach (a) and pyloric cecae (b). See caption in Figure 3.3.1 for information on data representation. Midgut and hindgut were not included in this figure as several fish spread over different time groups showed CCK-N mRNA expression below the detectable limit in q-PCR.*

3.4 Results of correlation between sample Ct value and RIN

Table 3.4 summarizes the results from the linear regression of sample Ct values and their corresponding RIN value obtained from q-PCR and RNA integrity assessment, respectively. All genes analyzed in pyloric cecae (S4) showed significant correlation at $p<0.05$. In midgut (S6); EF1α (reference gene), PYY and CCK-N showed significant correlation between Ct and RIN. In stomach (S2), GRLN-1 and GRLN-2 showed significant correlation with p values (<0.05). However in hindgut (S9), only CCK-L showed significant correlation ($p<0.05$). The coefficient of determination (r^2) showed low values for all genes ($r^2<31$).

Table 3.4 Correlation between RNA integrity and cycle threshold (Ct). *Results of Cycle threshold (Ct) values (y) plotted against RNA integrity (x). Data are represented for the different genes expressed in different segments of the GI tract (S2, S4, S6 and S9) showing; coefficient of determination (r^2) and regression p value. All samples underwent similar experimental treatment. Data were considered significant at α < 0.05, n.s. = non significant (α > 0.05). Significant values are shown in bold and underlined.*

	Stomach (S2)		Pyloric cecae (S4)		Midgut (S6)		Hindgut (S9)	
	r^2	p	r^2	p	r^2	p	r^2	p
EF1α	0.091	n.s.	0.290	**0.002**	0.306	**0.001**	0.082	n.s.
GRLN-1	0.144	**0.033**	0.134	**0.040**	0.001	n.s.	0.025	n.s.
GRLN-2	0.135	**0.042**	0.143	**0.033**	0.035	n.s.	0.037	n.s.
PYY	0.056	n.s.	0.309	**0.001**	0.298	**0.001**	0.025	n.s.
CCK-L	0.001	n.s.	0.146	**0.031**	0	n.s.	0.172	**0.018**
CCK-N	0.003	n.s.	0.210	**0.008**	0.174	**0.048**	0.047	n.s.

4. Discussion

4.1 Spatial mRNA expression of GI tract peptide hormones in Atlantic salmon

4.1.1 GRLN-1 and GRLN-2

The GI tract has been shown to be the main site of GRLN production in all vertebrates studied to date (Kaiya et al. 2008). This recently included Atlantic salmon (Murashita et al. 2009). The current study showed that GRLN-1 and GRLN-2 is expressed throughout the GI tract in Atlantic salmon (Figure 3.2.1 and 3.2.2) which correlates well with results from studies in other vertebrates (Kaiya et al. 2008; Murashita et al. 2009). GRLN-1 and GRLN-2 were both highest expressed in the stomach, moderately expressed in pyloric cecae and midgut, and lowest expressed in hindgut. No differences were found between GRLN-1 and GRLN-2 spatial mRNA expression, possibly indicating similar functions. The high levels of GRLN in Atlantic salmon stomach observed in the present study, is in agreement with previous studies in Atlantic salmon, rainbow trout, eel, catfish, tilapia (Murashita et al. 2009) (for more details on species comparison see; Kaiya et al. 2008).

In the current study, apparently higher GRLN mRNA expression for the two GRLN isoforms were found in pyloric cecae, midgut and hindgut compared to results presented in Murashita et al. (2009). In their work, Murashita et al. (2009) did not detect GRLN mRNA expression in Atlantic salmon hindgut after 32 amplification rounds using real time (RT)-PCR. However as RT-PCR results of GRLN expression in Atlantic salmon hindgut are only semi-quantitative, a direct comparison of the results should be avoided (Murashita et al. 2009). In addition fish size and consequently life stage differs and there may be physiological differences in the expression between juvenile fish in the present study and adult fish in Murashita et al. (2009).

In immature rainbow trout, regional studies on GRLN mRNA expression in the GI tract also showed a moderate GRLN expression in the intestine, but low expression in pyloric cecae (Kaiya et al. 2003a). GRLN mRNA expression has not been investigated in adult rainbow trout, however moderate GRLN levels in immature Atlantic salmon and rainbow trout could imply life stage differences in GRLN expression. Although high expression of GRLN in stomach was found in all fish studied by Kaiya et al. (2003a) one of the fish expressed very low levels of GRLN mRNA in the intestine. Thus, individual differences

GRLN expression may also be a factor to be considered when performing tissue distribution studies on GRLN. A moderate expression of GRLN in midgut and hindgut of Atlantic salmon thus agrees with studies in rainbow trout, but contrasts former studies in Atlantic salmon (Kaiya et al. 2003a; Murashita et al. 2009). The higher mRNA expression of GRLN in pyloric cecae compared with hindgut agrees with Murashita et al. (2009). In contrast, rainbow trout show significantly lower GRLN expression in pyloric cecae, indicating species specific tissue distribution of GRLN in salmonids.

Comparative studies in fish show high to moderate levels of GRLN in the intestine of eel, catfish, goldfish, and rainbow trout (Kaiya et al. 2008). Goldfish lacks a stomach, however the intestine was shown to be the main site of GRLN expression in this species (Unniappan et al. 2002). GRLN was not detected in the intestine of tilapia (Kaiya et al. 2003c). The degree of GI tract compartmentalization in the form of a well demarcated stomach may thus influence the tissue distribution pattern of GRLN. In general, GRLN is expressed in high levels in the stomach of fishes and in high to moderate levels in the intestine. However there are species specific variations and individual variations in GRLN tissue distribution, thus possibly indicating species specific functions of GRLN in GI tract. In addition developmental life stage may also influence the spatial GRLN distribution.

No differences in GRLN expression were found between fed and unfed fish groups. As these groups (fed/unfed fish) show the group average result of GRLN expression within 24 hours, there may not be a direct effect of GRLN-1/2 on the entire fish group. However potential short term effects of GRLN-1/2 on regulating digestion and appetite within this period may still exist, and will be discussed more in depth in section 4.2 and 4.3.

The specific function of GRLN in different areas of the GI tract is currently unknown. The higher expression in stomach of Atlantic salmon, suggest this region to be the most important in GRLN function. Intraperitoneal (IP) injections of GRLN have been shown to increase appetite in goldfish, while no effect was observed in rainbow trout (Unniappan et al. 2004; Jönsson et al. 2007). It is thus uncertain if GRLN act as an orexigenic factor in fish as observed in mammals (Kojima and Kangawa 2002). Studies in mammals suggest that GRLN mediate its main effects through vagal afferents and not through circulating levels (Date et al. 2002). Locally produced GRLN in the GI tract may thus have a more direct role in mediating the function of GRLN, in contrast to GRLN released into the circulation. The regional

distribution of GRLN in Atlantic salmon show similarities with rainbow trout, and thus indicates similarities in GRLN function in salmonids. This could include the effect of GRLN on appetite.

It is also possible that GRLN produced in the GI tract is released into the circulation and affect distant targets. IP injections of GRLN have been shown to increase circulating levels of GH in goldfish, channel catfish, rainbow trout and tilapia (Kaiya et al. 2003a; Unniappan and Peter 2004; Kaiya et al. 2005; Fox et al. 2007). GRLN synthesized and secreted by endocrine cells in the stomach and intestine may thus bind to GHS-R receptors in the pituitary and stimulate GH release. GHS-R receptors in salmonids have been identified in the pituitary of rainbow trout (Kaiya et al. 2009). It would thus be interesting to study GHS-R receptors in Atlantic salmon.

4.1.2 PYY

The highest expression of PYY mRNA was found in pyloric cecae and midgut, while lower levels were detected in hindgut and stomach (Figure 3.2.3) in accordance with Murashita et al. (2009). In contrast to Murashita et al. (2009), this study accurately quantifies regional PYY mRNA expression in Atlantic salmon GI tract.

Recently, Sundström et al. (2008) suggested a renaming of PY to PYYb. This naming will thus be used in this discussion as recommended in Murashita et al. (2009). Phylogenetic studies separate PYY into PYYa and PYYb type groups (Murashita et al. 2009). Atlantic salmon PYY is classified as PYYa type (Murashita et al. 2009). Semi-quantitative RT-PCR studies in tiger puffer fish (*Takifugu rubripes*) detected apparently high levels of PYYa and PYYb mRNA expression in intestine, similar to this current study (Sundstrom et al. 2008). However, Sundström et al. (2008) does not show the spatial tissue distribution of the two PYY types within the intestine. Quantitative spatial expression studies should thus be executed in tiger puffer fish in order to conclude on any similarities in PYYa expression with Atlantic salmon PYY. Q-PCR studies in yellowtail found high mRNA expression of PYYb in anterior intestine, -and lower expression in pyloric cecae, stomach and posterior intestine (Murashita et al. 2006). Although yellowtail PYYb belongs to the second teleost PYY group, PYYb show similar expression pattern with Atlantic salmon PYY in anterior intestine. However Atlantic salmon show higher levels of PYY mRNA in pyloric cecae, suggesting a species specific pattern of PYY mRNA expression.

In conclusion, studies on PYYa and PYYb distribution in fish GI tract all show a higher mRNA expression in anterior intestine and lower expression in stomach and hindgut. This is in contrast to mammals where PYY secreting cells are concentrated in distal intestine (Lundberg et al. 1982). Different expression patterns of PYY between fish and mammals suggest species specific functions of PYY in the GI tract. Also, the similarities in tissue distribution of PYYa and PYYb in fish possibly suggest similarities in function.

In the current study fed fish exhibits higher PYY mRNA expression compared to unfed fish in stomach (Figure 3.2.3). In conclusion, this may indicate a potential effect of PYY during feeding in Atlantic salmon, however this effect is minor as no temporal differences were observed in PYY mRNA expression in stomach during meal processing (Figure 3.3.3; a). The postprandial effect of PYY is discussed under section 4.3 and 4.4.

4.1.3 CCK-L and CCK-N

This study demonstrated differences in the regional distribution for CCK-L and CCK-N in Atlantic salmon GI tract. This was only indicated semi-quantitatively by Murashita et al. (2009). In the current study, CCK-L mRNA expression was detected in all GI tract regions studied (Figure 3.2.4). The highest expression of CCK-L mRNA was found in hindgut, whereas moderate expression was detected in midgut and pyloric cecae, and lowest expression in stomach. All results are in agreement with Murashita et al. (2009). Although CCK-L were only presented semi-quantitatively by Murashita et al. (2009), the band intensity are consistent with our results. In agreement with our results, Jensen et al. (2001) found high levels of trout CCK-L expression in anterior intestine and distal intestine. However, trout expresses higher levels of CCK-L in stomach and low levels in pyloric cecae. This finding contrast our results. A species specific distribution of CCK-L may thus exist in salmonids.

In this study, CCK-N mRNA expression was detected in stomach and pyloric cecae, while the expression of CCK-N in intestine showed low or no detectable levels (Figure 3.2.5). The low expression in intestine is in agreement with semi-quantitative results presented by Murashita et al. (2009). In contrast to Murashita et al. (2009), equal CCK-N levels were detected in stomach and pyloric cecae in this current experiment. Murashita et al. (2009) did not find expression of CCK-N in stomach, however the differences in assay specifications and fish size could have influenced this result (as previously discussed for GRLN). Thus, the use of more sensitive methodology in the present study seems to have enabled the detection of

CCK-N in stomach. All together, this may indicate that stomach and pyloric cecae are the main sites of CCK-N production in Atlantic salmon GI tract. Semi-quantitative studies in rainbow trout show apparently no CCK-N in rainbow trout stomach in contrast to our results (Jensen et al. 2001). CCK-N was mainly expressed in pyloric cecae in rainbow trout (Jensen et al. 2001). In conclusion, the results suggest an important role for CCK-N in pyloric cecae and possibly also in stomach of salmonids.

Studies in both rainbow trout and Atlantic salmon demonstrate different expression patterns for CCK. While CCK-L shows expression throughout the gut in both species, CCK-N is expressed mainly in pyloric cecae (and stomach in Atlantic salmon). The difference in regional expression between CCK-L and CCK-N may indicate specific roles including regulation of digestion and appetite. In addition, CCK-T which is found in trout was expressed in high levels in middle regions of the small intestine (Jensen et al. 2001). This could indicate the presence of additional CCK isoforms also in Atlantic salmon. In contrast to rainbow trout, Atlantic salmon exhibit higher expression of CCK-L in pyloric cecae, and CCK-N in stomach. Rainbow trout on the other hand expresses higher levels of CCK-L in stomach compared to Atlantic salmon. These results indicate species differences in the regional expression of CCK in salmonids.

In conclusion, the expression of CCK isoforms found in Atlantic salmon GI tract in the current study, agrees with previous studies indicating roles for CCK in inhibition of gastric emptying, inducing gallbladder contraction and stimulating pancreatic secretion (Aldman and Holmgren 1995; Einarsson et al. 1997).

Altogether this point to a difference in mRNA expression of GRLN, CCK and PYY as chyme is transported through the different GI tract compartments after feed ingestion.

4.2 Temporal changes in GI tract filling of feed and chyme, and gallbladder content in Atlantic salmon

In fed fish almost all ingested feeds was still present in the stomach 0.5 h after the termination of the meal (=hours past feeding, hpf) (Figure 3.1.1; a). Thereafter there was a rapid response in emptying of stomach content. These results demonstrate that the ingested feed are kept in the stomach for gastric digestion, and then emptied to proximal intestine for digestion and absorption by a gradual regulated mechanism. It is interesting to note that at 0.5 hpf when very little transfer apparently had taken place from stomach, the gallbladder was already nearly empty (Figure 3.1.2; a). At this time point minimal content was present in proximal intestine and pyloric cecae (Figure 3.1.1; a). Thus, the gallbladder appears to contract and release bile when feed is present in the stomach prior to significant chyme entry in the proximal intestine. These results may indicate feed-forward response of bile release in response to feed present in the stomach. This feed-forward response is possibly mediated by hormones in the stomach stimulated by luminal factors. In this current experiment all peptide hormones were expressed in stomach. The role of GI tract peptide hormones in regulating digestion will be discussed under section 4.3.

At 1.5 hpf, the stomach was still filled with feed and chyme, a time point characterized by an empty gallbladder. The gallbladder volume did not seem to increase before 9 hpf, and reached prefeeding levels only between 12-24 hpf. It is important to notice that the relationship between gallbladder volume/mass and secretion rates is more complex than what can be interpolated directly from the presently available data. The gallbladder weight (Figure 3.1.3; a) indicates that no bile is released into intestine from gallbladder from 1.5 hpf to 12 hpf, a period characterized by gastric evacuation. The emulsification of lipids in this period may thus take place by; 1) residues of bile still present in the intestine, 2) bile originating from other organs, or 3) re-circulation of bile absorbed in the distal intestine and transferred by circulation to proximal intestine via liver and directly secreted through the *Ductus choleductus*. It may be possible that a combination of these mechanisms exist in GI tract. The presently available data suggest that the hormonal regulation of gallbladder contraction mainly seems to occur within 1.5 hpf which represents the intestinal phase of digestion.

The gradual slower increase in luminal content in pyloric cecae, midgut and hindgut compared to stomach (Figure 3.1.1; a), imply a gradual transit of feed into intestine which most likely optimizes the capacity of intestine to digest and absorb nutrients. It is known that

gastric emptying takes place when the resistance to the flow of chyme through the pyloric sphincter and intestine is overcome (Olsson et al. 1999). A decrease in stomach content also allows ingestion of more feed and return of appetite. The current data for Atlantic salmon showed a concomitant increase in pyloric cecae content with decrease in stomach content, indicating gastric evacuation and relaxation of the pyloric sphincter. Although absorption and evacuation was not measured in this experiment, this may represent a loss of content observed in the graphs. The more gradual pattern of chyme filling observed in pyloric cecae, midgut and hindgut may thus include absorption. The pyloric cecae has been shown to absorb 50 % or more of amino acids, lipids and starch in Atlantic salmon and is thus considered to be the most important region for nutrient absorption (Krogdahl et al. 1999). However both proximal and distal intestine have also been shown to be involved in absorption (Krogdahl et al. 1999). Pyloric cecae serve to increase the absorptive area in fish and is surrounded by pancreatic tissue in Atlantic salmon (Buddington and Diamond 1987; Buddington et al. 1997). Filling of chyme into the cecae and mixing of chyme with pancreatic enzymes, have been suggested to occur by retrograde peristalsis in teleosts (Rønnestad et al. 2000). Taken together the observed increase of chyme in pyloric cecae at 0.5-9 hpf, together with reduction of overall content in the GI tract indicates release of pancreatic enzymes and absorption of nutrients.

Studies on gut transit in rainbow trout using chromic oxide feed indicator demonstrated that an increasing fraction of the meal were present and processed in pyloric cecae and midgut from 3 hpf and it reached a maximum at 12 hpf (Sveier et al. 1999). Differences in experimental conditions and estimation of GI content may have caused the difference between the data of Sveier et al. (1999) and the present study. In our experiment, weight measurements of GI tract content were used to estimate the compartmental distribution and transfer of ingested food/chyme, while Sveier et al. (1999) used an indicator in the feed.

One major limitation in the present study is the low water content in dry feed and the fluid balance that includes both swelling of the diets and release and absorption of fluids in the GI tract. The mass balance should therefore be done with precaution. Further, in the present experiment the content in pyloric cecae is not based on all the content in pyloric appendages as content was very difficult to remove from these cecae. The contents in the pyloric cecae were therefore probably underestimated (for more details, see Material and methods section). The fish used in the current experiment was ~45 g, while Sveier et al.

(1999) used ~695 g Atlantic salmon. The difference in fish size could also have caused a more rapid increase in content in proximal intestine and midgut in the current study.

At the same time, the present data is a good indicator of GI tract compartment filling. There is normally a combination of enteroendocrine cells and neuronal sensing (chemical and stretch) of luminal content (nutrients and volume). The gut transit data in the present study could therefore be expected to give a good indication as to the progress of the digestive process, including the gastric and intestinal phases, and the interpretation of the spatial and temporal mRNA expression of the peptide hormones during processing of a meal.

Altogether the increase in pyloric cecae content at 0.5-9 hpf followed by increased content in midgut and hindgut (1.5-12 hpf), indicate an increased expression of peptide hormones regulating pancreatic secretion, gallbladder contraction and gut motility. All peptide hormones analyzed in this current study was expressed in pyloric cecae region, suggesting a possible role of these in controlling gut secretion and motility. CCK-N was not detected in midgut and hindgut, however it may have an endocrine intra-GI tract. This will be discussed under section 4.3.

In the current experiment, all compartments were close to empty at 24 hpf, indicating the endpoint of digestion and evacuation of non digestible feedstuff and waste. This time scale is in agreement with Sveier et al. (1999). At this time the gallbladder weight is the same as prefeeding levels, indicating minimal digestion and refilling of the gallbladder. If GRLN, PYY or CCK are involved in regulating the digestive process, changes in expression could be expected to occur within the first 12-18 hours, when chyme is still present in GI tract.

Gastric evacuation is thought to be a good predictor of the return of appetite (Grove et al. 1978). In their study it was estimated that gastric evacuation of 80-90% of stomach content corresponded to an increase in appetite in rainbow trout (Grove et al. 1978). In the current experiment 80-90 % of stomach content was evacuated between 12-24 hpf. GRLN, CCK and PYY have been shown to change in expression and plasma levels according to meal ingestion (Peyon et al. 1999; Batterham and Bloom 2003; Unniappan et al. 2004). In conclusion, potential anorexigenic peptide hormones (PYY, CCKs) are expected to increase within the initial 12 or more hours, while potential orexigenic peptide hormones (GRLN) are expected to increase when the stomach is close to empty (12-24 hpf) and increases appetite.

4.3 Postprandial changes of GI tract peptide hormones

4.3.1 Postprandial regulation of digestion: GRLN-1 and GRLN-2

The distribution of GRLN throughout the GI tract of Atlantic salmon may suggest a more direct effect on the digestive process other than stimulation of GH and appetite. Studies have shown that GRLN stimulate gastric acid secretion in mammals (Masuda et al. 2000). Previous studies have localized GRLN producing cells in gastric glands in rainbow trout, Japanese eel and summer flounder indicating a possible role in the regulation of gastric secretion (Sakata et al. 2004; Kaiya et al. 2006; Breves et al. 2009). It is however uncertain if GRLN regulates gastric acid secretion in fish. Gastric acid production has however been shown to be regulated by gastrin and histamine in the presence of luminal factors in fish (Buddington and Krogdahl 2004). In this current study, no postprandial changes in GRLN mRNA were detected during the first 12 hours of digestion including stomach (Figure 3.3.1 and 3.3.2; a-d). The increase observed at 24 hpf in midgut and hindgut is concurrent with an empty gut (Figure 3.1.1, a). Together this may indicate an alternative mechanism of GRLN in regulating the digestive process. What should also be noticed is that the objective of the current study was to detect changes in mRNA expression. Thus possible effects of protein regulation are not included, but may exert an important step in GRLN regulation and function.

Studies in mammals and birds show that GRLN have a motor effect in the gut (Peeters 2005; Kitazawa et al. 2007). GRLN is structurally related to motilin which has a role in inducing the migrating motor complex (MMC) and accelerates gastric emptying in mammals (Vantrappen et al. 1979; Peeters 2005). MMC are spontaneous contractions which occur along certain patterns in the fasted state (Olsson and Holmgren 2001). Both the interstitial cells of cajal (ICC), the enteric nervous system and hormones have been implicated in the control of MMC (Olsson and Holmgren 2001). Studies in mammals recently suggested a role for GRLN in inducing MMC (reviewed by Peeters (2005)). Recently, both GRLN and motilin receptor genes were identified in the gut of zebrafish (Olsson et al. 2008). Although zebrafish do not have a stomach, immunohistochemical analysis detected GRLN receptor and motilin receptors throughout the gut of this species.

A contradictory aspect of this should however be mentioned as is discussed by Peeters (2005). While plasma GRLN levels typically decreases rapidly after feeding as seen in goldfish (Unniappan et al. 2004), there may not be high enough levels of GRLN in the blood

to induce MMC which is a phenomena occurring between meals, or enough to induce gastric emptying (Olsson and Holmgren 2001). The effect is thus suggested to be mediated via paracrine actions of GRLN on vagal afferents, vagal efferents or the enteric nervous system (Peeters 2005). Studies in zebrafish detected GRLN receptor in all muscle layers of the gut (Olsson et al. 2008). It was also shown that both GRLN and motilin had excitatory effects on gut motility in this species. These results may thus suggest a possibly role for GRLN in both paracrine and endocrine excitation of gut muscle activity.

In conclusion, the structural resemblance of GRLN and motilin, and the presence of both receptors in fish gut possibly indicate a role for GRLN in inducing MMC. The regional increase in GRLN mRNA expression observed in midgut and hindgut of fed fish when intestine is nearly empty (24 hpf) (Figure 3.1.1, a), and the effect of GRLN on fish gut motility strengthens the theory of GRLN involvement in MMC in Atlantic salmon. MMC has previously been described in trout and cod (Karila and Holmgren 1995; Olsson et al. 1999). GRLN mRNA expression did not increase at any of the time points in stomach or pyloric cecae region. Studies in zebrafish indicate a role for GRLN in motility throughout the GI tract (Olsson et al. 2008). However it is not possible to conclude on the regional effects of GRLN in Atlantic salmon. More studies should be conducted where the protein as well as the mRNA levels of GRLN is analyzed. Also, species specific responses may exist.

4.3.2   Postprandial regulation of digestion: PYY

PYY have been shown to have an inhibitory effect on pancreatic enzyme secretion, gallbladder release and intestinal motility in mammals (Batterham and Bloom 2003). In the current study, there was an increase in PYY mRNA expression at 1.5 hpf and a decrease at 3 hpf in hindgut (Figure 3.3.3; d) which occurred concurrent to gastric emptying and transit of chyme into intestine (Figure 3.1.1; a). These results may thus indicate a postprandial effect on PYY mRNA expression in hindgut. The initial increase in PYY mRNA expression at 1.5 hpf may suggest a possible role for PYY in regulating the digestive process. At 3 hpf and 24 hpf PYY mRNA expression decreases, which may suggest the main postprandial effect on PYY mRNA levels take place before 3 hpf. At 1.5 hpf minimal content is present in hindgut. However, it is possible that initial transit of chyme during this period triggers a rapid and transient response in mRNA expression. At 3 hpf chyme content in the hindgut increased, however the transient response in mRNA expression appeared to be over. It is possible that

processing and secretion of PYY peptides takes place although no changes in mRNA expression were observed at 3 hpf. If so, this agrees with mammalian studies where chyme directly stimulate PYY producing L cells (Adrian et al. 1985).

No postprandial changes in PYY mRNA expression were detected in stomach, pyloric cecae or midgut in Atlantic salmon in the present study (Figure 3.3.3; a, b and c). These results may however indicate an alternative mechanism of stimulating PYY mRNA expression other than direct stimulation by chyme. Studies in rats suggest a vagal stimulation of PYY producing L cells in the distal intestine by food in the proximal intestine (Fu-Cheng et al. 1997). In this study PYY plasma levels increased with peak values at 60 min after feeding, then decreased at 120 min. During this time food had not reached the PYY producing L cells. In the current experiment, PYY expression exhibits a similar short term response pattern after feeding. Thus, a vagal stimulation of PYY expression may be the case also in fish. However, future studies are needed to determine region specific response of PYY in Atlantic salmon.

PYY mRNA expression may also be regulated by other GI tract peptide hormones. Murashita et al. (2008) demonstrate that PYYb mRNA levels in yellowtail decrease after feeding while CCK mRNA expression increases. This is opposite to mammals where CCK has been shown to stimulate PYY release in the distal gut in response to lipids in the proximal gut (Lin et al. 2000a). In the current study CCK-L and PYY show opposite responses in mRNA expression when comparing CCK-L mRNA at 0.5 hpf and 24 hpf in midgut and 1.5 hpf in hindgut, with PYY mRNA in hindgut at the same time points (Figure 3.3.4; c, d and Figure 3.3.3; d, respectively). This could indicate an inhibitory effect of CCK-L in midgut and hindgut on PYY mRNA expression in hindgut.

The physiological effect of these results on digestion may be an initial stimulation of CCK by food in midgut, shown by an increase in CCK-L expression concurrent with an increase in stomach and midgut content. Increased secretion of CCK may then exert its effect on pancreatic secretion, gallbladder contraction and increased intestinal motility. CCK may thus inhibit PYY-inhibition of these effects in the initial phase of digestion, optimizing the digestive process. In contrast to CCK-L, CCK-N mRNA expression increases in stomach at 1.5 hpf. The different expression patterns of CCK-L and CCK-N may suggest different functions, also possibly in inhibition of PYY. It is possible that CCK and PYY are involved in a regulatory feedback loop in Atlantic salmon, similar to yellowtail (Murashita et al. 2008).

This feedback loop may possibly regulate the release of pancreatic secretion, gut motility and gallbladder contraction in response to feeding.

4.3.3 Postprandial regulation of digestion: CCK-L and CCK-N

The effect of CCK on digestion in Atlantic salmon has previously been described by Einarsson et al. (1997). In this study porcine CCK was IP injected and the release of pancreatic enzymes was measured. It was shown that CCK caused a dose dependent release of trypsin and chymotrypsin from the pancreas (Einarsson et al. 1997). In the present study CCK-N mRNA expression in stomach increased at 1.5 hpf, and mRNA levels of CCK-L in midgut increased at 0.5 hpf (Figure 3.3.5; a, and Figure 3.3.4; c, respectively). In this period, high content of feed and chyme was present in the stomach, and was also starting to be evacuated into pyloric region of intestine. The rapid increase in CCK-L levels, followed by CCK-N suggests a postprandial role in regulation of digestion, possibly involving secretion of pancreatic enzymes. Consequently, feed and chyme present in the GI tract may act in the direct stimulation of I-cells. Previous studies in mammals suggest that proteins present in the intestine serve as a substrate for proteases which normally degrades CCK-releasing factors (Liddle 1997). These studies may suggest that a similar mechanism exist in fish, with proteins in the feed indirectly stimulating CCK secretion.

Previous studies in Atlantic salmon show that CCK has a stimulatory effect on gallbladder discharge (Einarsson et al. 1997). In the present study, the increased expression of CCK-N in stomach and CCK-L in midgut correlated with a decrease in gallbladder content (Figure 3.1.2; a). The increased CCK-L mRNA at 0.5 hpf in midgut, was concurrent with a rapid reduction in gallbladder content, while CCK-N in stomach increased at 1.5 hpf when the gallbladder was nearly empty. Thus, increased CCK-L mRNA expression and subsequent CCK secretion from midgut could have induced release of bile in Atlantic salmon. There may be differences in response time or function of different CCK isoforms in this species. Consequently, CCK-L appears to show a more rapid response compared to CCK-N, and may thus have a more rapid excitatory role on gallbladder discharge.

In addition to potential stimulation of pancreatic secretion and gallbladder contraction, CCK may be involved in delaying gastric emptying. Studies in rainbow trout show that gastric emptying involves a lag phase before evacuation of chyme (Olsson et al. 1999). It was further demonstrated that IP injection of CCK-8 delayed gastric emptying, most likely by inhibiting

gastric motility and contracting smooth muscle cells in the pyloric sphincter (Olsson et al. 1999). In this experiment, no direct measurements were performed on gut motility or gastric evacuation of feed. Measurements of content of feed and chyme in fish sampled at different time points after a meal does however give an indication of the anterograde movement of gut content through the GI tract. Based on previous studies in fish, peptide hormones like CCK are expected to have a role in regulating the motility required for digestion and transfer of feed and chyme also in Atlantic salmon. The increase observed at 0.5 and 1.5 hpf in expression of both CCKs correlates with high content of feed and chyme in stomach. It may thus be possible that CCK-N and/or CCK-L is involved in delaying stomach evacuation.

It would be interesting to see the effect of IP injected Atlantic salmon CCK on motility patterns in the stomach and intestine of Atlantic salmon. It would also be interesting to identify how CCK mediates its effect in the GI tract. Olsson et al. (1999) suggest that CCK mediate its inhibitory effect on motility via a vago-vagal non-cholinergic pathway, while the excitatory effect of higher doses of CCK may be mediated via cholinergic pathways. Thus, it is good reason to believe that CCK mediates its effect by a similar mechanism in Atlantic salmon.

4.4 Regulation of appetite: postprandial variation in GRLN, CCK and PYY mRNA expression

4.4.1 GRLN-1 and GRLN-2 in appetite regulation

GRLN has been shown to undergo periprandial variation and increases appetite in both mammals and goldfish (Unniappan et al. 2004; Cummings 2006). In rainbow trout and Mozambique tilapia the role of GRLN in regulating appetite is uncertain (Riley et al. 2005; Jönsson et al. 2007; Shepherd et al. 2007; Fox et al. 2009). In this experiment, no short term effect of feeding was observed for GRLN-1 and GRLN-2 in Atlantic salmon GI tract within the first 6 hours after feeding (Figure 3.3.1, 3.3.2; a, b, c, d). However, higher expression of GRLN-1 and GRLN-2 at 24 hours after feeding was found in midgut and hindgut (Figure 3.3.1, 3.3.2; c, d). During this time period the stomach is almost empty, suggesting a possible return of appetite (Grove et al. 1978). The effect of increased GRLN at 24 hpf, could further indicate a role for GRLN in meal initiation in Atlantic salmon as observed for mammals, as

this time point is characterized by the normal scheduled feeding time (Cummings et al. 2001). More studies should be conducted to establish the effect of GI tract derived GRLN in initiating a meal in Atlantic salmon.

Studies in Mozambique tilapia did not detect periprandial variation in GRLN mRNA expression in the stomach of this species (Fox et al. 2009). In this study, GRLN mRNA levels were measured at 0.5, 2, 10 and 24 hpf in the stomach. No differences were found between fed and unfed Mozambique tilapia in this study. However, Mozambique tilapia does not express GRLN in intestine (Kaiya et al. 2003c). An increase in GRLN expression in Atlantic salmon at 24 hpf in midgut and hindgut might thus be a species specific response due to physiological differences in GRLN expression.

In contrast to what was observed in the present study, and for Mozambique tilapia, instant postprandial responses of GRLN mRNA was observed in goldfish (Unniappan et al. 2004). In goldfish, GRLN undergoes periprandial variation in mRNA expression in the GI tract (Unniappan et al. 2004). Goldfish GRLN mRNA expression decreased at 1- and 3 hpf, suggesting an appetite stimulatory role for GRLN. It should be noticed that in contrast to Atlantic salmon and Mozambique tilapia, goldfish do not have a stomach. The study by Unniappan et al. (2004) measured the total GRLN mRNA expression in goldfish gut, lacking region specific analysis of GRLN expression.

The previous discussion has focused on changes in GRLN mRNA expression. However, several studies report periprandial variation in GRLN plasma levels. Plasma levels of GRLN were not included in this experiment as the objective of this study was to detect changes in mRNA expression of peptide hormones. In goldfish, both GRLN expression levels and plasma levels decreased within 3 hours after feeding. However while unfed fish showed a slight decrease in GRLN expression an increase in plasma levels was observed within the same time scale. One possible explanation for this is that while expression studies of GRLN in gut tissue only detect differences in expression within the gut tissue, plasma levels represents GRLN produced by all tissues secreting GRLN into blood. The increase in plasma GRLN at time 0 in unfed goldfish, which indicate the normal scheduled feeding time, could thus suggest a role for GRLN in meal initiation.

However, Fox et al. (2009) reports no significant periprandial changes in plasma GRLN levels in Mozambique tilapia, concurrent with no changes in GRLN mRNA

expression. Also, no postprandial changes in plasma GRLN levels were observed for rainbow trout (Jönsson et al. 2007). No mRNA levels of GRLN are available for rainbow trout in the study of Jönsson et al. (2007). Although no short term effect was observed in GRLN mRNA expression in Atlantic salmon, studies in tilapia and rainbow trout indicate no postprandial change in GRLN plasma levels. However, since GRLN may exert its appetite effect through direct activation of vagal afferents, the measurements of mRNA expression might have indicated an effect not detected by assessment of GRLN plasma levels (Date et al. 2002). All together, the lack of short term postprandial changes in GRLN mRNA expression in Atlantic salmon indicates that GRLN may not be synthesized as mRNA by the GI tract to mediate short term regulation of feeding.

In addition to a role in appetite, IP injections of GRLN have been shown to stimulate the GH/IGF-1 axis in several fish species (Kaiya et al. 2003a; Unniappan and Peter 2004; Kaiya et al. 2005; Fox et al. 2007). Stimulation of the somatotrophic axis is however mediated through circulation. It is possible that GRLN has a similar role in Atlantic salmon, thus linking short term meal signals to longer term energy balance. Measurements of plasma GH/IGF-1 in rainbow trout, suggest no direct effect of GRLN on GH release (Jönsson et al. 2007). In this study GH levels were elevated in fasted fish, while plasma GRLN levels were suppressed. The lack of effect of GRLN in stimulating GH release may thus be a salmonid adaption.

4.4.2 CCK-L and CCK-N in appetite regulation

Postprandial increase in CCK-L mRNA expression was detected at 0.5 hpf (midgut), while a decrease was observed at 1.5 hpf (hindgut), 3 hpf (stomach) and 24 hpf (midgut). CCK-N mRNA expression was increased in stomach at 1.5 hpf. Altogether the results indicate a short term postprandial effect of CCK-L and CCK-N in Atlantic salmon GI tract. CCK has previously been shown to control appetite as a satiety signal in mammals (Gibbs et al. 1973; Chandra and Liddle 2007). However, little research on the role of CCK in regulating appetite in fish exists. Studies in goldfish and rainbow trout suggest a role for CCK in inhibiting appetite in these species (Himick and Peter 1994; Gelineau and Boujard 2001). In goldfish, CCK IR was detected in brain (including hypothalamic areas) and in anterior gut on nerve fibers and endocrine cells (Himick and Peter 1994). IP injection of sulphated CCK-8 in

goldfish decreased food intake in a dose dependent manner (Himick and Peter 1994). Altogether these studies suggest a role for peripheral CCK in decreasing food intake.

It has previously been shown in rainbow trout that CCK is released in response to an acidification of the proximal intestine and the presence of proteins and fats (Aldman and Holmgren 1995). Thus, it is possible that secreted CCK mediates a satiety action in salmonids.

In this current experiment, CCK-L showed higher expression in fed fish compared to unfed fish in midgut at 0.5 h after feeding. Himick and Peter (1994) demonstrate an immediately decrease in food intake when goldfish receive IP injection of CCK-8 30 min after feeding. Although feed intake was not monitored in this current experiment, an increase in CCK-L mRNA expression in Atlantic salmon midgut 30 min after feeding suggest a similar satiety action in these species. A higher mRNA expression of CCK-L was also observed in midgut at 24 hpf. At this time point, minimal chyme was present in this region. The potential role for increased CCK-L in regulation appetite at this time point is thus uncertain. In contrary to midgut, CCK-L exhibited no postprandial change in mRNA expression in Atlantic salmon hindgut at 0.5 hpf, and decreased at 1.5 hpf. Although CCK-L was expressed in low levels in stomach, a decreased mRNA expression was detected at 3 hpf. In conclusion, these results require further studies.

Although CCK-N exhibits a different regional tissue distribution than CCK-N, similarities exist in the postprandial regulation with respect to time. CCK-N mRNA levels were higher at 0.5 hpf, but instead of midgut tissue, this elevated expression was found in stomach tissue. Again this points to a region specific response for the different CCK isoforms. The elevation in mRNA expression of both CCK isoforms shortly after feeding, suggest a role for CCK as a satiety signal in Atlantic salmon. It is possible that CCK-N mediates a potential satiety effect in stomach in response to stomach filling. In contrast, CCK-L may possibly serve as an intestinal satiety signal, stimulated when food and chyme enters the midgut. Although the stomach is filled with chyme at 0.5 hpf, the increase in CCK-N in stomach at 1.5 hpf may provide a lag phase prior to mediating a potential satiety effect. In all cases CCK expression decreased at 3 hpf, indicating a rapid postprandial effect (within 1.5 hours) on mRNA expression.

In an experiment, rainbow trout were fed capsules containing CCK-1 receptor antagonist (Gelineau and Boujard 2001). The study showed a significant increase in appetite in fish provided CCK-1 receptor antagonist, indicating a role for CCK as a satiety signal trough CCK-1 receptors in rainbow trout. The present study is to our knowledge the first study that aims to describe the postprandial mRNA expression of CCK in GI tract of Atlantic salmon. It is possible that CCK have a satiety effect in Atlantic salmon similar to goldfish and rainbow trout. It is also unknown whether the distribution of CCK-1 receptors is similar for Atlantic salmon and rainbow trout. However, the two salmonid species show differences in regional CCK mRNA expression (as previously described). If the satiety effect of CCK is mediated through CCK-1 receptors on vagal afferents in Atlantic salmon as described for mammals, it may be activated by nearby CCK producing cells (Raybould 2007). It is thus possible that a region specific activation of CCK receptors and satiety exists for Atlantic salmon. As CCK mediates its satiety effect through CCK-1 receptors in mammals and trout, knowledge on tissue distribution of this receptor in Atlantic salmon would be valuable in describing the mechanism CCK mediates its action (Gelineau and Boujard 2001; Chandra and Liddle 2007).

4.4.3 PYY in appetite regulation

The physiological role of PYY on appetite regulation is not known for fish. However, the available information of PYY in regulating food intake is derived from mammalian studies. In mammals, PYY is characterized as a satiety signal based on a preprandial decrease and a postprandial increase in plasma concentration (Ueno et al. 2008). Both intravenous infusion and chronic administration of PYY_{1-36} in mammals decrease food intake (Batterham et al. 2003). In mammals plasma levels of PYY begin to rise within 15 min of the start of eating, with peak values within 90 min (1.5 hpf), and high levels kept until 6 h after a meal (Adrian et al. 1985).

A short term postprandial increase in PYY levels in mammalian hindgut 1.5 hpf is in agreement with our results. This could indicate a role for PYY in mediating a satiety effect from the hindgut of Atlantic salmon. The rapid response in PYY mRNA levels 1.5 hpf in hindgut may possibly have provided an appetite regulating signal in the form of increased PYY secretion. However, these results require future studies. The lack of postprandial effect on PYY mRNA levels in stomach, pyloric cecae or midgut may indicate a region specific

response to feeding within the GI tract. Although minimal chyme content were observed in hindgut at 1.5 hpf, chyme present in proximal pyloric cecae region could have initiated a neuronal signal which further stimulates PYY secreting cells in hindgut as seen in mammals (Fu-Cheng et al. 1997). The decrease in PYY expression observed at 3 hpf in hindgut, could however not be explained by this hypothesis as chyme is still present in proximal intestine at this time point. Consequently, PYY may thus initiate a rapid response to feeding within 1.5 hpf in Atlantic salmon.

Although fish PYYb belongs to a different phylogenetic group than fish PYYa, they both originate from a common PYY ancestor gene (Larhammar 1996; Murashita et al. 2009). The similar tissue distribution pattern between Atlantic salmon PYYa and yellowtail PYYb could imply similar physiological functions (Murashita et al. 2006; Murashita et al. 2009). Murashita et al. (2006) found decreased PYYb expression in anterior intestine of yellowtail 3 hpf, compared to fish fasted for 72 h. No such differences were found in this current experiment in anterior intestine of Atlantic salmon. This could indicate a species specific response to feeding by PYY. At time 3 h in this current experiment, unfed fish was fasted for 27 h, and maximum hours of fasting was 48 hours. Thus, differences in the experimental conditions could have affected the difference in results. In the yellowtail experiment only 3 hpf-fish were sampled, lacking a postprandial time scale. However, the lower expression of PYYb in fed yellowtail indicates an opposite effect in response to feeding compared to mammals (Murashita et al. 2006).

In conclusion, very limited knowledge exists on the effect of nutrient intake on PYY expression in fish. It is possible that PYY signals a satiety signal via Y2 receptors to hypothalamic brain centres involved in appetite regulation as is the case in mammals (Batterham et al. 2002). Recently NPY-receptors were identified in rainbow trout (Larsson et al. 2006). This study showed that Y7 receptors were more abundant in the intestine than Y2 receptors which were most abundant in gills. PYYa and PYYb bound to both receptors, indicating that PYY produced in GI tract signals through Y7 receptor. This also strengthens the theory of similar functions of PYYa and PYYb. No information exists on the effect of this binding. It is possible that Atlantic salmon PYY binds to nearby intestinal Y7 receptors in a paracrines manner if these receptors exist in this species. It is also possible that PYY produced in the intestine is transferred by the blood and binds to Y2 receptors on gills or distant targets including brain areas involved in feeding regulation.

4.5 Circadian variation in GRLN-1, GRLN-2, PYY, CCK-L and CCK-N

Food availability and meals can act as a powerful zeitgeber capable of synchronizing many biological rhythms in fish (SanchezVazquez et al. 1997). Goldfish synchronized to feeding at a particular schedule, overcame variations in light cycles and altered their activity pattern according to meals (SanchezVazquez et al. 1997). GRLN has been shown to change in a diurnal pattern in mammals, with levels rising throughout the day to a zenith at 01.00, then falling overnight to 09.00 (Cummings et al. 2001). In this experiment fish were fed once a day at the same time of day in two weeks prior to sampling. This period should have acclimated the fish to the feeding regime. Unfed fish were subsequently fasted for 24 hours prior to sampling. Results from the assessment of GI tract compartment filling and gallbladder content in unfed control group, show an empty GI tract (Figure 3.1.2; b) and gallbladder with overall high content (Figure 3.1.3; b) throughout the experimental period. Minimal effects of feed should thus have influenced the expression of the analyzed genes. The results from this current study showed no statistical effect of time alone on mRNA copy numbers of peptide hormones in unfed fish. Consequently, these results indicate minimal effect of diurnal rhythms on mRNA expression patterns of GI tract peptide hormones in Atlantic salmon. Although not statistical significant, the expression of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N in unfed fish shows minor variations in expression over time. In pyloric cecae, CCK-L expression increases from time 0 h (fish fasted for 24 hours) to 0.5 h. Thus, CCK-L may be an exception compared to the other genes. It is also possible that a circadian rhythm apart from a 24 h-profile exist in fish. However, in order to detect potential diurnal expression patterns, or alternative circadian profiles of GI tract derived peptide hormones, further studies are needed. In conclusion, no clear effect of diurnal rhythm on mRNA expression levels of GI tract peptide hormones was detected in Atlantic salmon.

5. Concluding remarks and future perspective

5.1 Concluding remarks

5.1.1 Spatial distribution of peptide hormones in Atlantic salmon GI tract

GRLN-1 and GRLN-2 expression was found throughout the GI tract in Atlantic salmon. Both isoforms exhibited the same spatial distribution. The highest GRLN expression was found in stomach, as seen in previous studies in vertebrates. Moderate expression was detected in pyloric cecae and midgut, and lowest expression was found in hindgut. PYY was expressed in all segments of the GI tract. The highest PYY expression was found in pyloric cecae and midgut, moderate expression in hindgut and lowest expression in stomach. CCK-L and CCK-N exhibits different tissue expression patterns, suggesting different functions in Atlantic salmon (see Figure 5.1). CCK-L exhibited highest mRNA levels in hindgut, with slightly lower expression in pyloric cecae and midgut. The lowest CCK-L expression was detected in stomach. CCK-N is mainly expressed in the stomach and pyloric cecae region, while only low expression levels were detected in midgut and hindgut. The spatial expression patterns of peptide hormones suggest involvement in regulation of digestive process and appetite.

5.1.2 Gastric filling and evacuation, GI tract transit of feed and chyme, and gallbladder release in Atlantic salmon

The stomach of Atlantic salmon was filled with feed and chyme at 0.5 hpf, shortly followed by gastric evacuation mainly between 0.5-12 hpf. At 24 hpf the stomach was empty. Pyloric cecae, midgut and hindgut show more gradual changes in content, indicating a regulated emptying of stomach content, and absorption of nutrients, however not assayed. At 24 hpf all GI tract compartments are empty, indicating the end point of digestion. The immediate release of gallbladder content within 0.5-1.5 hpf, suggest a rapid excitation of gallbladder contraction possibly induced by chyme in the proximal intestine or feed in the stomach. In conclusion these results indicate that the digestive process in Atlantic salmon is highly regulated by synchronizing events such as; gastric evacuation, GI tract peristalsis, digestive secretion, absorption and evacuation of non-digestible feed. GI tract peptide hormones are expected to be involved in this regulation.

5.1.3 Postprandial changes in mRNA expression of GI tract peptides and the possible involvement in regulating the digestive process and appetite in Atlantic salmon

Both CCK-L and CCK-N mRNA levels increased within 1.5 hpf in midgut and stomach, respectively, concurrent with an increase in feed and chyme in stomach and a decrease in gallbladder weight. This supports a possible role for CCK in regulating gastric evacuation, pancreas secretion and gallbladder contraction in Atlantic salmon (Figure 5.1). In addition, it is also possible that an increase in CCK mRNA expression in response to feeding is to mediate a satiety signal from the gut to the brain (Figure 5.1). PYY mRNA expression increases in 1.5 hpf, then decreases at 3 hpf. In this period chyme is evacuated into the pyloric region and is also present in midgut and hindgut, while the gallbladder is empty. These results support a role for PYY in regulating the digestive process, possibly involving GI tract motility and digestive secretion. In addition, PYY may also regulate appetite in Atlantic salmon (Figure 5.1). GRLN mRNA expression is not increased before 24 hpf, a time point characterized with minimal content of feed and chyme in the GI tract. Thus, GRLN may not have a short term postprandial effect on digestion, but may have a between-meal effect related to regulation of GI tract motility and a role in meal initiation (Figure 5.1). The mRNA expression results of CCK and PYY may further suggest that these peptide hormones are involved in a regulatory feedback loop where CCK possibly inhibits PYY. Consequently, CCK and PYY may have antagonistic effects on gallbladder contraction, pancreatic secretion and possibly GI tract motility in Atlantic salmon.

5.1.4 Diurnal influences and possible suggestions of species specific effects on mRNA expression of GI tract peptide hormones in Atlantic salmon

No clear diurnal rhythms were observed in mRNA expression of GRLN, PYY or CCK in unfed Atlantic salmon. However alternative circadian rhythms may still exist which may have a potential effect on GRLN, PYY and CCK mRNA levels or peptide levels. If this is the case, GRLN, PYY and CCK may be involved in long term energy homeostasis in Atlantic salmon.

Several differences in results were detected between this current experiment and mammals, and also between other fish species. Goldfish has been used as an extensive model organism when studying the effect of appetite regulating hormones. This species is in contrast to Atlantic salmon an omnivorous temperate freshwater fish that usually lives under relatively

constant environmental conditions (Volkoff et al. 2009). Atlantic salmon is a carnivorous euryhaline aquaculture species adapted to seasonal changes in temperature and light conditions. As fish are a highly diverse group of ectothermic species, the surrounding environment will influence the different species in a higher degree than mammals. Thus the difference in habitats, feeding habits and response to feeding suggest a species specific endocrine regulation of digestion and appetite in Atlantic salmon.

Figure 5.1. Suggested scheme of central and peripheral effects of gastrointestinal (GI) peptide hormones in Atlantic salmon based on their mRNA expression levels during processing of a single meal. *See next page for legend (page 71).*
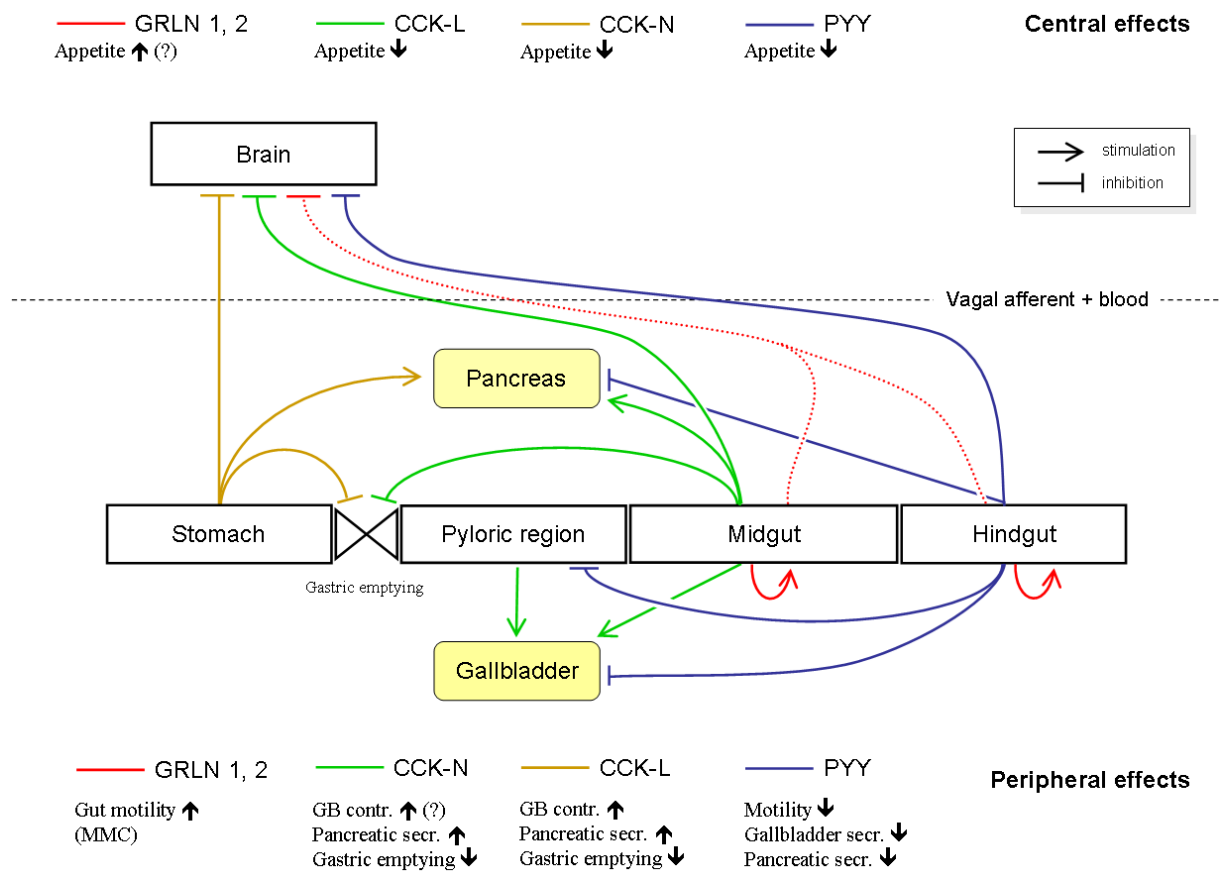
Figure 5.1. Suggested scheme of central and peripheral effects of gastrointestinal (GI) peptide hormones in Atlantic salmon based on their mRNA expression levels during processing of a single meal. *The GI tract is represented by four compartments including; stomach, pyloric region, midgut and hindgut with pancreas and gallbladder. The area between the stomach and pyloric region illustrates the pyloric sphincter and thus the control of gastric emptying. The origin site of the arrows represents a significant increase in mRNA expression of the representative peptide hormone in this compartment in the present experiment. The end of the arrow represents a suggested effect (stimulatory/inhibitory) of this peptide hormone on possible locations. Arrows arising and ending at the same target indicate local paracrines effects. The central effect of each peptide hormone is listed on top of the figure, while peripheral effects are listed on the bottom of the figure. Arrows pointing upwards next to a description indicates a possible stimulatory effect on this description, while arrows pointing downwards indicate a possible inhibitory effect. The dotted colored lines represent less supported effects. Colored lines crossing the dotted line running across the figure indicates indicate GI tract peptide hormones mediating a central effect on brain areas through vagal afferents or through the blood. The effect of GRLN (GRLN)- 1 and -2 are shown as one color coding (red) as no differences in mRNA expression were detected between the isoforms. MMC; migrating motor complex, GB; gallbladder, Secr.; Secretion, Contr.; Contraction.*

5.2   Future perspective

The current study is the first extensive description of the postprandial changes in mRNA expression of GRLN, PYY and CCK in Atlantic salmon. The results of these changes was then related to gastric evacuation, gut transit of feed and chyme and regulation of gallbladder contraction hence bile release. The data also indicate some of the roles served by these peptide hormones in the regulation and optimization of digestion and appetite in Atlantic salmon. However, in order to achieve a clearer understanding of the roles of GI tract peptide hormones in Atlantic salmon, future research should aim to assess protein levels as well as plasma concentrations, in addition to gene expression analysis of GI tract peptide hormones. This will enable to assess both their endocrine and paracrines roles. In addition experiments should be conducted to obtain information concerning the local effect of different peptides in the GI tract and both *in vivo* and *in vitro* studies could be included. Both GRLN, PYY and CCK have been described as brain-gut peptides. In the brain, the gut derived signals are involved in multiple redundant pathways regulating feed intake in order to maintain energy homeostasis (Volkoff et al. 2009). The distribution of GHS-R, CCK-R and NPY

receptors should be assessed to increase the knowledge of the regional effect of GRLN, PYY and CCK within Atlantic salmon GI tract, and in additional tissues including brain areas.

It would also be valuable to study the effect of both IP injected and centrally injected GRLN, PYY and CCK on appetite and digestion. In this case the use of recombinant peptides provides a valuable tool, not only in preparation of injections, but also by adding recombinant peptides into osmotic pumps. Insertion of osmotic pumps into Atlantic salmon can then be used to study the longer term (week-month) effects of peptide hormones on appetite and energy homeostasis, and potential alternative functions in this species.

The pathways GRLN, CCK and PYY are involved in when mediating a signal should also be studied to increase the knowledge of the roles of each peptide hormone. It has previously been shown that GRLN mediate its appetite stimulatory action via the orexigenic NPY in the goldfish brain (Miura et al. 2006). Further, CCK has been shown to mediate the effect of leptin in goldfish (Volkoff et al. 2003a). In goldfish leptin potentiates the satiety effect of CCK, and a blocking of CCK receptors attenuate leptin inhibition of NPY induced feeding (Volkoff et al. 2003a). PYY have been shown to inhibit NPY induced feeding in mammals, but limited information of PYY exist for fish (Vincent and le Roux 2008).

Several of the gut derived peptide hormones have been shown to respond differently according to meal composition and total calorie content. It should thus be of uttermost interest to the aquaculture industry to study the effect of feed with different compositions of proteins, lipids and carbohydrates on the digestive process and appetite in Atlantic salmon. An increased knowledge of the dynamic process of digestion and its complex regulation is needed in order to produce fish feeds with alternative ingredients that still maintain appetite and an efficient digestion in Atlantic salmon.

6. Methodological considerations

6.1 Gene expression

Q-PCR is considered to be the method of choice for detecting low abundance mRNA due to its high sensitivity (Bustin 2000). It is a highly sensitive method which may compromise the specificity of the reaction and detect false positives, as it does not distinguish between illegitimate transcripts and low abundance transcripts (Kwok and Higuchi 1989) .Several critical areas must be taken into consideration before analyzing the transcriptome such as; standardization of q-PCR protocols, assay design, reagents, template preparation and analytical methods (Freeman et al. 1999). In addition to operator variability; template quality, reverse transcription (RT) reaction and data analysis are all technical aspects which could bias the data interpretation (Bustin and Nolan 2004). The areas which will be discussed in this part include; sample acquisition, RNA isolation, DNase treatment, cDNA synthesis and qPCR. RNA quality and the reverse transcription reaction will be especially emphasized, as downstream results demands optimality of these factors.

6.1.1 Sample acquisition and storage

The first step towards qPCR is acquisition of biological material. The integrity and quality of RNA are the most critical determinants for reliable downstream qPCR results (Bustin and Nolan 2004). As RNA is unstable, the initial tissue preservation should be executed in a carefully manner. In this study, gut tissue were sampled quickly on dry ice after the removal of gut content. Samples wrapped in aluminum foil were then fresh frozen in liquid nitrogen. The frozen samples were kept at -80° C for storage. Prior to RNA isolation, samples were at all times kept on dry ice when not in -80° C freezer to avoid thawing.

6.1.2 Assessment of RNA quantity, quality and integrity

Prior to RNA isolation, tissue samples were weighed within a standardized range of 40-45 mg ± 5 %. 10 % of the samples had equal to or below 36 mg as total weight, for these samples all of the tissue was used. To estimate total RNA quantity and to ensure pure RNA samples, all samples were analyzed using NanoDrop ND-1000 spectrophotometer (Thermo Scientific), measuring sample absorbance. The A260/A280 gives an estimate of the RNA to protein ratio. As proteins and phenol absorb strongly around 280 nm, an impure sample will

have a lower A260/A280 value. A value of ~2.0 is accepted as pure for RNA, though this value may be influenced by pH and ionic strength in solvent (Wilfinger et al. 1997). All RNA was dissolved in Nuclease-Free Water (Ambion) and found to have A260/A280 value > 2.0 (results not shown). An overestimation of total RNA concentration may occur if DNA is present in the sample. This is caused by the absorbance of DNA at 260 nm (Imbeaud et al. 2005). All samples were DNase treated prior to cDNA synthesis to ensure RNA of high quality and correct quantity to be submitted to cDNA synthesis.

A secondary qualitative measure of A260/A230 was included for all samples. Both carbohydrates and phenol absorb at wavelength 230 nm, A260/A230 ratio should thus be above ~2.0-2.2 in order to classify a sample as pure (ThermoScientific 2009).All samples showed A260/A230 ratio above 2.2.

Although the results from the A260/A280 and A260/A230 ratios indicate a good quality sample in terms of pure RNA, these values do not give an estimate of RNA quality in terms of integrity; hence "RNA intactness". The RIN value obtained from Agilent 2100 Bioanalyzer (Agilent Technologies) was thus used a superior RNA quality estimate.

When handling RNA, RNases and long term storage under sub-optimal conditions can impair RNA integrity (Schoor et al. 2003). Thus, every RNA preparation should be tested for RNA integrity (Bustin and Nolan 2004). The RIN based Agilent method uses "state of the art technology", and is the most objective way of assessing RNA integrity (Bustin and Nolan 2004). Agilent 2100 uses microfluidic capillary electrophoresis technology, and in combination with a size standard, RNA bands can be estimated and contaminants ignored (Imbeaud et al. 2005). A RIN value of 1 corresponds to a completely degraded sample and a value of 10 indicates a fully intact sample (Mueller et al. 2000). Degraded RNA can affect later Ct values in q-PCR (Raeymaekers 1993; Imbeaud et al. 2005). It is also shown that RIN values and Ct values possess a negative relationship, where typically degraded RNA with low RIN value show higher Ct values (lower expression), (Fleige et al. 2006; Schroeder et al. 2006). A more fragmented sample demands a higher number of cycles to obtain cycle threshold. Fleige et al. (2006) suggest that all RIN values > 8 can be considered as optimal templates; RIN values 8 > 5 can result in sub optimal qPCR results, and degraded mRNA with RIN < 5 can interfere with PCR performance.

RIN values were obtained on all samples. 57 % of the samples showed partially degradation with total sample RIN average of 7.8 (±0.99 SD) (Appendix III, Figure AIII.2). There appeared to be a tissue/segment variation in RIN value, with stomach tissue having the most intact RNA with average RIN values of 8.9 (±0.68 SD). Tissue from pyloric cecae showed average RIN value of 7.0 (±0.53 SD). The midgut and hindgut both showed large variations within the group with average RIN values of 7.6 (±0.88 SD) and 7.5 (±0.76 SD), respectively (see Appendix III, Figure A.III.2). Consequently all samples were analyzed by linear regression plotting Ct values against RIN.

Table 3.4 lists the r^2 and p values of the linear regression, with p<0.05 considered significant correlation. The coefficient of determination r^2, is used as a measure of the straight line relationship with $r^2=1$, showing full explanation of the y value (Ct) by the regression line (Zar 1996). All genes analyzed in segment 4 (pyloric cecae) show significant p values at α = 0.05, although the r^2 show larger variation within the values for the different genes (Table 3.4). For a graphical presentation of the linear regression for all genes, see Appendix III, Figure A.III.3-10. The pyloric cecae are intervened with pancreatic tissue in Atlantic salmon which produces several enzymes including RNase. What can also be seen in Table 3.2 is that r^2 for PYY in pyloric cecae is higher compared with the other genes with 30.9% of variation in ct values explained by the regression line (p<0.05) followed by EF1α ($r^2=0.290$, p<0.05). An r^2 value of 0.309 should still be considered as a low value, indicating that the regression line explain the correlation between Ct and RIN for ~31% of the samples. These results are in contrast to the study executed by Fleige et al. (2006), where average $r^2 > 0.812$. In contrast to pyloric cecae, the stomach, midgut and hindgut each show significant values only for a few genes. No single gene show significant values for all tissues (Table 3.2). Consequently, the effect of assay is thought to have minor influence of Ct/RIN correlation. In conclusion, the results indicate a tissue specific effect on RIN, which is in accordance with Fleige et al. (2006). Furthermore, results from linear regression of Ct and RIN in the current study do not show an obvious relationship of low quality RNA and high Ct values, with the possible exception of pyloric cecae. The use of sub optimal RNA in q-PCR does not appear to affect Ct results in a critical manner.

On the basis of these findings, an experiment was set out to obtain the optimal sampling strategy for ensuring high quality RNA. In this experiment several GI tract sampling strategies were tested in both fed and unfed fish; 1) GI tract content were removed and tissue

cut on dry ice, 2) GI tract content were removed on bench top (room temperature) and the tissue cut on dry ice, and 3) the GI tract were immediately transferred to RNA *later®* (Ambion, USA) where the content was removed and the GI tract cut into segments. The second sampling strategy is identical to the sampling strategy applied in the present study. The results from this experiment should hopefully indicate which sampling strategy gives the highest RNA quality, and will clarify whether the current sampling strategy is optimal when sampling GI tissue (Valen et al., in prep).

6.1.3 Removal of genomical DNA

In this study all samples were DNase treated, thus minimal traces of DNA should be present in sample prior to reverse transcription. The effect of this step is somewhat questionable as oligo-DT primers used in cDNA synthesis poly(A)$^+$-selects mRNA. However, DNase treatment may have a negative effect on RNA integrity, hence RIN value (personal communication, Ann-Elise O. Jordal).

6.1.4 Reverse transcription: Synthesis of cDNA

The reverse transcription reaction was separated from the qPCR reaction and consequently the RNA- and DNA-dependent polymerases were added to different tubes. Also, leftover cDNA could be kept for long term storage. The conversion of RNA into single stranded cDNA is not a well understood step, and is considered to be the most uncertain step in gene expression analysis (Stahlberg et al. 2004). Sources of variation are; biological inherent variation in RNA, variable quality of RNA and hence stability, RNA template abundance, background nucleic acids, secondary and tertiary structure of mRNA, priming approaches and properties of the reverse transcriptase enzyme itself.

In the current study, SuperScript™ III First-Strand Synthesis System for RT-PCR was used. The SuperScript III Reverse Transcriptase enzyme is a version of M-MLV RT which has less RNAse H activity, and is the better choice if full length cDNA are to be amplified (Bustin 2000). Oligo(dT), which poly(A)$^+$-selects RNA, was used as primer in the cDNA synthesis. Oligo(dT) primers have the ability to maximize the number of mRNA molecules that can be analysed from a small sample of RNA (Bustin 2000). It is more specific than random priming, but as it requires full length RNA (with intact poly(A) tail), it can be less efficient when it comes to transcribing fragmented RNA (Bustin et al. 2005), especially

mRNA which is not intact in the 3' end (Kubista et al. 2006). As samples showed variable RIN values, different efficiencies in the RT step may be the case. However, as concluded earlier the use of sub optimal RNA in q-PCR does not appear to affect the Ct results in a critical manner.

In order to minimize sample to sample variation, samples were run in duplicate reactions taken from the same DNase treated RNA pool. According to Stahlberg et al. (2004), both inhibition that affect reaction efficiencies, and efficiency deviation that cause variation in copy number are eliminated by running multiple reactions in cDNA synthesis and averaging the measurement results. Stahlberg et al. (2004) show that by splitting the samples before reverse transcription a two times higher accuracy in sampling error occur, than by splitting the samples in the qPCR step.

A minus reverse transcriptase (-RT) control was included in each PCR run to check for genomic DNA contamination in the RNA sample. No signal was observed in the –RT, indicating minimal SYBR green background binding due to gDNA. To avoid variation caused by different stocks of reagents, reagents were pooled and then aliquoted to minimize stock-to-stock variations as far as possible.

6.1.5 Q-PCR

For the qPCR reaction, SYBR green I based Power SYBR$^®$ Green PCR Master Mix was used (Applied Biosystems). SYBR green I is a DNA binding dye, which binds in the minor groove of double-stranded DNA in a sequence independent way and the fluorescence increases over 100-fold when bound (Deprez et al. 2002). Melting curve analysis was performed to detect unwanted products (primer-dimers) that may occur because SYBR green I bind non-specifically to double-stranded DNA. By slowly increasing the temperature above T_m (melting temperature) of the amplicon, the fluorescence signal changes. Plotting fluorescence as a function of temperature can distinguish the characteristic peak of the product from artifacts that typically melt at lower temperatures (Bustin 2000). No primer-dimers were detected for any of the assays. The threshold cycle fluorescence level was set manually to 0.010, above background noise and within the exponential phase for all qPCR runs. As all cDNA samples were diluted the effect of potentially PCR inhibitory substances is reduced (Kubista et al. 2006). A non template control (NTC) and a between plate control (BPC), were included to detect contamination in mastermix and water, and to detect variations

between plates, respectively. No signal was observed in NTC control. The standard deviation of BPC for each gene was as follows; EF1α: 0.21, GRLN-1: 0.35, GRLN-2: 0.56, PYY: 0.49, CCK-L: 0.52 and CCK-N: 0.54.

The eukaryotic elongation factor 1α (EF1α) was included in the qPCR reaction as an endogenous control to compensate for intra- and inter-kinetic qPCR variations (Pfaffl 2004). Since the GI tract exists of several segments extracted from different individuals, normalizing the data against a constantly expressed reference gene may adjust for some of these differences. EF1α have been tested as a candidate reference gene in Atlantic salmon and shown to be a suitable choice (Olsvik et al. 2005).

6.1.6 Quantification

In this study absolute quantification was used. This quantification strategy relates qPCR signal (Ct value) to input copy number using an external calibration curve (Pfaffl 2004). Although this method produces highly specific, sensitive and reproducible data (Bustin 2000), the accuracy of absolute quantification in qPCR depends entirely on the accuracy of the standards (Pfaffl 2004). External calibration curves can be generated from recombinant RNA (recRNA) and recombinant DNA (recDNA) (Pfaffl and Hageleit 2001). Pfaffl et al. (2001) suggest that using recDNA calibration provides a better model than recRNA in quantifying mRNA, in terms of quantitative range, sensitivity, reproducibility and stability. The standards used for all assays were produced from recDNA by Dr. Koji Murashita (Murashita et al. 2009).

RecDNA does not undergo reverse transcription like the mRNA samples, thus variability of the qPCR results and amplification results may lead to difficulties when comparing results from the tested sample (Pfaffl 2004). CDNA efficiency was monitored as a function of input total RNA and linear regression performed (Appendix III, Figure A.III.1). This is important as the recDNA calibration curve model only measures the cDNA out of RT reaction, and this may not reflect the mRNA molecules in the native sample. The linear regression, r^2 value was 0.935, indicating a linear relationship between Ct and input RNA, but with a certain deviation from of a perfectly linear relationship (approaching $r^2 = 1$). This deviation could be caused by cDNA synthesis efficiency below 100 %, or inaccurate pipetting. In the current study, samples were run in parallels in cDNA synthesis which is thought to minimize the effect of suboptimal efficiency in RT reaction (Stahlberg et al. 2004).

6.2 Randomization

All tissue samples were randomized across all segments when isolating RNA and during DNase treatment and cDNA synthesis. When running RNA integrity analysis, samples were randomized according to http://randomization.com. In q-PCR, samples were run chronologically according to sample id. On the q-PCR plate, each sample was loaded into the same well location for the various assays, thus minimizing the effect of well variation on individual samples. Pipetting was done by one operator, thus minimizing operator variability.

6.3 Statistical methods

In the present study, parametric tests were chosen over nonparametric tests. The parametric tests make more assumptions than the nonparametric tests, but may give a more accurate and precise estimate, with the nonparametric tests requiring a larger sample size to achieve the same power (Walpole 2002). The underlying assumptions of parametric tests include random sampling from a normal distributed population, and equal variance between groups (Zar 1996).

All samples were randomly sampled in this study as far as possible and for each level of analysis and sampling. When it comes to testing for normality, the Shapiro-wilks W test was chosen due to its good power (Zar 1996). The test has been shown to be robust even for small sample sizes with n < 20 (Shapiro and Wilk 1965). Since some groups mean deviated from normality, the data were log transformed. After this only a few groups failed the normality assumption (see Appendix III).

In order to detect differences between means of unfed and fed fish groups at a given time point, a *t* test was used giving the two-tailed hypothesis:

H_0: $\mu_1 - \mu_2 = 0$ an H_A: $\mu_1 - \mu_2 \neq 0$. This parametric test is extremely robust despite non-normality and heterogeneity of variance (Zar 1996).

The assumption that samples come from a normally distributed population and show homogeneity of variance is true also for analysis of variance (ANOVA). Especially if *n* is equal for all groups (Zar 1996), as is the case for this study. The weaker non-parametric tests show lack in the robustness of normality and variance assumptions. For these reasons, ANOVA was the preferred choice.

When three or more samples are included in a group, the hypothesis that the samples came from a population with identical variances can be tested (Zar 1996). Due to its robustness against non-normality, Levene´s F-test was used to test for equality in variance (Zar 1996).

In the current study statistical tests were at times run on mRNA expression data for three fish, which is a minimum number of individuals required for statistical analysis in several statistical tests. However, originally six fish were sampled at each time point of the experiment. The reason for the reduction in fish number was due to fish in fed fish group which had not eaten and had large gallbladders (and vice versa for unfed fish control), and consequently would have biased the results. In addition the aspects of running costs associated with q-PCR and time available during this thesis limits the number of fish in each time group.

7. References

Adrian, T. E., G. L. Ferri, A. J. Bacaresehamilton, H. S. Fuessl, J. M. Polak and S. R. Bloom (1985). "Human distribution and release of a putative new gut hormone, peptide-YY." Gastroenterology **89**(5): 1070-1077.

Adrian, T. E., G. R. Sagor, A. P. Savage, A. J. Bacaresehamilton, G. M. Hall and S. R. Bloom (1986). "Peptide YY kinetics and effects on blood-pressure and circulating pancreatic and gastrointestinal hormones and metabolites in man." Journal of Clinical Endocrinology and Metabolism **63**(4): 803-807.

Aldman, G. and S. Holmgren (1995). "Intraduodenal Fat and Amino Acids Activate Gallbladder Motility in the Rainbow Trout, Oncorhynchus mykiss." General and Comparative Endocrinology **100**(1): 27-32.

Amole, N. and S. Unniappan (2009). "Fasting induces preproghrelin mRNA expression in the brain and gut of zebrafish, Danio rerio." General and Comparative Endocrinology **161**(1): 133-137.

AppliedBiosystems. (2003). "Creating standard curves with genomic DNA or plasmid DNA templates for use in quantitative PCR." from http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf.

Ariyasu, H., K. Takaya, T. Tagami, Y. Ogawa, K. Hosoda, T. Akamizu, M. Suda, T. Koh, K. Natsui, S. Toyooka, G. Shirakami, T. Usui, A. Shimatsu, K. Doi, H. Hosoda, M. Kojima, K. Kangawa and K. Nakao (2001). "Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans." Journal of Clinical Endocrinology and Metabolism **86**(10): 4753-4758.

Arvat, E., L. Di Vito, F. Broglio, M. Papotti, G. Muccioli, C. Dieguez, F. F. Casanueva, R. Deghenghi, F. Camanni and E. Ghigo (2000). "Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans." Journal of Endocrinological Investigation **23**(8): 493-495.

Baeverfjord, G. and A. Krogdahl (1996). "Development and regression of soybean meal induced enteritis in Atlantic salmon, Salmo salar L, distal intestine: A comparison with the intestines of fasted fish." Journal of Fish Diseases **19**(5): 375-387.

Bahuaud, D., T. K. Østbye, B. E. Torstensen, M. B. Rørå, R. Ofstad, E. Veiseth, M. S. Thomassen and B. Ruyter (2009). "Atlantic salmon (Salmo salar) muscle structure integrity and lysosomal cathepsins B and L influenced by dietary n-6 and n-3 fatty acids." Food Chemistry **114**(4): 1421-1432.

Bail, P.-Y. L. and G. Ruf (1997). "What hormones may regulate food intake in fish?" Aquat. Living Resour. **10**(6): 371-379.

Batterham, R. L. and S. R. Bloom (2003). The gut hormone peptide YY regulates appetite. Melanocortin systems. R. D. Cone, New York Acad Sciences. **994:** 162-168.

Batterham, R. L., M. A. Cohen, S. M. Ellis, C. W. Le Roux, D. J. Withers, G. S. Frost, M. A. Ghatei and S. R. Bloom (2003). "Inhibition of food intake in obese subjects by peptide YY3-36." New England Journal of Medicine **349**(10): 941-948.

Batterham, R. L., M. A. Cowley, C. J. Small, H. Herzog, M. A. Cohen, C. L. Dakin, A. M. Wren, A. E. Brynes, M. J. Low, M. A. Ghatei, R. D. Cone and S. R. Bloom (2002). "Gut hormone PYY3-36 physiologically inhibits food intake." Nature **418**(6898): 650-654.

Batterham, R. L., H. Heffron, S. Kapoor, J. E. Chivers, K. Chandarana, H. Herzog, C. W. Le Roux, E. L. Thomas, J. D. Bell and D. J. Withers (2006). "Critical role for peptide YY in protein-mediated satiation and body-weight regulation." Cell Metabolism **4**(3): 223-233.

Bednarek, M. A., S. D. Feighner, S. S. Pong, K. K. McKee, D. L. Hreniuk, M. V. Silva, V. A. Warren, A. D. Howard, L. H. Y. Van der Ploeg and J. V. Heck (2000). "Structure-function studies on the new growth hormone-releasing peptide, ghrelin: Minimal sequence of ghrelin necessary for

activation of growth hormone secretagogue receptor 1a." Journal of Medicinal Chemistry **43**(23): 4370-4376.

Bell, J. G., J. McEvoy, D. R. Tocher, F. McGhee, P. J. Campbell and J. R. Sargent (2001). "Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism." Journal of Nutrition **131**(5): 1535-1543.

Bermúdez, R., F. Vigliano, M. I. Quiroga, J. M. Nieto, G. Bosi and C. Domeneghini (2007). "Immunohistochemical study on the neuroendocrine system of the digestive tract of turbot, Scophthalmus maximus (L.), infected by Enteromyxum scophthalmi (Myxozoa)." Fish & Shellfish Immunology **22**(3): 252-263.

Bosi, G., A. P. Shinn, L. Giari, E. Simoni, F. Pironi and B. S. Dezfuli (2005). "Changes in the neuromodulators of the diffuse endocrine system of the alimentary canal of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), naturally infected with *Eubothrium crassum* (Cestoda)." Journal of Fish Diseases **28**(12): 703-711.

Bottcher, G., E. Ekblad, R. Ekman, R. Hakanson and F. Sundler (1993). "Peptide-YY - A neuropeptide in the gut - immunocytochemical and immunocytochemical evidence." Neuroscience **55**(1): 281-290.

Breves, J. P., P. A. Veillette and J. L. Specker (2009). "Ghrelin in the summer flounder: Immunolocalization to the gastric glands and action on plasma cortisol levels." Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology **152**(2): 268-272.

Buchan, A. M. J., J. M. Polak, E. Solcia, C. Capella, D. Hudson and A. G. E. Pearse (1978). "Electron immunohistochemical evidence for human intestinal I cells as source of CCK." Gut **19**(5): 403-407.

Buddington, R. K. and J. M. Diamond (1987). "Pyloric ceca of fish: a "new" absorptive organ." American Journal of Physiology and Gastrointestinal Liver Physiology **252**(1): G65-76.

Buddington, R. K. and A. Krogdahl (2004). "Hormonal regulation of the fish gastrointestinal tract." Comparative biochemistry and physiology a-molecular & integrative physiology **139**(3): 261-271.

Buddington, R. K., A. Krogdahl and A. M. BakkeMcKellep (1997). "The intestines of carnivorous fish: structure and functions and the relations with diet." Acta physiologica scandinavica **161**(638): 67-80.

Bustin, S. A. (2000). "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays." Journal of Molecular Endocrinology **25**(2): 169-193.

Bustin, S. A., V. Benes, T. Nolan and M. W. Pfaffl (2005). "Quantitative real-time RT-PCR--a perspective." J Mol Endocrinol **34**(3): 597-601.

Bustin, S. A. and T. Nolan (2004). "Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction." J Biomol Tech **15**(3): 155-166.

Cerda-Reverter, J. M. and D. Larhammar (2000). "Neuropeptide Y family of peptides: Structure, anatomical expression, function, and molecular evolution." Biochemistry and cell biology **78**(3): 371-392.

Cerda-Reverter, J. M., G. Martinez-Rodriguez, I. Anglade, O. Kah and S. Zanuy (2000). "Peptide YY (PYY) and fish pancreatic peptide Y (PY) expression in the brain of the sea bass (*Dicentrarchus labrax*) as revealed by in situ hybridization." Journal of Comparative Neurology **426**(2): 197-208.

Chan, C. B. and C. H. K. Cheng (2004). "Identification and functional characterization of two alternatively spliced growth hormone secretagogue receptor transcripts from the pituitary of black seabream Acanthopagrus schlegeli." Molecular and Cellular Endocrinology **214**(1-2): 81-95.

Chandra, R. and R. A. Liddle (2007). "Cholecystokinin." Current Opinion in Endocrinology, Diabetes and Obesity **14**(1): 63-67.

Chaudhri, O., C. Small and S. Bloom (2006). "Gastrointestinal hormones regulating appetite." Philosophical Transactions of the Royal Society B-Biological Sciences **361**(1471): 1187-1209.

Chomczynski, P. (1993). "A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples." Biotechniques **15**(3): 532-534, 536-537.

Clements, K. D. and D. Raubenheimer (2006). The Physiology of fishes. Marine biology series. D. H. Evans and J. B. Claiborne. Boca Raton, Fla., CRC/Taylor & Francis**:** 47-74.

Cummings, D. E. (2006). "Ghrelin and the short- and long-term regulation of appetite and body weight." Physiology & Behavior **89**(1): 71-84.

Cummings, D. E., J. Q. Purnell, R. S. Frayo, K. Schmidova, B. E. Wisse and D. S. Weigle (2001). "A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans." Diabetes **50**(8): 1714-1719.

Date, Y., M. Kojima, H. Hosoda, A. Sawaguchi, M. S. Mondal, T. Suganuma, S. Matsukura, K. Kangawa and M. Nakazato (2000). "Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans." Endocrinology **141**(11): 4255-4261.

Date, Y., N. Murakami, K. Toshinai, S. Matsukura, A. Niijima, H. Matsuo, K. Kangawa and M. Nakazato (2002). "The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats." Gastroenterology **123**(4): 1120-1128.

Deprez, R. H. L., A. C. Fijnvandraat, J. M. Ruijter and A. F. M. Moorman (2002). "Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions." Analytical Biochemistry **307**(1): 63-69.

Einarsson, S., P. S. Davies and C. Talbot (1997). "Effect of exogenous cholecystokinin on the discharge of the gallbladder and the secretion of trypsin and chymotrypsin from the pancreas of the Atlantic salmon, Salmo salar L." Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **117**(1): 63-67.

Espe, M., A. Lemme, A. Petri and A. El-Mowafi (2006). "Can Atlantic salmon (*Salmo salar*) grow on diets devoid of fish meal?" Aquaculture **255**(1-4): 255-262.

FAO. (2007). "Cultured aquatic species information programme, *Salmo salar*: Statistics." from http://www.fao.org/fishery/culturedspecies/Salmo_salar/en#tcN900D6.

Feldman, M. and C. T. Richardson (1986). "Role of thought, sight, smell, and taste of food in the cephalic phase of gastric-acid secretion in humans." Gastroenterology **90**(2): 428-433.

Fleige, S., V. Walf, S. Huch, C. Prgomet, J. Sehm and M. W. Pfaffl (2006). "Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR." Biotechnology Letters **28**(19): 1601-1613.

Fox, B. K., J. P. Breves, T. Hirano and E. G. Grau (2009). "Effects of short- and long-term fasting on plasma and stomach ghrelin, and the growth hormone/insulin-like growth factor I axis in the tilapia, Oreochromis mossambicus." Domestic Animal Endocrinology **37**(1): 1-11.

Fox, B. K., L. G. Riley, C. Dorough, H. Kaiya, T. Hirano and E. G. Grau (2007). "Effects of homologous ghrelins on the growth hormone/insulin-like growth factor-I axis in the Tilapia, Oreochromis mossambicus." Zoological Science **24**(4): 391-400.

Francis, G., H. P. S. Makkar and K. Becker (2001). "Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish." Aquaculture **199**(3-4): 197-227.

Freeman, W. M., S. J. Walker and K. E. Vrana (1999). "Quantitative RT-PCR: Pitfalls and potential." Biotechniques **26**(1): 112-+.

Fu-Cheng, X., Y. Anini, J. Chariot, N. Castex, J.-P. Galmiche and C. Rozé (1997). "Mechanisms of peptide YY release induced by an intraduodenal meal in rats: neural regulation by proximal gut." Pflügers Archiv European Journal of Physiology **433**(5): 571-579.

Gelineau, A. and T. Boujard (2001). "Oral administration of cholecystokinin receptor antagonists increase feed intake in rainbow trout." Journal of Fish Biology **58**(3): 716-724.

Gibbs, J., R. C. Young and G. P. Smith (1973). "Cholecystokinin decreases food intake in rats." Journal of Comparative and Physiological Psychology **84**(3): 488-495.

Gnanapavan, S., B. Kola, S. A. Bustin, D. G. Morris, P. McGee, P. Fairclough, S. Bhattacharya, R. Carpenter, A. B. Grossman and M. Korbonits (2002). "The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans." Journal of Clinical Endocrinology and Metabolism **87**(6): 2988-2991.

Grandt, D., M. Schimiczek, C. Beglinger, P. Layer, H. Goebell, V. E. Eysselein and J. R. Reeve (1994). "2 Molecular forms of Peptide YY (PYY) are abundant in human blood - characterization of a radioimmunoassay recognizing PYY-1-36 and PYY-3-36." Regulatory Peptides **51**(2): 151-159.

Grove, D. J., L. G. Loizides and J. Nott (1978). "Satiation amount, frequency of feeding and gastric emptying rate in Salmo gairdneri." Journal of Fish Biology **12**(5): 507-516.

Guo, Y. S., M. Fujimura, F. Lluis, Y. Tsong, G. H. Greeley, Jr and J. C. Thompson (1987). "Inhibitory action of peptide YY on gastric acid secretion." Am J Physiol Gastrointest Liver Physiol **253**(3): 298-302.

Hemre, G.-I., K. Sandnes, Ø. Lie, O. Torrissen and R. Waagbø (1995). "Carbohydrate nutrition in Atlantic salmon, *Salmo salar* L.: growth and feed utilization." Aquaculture Research **26**(3): 149-154.

Himick, B. A. and R. E. Peter (1994). "CCK/Gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish." American Journal of Physiology **267**(3): 841-851.

Hoentjen, F., W. P. M. Hopman and J. Jansen (2001). "Effect of circulating peptide YY on gallbladder emptying in humans." Scandinavian Journal of Gastroenterology **36**(10): 1086-1091.

Holst, J. J., J. Fahrenkrug, F. Stadil and J. F. Rehfeld (1996). "Gastrointestinal endocrinology." Scandinavian Journal of Gastroenterology **31**: 27-38.

Hosoda, H., M. Kojima, H. Matsuo and K. Kangawa (2000). "Ghrelin and des-acyl ghrelin: Two major forms of rat ghrelin peptide in gastrointestinal tissue." Biochemical and Biophysical Research Communications **279**(3): 909-913.

Imbeaud, S., E. Graudens, V. Boulanger, X. Barlet, P. Zaborski, E. Eveno, O. Mueller, A. Schroeder and C. Auffray (2005). "Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces." Nucleic Acids Research **33**(6): e56 (51-12).

Ivy, A. C. and E. Oldberg (1928). "A hormone mechanism for gallbladder contraction and evacuation." Am J Physiol **86**(3): 599-613.

Jensen, H., I. J. Rourke, M. Moller, L. Jonson and A. H. Johnsen (2001). "Identification and distribution of CCK-related peptides and mRNAs in the rainbow trout, *Oncorhynchus mykiss*." Biochimica Et Biophysica Acta-Gene Structure and Expression **1517**(2): 190-201.

Johnsen, A. H. (1998). "Phylogeny of the Cholecystokinin/Gastrin Family." Frontiers in Neuroendocrinology **19**(2): 73-99.

Jonsson, A. C., S. Holmgren and B. Holstein (1987). "Gastrin/CCK-like immunoreactivity in endocrine cells and nerves in the gastrointestinal tract of the cod, Gadus morhua, and the effect of peptides of the gastrin/CCK family on cod gastrointestinal smooth muscle." General Comparative Endocrinology **66**(2): 190-202.

Jönsson, E., A. Forsman, I. E. Einarsdottir, H. Kaiya, K. Ruohonen and B. T. Björnsson (2007). "Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake." Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology **147**(4): 1116-1124.

Kaiya, H., M. Kojima, H. Hosoda, S. Moriyama, A. Takahashi, H. Kawauchi and K. Kangawa (2003a). "Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout." Endocrinology **144**(12): 5215-5226.

Kaiya, H., M. Kojima, H. Hosoda, L. G. Riley, T. Hirano, E. G. Grau and K. Kangawa (2003b). "Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity." Journal of Endocrinology **176**(3): 415-423.

Kaiya, H., M. Kojima, H. Hosoda, L. G. Riley, T. Hirano, E. G. Grau and K. Kangawa (2003c). "Identification of tilapia ghrelin and its effects on growth hormone and prolactin release in the tilapia, Oreochromis mossambicus." Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology **135**(3): 421-429.

Kaiya, H., M. Miyazato, K. Kangawa, R. E. Peter and S. Unniappan (2008). "Ghrelin: A multifunctional hormone in non-mammalian vertebrates." Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology **149**(2): 109-128.

Kaiya, H., T. Mori, M. Miyazato and K. Kangawa (2009). "Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, Oncorhynchus mykiss." Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology **153**(4): 438-450.

Kaiya, H., B. C. Small, A. L. Bilodeau, B. S. Shepherd, M. Kojima, H. Hosoda and K. Kangawa (2005). "Purification, cDNA cloning, and characterization of ghrelin in channel catfish, Ictalurus punctatus." General and Comparative Endocrinology **143**(3): 201-210.

Kaiya, H., T. Tsukada, S. Yuge, H. Mondo, K. Kangawa and Y. Takei (2006). "Identification of eel ghrelin in plasma and stomach by radioimmunoassay and histochemistry." General and Comparative Endocrinology **148**(3): 375-382.

Kamisaka, Y., O. Drivenes, T. Kurokawa, M. Tagawa, I. Ronnestad, M. Tanaka and J. V. Helvik (2005). "Cholecystokinin mRNA in Atlantic herring, Clupea harengus - molecular cloning, characterization, and distribution in the digestive tract during the early life stages." Peptides **26**(3): 385-393.

Karila, P. and S. Holmgren (1995). "Enteric reflexes and nitric oxide in the fish intestine." Journal of Experimental Biology **198**(11): 2405-2412.

Katschinski, M. (2000). "Nutritional implications of cephalic phase gastrointestinal responses." Appetite **34**(2): 189-196.

Kawakoshi, A., H. Kaiya, L. G. Riley, T. Hirano, E. G. Grau, M. Miyazato, H. Hosoda and K. Kangawa (2007). "Identification of a ghrelin-like peptide in two species of shark, Sphyrna lewini and Carcharhinus melanopterus." General and Comparative Endocrinology **151**(3): 259-268.

Kitazawa, T., H. Kaiya and T. Taneike (2007). "Contractile effects of ghrelin-related peptides on the chicken gastrointestinal tract in vitro." Peptides **28**(3): 617-624.

Koda, S., Y. Date, N. Murakami, T. Shimbara, T. Hanada, K. Toshinai, A. Niijima, M. Furuya, N. Inomata, K. Osuye and M. Nakazato (2005). "The role of the vagal nerve in peripheral PYY3-36-induced feeding reduction in rats." Endocrinology **146**(5): 2369-2375.

Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo and K. Kangawa (1999). "Ghrelin is a growth-hormone-releasing acylated peptide from stomach." Nature **402**(6762): 656-660.

Kojima, M., H. Hosoda, H. Matsuo and K. Kangawa (2001). "Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor." Trends in Endocrinology and Metabolism **12**(3): 118-122.

Kojima, M. and K. Kangawa (2002). "Ghrelin, an orexigenic signaling molecule from the gastrointestinal tract." Current Opinion in Pharmacology **2**(6): 665-668.

Korbonits, M., S. A. Bustin, M. Kojima, S. Jordan, E. F. Adams, D. G. Lowe, K. Kangawa and A. B. Grossman (2001). "The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors." Journal of Clinical Endocrinology and Metabolism **86**(2): 881-887.

Kozaka, T., Y. Fujii and M. Ando (2003). "Central effects of various ligands on drinking behavior in eels acclimated to seawater." Journal of Experimental Biology **206**(4): 687-692.

Krogdahl, A., G. I. Hemre and T. P. Mommsen (2005). "Carbohydrates in fish nutrition: digestion and absorption in postlarval stages." Aquaculture Nutrition **11**(2): 103-122.

Krogdahl, Å. (2001). Fordøyelsessystemet hos kaldtvannsfisk - Oppbygging og funksjon. Fiskeernæring. R. Waagbø, M. Espe, K. Hamre and Ø. Lie. Bergen, Kystnæringen forl. & bokklubb**: 11-35.

Krogdahl, Å., A. M. Bakke-McKellep and G. Baeverfjord (2003). "Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.)." Aquaculture Nutrition **9**(6): 361-371.

Krogdahl, Å., S. Nordrum, M. Sørensen, L. Brudeseth and C. Røsjø (1999). "Effects of diet composition on apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal disaccharidase activities and plasma nutrient concentration in Atlantic salmon, *Salmo salar* L." Aquaculture Nutrition **5**(2): 121-133.

Kubista, M., J. M. Andrade, M. Bengtsson, A. Forootan, J. Jonak, K. Lind, R. Sindelka, R. Sjoback, B. Sjogreen, L. Strombom, A. Stahlberg and N. Zoric (2006). "The real-time polymerase chain reaction." Mol Aspects Med **27**(2-3): 95-125.

Kurokawa, T. and T. Suzuki (2002). "Development of neuropeptide Y-related peptides in the digestive organs during the larval stage of Japanese flounder, Paralichthys olivaceus." General and Comparative Endocrinology **126**(1): 30-38.

Kurokawa, T., T. Suzuki and H. Hashimoto (2003). "Identification of gastrin and multiple cholecystokinin genes in teleost." Peptides **24**(2): 227-235.

Kwok, S. and R. Higuchi (1989). "Avoiding false positives with PCR." Nature **339**(6221): 237-238.

Larhammar, D. (1996). "Evolution of neuropeptide Y, peptide YY and pancreatic polypeptide." Regulatory Peptides **62**(1): 1-11.

Larhammar, D., C. Soderberg, A. G. Blomqvist, I. Lundell and L. Brodin (1995). "Evolution of the neuropeptide Y-peptide YY family." Netherlands Journal of Zoology **45**(1-2): 15-17.

Larsson, T. A., E. T. Larson, R. Fredriksson, J. M. Conlon and D. Larhammar (2006). "Characterization of NPY receptor subtypes Y2 and Y7 in rainbow trout *Oncorhynchus mykiss*." Peptides **27**(6): 1320-1327.

Liddle, R. A. (1997). "Cholecystokinin cells." Annual Review of Physiology **59**: 221-242.

Lin, H. C., W. Y. Chey and X. T. Zhao (2000a). "Release of distal gut peptide YY (PYY) by fat in proximal gut depends on CCK." Peptides **21**(10): 1561-1563.

Lin, X. W., H. Volkoff, Y. Narnaware, N. J. Bernier, P. Peyon and R. E. Peter (2000b). "Brain regulation of feeding behavior and food intake in fish." Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology **126**(4): 415-434.

Lluis, F., G. Gomez, M. Fujimura, G. H. Greeley and J. C. Thompson (1987). "Peptide-YY inhibits nutrient-stimulated, hormonal-stimulated, and vagally-stimulated pancreatic exocrine secretion." Pancreas **2**(4): 454-462.

Lu, S., J. L. Guan, Q. P. Wang, K. Uehara, S. Yamada, N. Goto, Y. Date, M. Nakazato, M. Kojima, K. Kangawa and S. Shioda (2002). "Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus." Neuroscience Letters **321**(3): 157-160.

Lundberg, J. M., K. Tatemoto, L. Terenius, P. M. Hellstrom, V. Mutt, T. Hokfelt and B. Hamberger (1982). "Localization of peptide-YY (PYY) in gastrointestinal endocrine-cells and effects on intestinal blood-flow and motility." Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences **79**(14): 4471-4475.

Luyer, M. D., J. W. M. Greve, M. h. Hadfoune, J. A. Jacobs, C. H. Dejong and W. A. Buurman (2005). "Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve." J. Exp. Med. **202**(8): 1023-1029.

Masuda, Y., T. Tanaka, N. Inomata, N. Ohnuma, S. Tanaka, Z. Itoh, H. Hosoda, M. Kojima and K. Kangawa (2000). "Ghrelin stimulates gastric acid secretion and motility in rats." Biochemical and Biophysical Research Communications **276**(3): 905-908.

Matsuda, K., T. Miura, H. Kaiya, K. Maruyama, S. I. Himakura, M. Uchiyama, K. Kangawa and S. Shioda (2006a). "Regulation of food intake by acyl and des-acyl ghrelins in the goldfish." Peptides **27**(9): 2321-2325.

Matsuda, K., T. Miura, H. Kaiya, K. Maruyama, M. Uchiyama, K. Kangawa and S. Shioda (2006b). "Stimulatory effect of n-octanoylated ghrelin on locomotor activity in the goldfish, *Carassius auratus*." Peptides **27**(6): 1335-1340.

McCormick, S. D., J. M. Shrimpton, J. B. Carey, M. F. O'Dea, K. E. Sloan, S. Moriyama and B. T. Bjornsson (1998). "Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol." Aquaculture **168**(1-4): 221-235.

Mentlein, R., P. Dahms, D. Grandt and R. Kruger (1993). "Proteolytic processing of neuropeptide-Y and peptide-YY by dipeptidyl peptidase-IV." Regulatory Peptides **49**(2): 133-144.

Miura, T., K. Maruyama, S. I. Shimakura, H. Kaiya, M. Uchiyama, K. Kangawa, S. Shioda and K. Matsuda (2006). "Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*." Neuroscience Letters **407**(3): 279-283.

Mondal, M. S., Y. Date, H. Yamaguchi, K. Toshinai, T. Tsuruta, K. Kangawa and M. Nakazato (2005). "Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus." Regulatory Peptides **126**(1-2): 55-59.

Moran, T. H. and K. P. Kinzig (2004). "Gastrointestinal satiety signals - II. Cholecystokinin." American Journal of Physiology-Gastrointestinal and Liver Physiology **286**(2): 183-188.

Murashita, K., H. Fukada, H. Hosokawa and T. Masumoto (2006). "Cholecystokinin and peptide Y in yellowtail (Seriola quinqueradiata): Molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting." General and Comparative Endocrinology **145**(3): 287-297.

Murashita, K., H. Fukada, I. Rønnestad, T. Kurokawa and T. Masumoto (2008). "Nutrient control of release of pancreatic enzymes in yellowtail (Seriola quinqueradiata): Involvement of CCK and PY in the regulatory loop." Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology **150**(4): 438-443.

Murashita, K., T. Kurokawa, T. O. Nilsen and I. Rønnestad (2009). "Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (Salmo salar): Molecular cloning and tissue expression." General and Comparative Endocrinology **160**(3): 223-235.

Mustonen, A. M., P. Nieminen and H. Hyvarinen (2002). "Leptin, ghrelin, and energy metabolism of the spawning burbot (*Lota lota*)." Journal of Experimental Zoology **293**(2): 119-126.

Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa and S. Matsukura (2001). "A role for ghrelin in the central regulation of feeding." Nature **409**(6817): 194-198.

Narnaware, Y. K. and R. E. Peter (2001). "Effects of food deprivation and refeeding on neuropeptide Y (NPY) mRNA levels in goldfish." Comparative Biochemistry and Physiology - B: Biochemistry and molecular biology **129**(2-3): 633-637.

Naylor, R. L., R. W. Hardy, D. P. Bureau, A. Chiu, M. Elliott, A. P. Farrell, I. Forster, D. M. Gatlin, R. J. Goldburg, K. Hua and P. D. Nichols (2009). "Feeding aquaculture in an era of finite resources." Proceedings of the National Academy of Sciences of the United States of America **106**(36): 15103-15110.

Nordrum, S., A. M. Bakke-McKellep, Å. Krogdahl and R. K. Buddington (2000). "Effects of soybean meal and salinity on intestinal transport of nutrients in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **125**(3): 317-335.

Olli, J. J., K. Hjelmeland and A. Krogdahl (1994). "Soybean trypsin-inhibitors in diets for Atlantic salmon (*Salmo salar*) - effects on nutrient digestibility and trypsin in pyloric ceca homogenate and intestinal content." Comparative Biochemistry and Physiology a-Physiology **109**(4): 923-928.

Olsson, C., G. Aldman, A. Larsson and S. Holmgren (1999). "Cholecystokinin affects gastric emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss*." Journal of Experimental Biology **202**(2): 161-170.

Olsson, C., J. D. Holbrook, G. Bompadre, E. Jonsson, C. H. V. Hoyle, G. J. Sanger, S. Hohngren and P. L. R. Andrews (2008). "Identification of genes for the ghrelin and motilin receptors and a novel related gene in fish, and stimulation of intestinal motility in zebrafish (*Danio rerio*) by ghrelin and motilin." General and Comparative Endocrinology **155**(1): 217-226.

Olsson, C. and S. Holmgren (2001). "The control of gut motility." Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology **128**(3): 479-501.

Olsvik, P. A., K. K. Lie, A. E. Jordal, T. O. Nilsen and I. Hordvik (2005). "Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon." BMC Mol Biol **6**: 21.

Pankhurst, N. W., S. L. Ludke, H. R. King and R. E. Peter (2008). "The relationship between acute stress, food intake, endocrine status and life history stage in juvenile farmed Atlantic salmon, Salmo salar." Aquaculture **275**(1-4): 311-318.

Parhar, I. S., H. Sato and Y. Sakuma (2003). "Ghrelin gene in cichlid fish is modulated by sex and development." Biochemical and Biophysical Research Communications **305**(1): 169-175.

Peeters, T. L. (2005). "Ghrelin: A new player in the control of gastrointestinal functions." Gut **54**(11): 1638-1649.

Peyon, P., X. W. Lin, B. A. Himick and R. E. Peter (1998). "Molecular cloning and expression of cDNA encoding brain preprocholecystokinin in goldfish." Peptides **19**(2): 199-210.

Peyon, P., H. Saied, X. Lin and R. E. Peter (1999). "Postprandial, seasonal and sexual variations in cholecystokinin gene expression in goldfish brain." Molecular Brain Research **74**(1-2): 190-196.

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. A-Z of quantitative PCR. S. Bustin. La Jolla, Ca, USA, International University Line (IUL)**:** 87-112.

Pfaffl, M. W. and M. Hageleit (2001). "Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR." Biotechnology Letters **23**(4): 275-282.

Raeymaekers, L. (1993). "Quantitative PCR: theoretical considerations with practical implications." Anal Biochem **214**(2): 582-585.

Ran, X. Q., W. S. Li and H. R. Lin (2004). "Rat ghrelin stimulates GH release and GH mRNA expression in the pituitary of orange-spotted grouper, *Epinephelus coioides*." Fish Physiology and Biochemistry **30**(2): 95-102.

Raybould, H. E. (2007). "Mechanisms of CCK signaling from gut to brain." Current Opinion in Pharmacology **7**(6): 570-574.

Riley, L. G., B. K. Fox, H. Kaiya, T. Hirano and E. G. Grau (2005). "Long-term treatment of ghrelin stimulates feeding, fat deposition, and alters the GH/IGF-I axis in the tilapia, *Oreochromis mossambicus*." General and Comparative Endocrinology **142**(1-2): 234-240.

Rumsey, G. L., A. K. Siwicki, D. P. Anderson and P. R. Bowser (1994). "Effect of soybean protein on serological response, non-specific defense mechanisms, growth, and protein utilization in rainbow trout." Veterinary Immunology and Immunopathology **41**(3-4): 323-339.

Rønnestad, I., C. R. Rojas-Garcia and J. Skadal (2000). "Retrograde peristalsis; a possible mechanism for filling the pyloric caeca?" Journal of Fish Biology **56**(1): 216-218.

Sakata, I., T. Mori, H. Kaiya, M. Yamazaki, K. Kangawa, K. Inoue and T. Sakai (2004). "Localization of ghrelin-producing cells in the stomach of the rainbow trout (*Oncorhynchus mykiss*)." Zoological Science **21**(7): 757-762.

SanchezVazquez, F. J., J. A. Madrid, S. Zamora and M. Tabata (1997). "Feeding entrainment of locomotor activity rhythms in the goldfish is mediated by a feeding-entrainable circadian oscillator." Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology **181**(2): 121-132.

Schoor, O., T. Weinschenk, J. Hennenlotter, S. Corvin, A. Stenzl, H. G. Rammensee and S. Stevanovic (2003). "Moderate degradation does not preclude microarray analysis of small amounts of RNA." Biotechniques 35(6): 1192-1198.

Schroeder, A., O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow and T. Ragg (2006). "The RIN: an RNA integrity number for assigning integrity values to RNA measurements." BMC Molecular Biology 7(3): 1-14.

Seoane, L. M., S. Tovar, R. Baldelli, E. Arvat, E. Ghigo, F. F. Casanueva and C. Dieguez (2000). "Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats." European Journal of Endocrinology 143(5): R7-R9.

Shapiro, S. S. and M. B. Wilk (1965). "An analysis of variance test for normality (complete samples)." Biometrika 52(3-4): 591-611.

Shepherd, B. S., J. K. Johnson, J. T. Silverstein, I. S. Parhar, M. M. Vijayan, A. McGuire and G. M. Weber (2007). "Endocrine and orexigenic actions of growth honnone secretagogues in rainbow trout (Oncorhynchus mykiss)." Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 146(3): 390-399.

Silverthorn, D. U. (2004). Digestion. Human physiology: an integrated approach. D. U. Silverthorn. San Francisco, Pearson Education Inc, Benjamin Cummings: 659-694.

Soderberg, C., V. A. Pieribone, J. Dahlstrand, L. Brodin and D. Larhammar (1994). "Neuropeptide role of both peptide YY and neuropeptide-Y in vertebrates suggested by abundant expression in their messenger-RNAs in a cyclostom brain." Journal of Neuroscience Research 37(5): 633-640.

Soderberg, C., A. Wraith, M. Ringvall, Y. L. Yan, J. H. Postlethwait, L. Brodin and D. Larhammar (2000). "Zebrafish genes for neuropeptide Y and peptide YY reveal origin by chromosome duplication from an ancestral gene linked to the homeobox cluster." Journal of Neurochemistry 75(3): 908-918.

Spiller, R. C., I. F. Trotman, B. E. Higgins, M. A. Ghatei, G. K. Grimble, Y. C. Lee, S. R. Bloom, J. J. Misiewicz and D. B. A. Silk (1984). "The ileal break - inhibition of jejunal motility after ileal fat perfusion in man." Gut 25(4): 365-374.

Stahlberg, A., J. Hakansson, X. J. Xian, H. Semb and M. Kubista (2004). "Properties of the reverse transcription reaction in mRNA quantification." Clinical Chemistry 50(3): 509-515.

Sundstrom, G., T. A. Larsson, S. Brenner, B. Venkatesh and D. Larhammar (2008). "Evolution of the neuropeptide Y family: New genes by chromosome duplications in early vertebrates and in teleost fishes." General and Comparative Endocrinology 155(3): 705-716.

Sveier, H., E. Wathne and E. Lied (1999). "Growth, feed and nutrient utilisation and gastrointestinal evacuation time in Atlantic salmon (Salmo salar): the effect of dietary fish meal particle size and protein concentration." Aquaculture 180(3-4): 265-282.

Takaya, K., H. Ariyasu, N. Kanamoto, H. Iwakura, A. Yoshimoto, M. Harada, K. Mori, Y. Komatsu, T. Usui, A. Shimatsu, Y. Ogawa, K. Hosoda, T. Akamizu, M. Kojima, K. Kangawa and K. Nakao (2000). "Ghrelin strongly stimulates growth hormone (GH) release in humans." Journal of Clinical Endocrinology and Metabolism 85(12): 4908-4911.

Taniaka-Shintani, M. and M. Watanabe (2005). "Distribution of ghrelin-immunoreactive cells in human gastric mucosa: comparison with that of parietal cells." Journal of Gastroenterology 40(4): 345-349.

Tatemoto, K. (1982). "Isolation an characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion." Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences 79(8): 2514-2518.

Tatemoto, K. and V. Mutt (1980). "Isolation of 2 novel candidate hormones using a chemical method for finding naturally-ocurring polypeptides." Nature 285(5764): 417-418.

Terova, G., S. Rimoldi, G. Bernardini, R. Gornati and M. Saroglia (2008). "Sea bass ghrelin: Molecular cloning and mRNA quantification during fasting and refeeding." General and Comparative Endocrinology **155**(2): 341-351.

ThermoScientific. (2009). "260/280 and 260/230 ratios." from www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf.

Torstensen, B. E., M. Espe, M. Sanden, I. Stubhaug, R. Waagbo, G. I. Hemre, R. Fontanillas, U. Nordgarden, E. M. Hevroy, P. Olsvik and M. H. G. Berntssen (2008). "Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends." Aquaculture **285**(1-4): 193-200.

Tschop, M., D. L. Smiley and M. L. Heiman (2000). "Ghrelin induces adiposity in rodents." Nature **407**(6806): 908-913.

Ueno, H., H. Yamaguchi, M. Mizuta and M. Nakazato (2008). "The role of PYY in feeding regulation." Regulatory Peptides **145**(1-3): 12-16.

Unniappan, S., L. F. Canosa and R. E. Peter (2004). "Orexigenic actions of ghrelin in goldfish: Feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections." Neuroendocrinology **79**(2): 100-108.

Unniappan, S., X. W. Lin, L. Cervini, J. Rivier, H. Kaiya, K. Kangawa and R. E. Peter (2002). "Goldfish ghrelin: Molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake." Endocrinology **143**(10): 4143-4146.

Unniappan, S. and R. E. Peter (2004). "In vitro and in vivo effects of ghrelin on luteinizing hormone and growth hormone release in goldfish." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **286**(6): R1093-R1101.

Vantrappen, G., J. Janssens, T. L. Peeters, S. R. Bloom, N. D. Christofides and J. Hellemans (1979). "Motilin and the interdigestive migrating complex in man." Digestive Diseases and Sciences **24**(7): 497-500.

Vincent, R. P. and C. W. le Roux (2008). "The satiety hormone peptide YY as a regulator of appetite." Journal of Clinical Pathology **61**(5): 548-552.

Volkoff, H. (2006). "The role of neuropeptide Y, orexins, cocaine and amphetamine-related transcript, cholecystokinin, amylin and leptin in the regulation of feeding in fish." Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology **144**(3): 325-331.

Volkoff, H., L. F. Canosa, S. Unniappan, J. M. Cerda-Reverter, N. J. Bernier, S. P. Kelly and R. E. Peter (2005). "Neuropeptides and the control of food intake in fish." General Comparative Endocrinology **142**(1-2): 3-19.

Volkoff, H., A. J. Eykelbosh and R. E. Peter (2003a). "Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting." Brain Research **972**(1-2): 90-109.

Volkoff, H., A. Joy Eykelbosh and R. Ector Peter (2003b). "Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting." Brain Research **972**(1-2): 90-109.

Volkoff, H., M. Xu, E. MacDonald and L. Hoskins (2009). "Aspects of the hormonal regulation of appetite in fish with emphasis on goldfish, Atlantic cod and winter flounder: Notes on actions and responses to nutritional, environmental and reproductive changes." Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology **153**(1): 8-12.

Walpole, R. E., Myers, Raymond H., Myers, Sharon L., Ye, Keying (2002). Probability & statistics for engineers & scientists. Upper Saddle River, N.J., Prentice-Hall.

Wilfinger, W. W., K. Mackey and P. Chomczynski (1997). "Effect of pH and ionic strength on the spectro-photometric assessment of nucleic acid purity." Biotechniques **22**(3): 474-477.

Wren, A. M., C. J. Small, H. L. Ward, K. G. Murphy, C. L. Dakin, S. Taheri, A. R. Kennedy, G. H. Roberts, D. G. A. Morgan, M. A. Ghatei and S. R. Bloom (2000). "The novel hypothalamic peptide

ghrelin stimulates food intake and growth hormone secretion." <u>Endocrinology</u> **141**(11): 4325-4328.

Yada, T., H. Kaiya, K. Mutoh, T. Azuma, S. Hyodo and K. Kangawa (2006). "Ghrelin stimulates phagocytosis and superoxide production in fish leukocytes." <u>Journal of Endocrinology</u> **189**(1): 57-65.

Yeung, C. M., C. B. Chan, N. Y. S. Woo and C. H. K. Cheng (2006). "Seabream ghrelin: cDNA cloning, genomic organization and promoter studies." <u>Journal of Endocrinology</u> **189**(2): 365-379.

Zar, J. H. (1996). <u>Biostatistical analysis</u>. Upper Saddle River, N.J., Prentice-Hall.

Appendix I

Applied equipment

Bio freezer: Forma ULT – 86 C Freezer (Thermo Scientific, USA)

Scale: Mettler AC88 DeltaRange® (Mettler Toledo AS, Switzerland)

Centrifuge: Eppendorf Centrifuge 5415R (Heracus Instruments, Germany)

Tissue homogenizer: FastPrep™ FP120, BIO101 ThermoSavant (Q-BIOgene, USA)

Vortex: Test Tube Shaker (Wilmington, USA)

Heat block: Techne DRI-BLOCK, DB-3D (Techne, USA) and VWR™ Digital Heatblock (Henry Troemner LLC, USA)

PCR machine: Gene Amp PCR systems 2700 (ABI, USA)

Real-Time RT-PCR machine: Chromo4™ Continuous Fluorescence Detector (Bio-Rad, Hercules, CA, USA)

Spectrophotometer: NanoDrop ND-1000 (Thermo Scientific, USA)

Electrophoresis equipment: Agilent 2100 Bioanalyzer (Agilent Technologies, USA)

Pipettes: PipetMan (Gilson, France)

Pipette tips: Gilson Diamond® Filter tips (Gilson, France)

Vials: 1.5ml Microcentrifuge tubes (Axygen, USA) and 0.6 ml PCR tubes (Axygen, USA)

qPCR plates: Hard-Shell Thin-Wall 96-Well Skirted PCR Plates

Applied chemicals

TRI-Reagent®: Batch # 087K0752 (Sigma, USA)

Chloroform: Batch # 055K0070 (Sigma, USA)

Isopropanol: Batch # 103K3724 (Sigma, USA)

Nuclease-Free Water: Lot # 0809008 (Ambion, USA)

Ethanol: 1000 ml Absolutt Prima (Arcus, Norway)

Sodium Acetate: 3 M NaAc pH (5.5), Lot # 0708003, (Ambion, USA)

DNase kit: TURBO DNA-*free* Kit™, Lot # 0809014, (Ambion, USA)

Reverse transcription kit: Superscript III First-Strand Synthesis System, Lot # 495661 (Invitrogen, USA)

Primer for cDNA synthesis: Oligo(dT) (Sigma, USA)

Real-time qPCR mastermix: Power SYBR$^®$ Green PCR Master Mix (Applied Biosystems), Lot # 0902071

RNA integrity kit: RNA 6000 Pico LabChip kit (Agilent Technologies, USA), Lot # MB18BK04

Appendix II

Standard curves used in calculation of mRNA copy numbers

Figure A.II.1: *Standard calibration curve for GRLN-1. The GRLN-1 standard curve is constructed with serial 10-fold dilutions of GRLN-1 (recDNA), ranging from 1 x 10² to 1 x 10¹⁰ (copies/ul). Each standard dilution was run in duplicate reactions in qPCR. Determined Ct values were averaged and plotted against the logarithm of their initial copy numbers. A linear regression through these point results in a standard curve with: y = -3.9685x + 43.512, R² = 0.9984.*
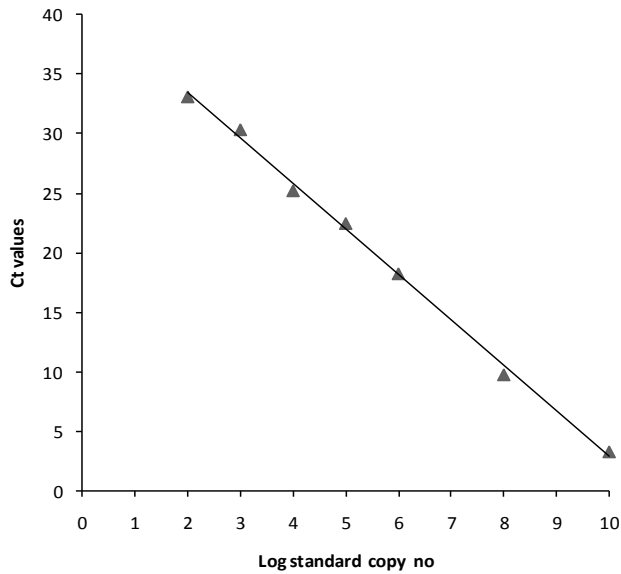
Figure A.II.2: *Standard calibration curve for GRLN-2. The GRLN-2 standard curve is constructed with serial 10-fold dilutions of GRLN-2 (recDNA), ranging from 1 x 10^2 to 1 x 10^{10} (copies/ul). Each standard dilution was run in duplicate reactions in qPCR. Determined Ct values were averaged and plotted against the logarithm of their initial copy numbers. A linear regression through these point results in a standard curve with: y = -3.8163x + 41.066, R^2 = 0.9972.*
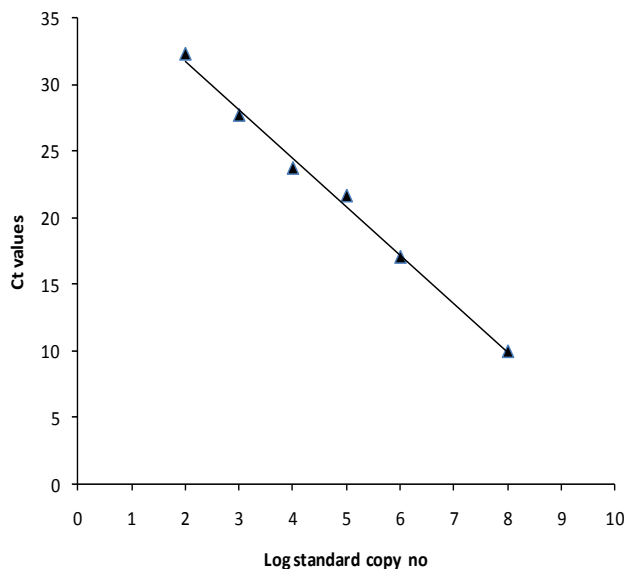
Figure A.II.3: *Standard calibration curve for PYY. The PYY standard curve is constructed with serial 10-fold dilutions of PYY (recDNA), ranging from 1 x 10^2 to 1 x 10^{10} (copies/ul). Each standard dilution was run in duplicate reactions in qPCR. Determined Ct values were averaged and plotted against the logarithm of their initial copy numbers. A linear regression through these point results in a standard curve with: y = -3.9685x + 43.512, R^2 = 0.9984.*
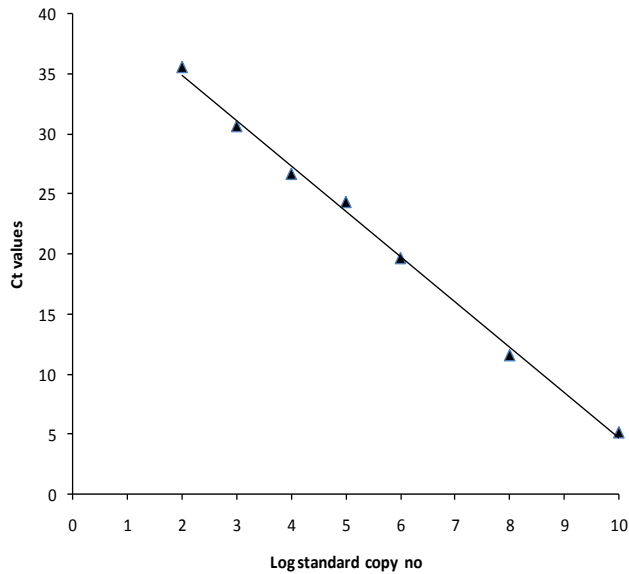
Figure A.II.4: *Standard calibration curve for CCK-L. The CCK-L standard curve is constructed with serial 10-fold dilutions of CCK-L (recDNA), ranging from 1 x 10^2 to 1 x 10^{10} (copies/ul). Each standard dilution was run in duplicate reactions in qPCR. Determined Ct values were averaged and plotted against the logarithm of their initial copy numbers. A linear regression through these point results in a standard curve with: y = -3.7811x + 42.463, R^2 = 0.9965.*
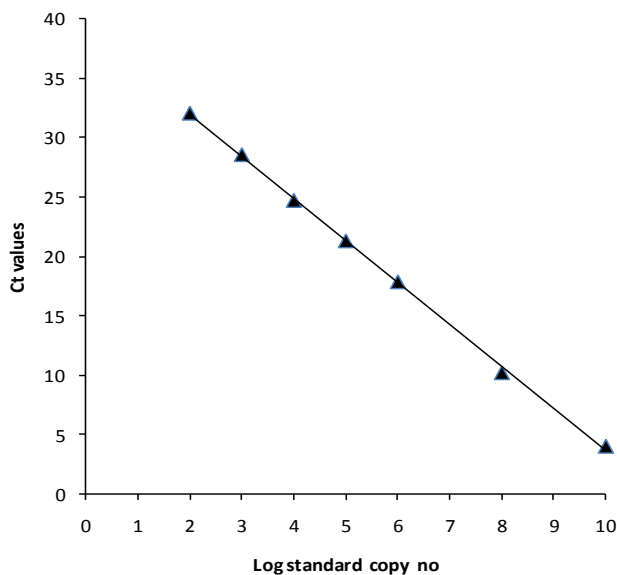
Figure A.II.5: *Standard calibration curve for CCK-N. The CCK-N standard curve is constructed with serial 10-fold dilutions of CCK-N (recDNA), ranging from 1 x 10^2 to 1 x 10^{10} (copies/ul). Each standard dilution was run in duplicate reactions in qPCR. Determined Ct values were averaged and plotted against the logarithm of their initial copy numbers. A linear regression through these point results in a standard curve with: y = -3.5341x + 39.012, R^2 = 0.9992.*
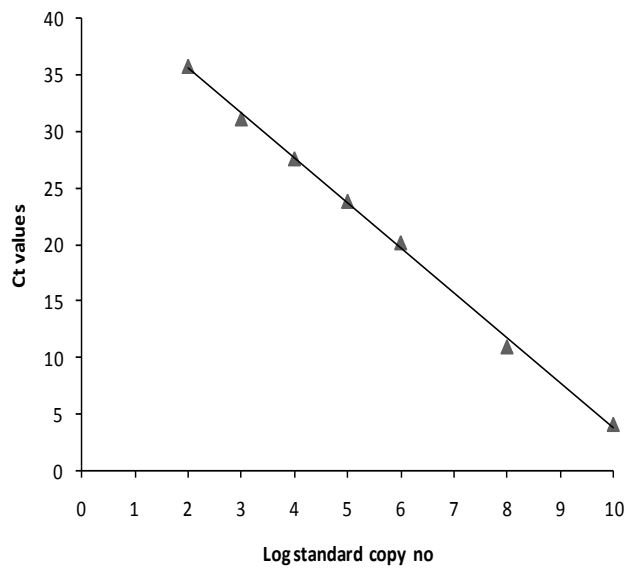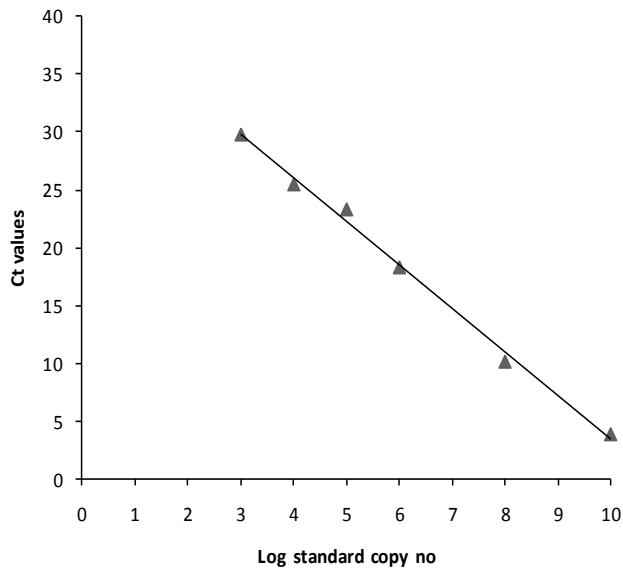
Figure A.II.6: *Standard calibration curve for EF1α. The EF1α standard curve is constructed with serial 10-fold dilutions of EF1α (recDNA), ranging from 1 x 10^3 to 1 x 10^{10} (copies/ul). Each standard dilution was run in duplicate reactions in qPCR. Determined Ct values were averaged and plotted against the logarithm of their initial copy numbers. A linear regression through these point results in a standard curve with: y = -3.7621x + 41.102, R^2 = 0.995.*

Appendix III

Statistics

Testing of normality

Table A.III.1: *Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for unfed fish, segment 2 (stomach). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.*

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0h	W = 0.861	W = 0.902	W = 0.989	W = 0.973	W = 0.946
Time 0.5h	W = 0.991	W = 0.795	W = 0.999	W = 0.944	W = 0.948
Time 1.5h	W = 0.984	W = 0.993	W = 0.864	W = 0.953	W = 0.820
Time 3h	W = 0.795	W = 0.970	W = 0.785	**W = 0.753**	W = 0.953
Time 6h	W = 0.982	W = 1	W = 0.928	W = 0.970	W = 0.941
Time 24h	W = 0.855	W = 0.831	**W = 0.972**	W = 0.860	W = 0.970

Table A.III.2: *Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for unfed fish, segment 4 (pyloric cecae). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.*

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0h	W = 0.944	W = 0.891	W = 0.787	W = 1	W = 0.980
Time 0.5h	W = 0.986	**W = 0.772**	W = 0.967	W = 0.960	W = 0.997
Time 1.5h	W = 0.924	W = 0.885	W = 0.777	W = 0.868	W = 0.965
Time 3h	W = 0.962	W = 0.968	W = 0.777	W = 0.941	W = 0.991
Time 6h	W = 0.959	W = 0.983	W = 0.893	W = 0.977	W = 0.991
Time 24h	W = 0.973	W = 0.879	W = 0.898	W = 0.918	W = 0.874

Table A.III.3: *Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for unfed fish, segment 6 (midgut). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.*

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0h	W = 0.869	W = 0.890	W = 0.973	W = 0.873	W = -
Time 0.5h	W = 0.977	W = 0.992	W = 0.776	W = 0.963	W = -
Time 1.5h	W = 0.964	W = 0.996	W = 0.880	W = 0.844	W = -
Time 3h	W = 0.988	W = 0.998	W = 0.982	W = 0.912	W = -
Time 6h	W = 0.863	**W = 0.791**	W = 0.992	W = 0.871	W = -
Time 24h	W = 0.853	W = 0.988	W = 0.996	W = 0.818	W = -

Table A.III.4: *Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for unfed fish, segment 9 (hindgut). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.*

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0h	W = 0.826	W = 0.839	W = 0.789	W = 0.801	W = -
Time 0.5h	W = 0.956	W = 0.955	W = 0.906	W = 0.842	W = -
Time 1.5h	W = 0.983	W = 1	W = 0.954	**W = 0.765**	W = -
Time 3h	W = 0.992	W = 0.993	W = 0.833	W = 0.888	W = -
Time 6h	W = 0.784	W = 0.962	W = 0.883	**W = 0.763**	W = -
Time 24h	W = 0.923	W = 0.926	W = 0.992	W = 0.962	W = -

Table A.III.5: *Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for fed fish, segment 2 (stomach). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.*

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0.5h	W = 0.982	W = 0.910	W = 0.910	W = 0.997	W = 0.865
Time 1.5h	W = 0.995	W = 0.928	W = 0.900	W = 0.985	W = 0.987
Time 3h	**W = 0.752**	W = 0.807	W = 0.961	W = 0.985	W = 0.835
Time 6h	W = 0.998	**W = 0.754**	W = 0.906	W = 0.995	W = 0.840
Time 24h	W = 0.865	W = 0.924	**W = 0.757**	W = 0.861	W = 0.863

Table A.III.6: *Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for fed fish, segment 4 (pyloric cecae). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.*

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0.5h	W = 0.978	W = 0.972	W = 0.824	W = 0.953	W = 0.996
Time 1.5h	W = 0.871	W = 0.902	W = 0.990	W = 0.773	W = 0.969
Time 3h	W = 0.845	**W = 0.754**	W = 0.871	W = 0.979	W = 0.923
Time 6h	W = 0.954	W = 0.945	W = 0.975	W = 0.958	W = 0.996
Time 24h	W = 0.976	W = 0.976	W = 0.855	W = 0.910	W = 0.975

Table A.III.7: Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for fed fish, segment 6 (midgut). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0.5h	W = 0.988	W = 0.999	W = 0.999	W = 0.915	W = -
Time 1.5h	W = 0.998	W = 0.866	W = 0.915	W = 0.970	W = -
Time 3h	W = 0.997	W = 1	W = 0.906	W = 0.979	W = -
Time 6h	W = 0.930	W = 0.883	W = 0.810	W = 0.850	W = -
Time 24h	W = 0.994	W = 0.982	W = 0.985	W = 0.944	W = -

Table A.III.8: Shapiro-Wilks test of goodness of fit on log transformed normalized expression data for fed fish, segment 9 (hindgut). W=1 if data are perfectly normal in distribution. Underlined and bold W values are significantly smaller than 1 (p<0.05), indicating rejection of the normal distribution hypothesis.

	GRLN-1	GRLN-2	PYY	CCK-L	CCK-N
Time 0.5h	W = 0.984	W = 0.983	W = 0.804	W = 0.998	W = -
Time 1.5h	W = 1	W = 0.829	**W = 0.753**	W = 0.965	W = -
Time 3h	W = 1	W = 0.941	W = 0.798	W = 0.919	W = -
Time 6h	W = 0.857	W = 0.837	W = 0.922	W = 0.805	W = -
Time 24h	W = 0.955	W = 0.996	W = 0.995	W = 0.863	W = -

Tissue distribution of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N mRNA in Atlantic salmon GIT: Factorial ANOVA results

Table A.III.9: Factorial ANOVA of GRLN-1 mRNA tissue distribution in stomach, pyloric cecae, midgut and hindgut (segments) of unfed and fed (group) Atlantic salmon

	SS	DF	MS	F	p
Intercept	126.977	1	126.977	2637.796	<<0.001
Segment	19.460	3	6.487	134.749	<<0.001
group	0.129	1	0.129	2.680	0.104
Segment*group	0.037	3	0.012	0.255	0.858
Error	5.969	124	0.048		

Table A.III.10: Factorial ANOVA of GRLN-2 mRNA tissue distribution in stomach, pyloric cecae, midgut and hindgut (segments) of unfed and fed (group) Atlantic salmon

	SS	DF	MS	F	p
Intercept	151.6494	1	151.6494	2831.984	<<0.001
Segment	30.7324	3	10.2441	191.305	<<0.001
group	0.4021	1	0.4021	7.508	<0.01
Segment*group	0.1181	3	0.0394	0.735	0.533
Error	6.6401	124	0.0535		

Table A.III.11: Factorial ANOVA of PYY mRNA tissue distribution in stomach, pyloric cecae, midgut and hindgut (segments) of unfed and fed (group) Atlantic salmon

	SS	DF	MS	F	p
Intercept	1172.915	1	1172.915	29094.31	<<0.001
Segment	25.856	3	8.619	213.78	<<0.001
group	0.072	1	0.072	1.79	0.183
Segment*group	0.402	3	0.134	3.32	<0.05
Error	4.999	124	0.040		

Table A.III.12: Factorial ANOVA of CCK-L mRNA tissue distribution in stomach, pyloric cecae, midgut and hindgut (segments) of unfed and fed (group) Atlantic salmon

	SS	DF	MS	F	p
Intercept	1591.657	1	1591.657	60158.325	<<0.001
Segment	26.644	3	8.881	335.680	<<0.001
group	0.000	1	0.000	0.001	0.975
Segment*group	0.447	3	0.149	5.629	<0.01
Error	3.281	124	0.026		

Table A.III.13: Factorial ANOVA of CCK-N mRNA tissue distribution in stomach, pyloric cecae, midgut and hindgut (segments) of unfed and fed (group) Atlantic salmon

	SS	DF	MS	F	p
Intercept	908.096	1	908.096	6744.100	<<0.001
Segment	0.013	1	0.013	0.096	0.758
Group	0.381	1	0.381	2.829	0.098
Segment*Group	0.178	1	0.178	1.320	0.255
Error	8.348	62	0.135		

Tukey HSD post-hoc test results:

Table A.III.14: Tukey HSD test results for log transformed GRLN-1 mRNA data in stomach (S2), pyloric cecae (S4), midgut (S6) and hindgut (S9) for unfed and fed fish. Non significant p values are listed as n.s.

Error: Between MS = 0.04814, df = 124.00

Cell No.	Segment (S)	group	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}
1		Unfed fish		n.s	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
2	2	Fed fish	n.s		<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
3	4	Unfed fish	<<0.001	<<0.001		n.s	n.s	n.s	<<0.001	<<0.001
4	4	Fed fish	<<0.001	<<0.001	n.s		n.s	n.s	<<0.001	<<0.001
5	6	Unfed fish	<<0.001	<<0.001	n.s	n.s		n.s	<<0.001	<0.05
6	6	Fed fish	<<0.001	<<0.001	n.s	n.s	n.s		<<0.001	<<0.001
7	9	Unfed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001		n.s
8	9	Fed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	n.s	

Table A.III.15: Tukey HSD test results for log transformed GRLN-2 mRNA data in stomach (S2), pyloric cecae (S4), midgut (S6) and hindgut (S9) for unfed and fed fish. Non significant p values are listed as n.s.

Error: Between MS = ,03617, df = 124,00

Cell No.	Segment (S)	group	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}
1	2	Unfed fish		n.s	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
2	2	Fed fish	n.s		<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
3	4	Unfed fish	<<0.001	<<0.001		n.s	n.s	n.s	<<0.001	<<0.001
4	4	Fed fish	<<0.001	<<0.001	n.s		n.s	n.s	<<0.001	<<0.001
5	6	Unfed fish	<<0.001	<<0.001	n.s	n.s		n.s	<<0.001	<<0.001
6	6	Fed fish	<<0.001	<<0.001	n.s	n.s	n.s		<<0.001	<<0.001
7	9	Unfed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001		n.s
8	9	Fed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	n.s	

Table A.III.16: Tukey HSD test results for log transformed PYY mRNA data in stomach (S2), pyloric cecae (S4), midgut (S6) and hindgut (S9) for unfed and fed fish. Non significant p values are listed as n.s.

Error: Between MS = ,04031, df = 124,00

Cell No.	Segment (S)	group	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}
1	2	Unfed fish		<0.05	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
2	2	Fed fish	<0.05		<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
3	4	Unfed fish	<<0.001	<<0.001		n.s	n.s	n.s	<<0.001	<<0.001
4	4	Fed fish	<<0.001	<<0.001	n.s		n.s	n.s	<<0.001	<<0.001
5	6	Unfed fish	<<0.001	<<0.001	n.s	n.s		n.s	<<0.001	<<0.001
6	6	Fed fish	<<0.001	<<0.001	n.s	n.s	n.s		<<0.001	<<0.001
7	9	Unfed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001		n.s
8	9	Fed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	n.s	

Table A.III.18: Tukey HSD test results for log transformed CCK-L mRNA data in stomach (S2), pyloric cecae (S4), midgut (S6) and hindgut (S9) for unfed and fed fish. Non significant p values are listed as n.s.

Error: Between MS = ,02646, df = 124,00

Cell No.	Segment (S)	group	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}
1	2	Unfed fish		0,059	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
2	2	Fed fish	0,059		<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001
3	4	Unfed fish	<<0.001	<<0.001		n.s	n.s	n.s	<<0.001	<<0.001
4	4	Fed fish	<<0.001	<<0.001	n.s		<0.01	n.s	<<0.001	<<0.001
5	6	Unfed fish	<<0.001	<<0.001	n.s	<0.01		n.s	<<0.001	<<0.001
6	6	Fed fish	<<0.001	<<0.001	n.s	n.s	n.s		<<0.001	<<0.001
7	9	Unfed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001		n.s
8	9	Fed fish	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	<<0.001	n.s	

Postprandial effect on mRNA expression of GRLN-1, GRLN-2, PYY, CCK-L and CCK-N in Atlantic salmon GIT: Main effect ANOVA

Main effects ANOVA

Table A.III.19: Main effects ANOVA for log transformed CCK-L copy no in segment 2 (stomach) of Atlantic salmon with time (h) and fish groups (unfed/fed) as effects. Non significant values are listed as n.s.

Effect	SS	DF	MS	F	p
Intercept	534.770	1	534.770	27135.926	<<0.001
Time:	0.029	5	0.006	0.299	n.s
Group	0.206	1	0.202	10.477	<0.01
Error	0.512	26	0.020		

Table A.III.20: Main effects ANOVA for log transformed CCK-L copy no in segment 4 (pyloric cecae) of Atlantic salmon with time (h) and fish groups (unfed/fed) as effects. Non significant values are listed as n.s.

	SS	DF	MS	F	p
Intercept	335.970	1	335.870	5824.966	<<0.001
Time	0.804	5	0.161	2.787	<0.05
Group	0.013	1	0.013	0.226	n.s
Error	1.500	26	0.058		

Table A.III.21: Main effects ANOVA for log transformed GRLN-2 copy no in segment 6 (midgut) of Atlantic salmon with time (h) and fish groups (unfed/fed) as effects. Non significant values are listed as n.s.

Effect	SS	DF	MS	F	p
Intercept	36.700	1	36.700	850.468	<<0.001
Time:	0.164	5	0.033	0.761	n.s
Group	0.247	1	0.247	5.716	<0.05
Error	1.122	26	0.043		

Table A.III.22: Main effects ANOVA for log transformed PYY copy no in segment 6 (midgut) of Atlantic salmon with time time (h) and fish groups (unfed/fed) as effects. Non significant values are listed as n.s.

Effect	SS	DF	MS	F	p
Intercept	201.484	1	201.484	173407.129	<<0.001
Time:	0.016	5	0.003	2.780	<0.05
Group	0	1	0	0.099	n.s
Error	0.030	26	0.001		

Table A.III.23: Main effects ANOVA for log transformed CCK-L copy no in segment 6 (midgut) of Atlantic salmon with time (h) and fish groups (unfed/fed) as effects. Non significant values are listed as n.s.

Effect	SS	DF	MS	F	p
Intercept	349.819	1	349.819	48114.319	<<0.001
Time:	0.054	5	0.011	1.498	n.s
Group	0.063	1	0.063	8.691	<0.01
Error	0.189	26	0.007		

Tukey HSD post hoc tests for significant values in main effects ANOVA

Table A.III.24: Tukey HSD test for log transformed CCK-L copy no data in segment 2 (somach) of Atlantic salmon

Cell No.	Group	{1}	{2}
1	Unfed fish		<0.01
2	Fed fish	<0.01	

Table A.III.25: Tukey HSD test for log transformed CCK-L copy no in segment 4 (pyloric cecae) with time as main effect. Non significant values are listed as n.s.

Cell No.	Time:	{1}	{2}	{3}	{4}	{5}	{6}
1	0		<0.05	n.s	n.s	n.s	<0.05
2	0,5	<0.05		n.s	n.s	n.s	n.s
3	1,5	n.s	n.s		n.s	n.s	n.s
4	3	n.s	n.s	n.s		n.s	n.s
5	6	n.s	n.s	n.s	n.s		n.s
6	24	<0.05	n.s	n.s	n.s	n.s	

Table A.III.26: Tukey HSD test for log transformed GRLN-2 copy no data in segment 6 (midgut) of Atlantic salmon

Cell No.	Group	{1}	{2}
1	Unfed fish		<0.05
2	Fed fish	<0.05	

Table A.III.27: Tukey HSD test for log transformed PYY copy no in segment 6 (midgut) of Atlantic salmon. Non significant values are listed as n.s.

Cell No.	Time:	{1}	{2}	{3}	{4}	{5}	{6}
1	0		<0.05	n.s	n.s	n.s	n.s
2	0,5	<0.05		n.s	n.s	n.s	n.s
3	1,5	n.s	n.s		n.s	n.s	n.s
4	3	n.s	n.s	n.s		n.s	n.s
5	6	n.s	n.s	n.s	n.s		n.s
6	24	n.s	n.s	n.s	n.s	n.s	

Table A.III.28: Tukey HSD test for log transformed CCK-L copy no data in segment 6 (midgut) of Atlantic salmon

Cell No.	Group	{1}	{2}
1	Fasted fish		<0.01
2	Fed fish	<0.01	

Postprandial effect on GRLN-1, GRLN-2, PYY, CCK-L and CCK-N mRNA expression: T-test results

Table A.III.29: *T-test of log transformed GRLN-1 mRNA normalized expression in S6 at time:=24. Groups were treated as independent samples. Group 1=Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-1.088	-0.852	-13.535	4	<<0.001	0.029	0.010

Table A.III.30: *T-test of log transformed GRLN-1 mRNA normalized expression in S9 at time:=24. Groups were treated as independent samples. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-1.483	-1.115	-7.078	4	<0.01	0.052	0.074

Table A.III.31: *T-test of log transformed GRLN-2 mRNA normalized expression in S6 at time:=24. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-1.178	-0.846	-6.515	4	<0.01	0.078	0.042

Table A.III.32: *T-test of log transformed GRLN-2 mRNA normalized expression in S9 at time:=24. Groups were treated as independent samples. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-1.851	-1.312	-9.558	4	<<0.001	0.065	0.073

Table A.III.33: *T-test of log transformed PYY mRNA normalized expression in S9 at time:=1.5. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-3.036	-2.916	-4.210	4	<0.05	0.048	0.013

Table A.III.34: *T-test of log transformed PYY mRNA normalized expression in S9 at time:=3. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-2.917	-3.084	4.059	4	<0.05	0.062	0.036

Table A.III.35: *T-test of log transformed PYY mRNA normalized expression in S9 at time:=24. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-2.957	-3.004	3.100	4	<0.05	0.024	0.011

Table A.III.36: *T-test of log transformed CCK-L mRNA normalized expression in S2 at time:=3. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-4.102	-4.397	2.900	4	<0.05	0.112	0.135

Table A.III.37: *T-test of log transformed CCK-L mRNA normalized expression in S6 at time:=0.5. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-3.558	-3.362	-4.996	4	<0.01	0.014	0.067

Table A.III.38: *T-test of log transformed CCK-L mRNA normalized expression in S6 at time:=24. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-3.450	-3.267	-3.270	4	<0.05	0.095	0.017

Table A.III.39: *T-test of log transformed CCK-L mRNA normalized expression in S9 at time:=1.5. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-2.959	-3.018	2.906	4	<0.05	0.033	0.013

Table A.III.40: *T-test of log transformed CCK-N mRNA normalized expression in S2 at time:=1.5. Groups were treated as independent variables. Group 1= Unfed fish (n=3), Group 2=Fed fish (n=3).*

	Mean group 1	Mean group 2	t-value	df	p	Std.Dev. Group 1	Std.Dev. Group 2
Fasted fish: vs. Fed fish:	-4.115	-3.406	-2.846	4	<0.05	0.423	0.087

cDNA synthesis efficiency
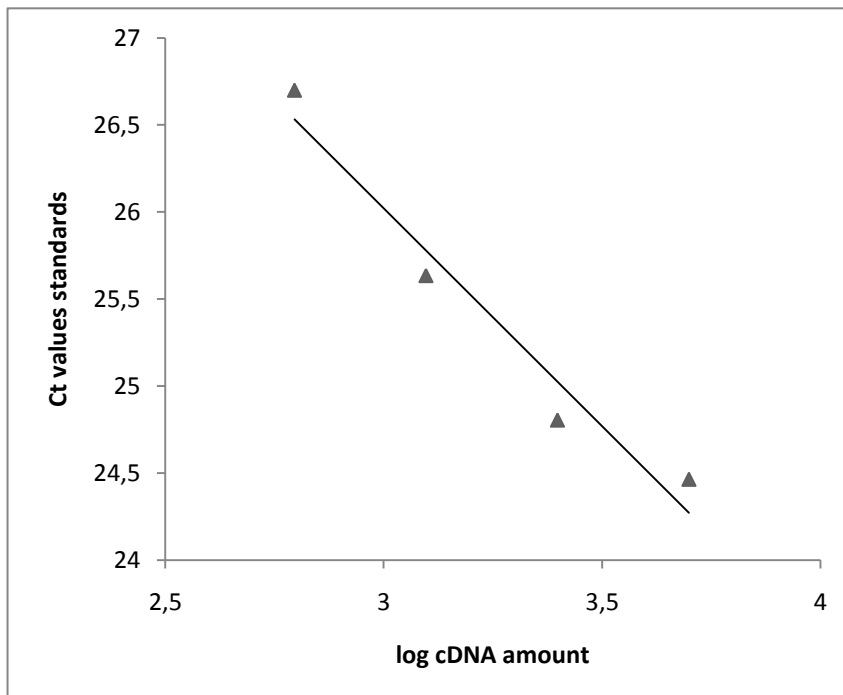
Figure A.III.1 *cDNA synthesis efficiency. Data are represented as qPCR Ct values (y-axis) of a random gene (GRLN-1) plotted against the logarithm of a standard dilution series of RNA from 12 random samples into cDNA synthesis (x-axis). The four different dilutions (D1-D4) were run in parallel reactions into reverse transcription (RT) reaction with the following amount of total RNA (estimated with Nanodrop): D1=5000 ng, D2=2500 ng, D3=1250 ng and D4=625 ng. The qPCR reaction gave the following Ct values ±SD(standard deviation): D1=24.47±0.35, D2=24.81±0.01, D3=25.64±0.08 and D4=26.70±0.14. The slope of the RNA dilution curve was calculated by linear regression: y = -2.508x + 33.56 and the coefficient of determination (r²) = 0.935.*

RNA integrity assessment
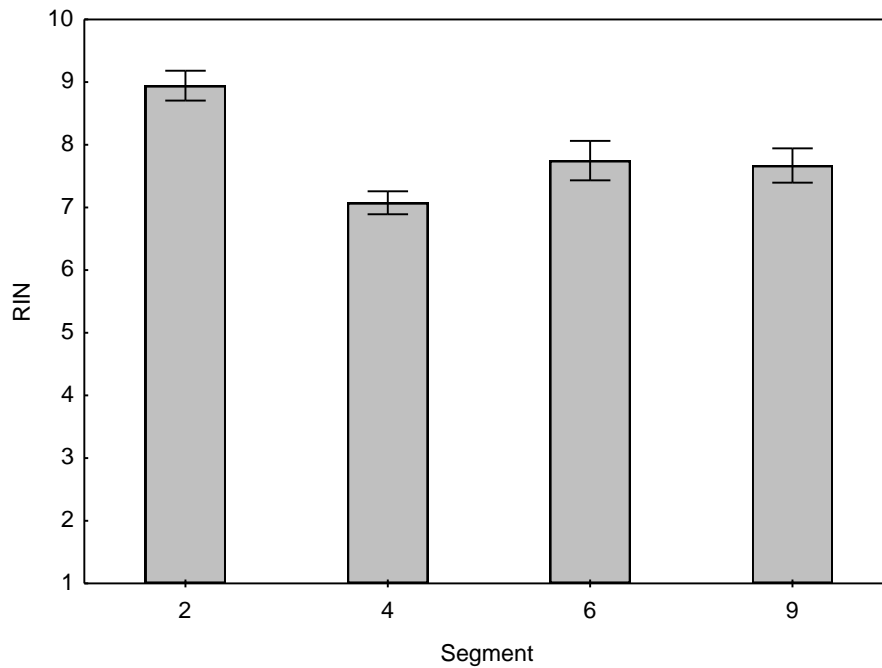
Figure A.III.2: *RNA integrity numbers (RIN) for different segments in Atlantic salmon GI tract. The y-axis show RIN categories 1 to 10, where 10 is a fully intact sample and 1 is a totally degraded sample. The x-axis show the mean combined results for unfed and fed fish groups in different segments of the GI tract, including stomach tissue (Segment 2); n=32, pyloric cecae (Segment 4); n=32, midgut (Segment 6); n=33 and hindgut (Segment 9); n=32. Error bars express ±SD.*
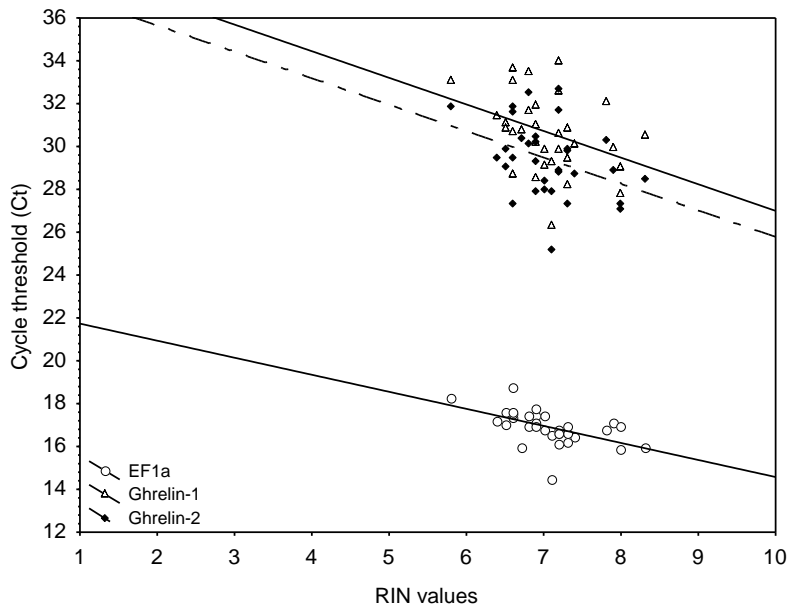
Linear regression of Ct against RIN
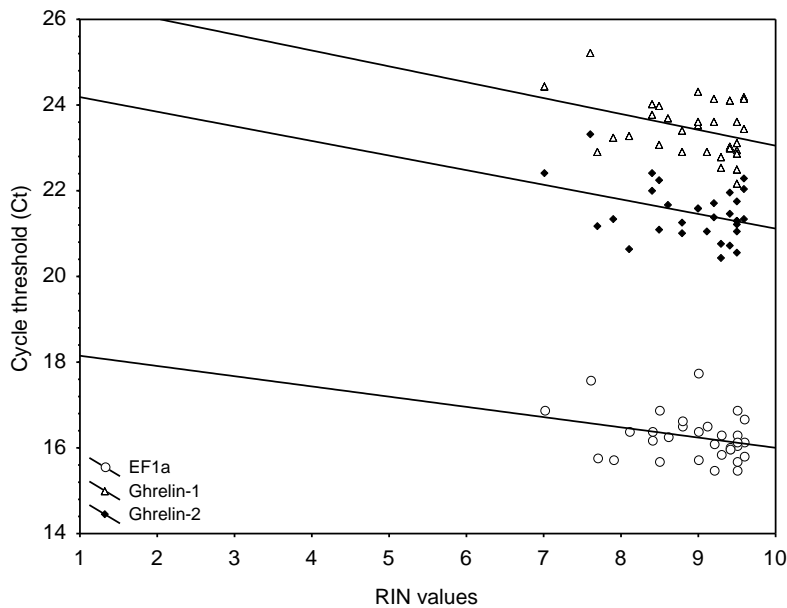
Figure A.III.3: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in stomach tissue of Atlantic salmon (segment 2). The results are represented as average Ct of two parallels from q-PCR absolute expression for EF1a reference gene (n=32), GRLN-1 (n= 32) and GRLN-2 (n=32). The linear regression lines show the following values; EF1a: y = -0.239x + 18.39, GRLN-1: y = -0.370x + 26.76 and GRLN-2: y = -0.341x + 24.53. The r^2 and p values of regression are shown in Table 3.4.
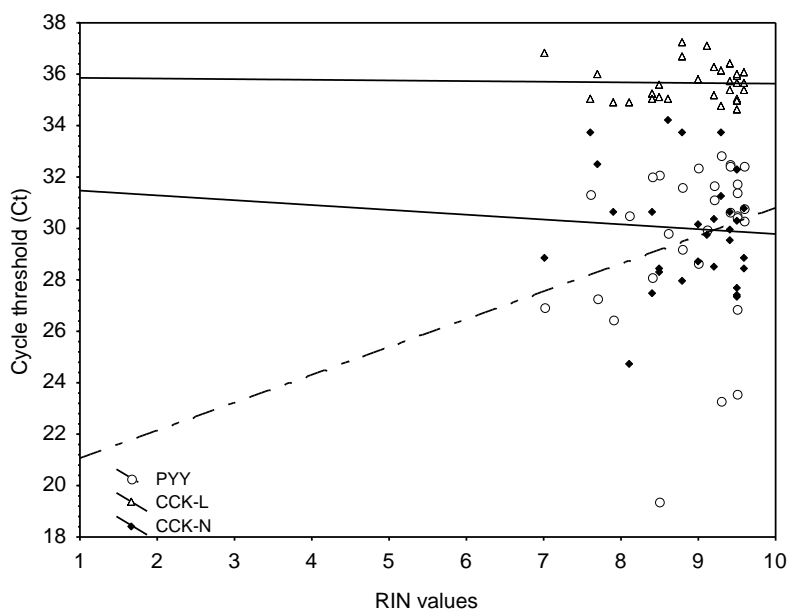
Figure A.III.4: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in stomach tissue of Atlantic salmon (segment 2). The results are represented as average Ct of two parallels from q-PCR absolute expression for PYY (n= 32), CCK-L (n= 32) and CCK-N (n= 32). The linear regression lines show the following values; PYY: y = 1.081x + 19.99, CCK-L: y = -0.025x + 35.88 and CCK-N: y = -0.188x + 31.66. The r^2 and p values of regression are shown in Table 3.4.
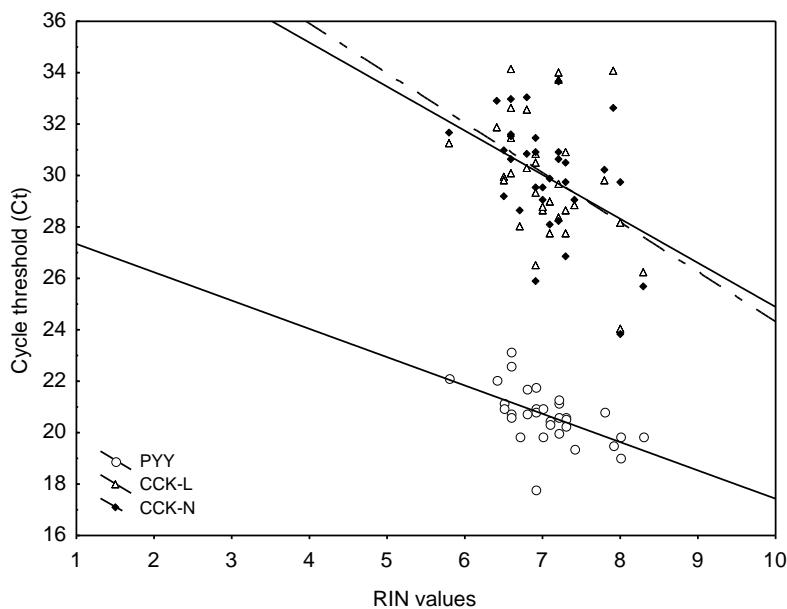
Figure A.III.5: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in pyloric cecae tissue of Atlantic salmon (segment 4). The results are represented as average Ct of two parallels from qPCR absolute expression for EF1a reference gene (n= 32), GRLN-1 (n=32) and GRLN-2 (n= 32). The linear regression lines show the following values; EF1a: y = -0.795x + 22.53, GRLN-1: y = -1.242x + 39.42 and GRLN-2: y = -1.233x + 38.12. The r^2 and p values of regression are shown in Table 3.4.

Figure A.III.6: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in pyloric cecae tissue of Atlantic salmon (segment 4). The results are represented as average Ct of two parallels from qPCR absolute expression for PYY (n= 32), CCK-L (n= 32) and CCK-N (n= 32). The linear regression lines show the following values; PYY: y = -1.101x + 28.44, CCK-L: y = -1.714x + 42.03 and CCK-N: y = -1.933x + 43.64. The r^2 and p values of regression are shown in Table 3.4.
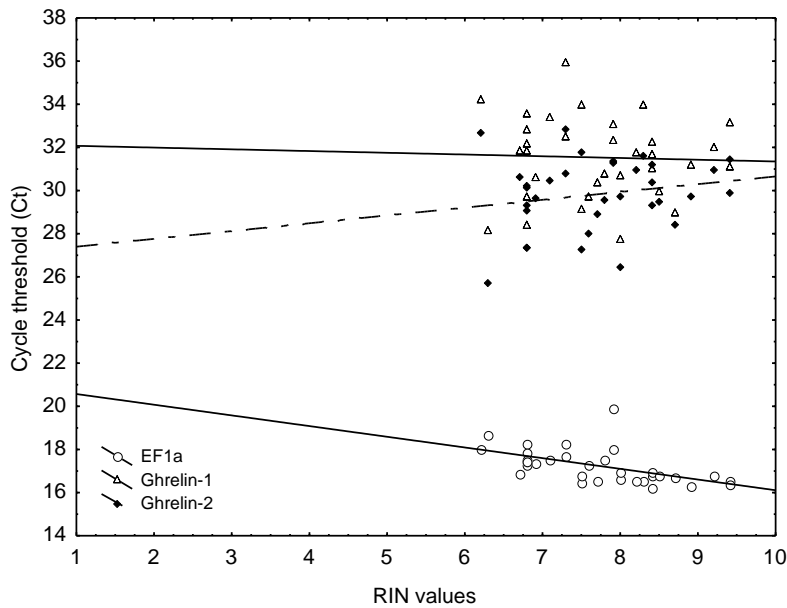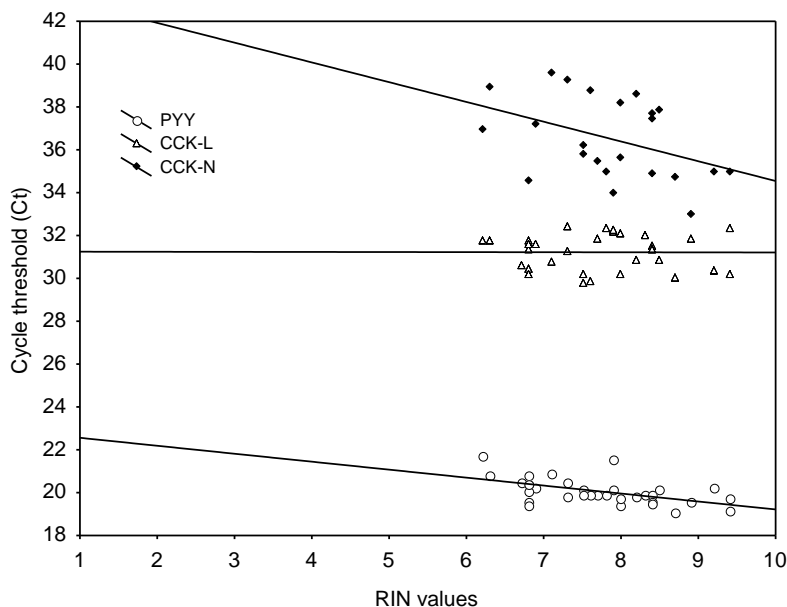
Figure A.III.7: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in midgut tissue of Atlantic salmon (segment 6). The results are represented as average Ct of two parallels from qPCR absolute expression for EF1a reference gene (n= 33), GRLN-1 (n= 33) and GRLN-2 (n=33). The linear regression lines show the following values; EF1a: y = -0495x + 21.07, GRLN-1: y = -0.080x + 32.15 and GRLN-2: y = 0.362x + 27.04. The r^2 and p values of regression are shown in Table 3.4.

Figure A.III.8: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in midgut tissue of Atlantic salmon (segment 4). The results are represented as average Ct of two parallels from qPCR absolute expression for PYY (n= 33), CCK-L (n= 33) and CCK-N (n= 33). The linear regression lines show the following values; PYY: y = -0.372x + 22.93, CCK-L: y = -0.004x + 31.25 and CCK-N: y = -0.922x + 43.77. The r^2 and p values of regression are shown in Table 3.4.
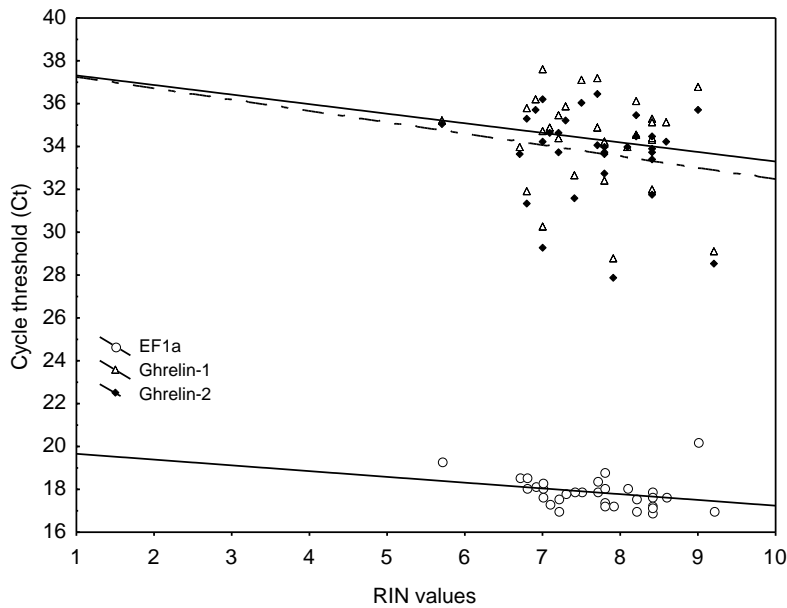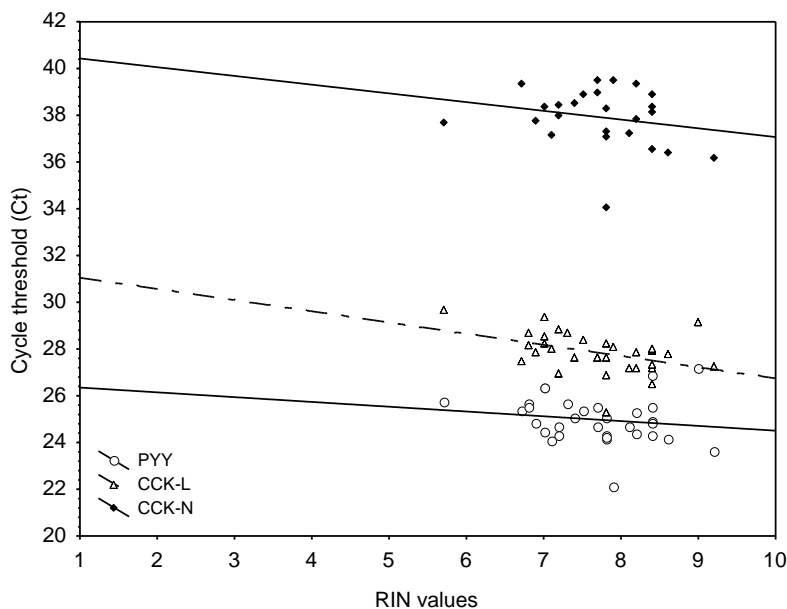
Figure A.III.9: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in hindgut tissue of Atlantic salmon (segment 9). The results are represented as average Ct of two parallels from qPCR absolute expression for EF1a reference gene (n= 32), GRLN-1 (n=32) and GRLN-2 (n=32). The linear regression lines show the following values; EF1a: y = -0.269x + 19.93, GRLN-1: y = -0.446x + 37.76 and GRLN-2: y = -0.530x + 37.78. The r^2 and p values of regression are shown in Table 3.4.

Figure A.III.10: Correlation between cycle threshold value (Ct) and RNA integrity number (RIN) in hindgut tissue of Atlantic salmon (segment 9). The results are represented as average Ct of two parallels from qPCR absolute expression for PYY (n= 32), CCK-L (n= 32) and CCK-N (n= 32). The linear regression lines show the following values; PYY: y = -0.205x + 26.56, CCK-L: y = -0.478x + 31.53 and CCK-N: y = -0.374x + 40.80. The r^2 and p values of regression are shown in Table 3.4.
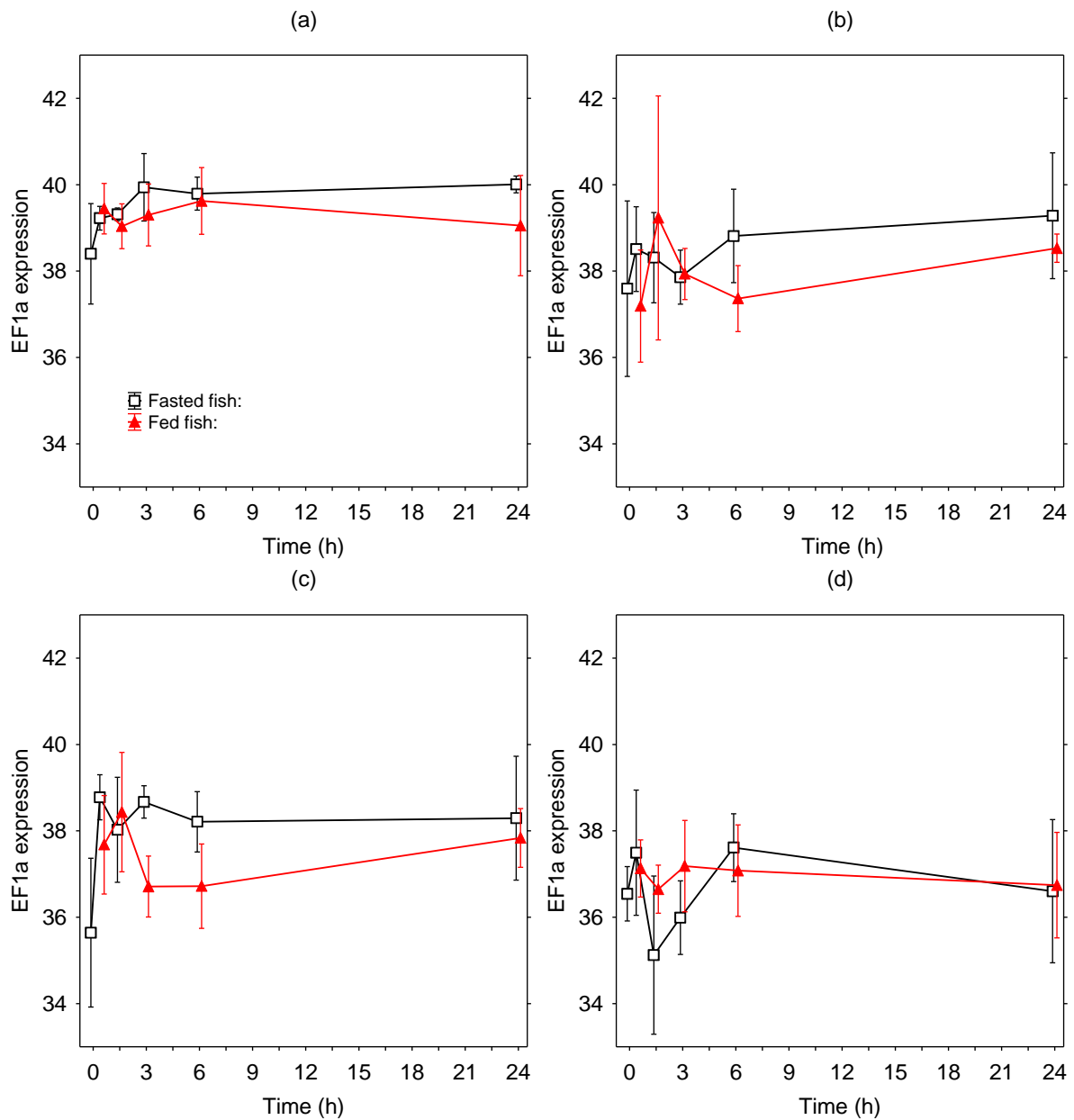
Postprandial effects and tissue distribution of EF1α

Figure A.III.11: *EF1a expression in Atlantic salmon gut tissue. Data are presented as mean (n=3) calculated copy number (±S.D) for fasted fish (black square) and fed fish (red triangle) at time (h); 0.5, 1.5, 3, 6 and 24. Time 0 is represented for fasted fish only, as this marks the start of the experiment. a) Represents results for stomach tissue (S2), b) represents results for pyloric cecae (S4), c) represents result for midgut (S6) and d) represents result for hindgut (S9). Mean values with an asterisk above show significant differences between fish groups at a given time point (p<0.5).*
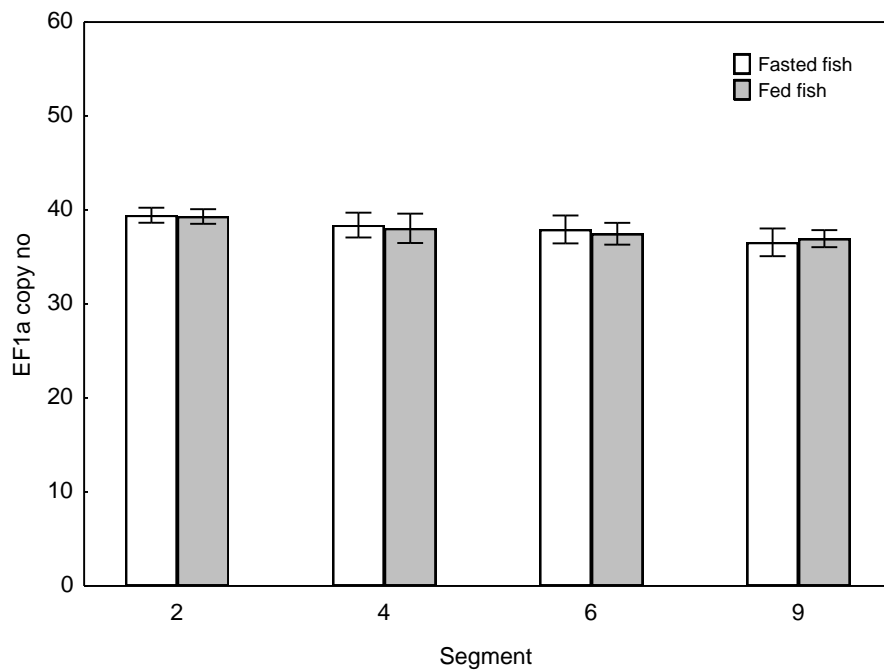
Figure A.III.12: *Segmental distribution of EF1a reference gene mRNA in Atlantic salmon GI tract. Data are represented as mean calculated copy number for fasted fish (n= 18), and fed fish (n= 15) ± SD, for stomach (S2), pyloric cecae (S4), midgut (S6) and hindgut (S9).*

Appendix IV

Composition of feed used in the postprandial experiment

Table AIV1. Dietary ingredients, chemical composition and energy of the feed used. Values provided by the producer (EWOS, Norway).

	EWOS Micro 50
Ingredients (g kg^{-1})	
Fish meal	471
Wheat	140
Plant proteins	200
Fish oil	185
Premix (vitamin, minerals)	2
Composition (g kg^{-1})	
Protein	48
Fat	24
Water	8
Ash	8.5
Fiber	1
Extracts	12
Gross energy (MJ kg^{-1})	22.9