ENDOCRINE REGULATION OF APPETITE AND GROWTH IN ATLANTIC COD (GADUS MORHUA)

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ENDOCRINE REGULATION OF APPETITE AND GROWTH IN ATLANTIC COD (*GADUS MORHUA*)

by

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Abstract

Atlantic cod, *Gadus morhua*, is an important commercial fisheries species in many North Atlantic countries. Due to the recent decline in cod populations worldwide, Atlantic cod has become an emerging species in aquaculture in Canada, in particular New Brunswick, and Newfoundland and Labrador. The well-developed resistance to long-term starvation also makes cod a good model for the study of growth- and appetite-related hormones under both short-and long-term nutritional changes. The endocrinal regulation of food intake and energy homeostasis involves a two-way communication of hormones existing in central nervous system and several peripheral organs. I aimed to identify and characterizing genes coding for two growth-related factors (PACAP and SS), as well as one central (OX) and two peripheral (ghrelin and GRP) appetite-related factors in Atlantic cod. Multiple alignments and phylogenetic analyses show that the amino acid sequences all 5 peptides appear relatively conserved among fish, at least among teleosts. The typical expression patterns reflect their functional regions and are highly similar to those of other teleosts reported so far, with high forebrain expression levels for brain peptides and high stomach expression levels for gut peptides. For all the peptides in the present study, an early appearance in development probably indicates a crucial role in development. The apparent differences of expression for PPSS 1, OX and NPY before and after first larval feeding suggest that growth and appetite in fish are already under strict endocrine regulation at very early stages of life. The different expression patterns displayed by PPSS 1 and PRP/PACAP during fasting and re-feeding indicate that they might have distinct functions in the regulation of feeding and metabolism of Atlantic cod.

OX and NPY both display periprandial changes and appear to be more affected by re-feeding than a long fasting period, while an increase in CART expression was induced during long-term food deprivation. Ghrelin displayed periprandial changes in expression but was not affected by fasting, while GRP did not display periprandial changes but was affected by food ration. One month exposure to different photoperiods did not affect food intake, body length or growth condition factors in juvenile cod. No overall orexigenic or anorexigenic tendency is shown in juvenile cod, though hypothalamic expressions of both NPY, an appetite stimulator, and CART, a feeding inhibitor, increase in cod exposed to complete darkness compared to fish in constant light. QPCR showed no significant effects of photoperiod on the expression levels of gut ghrelin and GRP. In general, the interaction displayed in Atlantic cod between brain and gut peptites with appetite and nutritional status may help further understanding of the endocrinal control of appetite and the effects of environmental factors, such as photoperiods, in teleosts.

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Abbreviations:

AgRP (Agouti-related peptide)

ARC (Arcuate nucleus)

BBS (Bombesin)

CCK (Cholecystokinin)

CART (Cocaine- and amphetamine-regulated transcript)

CRH (Corticotropin-releasing hormone)

DMH (Dorsomedial hypothalamus)

GCF (Growth condition factor)

GRP (Gastrin-releasing peptide)

GPCR (G-protein coupled receptors)

GH (Growth hormone)

GHRH (Growth hormone releasing hormone)

GHS (Growth-hormone secretagogue)

GI (Gastro-intestinal)

hpf (Hour post fertilization)

IGF-I (Insulin-like growth factor-I)

IP (Introperitoneal)

ICV (Introcerebraventricular)

IR (Iimmunoreactivity)

LHA (Lateral hypothalamus area)

MCH (Melanin concentrating hormone)

MCR (Melanocortin receptor)

MSH (Melanocyte stimulating hormone)

NJ (neighbor-joining method)

NMB (Neuromedin B)

NPY (Neuropeptide Y)

OX (Orexin)

PRP (PACAP-related peptide)

PVN (Paraventricular nucleus)

Q-RT-PCR (Quantification reverse transcriptase polymerase chain reaction)

RT (Reverse transcriptase)

RACE (Rapid Amplification of cDNA Ends)

PACAP (Pituitary adenylate cyclase activating peptide)

POMC (Proopiomelanocortin)

SEM (Standard error of means)

sGH (salmone Growth hormone)

SS (Somatostatin)

Chapter 1

General introduction

1.1. Hormonal regulation of growth and appetite in vertebrates

Body weight depends on a tight balance between caloric intake and energy expenditure. In general, animals gain weight when overfed for several weeks, but when permitted free access to food, they eat less and their body weight sharply falls to normal values. Conversely, animals lose weight under starvation, but when permitted to re-feed freely, they spontaneously increase their food intake in order to "catch up" for lost weight. In addition to food availability, various intrinsic factors, including age, gender and stress, as well as environmental factors such as temperature and photoperiod, can induce changes in feeding behavior. These variations in food intake and the maintenance of body weight within relatively narrow limits are mediated by a number of different signals and by both short-term and long-term mechanisms.

The central nervous system and several peripheral organs (such as gut, pancreas and adipose tissues) are involved in a two-way communication that regulates food intake and energy homeostasis (**Figure 1.1**). In mammals, the arcuate nucleus (ARC) is an important hypothalamic nucleus that plays a primary role in the control of appetite and in the interactions between peripheral organs and the brain. Two distinct neuronal populations in the ARC control food intake in opposite ways. One group contains neurons expressing the orexigenic peptides (feeding stimulators) neuropeptide Y (NPY) and agouti-related peptide (AgRP), while the other group contains the anorexigenic

peptides (feeding inhibitors) cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) (Schwartz et al., 2000). Other brain areas involved in the control of food intake are located downstream of the ARC. The paraventricular nucleus (PVN) produces anorexigenic peptides such as thyrotropin releasing hormone (TRH),

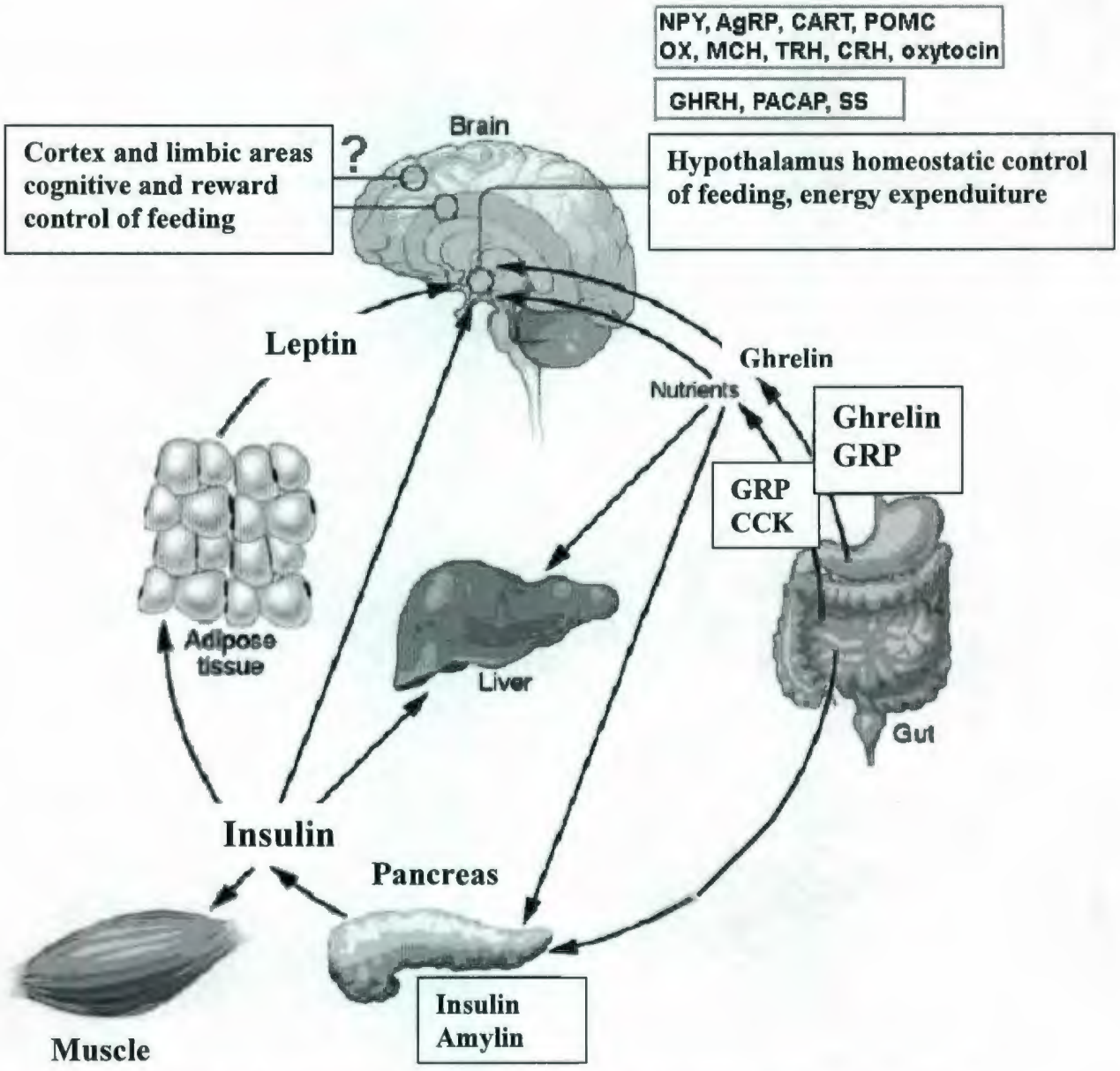


Figure 1.1 Schematic illustration of peptides secreted by the central nervous system and peripheral organs regulating energy homeostasis. (Ahima, 2008)

corticotrophin releasing hormone (CRH) and oxytocin. The lateral hypothalamic area (LHA) and perifornical area (PFA) contain the orexigenic peptides melanin-concentrating hormone (MCH) and orexins (OXs). The gut is also able to modulate feeding: mechano- and chemo-receptors located within the gastrointestinal tract sense the presence of food and convey signals to the brain either via the vagus nerve or through endocrine mechanisms to regulate short-term appetite and satiety. This short-term regulation, on a meal-to-meal basis, is performed by several hormones from the endocrine gastrointestinal cells, such as cholecystokinin (CCK) and gastrin-releasing peptide (GRP), which act to inhibit the expression and release of central food-intake stimulating factors (NPY and AgRP), thereby inducing satiety through inhibition of the PVN. In contrast, during fasting, the gastrointestinal tract releases the appetite-stimulating factor ghrelin. Ghrelin promotes the expression and release of hypothalamic NPY and AgRP thus driving food searching and ingesting behavior (Seoane et al., 2003). The balance and interactions between anorexigenic and orexigenic factors originating from the GI tract appears to play an important role in the short-term regulation of food intake. In addition to this short-term regulation, a long-term regulation of food intake is also important in maintaining food intake and energy balance and is maintained by peripheral hormones such as insulin, amylin and leptin. Insulin secretion from islet β cells of the endocrine pancreas stimulated by food ingestion and insulin levels in the systemic circulation are proportional to both body fat content and to recent carbohydrate and protein intake (Woods et al., 1985). Insulin interacts with several hypothalamic neuropeptides involved in the regulation of feeding behavior, including NPY and MCH, to decrease food intake and adipose stores

(Schwartz et al., 1999; Schwartz et al., 2000). Amylin is co-secreted with insulin by pancreatic β cells in response to food intake (Lutz, 2005). In mammals, amylin decreases food intake and treatments with amylin antagonists increase food intake and body weight (Mollet et al., 2004; Rushing et al., 2001). In mammals, another key factor in the long-term regulation is the adipocyte hormone leptin, which is produced and released in proportion to body fat mass. Leptin inhibits food intake by inhibiting NPY-containing neurons (Mercer et al., 1997) and stimulating POMC-containing neurons in the hypothalamus (Cowley et al., 2001). A number of metabolic products, including glucose, amino acids, and fatty acids, are also important cues for regulating food intake. An impairment of any of the above regulating systems (short-term and long-term) may result in disorders of feeding behavior and weight change.

Growth hormone (GH) has long been identified as the main regulator of somatic growth in vertebrates. GH is synthesized and secreted from somatotrophs in the anterior pituitary under a "dual regulator" control system that consists of two hypothalamic hormones, stimulatory growth hormone releasing hormone (GHRH) and the inhibitory somatostatin (SS). Whether this "dual regulator" system represents an overly simplistic model in mammals is a question that has been often raised, as new research shows an important role for other neuroendocrine regulators such as pituitary adenylate cyclase-activating polypeptide (PACAP) and ghrelin in the control of GH secretion (Bona and Bellone, 2003; Jarry et al., 1992; Murakami et al., 1996). In addition, new evidence suggests that other factors, including appetite-regulating peptides (such as NPY, CCK, GRP, galanin and leptin), neurotransmitters (such as dopamine), metabolic hormones

(such as thyroid hormone and corticosteroid) and sex steroid hormones are also involved in the regulation of GH secretion.

To date, molecular and behavioral evidence indicates that the regulation of food intake and growth has been relatively well conserved along the vertebrate lineage, at least between the piscine and the mammalian groups. Early studies using either electrical stimulation or lesion of specific brain areas have shown that the hypothalamus is involved in the control of food intake in fish as in mammals. Extra-hypothalamic regions (olfactory areas and ventral telecephalon) also appear to be involved in feeding in teleosts (Volkoff et al., 2005). In addition, in fish, a number of homologs of mammalian appetite- and growth-regulating peptides have been isolated or their sequences deduced from cloned cDNA sequences (Rousseau and Dufour, 2007; Volkoff et al., 2005). These are described in subsequent sections.

1.2. Appetite-related hormones

1.2.1. Hormones from the central nervous system

1.2.1.1. Neuropeptide Y (NPY) and agouti-related peptide (AgRP)

Neuropeptide Y, discovered over 20 years ago by Tatemoto and Mutt, is a 36 amino acid peptide that belongs to the pancreatic polypeptide family (Tatemoto et al., 1982). Its name derives from the single-letter code (Y) for the amino acid tyrosine since it contains several tyrosine residues including an amidated C-terminal tyrosine residue. It is one of the most abundant peptides found in the brain and one of the most evolutionarily conserved peptides. In mammals, NPY plays an essential role in the control of food

intake and body weight. Centrally administered NPY causes robust increases in food intake and body weight, and chronic NPY administration can eventually produce obesity (Clark et al., 1984). NPY activates a population of at least six G protein-coupled Y receptors, and its orexigenic effects are predominantly mediated through Y1 and Y5 receptors (Beck, 2006). Inhibition of NPY synthesis by antisense oligonucleotides or blockade of NPY action by NPY antibodies results in suppression of food intake (Chamorro et al., 2002). The absence of NPY in NPY knockout mice is associated with a pronounced delay in the initiation of feeding (Sindelar et al., 2005).

NPY is also known to regulate feeding in fish, as both central and peripheral injections of mammalian or fish NPY increase food intake in goldfish in a dose-dependent manner (Lopez-Patino et al., 1999; Narnaware et al., 2000), catfish (Silverstein and Plisetskaya, 2000) and rainbow trout (Aldegunde and Mancebo, 2006). NPY also undergoes periprandial variations in goldfish (Narnaware et al., 2000) and Atlantic cod (Kehoe and Volkoff, 2007), with a peak in brain NPY mRNA levels around feeding time. Fasting induces an increase in NPY hypothalamic expression in goldfish (Narnaware and Peter, 2001) and salmon (Silverstein et al., 1998) and re-feeding food-deprived goldfish reverses these effects (Narnaware and Peter, 2001). However, no significant differences are detected in forebrain of cod after one week of starvation (Kehoe and Volkoff, 2007). In goldfish, central administration of a Y1-receptor antagonist decreases food intake, whereas central injections with either a Y1- or a Y5-receptor agonist induce an increase in food intake (Narnaware and Peter, 2001). However, recent molecular evidence shows that Y1 and Y5 receptors are absent in both fugu and zebrafish, suggesting that these two receptor genes have been lost in some fish and that other NPY receptor subtypes mediate

the action of NPY on food intake (Larhammar and Salaneck, 2004). In fish, NPY is also involved in cardiovascular function (Preston et al., 1998; Shahbazi et al., 2002), in reproduction (Peng et al., 1994) and in the immune response (Volkoff and Peter, 2004).

AgRP is another potent orexigenic peptide in mammals. AgRP influences food intake mainly through the competitive antagonism of central melanocortin-4 receptors (MC4R) (Rossi et al., 1998). AgRP, at least in part, is a downstream physiological mediator of the central effects of leptin, because co-injections of AgRP and leptin into the rat hypothalamus block a leptin-induced decrease in food intake and bodyweight (Ebihara et al., 1999). Also, its release by ARC is inhibited by leptin infusion, while its expression is up-regulated in *ob/ob* leptin-deficient mice. A single intracerebroventricular (ICV) dose of AgRP results in long-term stimulation of food intake for up to a week, whereas other orexigenic agents such as NPY, MCH, OX and galanin only exert their effects for a few hours (Hagan et al., 2000). As the long-term effects of AgRP cannot be explained by its continued occupancy of the MC4R (Hagan et al., 2000), it has been suggested that these effects are accomplished by recruitment of extrahypothalamic sites that are involved in emotional aspects of food intake, *e.g.* hedonic aspect of eating behavior. Alternatively, the long-term actions of AgRP might be explained by a recruitment of the OX system, as the c-fos immunoreactivity (ir) pattern in the brain following ICV administration of OX injection is similar to that observed for AgRP (Mullett et al., 2000).

Although the physiological role of AgRP in fish is not known, fasting up-regulates hypothalamic AgRP mRNA levels in both goldfish (Cerda-Reverter and Peter, 2003) and zebrafish (Song et al., 2003), suggesting a possible role of AgRP in the control of food intake in fish.

1.2.1.2. Cocaine- and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC)

CART is a novel neuropeptide that has been shown to have a role in the control of feeding behavior in mammals. Centrally injected CART decreases food intake in both normal and fasted rats (Couceyro and Fritz, 2003; Edwards et al., 2000; Kristensen et al., 1998; Sun et al., 2004), while central administration of CART antibodies stimulates food intake (Kristensen et al., 1998; Lambert et al., 1998). Chronic administration of CART decreases food intake and body weight in a dose-dependent manner (Baker et al., 2000; Rohner-Jeanrenaud et al., 2002). Fasting induces a decrease in both CART mRNA levels (Kristensen et al., 1998) and the number of CART-expressing neurons in the ARC (McAlister and Van Vugt, 2004). CART peptide levels are also found to decrease during fasting in several brain regions, including the hypothalamus (Vicentic et al., 2005). The anorexigenic action of CART seems to be mediated by the central release of Glucagon-Like Peptide 1 (GLP-1), since blockade of GLP-1 receptors inhibits CART-induced hypophagia (Aja et al., 2001). CART-deficient mice display increased food intake and increased fat mass when fed a high calorie diet (Asnicar et al., 2001). Both *ob/ob* and *fa/fa* mice show reduced CART expression, indicating that leptin inhibits food intake in part through the CART pathway (Vettor et al., 2002). A missense mutation in the CART gene has recently been described, which causes severe obesity and reduction of resting energy expenditure in humans (del Giudice et al., 2001).

However, CART may have a dual effect on eating behavior, likely dependent on different sites of action in the hypothalamus. In rodents, 95% of CART neurons located in the LHA co-express the orexigenic peptide MCH (Kong et al., 2003), while in humans,

CART is absent from alpha-MSH-synthesizing neurons, but expressed in approximately one third of NPY/AgRP neurons in the infundibular nucleus of brain (Menyhert et al., 2007). This co-localization of CART with orexigenic neurons suggests that CART might also have an orexigenic role. Indeed, recent data show that although ICV administration of CART inhibits feeding, direct injections of CART into different hypothalamic areas of 24-hour fasted rats significantly increase food intake one or two hours post-injection (Abbott et al., 2001).

Two forms of CART, CART I and CART II, have been reported in goldfish, while only one form of CART has to date been found in other species. Several lines of evidence point to a role for CART in feeding in fish. ICV injection of human CART decreases food consumption in goldfish (Volkoff and Peter, 2000). Periprandial changes in brain CART expression have been shown for CART I, but not CART II, in goldfish and in Atlantic cod (Kehoe and Volkoff, 2007). Food deprivation induces a decrease in hypothalamic CART I mRNA levels and in CART II mRNA expression in the olfactory bulbs of goldfish (Volkoff and Peter, 2000), CART mRNA forebrain levels in Atlantic cod (Kehoe and Volkoff, 2007) and brain mRNA in catfish (Kobayashi et al., 2008). In catfish, hypothalamic CART mRNA levels are lower in families with fast growth rate (and higher feeding levels) compared to those with slow growth rate (Kobayashi et al., 2008). Administration of CART inhibits both NPY- and OX A-stimulated food intake in goldfish, suggesting an inhibitory action of CART on both the NPY and OX-A systems. Leptin and CART may also have a synergistic interaction in reducing food intake (Volkoff and Peter, 2001a).

POMC mRNA encodes a large precursor protein, which is cleaved into melanocyte-stimulating hormones (α -, β-, and g-MSH), adrenocorticotrophic hormone (ACTH), and β-endorphin (Cone, 2005). One or more of the POMC products has been shown to regulate feeding. α-MSH appears to be the predominant ligand involved in appetite regulation in mammals by acting on two melanocortin receptor subtypes (MC3-R and MC4-R) (Ludwig et al., 1998). Mice lacking whole POMC gene show marked obesity and increased food intake, accompanied by a marked increase in OX mRNA levels in the LHA, which is due to the loss of α-MSH activity (Lopez et al., 2007). Central administration of α-MSH and other MCR agonists (*e.g.*, melatonin II, MT II) decreases food intake and body weight (Grill et al., 1998; McMinn et al., 2000). Conversely, treatments with receptor antagonists increase food intake and body weight (Raposinho et al., 2000), and continuous infusion of a MC4-R selective antagonist induces hyperphagia and severe obesity (Kask et al., 1999). Low doses of α-MSH and MTII ICV injection significantly reduce ghrelin-dependent hyperphagia (Olszewski et al., 2007), suggesting a potential interaction between the two systems. To date, little data is available on the melanocortin system in fish and its role on food intake regulation. In fish, POMC mRNA is expressed in the mediobasal hypothalamus, a brain area associated with food intake and neuroendocrine regulation. When ICV injected in goldfish, MCRs agonists inhibit food intake whereas antagonists stimulate food intake, suggesting that melanocortins exert a tonic inhibitory effect on food intake (Cerda-Reverter et al., 2003a; Cerda-Reverter et al., 2003b). This effect appears to be mediated by central MC4R signalling (Cerda-Reverter et al., 2003a). However, fasting does not cause changes in POMC gene expression in goldfish (Cerda-Reverter et al., 2003b).

1.2.1.3. Orexin (OX)

Orexins (OXs) consist of two peptides, orexin A (OX-A) and orexin B (OX-B), derived from proteolytic processing of a single precursor molecule called prepro-orexin. In mammals, OXs act on two closely related orphan G protein-coupled receptors, termed orexin1 receptor (OX_1R) and orexin2 receptor (OX_2R). OX_1R is selective for OX-A, while OX_2R is a non-selective receptor for both OX-A and OX-B (Sakurai et al., 1998).

Orexins have been shown to have a physiological role in the regulation of food intake. In rats, ICV injections of synthetic OX increase food intake (Dube et al., 1999; Haynes et al., 1999), while IP injections of selective OX_1R antagonists (Ishii et al., 2005) or intracisternal injections of OX antibodies (Yamada et al., 2000) inhibit food intake and cause body weight loss. Fasting-induced food intake in rats is dramatically reduced by central, but not peripheral, administration of OX-A antibodies (Yamada et al., 2000). Furthermore, hypophagia is also observed in rats treated with antibodies directed at the OX_1R (Rodgers et al., 2001) as well as in mice with a targeted genetic ablation of the OX gene (Hara et al., 2001). Nutritional status and food availability both appear to induce pronounced changes in OX neurons. Fasting- or insulin-induced hypoglycaemia both increase hypothalamic OX mRNA expression (Cai et al., 1999; Griffond et al., 1999). Leptin administration inhibits fasting-induced increases in hypothalamic OX mRNA (Lopez et al., 2000), suggesting an interaction between leptin and OXs. Both obese Zucker rats and *ob/ob* rats display increased NPY levels with down-regulation of NPY receptors and diminished levels of OX with up-regulation of OX receptors (Beck et al., 2001), which might be linked to the absence of functional leptin signaling in these rats. It has been suggested that the decrease in the OX tone might participate in a counter

regulatory system necessary to limit the possible noxious effects of NPY on food intake and body weight (Beck et al., 2001). Besides appetite regulation, OXs have also been shown to be involved in several physiological processes such as gastric secretion (Takahashi et al., 1999), and the control of sleep and wakefulness (Burlet et al., 2002; Chemelli et al., 1999).

OXs also appear to have a role in the regulation of feeding in fish. In goldfish, ICV injections of either human OX-A or OX-B cause a significant increase in appetite (Volkoff et al., 1999), and the number of cells showing orexin-like ir in the hypothalamus increases in fasted fish and decreases in glucose-injected fish (Nakamachi et al., 2006). OX mRNA levels have been found to increase during food deprivation in both goldfish (Nakamachi et al., 2006) and zebrafish (Novak et al., 2005) brain. In goldfish, OX appears to interact with several other appetite-related peptides, including NPY (Volkoff and Peter, 2001b), and ghrelin (Miura et al., 2007). ICV injections with NPY or ghrelin receptor antagonists block the appetite-stimulating effects of OX-A, and blocking OX receptors decreases both NPY and ghrelin-induced feeding. In sea perch, OX-A-immunoreactive cells in the pituitary correspond to cells immunostained with antiserum against salmon sGH, suggesting that orexin might also interact with growth -related peptides in fish (Suzuki et al., 2007).

1.2.2. Peripheral systems

1.2.2.1. Ghrelin

Ghrelin is a recently discovered hormone that appears to represent a link between the short-term regulation of food intake and the long-term regulation of growth

and metabolism. Predominantly produced by the stomach in response to hunger and starvation, ghrelin is released into the general circulation and serves as a peripheral signal that acts on the central nervous system to stimulate feeding and reduce energy expenditure (Cummings et al., 2001). Ghrelin also represents the third pathway, in addition to growth hormone releasing hormone (GHRH) and somatostatin (SS), involved in regulating growth hormone (GH) release from pituitary (also see section **1.3.2.**). To date, two major forms of ghrelin have been identified in tissues and plasma of vertebrates, acyl-modified and des-acyl ghrelin. The major form of the acyl modification of ghrelin is the presence of an octanoic acid (C8) in vertebrates, with the exception of tilapia, where the decanoic acid (C10) modified ghrelin is the major form (Kawakoshi et al., 2007). The non-acylated ghrelin has a long half life and is the major form in the blood. The post-translational fatty acid modification at 3-Ser of the ghrelin molecule is essential for its transport across the blood brain barrier and for it to bind to its receptor (Banks et al., 2002). Gastric vagal afferents may also convey ghrelin's signals to the brain (Date et al., 2002).

Ghrelin has been identified in several fish species. All fish ghrelins identified to date exhibit an amide structure at the C-terminus, with the exception of shark ghrelin, which is similar to that of tetrapod ghrelins. This conservation in C-terminus structural modifications in teleost ghrelins indicates that they might have a specific functional role in teleosts (Kaiya et al., 2005; Matsumoto et al., 2001). The acyl modification of ghrelin is conserved and appears to be essential for the biological function of ghrelin in fish as in mammals. Indeed, in goldfish, administration of goldfish acylated ghrelin, but not des-acyl ghrelin, stimulates food intake and locomotor activity (Matsuda et al., 2006a;

Matsuda et al., 2006b). Recently, an appetite-inhibiting hormone, obestatin, has been reported to be present in the C-terminal peptide of the ghrelin precursor in mammals (Zhang et al., 2005). However, obestatin does not appear to be present in fish.

Chronic central administration of ghrelin significantly increases food intake and body weight in part by stimulating NPY/AgRP neurons in the hypothalamus (Kamegai et al., 2001; Seoane et al., 2003). The actions of ghrelin on feeding are also in part mediated by the OX system. Ghrelin-containing axonal terminals make direct synaptic contacts with OX-producing neurons in the ARC and LHA, and ICV injection of ghrelin induces c-fos expression in OX-containing neurons. In addition, ghrelin-induced feeding is attenuated by pretreatment with OX antibodies (Toshinai et al., 2003). One of the key regulating factors of plasma ghrelin levels are food intake and nutritional status. Plasma ghrelin levels are elevated under starvation and decrease following food intake, in response to an increase in glycemia. Plasma ghrelin levels decrease more drastically and faster following a meal rich in carbohydrates rather than in fats, while a meal rich in proteins has variable effects. Plasma ghrelin levels are also negatively correlated to body mass index (BMI). Indeed, plasma ghrelin levels increase in anorexia nervosa and cachexia, and decrease in obesity (De Vriese and Delporte, 2008). Ghrelin has other physiological actions in addition to feeding regulation, including the control of acid secretion, and the regulation of sleep and anxiety (Seoane et al., 2004).

To date, the role of ghrelin in the regulation of feeding and metabolism in fish remains unclear. Although both central and peripheral administration of ghrelin stimulate food intake in goldfish (Matsuda et al., 2006b; Unniappan et al., 2004) and IP injection of GH secretagogues (agonists of ghrelin receptor) increase food intake in rainbow trout

(Shepherd et al., 2007), a single IP injection of trout ghrelin did not affect voluntary food intake in rainbow trout (Jonsson et al., 2007). In long-term ghrelin-treated tilapia, food consumption increases significantly in those animals that receive ghrelin-C10 but not ghrelin-C8 (Riley et al., 2005). Both ghrelin mRNA expression and ghrelin peptide levels are affected by feeding in fish. Serum ghrelin levels and ghrelin mRNA expression in both hypothalamus and gut show postprandial decreases in goldfish (Unniappan et al., 2004). A 7 days fasting induces significant increases in ghrelin hypothalamic and gut mRNA expressions in goldfish (Unniappan et al., 2004) but does not affect stomach ghrelin mRNA levels in Nile tilapia (Parhar et al., 2003). One month of starvation significantly increases stomach ghrelin mRNA levels in seabass and 3 weeks of refeeding returns the ghrelin expression back to normal levels (Terova et al., 2008). A significant reduction in plasma ghrelin levels are observed in burbot after a two-week fasting (Nieminen et al., 2003) and in rainbow trout after 1-3 weeks fasting (Jonsson et al., 2007). However, fasting for 7 days has no effect on plasma ghrelin concentrations in tilapia, suggesting that that ghrelin does not act as a hunger signal in short-term fasted tilapia (Riley et al., 2008). It appears that in goldfish as in mammals, ghrelin could be acting through the NPY and OX pathways. Central, but not peripheral, administration of ghrelin at a dose sufficient to stimulate food intake increases brain NPY mRNA expression (Miura et al., 2006) and the stimulation of food intake induced by ICV or IP injection of ghrelin is suppressed by ICV pre-injection of a NPY Y1 receptor antagonist (Miura et al., 2006), suggesting an interaction between ghrelin and NPY. Pretreatment with a selective OX receptor-1 antagonist inhibits ghrelin-induced feeding, and OX mRNA expression in the diencephalon increases in response to ICV injection of ghrelin

(Miura et al., 2007), suggesting an interaction between ghrelin and OXs. It has also been suggested that ghrelin might be involved in promoting gastric emptying, in inhibiting water intake, and in regulating the immune and reproductive systems (Riley et al., 2008).

1.2.2.2. Bombesin (BBS) and gastrin releasing peptide (GRP)

Bombesin (BBS) is a tetradecapeptide originally purified from the skin of the European frog (*Bombina bombina*) (Anastasi, 1971). Later, two BBS-like peptides, GRP and neuromedin B (NMB) were purified from mammalian tissues and found to be widely distributed in the gastrointestinal tract and brain. All BBS-like peptides share a similar carboxyl-terminal (C-terminal) heptapeptide and, based on common amino acids in their C-terminal region, they are divided into three subfamilies, the bombesin-related peptides (BRP) family (BBS, ranatensin, and phyllolitorin), the NMB family and the GRP family (Nagalla et al., 1996). Several BBS-like peptides have been shown to affect feeding (Fekete et al., 2002; Fekete et al., 2007; Gibbs et al., 1979).

BBS-like peptides injected into different regions of the brain decrease food intake (de Beaurepaire and Suaudeau, 1988; Kyrkouli et al., 1987; Stuckey and Gibbs, 1982). ICV injections of either BBS or GRP, but not NMB, dose-dependently decrease food intake in male Wistar rats fasted for 17 h (Yamauchi et al., 1998). However, brief vena caval infusions of GRP and NMB significantly reduce meal size and duration in spontaneously feeding rats (Rushing et al., 1996). GRP/NMC ir increases in the antrum of the stomach after food ingestion, and it has been hypothesized that GRP/NMC act as negative feedback satiety signal through both neuronal (via the vagus) and humoral effects (Kateb and Merali, 1992). BBS-like peptides are usually more potent in inhibiting

food intake after ICV than after peripheral administration. GRP/BBS also potently delays gastric emptying (Yegen et al., 1996), but the extent to which these effects on GI motility contribute to the reduction of food intake is uncertain.

To date, BBS/GRP has only been isolated in few fish species, including goldfish, trout, dogfish, zebrafish and shark (Conlon et al., 1987; Jensen and Conlon, 1992; Volkoff et al., 2000). Little is known about the role of BBS-like peptides in the regulation of food intake in fish. In goldfish, BBS/GRP binding sites are located in nuclei of the hypothalamic feeding area, which supports a role for BBS/GRP-like peptides in the regulation of feeding behavior in fish (Himick et al., 1995). BBS decreases feeding when injected IP in carp (Beach et al., 1988) and following both IP and ICV injections in goldfish (Himick and Peter, 1994a). However, in rainbow trout, GRP plasma levels are low, do not display periprandial changes and are not influenced by meal size (Jonsson et al., 2007).

1.2.2.3. Cholecystokinin (CCK)

In mammals, CCK is released postprandially from endocrine cells of the small intestine and inhibit gastric emptying, pancreatic exocrine secretions and food intake (Wank, 1995). CCK-1 receptors are responsible for the anorectic actions of CCK (Moran et al., 1992; Moran et al., 1998). Interestingly, CCK knock-out mice have normal food intake body weight, and body mass, suggesting other complementary pathways regulate food intake when CCK is absent (Lo et al., 2008). In mammals, CCK interacts with other appetite regulators. IP administration of CCK reduces fasting-induced hyperphagia, and increases OX-A content in the posterior brainstem of 48 h fasted rats (Gallmann et al.,

2006), which suggests a relationship between peripheral CCK and the hypothalamic OX neurons exists. CCK also seems to modulate DMH NPY signaling as immunohistochemical studies show that CCK1 receptors and NPY are co-localized in DMH neurons, and administration of CCK into the DMH down-regulates NPY gene expression and inhibits food intake in intact rats (Bi et al., 2004; Chen et al., 2007).

CCK is also involved in the regulation of feeding of fish. In goldfish, both peripheral and central injections of CCK suppress food intake (Himick and Peter, 1994b; Volkoff et al., 2003), and rainbow trout treated with CCK receptor antagonists eat significantly more than controls (Gelineau and Boujard, 2001). In yellowtail fish, CCK mRNA levels in the anterior intestine are significantly higher in fed fish than those in fasting fish (Murashita et al., 2006). Periprandial changes in CCK mRNA brain expression have also been shown in goldfish with elevated level 2h after a meal (Peyon et al., 1998). In goldfish, blockade of CCK brain receptors results in an inhibition of leptin-induced hypophagia and centrally injected leptin potentiates the satiating actions of CCK, suggesting a synergistic interaction between leptin and peripheral CCK in fish (Volkoff et al., 2003). Blockade of CCK brain receptors also results in attenuation of the inhibiting action of leptin on both NPY- and OX A-induced feeding and CCK appears to mediate in part the effects of leptin on food intake, suggesting that the actions of leptin are mediated in part by CCK through NPY and OX pathways (Volkoff et al., 2003).

1.2.2.4. Leptin

Leptin, secreted primarily by adipocytes, regulates food intake and energy expenditure in mammals. Circulating leptin levels are elevated in obesity states and

decreased in response to food restriction and fasting. Both *ob/ob* (leptin deficient) and *db/db* (leptin receptor deficient) mice show obesity and hyperphagia, and central administration of leptin to *ob/ob* mice completely reverses the body weight and metabolic phenotypes (Friedman and Halaas, 1998). Leptin appears to act on other appetite-related systems. Leptin stimulates hypothalamic POMC/CART neurons and inhibits NPY/AgRP neurons (Pinto et al., 2004), and blocks OX-induced food intake (Zhu et al., 2002) as well as the fasting-induced increases in hypothalamic OX mRNA expression (Lopez et al., 2000) .

Besides its role on feeding, leptin also takes part in controlling GH secretion. Leptin has a potent stimulatory effect on both spontaneous pulsatile GH secretion and the GH response to GHRH (Tannenbaum et al., 1998). ICV infusions of leptin stimulate GHRH and inhibit SS release (Cocchi et al., 1999; Tannenbaum et al., 1998). Leptin treatment abolishes fasting-induced inhibition of GH secretion, and central infusion of leptin antiserum suppresses pulsatile GH release in fed rats (Carro et al., 1997).

Leptin has only recently been isolated in fish. Its sequence has only been identified for a small number of species, including carp, pufferfish, salmon, and medaka (Huising et al., 2006; Kurokawa et al., 2005). The relatively late characterization of leptin in fish is partly due to low amino acid identities between fish and mammals (10-15%) and even between fish (10-48%) (Huising et al., 2006; Kurokawa et al., 2005). In contrast to mammals, where leptin is predominantly expressed in the subcutaneous adipose tissue, the liver appears to be the major site for leptin expression in carp and pufferfish (Volkoff et al., 2003). In goldfish, both central and peripheral injection of mammalian leptin reduce food intake (de Pedro et al., 2006) and chronic IP treatment of leptin reduces food

intake, body weight gain, specific growth rate and food efficiency ratio (Volkoff et al., 2003). In goldfish, central injections of murine leptin inhibit both NPY- and OX-A induced food intake (Volkoff and Peter, 2001a), reinforce CART-induced inhibition of feeding behavior and food intake (Volkoff and Peter, 2001a) and increase CART mRNA expression in different brain regions (Baker et al., 2000), which suggests interactions between leptin and other feeding regulators exist in fish. However, long-term of peripheral treatment of immature coho salmon with human leptin has no effect on growth, energy stores, or plasma levels of IGF-I, insulin, and GH, suggesting its functions might be quite different in some fish (Isaksson et al., 1987). In rainbow trout, IP injections of recombinant trout leptin suppress food intake and induce a decrease in hypothalamic NPY mRNA levels and an increase in POMC mRNA levels compared with vehicle-injected controls (Murashita et al., 2006). The role of leptin in the regulation of feeding, growth and metabolism in fish remains unclear and needs further investigation.

1.2.3. Summary

The regulation of food intake has been relatively well conserved and defined in mammals as the same appetite-regulating peptide homologs are present in same tissues and appear to have similar actions (**Fig 1.2**). Recent reports show that appetite-regulating peptide homologs have been found in fish to have similar function (**Fig 1.3**). However, increasing evidence suggests that in fish, species specific differences might exist. In order to characterize an appetite-regulating peptide in a specific fish, *e.g* Atlantic cod, one must first find out those genes and take into account not only the actions of the hormone and its interactions with other appetite-related hormones and other environmental factors,

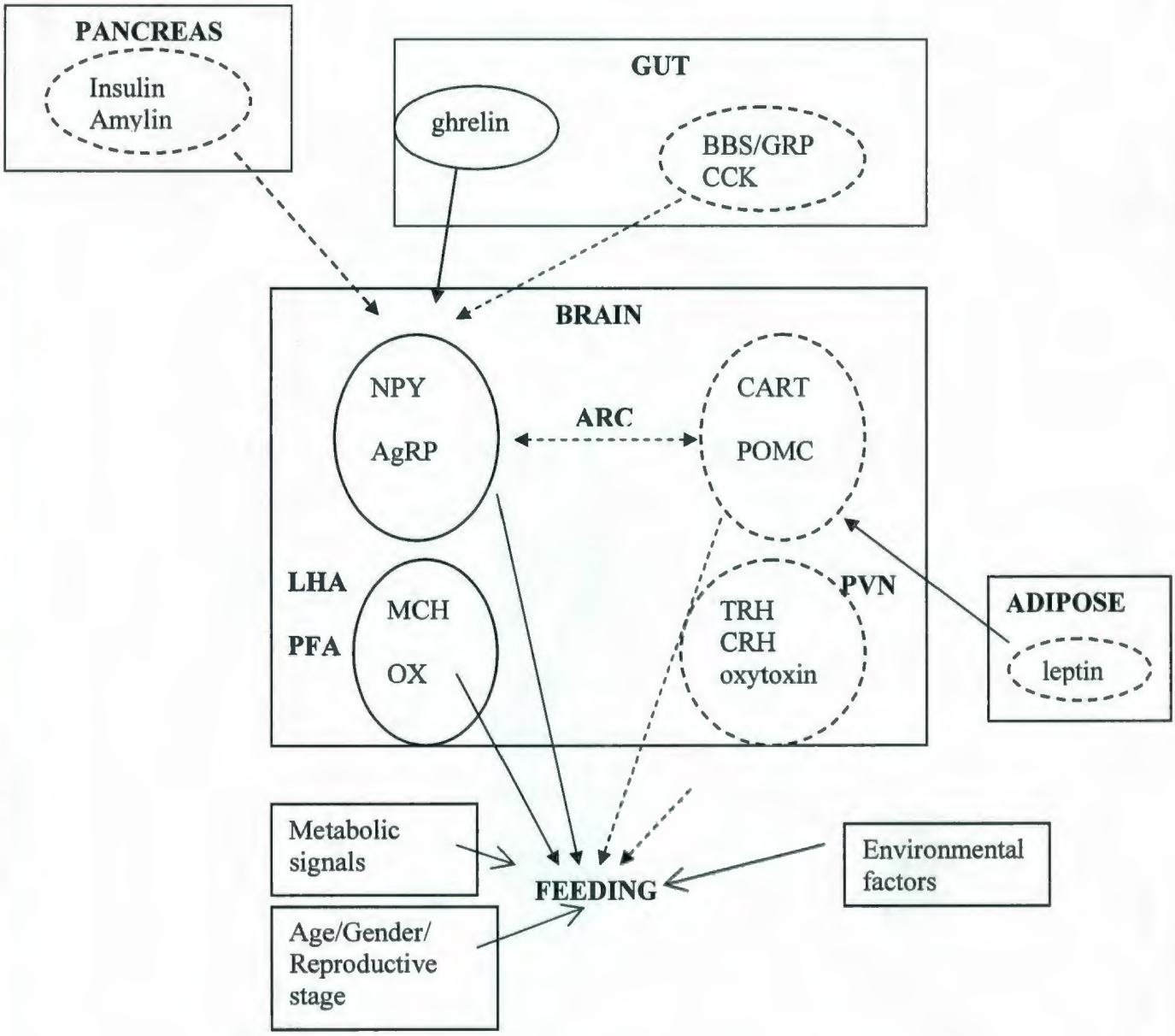


Figure 1.2. Simplified paradigm for the regulation of food intake in mammals. Orexigenic peptides are indicated by plain circles and anorexigenic peptides are indicated by dotted circles. Stimulating effects are represented by plain arrows and inhibiting effects are represented by dotted arrows.

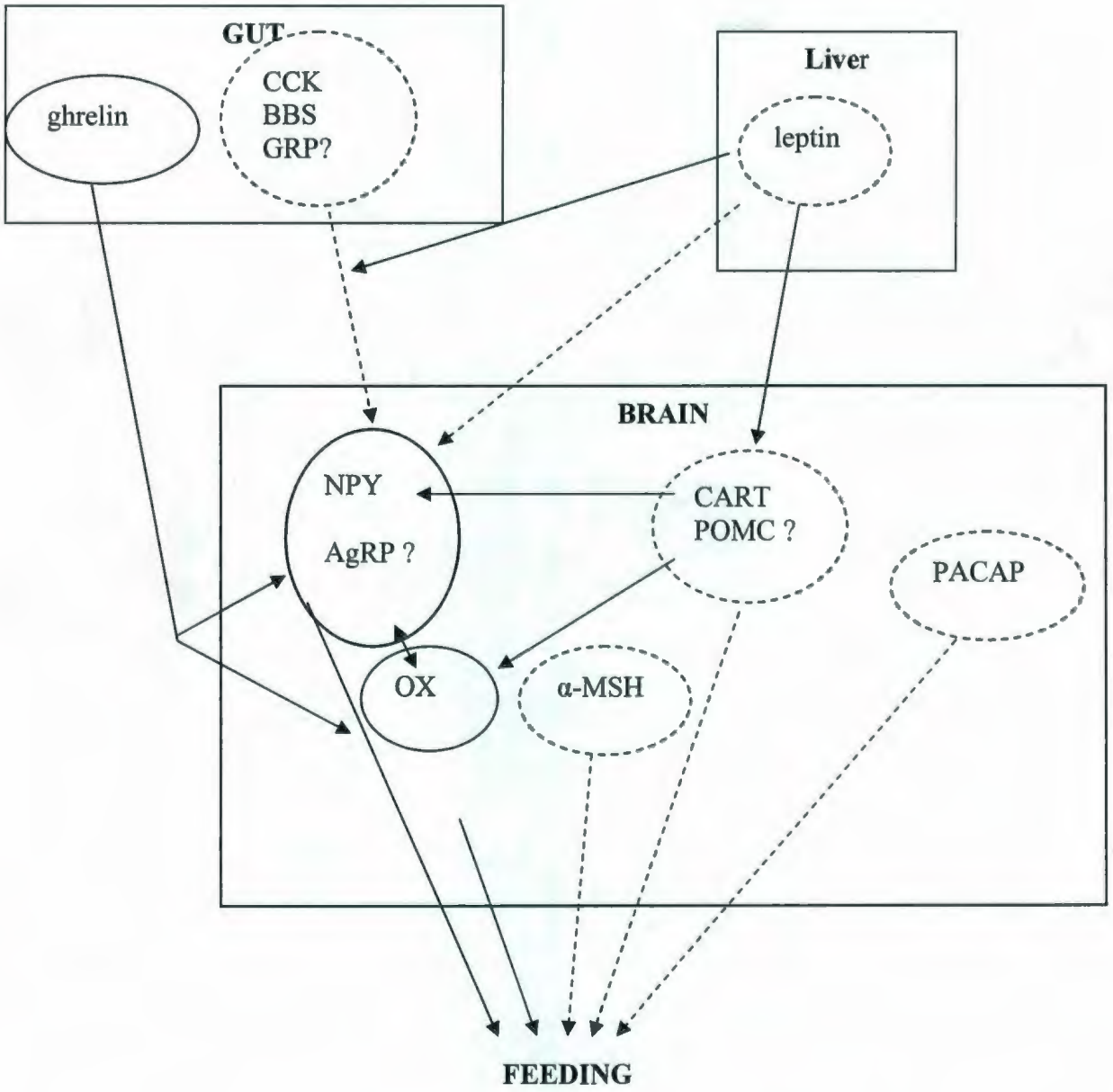


Figure 1.3. Simplified paradigm for the regulation of food intake in fish. Orexigenic peptides are indicated by plain circles and anorexigenic peptides are indicated by dotted circles. Stimulating effects are represented by plain arrows and inhibiting effects are represented by dotted arrows.

1.3. Growth-related hormones

1.3.1. Somatotrophic axis

GH is synthesized and secreted from somatotrophs in the anterior pituitary, and is the major regulator of apical growth in vertebrates. GH exerts its action either through direct effects by binding its receptor on target cells or through indirect effects by stimulating the secretion of insulin-like growth factor-I (IGF-I) by the liver and other tissues (Leshin et al., 1994). In mammals, GH secretion has long been known to be regulated by the interplay of two major hypothalamic neuropeptides, the stimulatory GHRH and the inhibitory SS. Hypothalamic GHRH and SS neurons directly innervate the median eminence, and release their hormones into the hypophyseal portal blood (Wagner et al., 1998). The amounts secreted and the ratio of GHRH/SS determine the GH release patterns by the pituitary (Baker et al., 1993; Giustina and Veldhuis, 1998; Liu et al., 1993). In turn, the secretions of GHRH and SS are regulated by a negative feedback from blood concentrations of both GH and its downstream effector IGF-I (Patel, 1999). In mammals, two biologically active forms, SS-14 and its NH2-terminal extension of 14 amino acids, SS-28, are produced by alternative cleavage from the same precursor, preprosomatostatin 1 (PPSS 1) (Tostivint et al., 2004). In mammals, cortistatin (CST), which is a homolog of the SS 2 variant of non-mammalian species, also acts as an inhibitor of GH secretion (Tam et al., 2007).

Pituitary adenylate cyclase-activating polypeptide (PACAP), a peptide structurally related to GHRH, might also be involved in regulating GH secretion. In mammals, post-translational processing of the PACAP precursor generates two

biologically active forms, PACAP38 and PACAP27, and a peptide called PACAP-related peptide (PRP), whose biological functions remain unknown (Chance et al., 1995; Morley et al., 1992).

PACAP and SS might also be involved in the regulation of feeding. ICV injection of PACAP reduces food intake in rodents (Levine and Morley, 1982; Lotter et al., 1981). The effects of SS on feeding are inhibitory, although it appears to reduce food intake only in animals with a mild degree of hunger (Lee et al., 2007). The mechanisms by which SS might act to reduce appetite are still uncertain.

Recent evidence indicates that in non-mammals, peptides previously known as "GHRH-like peptides" are in fact counterparts of mammalian PRPs, while a "real" GHRH is present as a separate gene (Gelineau and Boujard, 2001). More research is needed to demonstrate the role of GHRH in the regulation of GH secretion in fish. In fish, four different cDNAs (PPSS 1, PPSS 2, PPSS 3 and PPSS 4) encoding preprosomatostatin (PPSS) peptides have been identified to date, and PPSS mRNAs or SS proteins and alternative variants have been isolated from over 20 species (Canosa et al., 2007). Several *in vitro* and *in vivo* studies have shown that SS-14-1 regulates pituitary function in teleost fish, as it inhibits both basal GH secretion and GH secretion stimulated by other factors (Sherwood et al., 2000). The physiological effects of the other various fish SS isoforms on GH regulation remain unclear, since some inhibit GH release whereas others do not.

Pituitary PACAP, a structurally related peptide that evolved with GHRH along the glucagons lineage, has been proposed to be the ancestral GHRH in lower vertebrates (Montero et al., 1998; Olszewski et al., 2007; Wong et al., 1998; Wong et al., 2005).

PACAP stimulates GH release and GH mRNA expression by direct actions at the pituitary level via PACAP receptor type 1 (PAC1-R) in goldfish, European eel and common carp (Matsuda et al., 2005b).

PACAP and SS have also been found to be involved in the regulation of feeding in fish. Both centrally and peripherally injected PACAP suppress food intake in goldfish (Miura et al., 2007), and these actions might be mediated by the stimulation of POMC and corticotropin-releasing hormone (CRH) pathways in the brain (Canosa et al., 2004). In goldfish brain, SS mRNA has been identified in nuclei associated with feeding behavior as well as various sensory regions which project to the hypothalamus, adequately positioning SS for a putative role in feeding (Very et al., 2001). Implantation of rainbow trout with SS 1 has no effect on food intake, but significantly reduces food conversion (Kamegai et al., 2004).

1.3.2. Ghrelin: a third pathway of GH regulation

Ghrelin is a natural ligand of the growth hormone secretagogue (GHS)-receptor (GHS-R), and functions primarily as a GH-releasing hormone and an orexigen, although it also has several other biological actions. Ghrelin represents a third pathway for the regulation of GH release from the pituitary in addition to GHRH and SS. Ghrelin is produced mainly by the stomach, but it is also synthesized in the hypothalamus and pituitary and several other tissues. Ghrelin secreted by the pituitary stimulates the release of GH by directly acting at the level of the pituitary to optimize somatotroph responsiveness to GHRH (Korbonits et al., 1999; Tannenbaum et al., 2003; Wren et al., 2002). Ghrelin secreted by the stomach stimulates GH release both directly from the

pituitary gland and through stimulation of the hypothalamus by binding to an orphan G-protein coupled receptor, growth hormone secretagogue-receptor 1a (GHS-R1a). Ghrelin can act directly on pituitary cells to stimulate GH release or increase GHRH-stimulated cAMP production. Ghrelin can also act on GHRH-producing neurons in the ARC to increase GHRH release, but does not appear to alter hypothalamic SS release (Yamazaki et al., 2002). Conversely, SS has been shown to inhibit the effects of ghrelin on GH release in mammals (Kineman et al., 1999). An intact GHRH system appears to be necessary for ghrelin to maintain its maximal stimulatory effect on GH release (Sun et al., 2003). Interestingly, body size, body weight and body composition, glucose, insulin and leptin concentrations (Wortley et al., 2004) as well as plasma GH concentrations (Fox et al., 2007; Kaiya et al., 2003c) are similar in ghrelin-null, GHSR-null and wild-type mice, suggesting that compensatory pathways may exist in the mutant mice.

In fish, ghrelin can also act as a potent GH stimulator in the pituitary. Experiments conducted either *in vitro* (cultured pituitary cells) or *in vivo* (injections) have shown that the release of GH is simulated by ghrelin or GH secretagogues in tilapia (Unniappan and Peter, 2004), goldfish (Chan et al., 2004), seabream (Kaiya et al., 2005), channel catfish (Ran et al., 2004), orange-spotted grouper (Kaiya et al., 2003a) and rainbow trout (Unniappan and Peter, 2004). Ghrelin-stimulated GH release from goldfish pituitary cells is inhibited by SS-14 (Canosa et al., 2005) and IP injections of ghrelin cause a significant reduction in prepro-SS-3 mRNA expression in the goldfish brain, indicating possible interactions between ghrelin and SS in the regulation of GH release in goldfish (Zhao and Wang, 2006).

1.3.3 Summary

The regulation of growth has been well conserved and defined in mammals for century (**Fig 1.4**). Fish have shown homologs of peptides and similar function (**Fig 1.5**). However, evolutionary ancient vertebrates, multi-copied of genes, versatile habitats and unlimited growth make the growth regulation study more complex in fish.

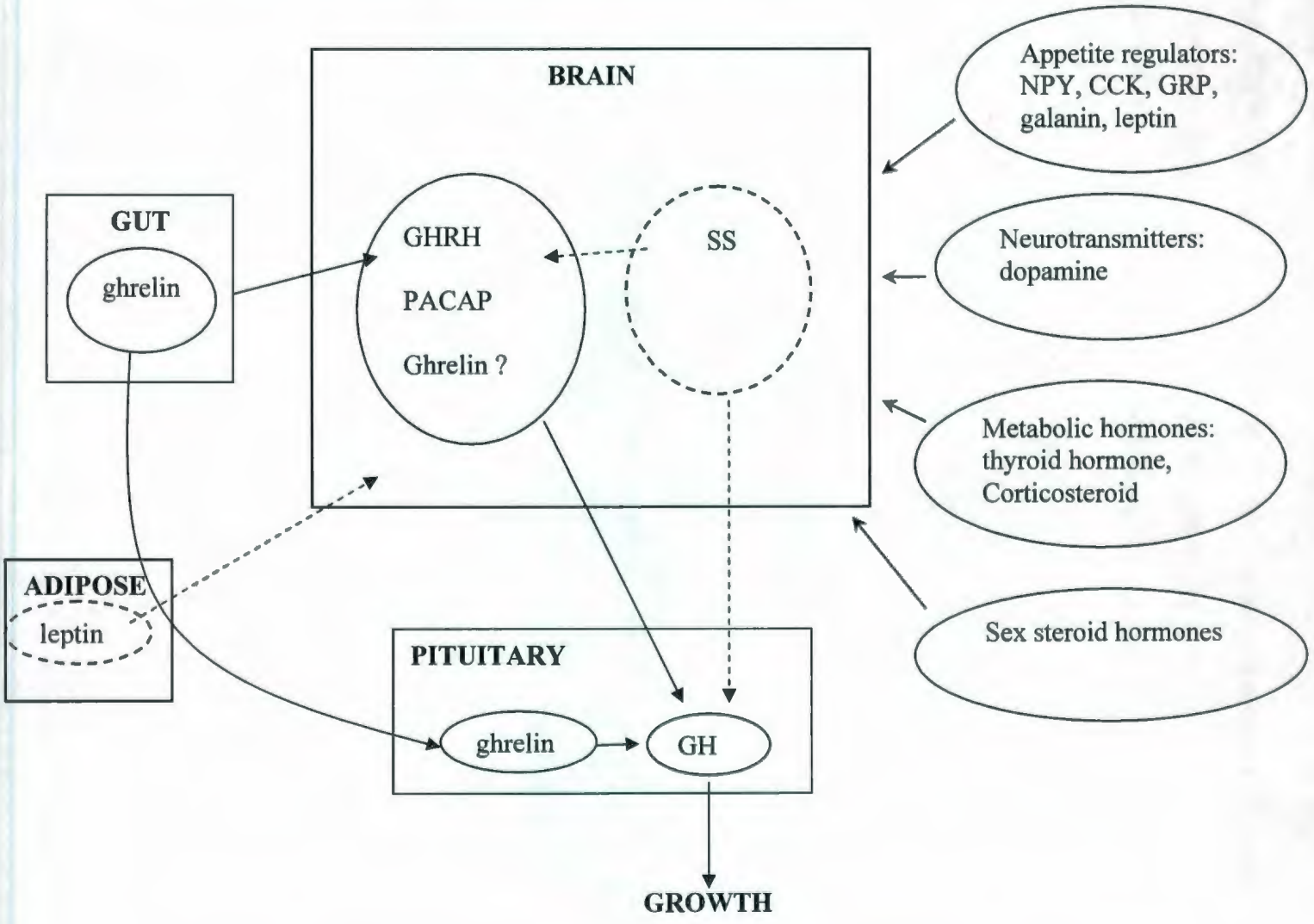


Figure 1.4. Simplified paradigm for the regulation of growth in mammals. Orexigenic peptides are indicated by plain circles and anorexigenic peptides are indicated

by dotted circles. Stimulating effects are represented by plain arrows and inhibiting effects are represented by dotted arrows.

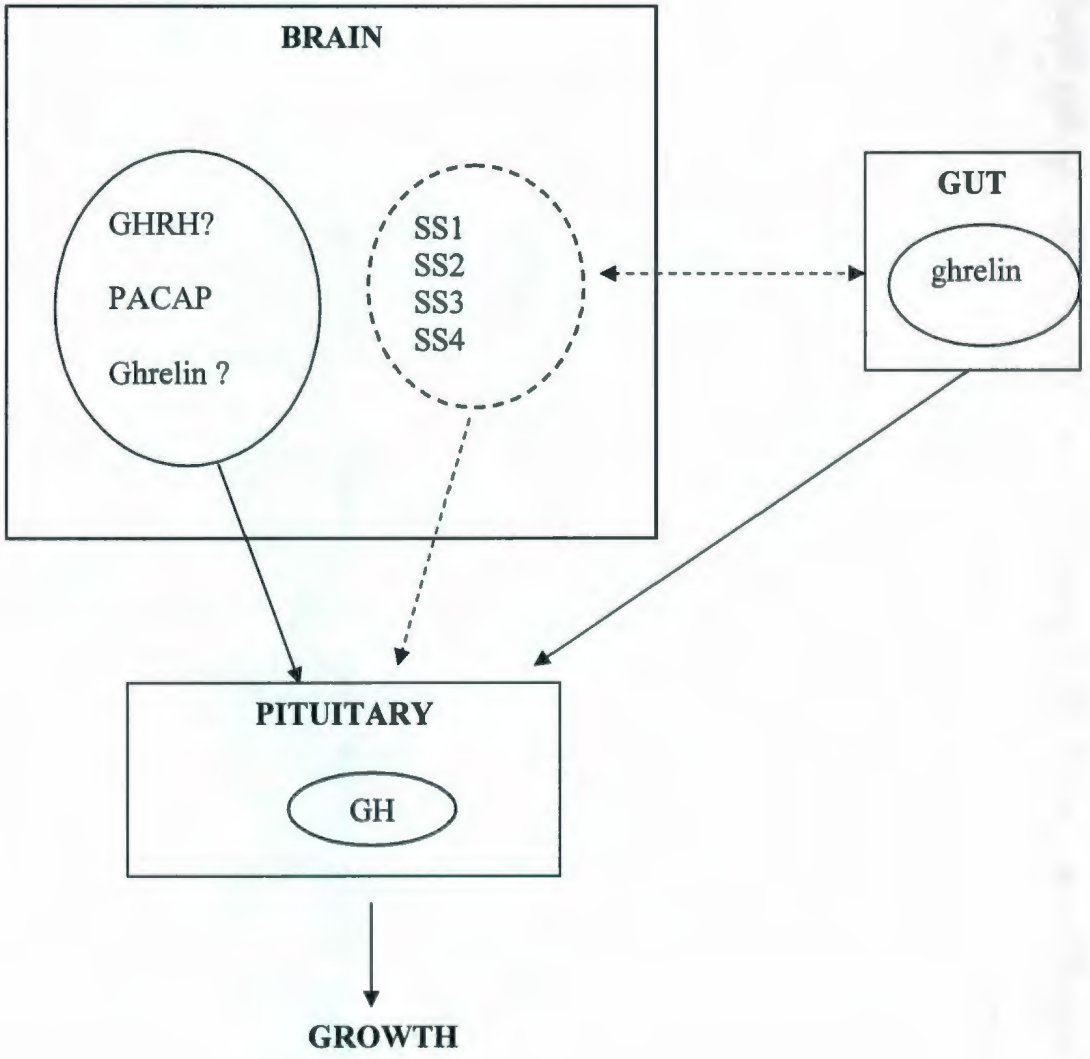


Figure 1.5. Simplified paradigm for regulation of growth in fish. Orexigenic peptides are indicated by plain circles and anorexigenic peptides are indicated by dotted circles. Stimulating effects are represented by plain arrows and inhibiting effects are represented by dotted arrows.

1.4. Environmental factors

Many species in temperate and cold climates display profound seasonal cycles in food intake and body weight, which may be the result of changes in environmental factors, such as temperature, photoperiod and food availability. Appetite-related hormones are likely mediators of these environment-induced changes in food intake or body weight.

In mammals, several hormones (*e.g.* leptin, ghrelin, AgRP, POMC and NPY) have been shown to be affected by photoperiod. Short day (SD) voles show lower levels of serum leptin than long day (LD) voles, which is correlated positively with body fat mass and negatively with gross energy intake (Mercer et al., 2000). In Siberian hamsters, exposure to short photoperiods for 18 weeks results in down-regulation of POMC and leptin receptor mRNA expression levels and up-regulation of AgRP mRNA levels (Mercer et al., 2003). Compared to controls kept in a LD photoperiod, Siberian hamsters show elevated CART mRNA throughout the ARC that depends on age and gender, when exposed to a SD photoperiod for 14 or 21 days (Harrison et al., 2008). Photoperiod has also been shown to influence the actions of ghrelin on appetite and reproduction. Central ghrelin injections increase voluntary food intake 2-fold in the first hour after administration in LD but not in SD, and decrease luteinizing hormone (LH) pulse frequency and amplitude in SD but not in LD, and ghrelin-induced GH release is 1.5-fold larger in LD than in SD (Archer et al., 2005). Photoperiod can also influence the response of hypothalamic appetite-related peptides to glucose. In LD, but not in SD, the hypothalamic ARC gene expression is lower for NPY and AgRP and higher for POMC,

in glucose- than saline-infused sheep (Kohbara et al., 2000; Noble et al., 2005; Sunuma et al., 2007; Tucker et al., 2006).

Feeding activity has been shown to be affected by photoperiod and light regimens in a number of fish species (Canavate et al., 2006), although exceptions have been reported in sole (Reddy and Leatherland, 2003a). Plasma GH, thyroid hormone and cortisol concentrations are affected by light regimens in rainbow trout (Nordgarden et al., 2007) and plasma GH is elevated in Atlantic salmon under increased photoperiod (Davis and McEntire, 2006). In contrast, photoperiod does not affect circulating IGF-I levels in sunshine bass (Beaulieu and Guderley, 1998). These data suggest a role for appetite regulating factors that stimulate the GH related pathways, at least in salmonids. The specific endocrine mechanisms behind these changes remain unclear and will require further investigation.

1.5 Atlantic cod

Atlantic cod, *Gadus morhua*, is a bony ray-finned fish (class Osteichthyes, subclass Actinopterygii) belonging to the Gadiform order and the Gadidae family. Atlantic cod represents a good model for the study of growth- and appetite-related hormones. First, as a result of a decrease in food availability during the prolonged winter periods, this species has developed a resistance to starvation and has been shown to be able to survive up to 16 weeks of fasting (Beaulieu and Guderley, 1998). The endocrine mechanisms regulating these adaptations are largely unknown and examining them might reveal new patterns for the regulation of feeding in vertebrates. Second, for hundreds of years, Atlantic cod has been an important commercial fisheries species in many North Atlantic countries and

been often referred to as "beef in the ocean". Due to the recent decline in cod populations worldwide, and a collapse in cod stocks in the early 1990s, Atlantic cod has become an emerging species in aquaculture in Canada, in particular New Brunswick, and Newfoundland and Labrador. Canada's first farmed cod was harvested from a site in Hermitage Bay, Newfoundland and Labrador, in 2003. To date, however, the cod farming industry is still developing and facing problems. Further research is still needed to optimize growth and health of cod. In particular, there is a need to understand feeding mechanisms in cod, to develop diets to promote feeding and growth and stress resistance in juvenile fish and to optimize the weaning period where cod learn to go from live feed to dry pelleted feed. A better understanding of the endocrine regulation of feeding and growth in Atlantic cod could provide valuable information that could be used in the aquaculture industry.

1.6 Summaries and objectives of the thesis

In summary, food intake (**Figures 1.2 and 1.3**) and growth (**Figures 1.4 and 1.5**) regulation involves intricate networks of hormones produced by both brain and peripheral organs in vertebrates. Fish, in particular, show specific patterns due to their variable physiological states (*e.g.* nutritional status and developing stages) as well as changing habitat environments (*e.g.* photoperiod, temperature, salinity...). The experiments outlined in this thesis aim at identifying and characterizing genes coding for two (PACAP and SS) growth-related factors, as well as one central (OX) and two peripheral (ghrelin and GRP) appetite-related factors in Atlantic cod, a commercially important marine fish. Studies were designed to investigate their potential roles in growth and food intake under

different nutritional status and environmental changes (*i.e* photoperiod). Two other previously cloned central appetite-related factors, NPY and CART were also examined to help understand the endocrine mechanism behind long-lasting resistance of Atlantic cod to food shortage during the winter.

Chapter 2

Molecular characterization of growth-related hormones (PACAP and PPSS 1) and appetite-related hormones (OX, ghrelin and GRP) in Atlantic cod

2.1. Introduction

Growth hormone-releasing hormone (GHRH), pituitary adenylate cyclase-activating polypeptide (PACAP) and somatostatin (SS) are major hypothalamic hormones and regulate growth hormone (GH) secretion from the pituitary (Tam et al., 2007).

In mammals, post-translational processing of the PACAP precursor generates two biologically active forms, PACAP38 and PACAP27, and a peptide called PACAP-related peptide (PRP), whose biological functions remain unknown (Tam et al., 2007). GHRH-like peptides and PACAP have been identified in a number of fish species. It was originally hypothesized that the GHRH-like/PACAP gene in fish represented an ancestral gene that had given rise to both PRP/PACAP and GHRH genes in higher vertebrates following a gene duplication event (Lee et al., 2007). Recent evidence indicates that GHRH-like peptides found in non-mammals are in fact counterparts of mammalian PRPs, while a "real" GHRH that is capable of stimulating GH release is distinctly present in another gene, as is the case in mammalian species (Patel, 1999).

In mammals, two biologically active forms, SS-14 and SS-28 with its NH2-terminal extension of 14 amino acids, are produced by alternative cleavage from the same precursor, designated as preprosomatostatin 1 (PPSS 1) (Gelineau and Boujard, 2001). In fish, four different cDNAs coding for preprosomatostatin (PPSS) peptides have been

identified to date, and SS proteins or mRNAs encoding PPSSs have been isolated from over 20 species (Klein and Sheridan, 2007). In addition to PPSS1, a second preprosomatostatin (PPSS 2) encodes a peptide with [Pro2]-SS-14 at its C-terminus (denoted SS-14-2) (Tostivint et al., 2008). Most teleost fish possess a third somatostatin precursor, preprosomatostatin 3 (PPSS 3), containing the [Tyr7, Gly10]-SS-14 at its C-terminus (denoted SS-14-3). PPSS 3 appears to be processed into SS-14-2 and other forms including SS-25-3, SS-27-3 and SS-28-3. Recently, a fourth preprosomatostatin (PPSS 4) encoding a peptide with [Tyr6, Ser10, Arg11, Ala13] at its C-terminus has be identified as paralogue of SS3 and only occurs in species of the group ostariophysi (e.g. catfish and carps) (de Lecea, 2005). Cortistatin (CST), a peptide found in the mammalian cerebral cortex, has a strong structural similarity to somatostatin and binds with high affinity to all SS receptor subtypes (Tostivint et al., 2004). Both procortistatin (PCST) and PPSS 2 characteristically bear a proline in the second position in the 14 amino-acid C-terminus peptide. Recent comparative genomic studies suggest that mammalian CST is an orthologue of the SS 2 variant of non-mammalian species (Gottero et al., 2004). In mammals, the two known forms of CST, CST-17 and CST-14, both have the same inhibitory effects as SS on GH and insulin secretion (de Lecea et al., 1998).

Orexins consist of two peptides, orexin A (OX-A) and orexin B (OX-B), derived from a single precursor molecule by proteolytic processing, called prepro-orexin (prepro-OX). Orexins (OXs) were first discovered in 1998 in a study using directional tag PCR subtraction of hypothalamic rat mRNAs (Sakurai et al., 1998). OXs act on two closely related orphan G protein-coupled receptors, termed orexin1 receptor (OX_1R) and orexin2 receptor (OX_2R). OX_1R is selective for OX-A, while OX_2R is a non-selective receptor for

both OX-A and OX-B. The name "orexin", from the Greek word *orexis*, meaning appetite, was given to these peptides due to their localization in areas in the brain recognized as a feeding center in mammals, such as the dorsal and lateral hypothalamic areas and the perifornical hypothalamus, which suggested a role of these peptides in the regulation of feeding (Ohkubo et al., 2002). The structure and function of OXs have been well-studied in mammals, but relatively few studies have been published on OXs in non-mammalian species, including birds (Shibahara et al., 1999; Singletary et al., 2005; Yamamoto et al., 2004), amphibians (Faraco et al., 2006; Novak et al., 2005; Volkoff et al., 1999; Volkoff and Peter, 2001b), and fish (Faraco et al., 2006). In fish, OX sequences are only available for zebrafish, pufferfish, stickleback and medaka (Anastasi, 1971).

GRP and bombesin (BBS) are structurally related peptides that have similar biological actions. BBS is a tetradecapeptide originally purified from the skin of the European frog (*Bombina bombina*) (Nagalla et al., 1996). Two BBS-like peptides, GRP and neuromedin B (NMB) were later purified from mammalian tissues and found to be widely distributed in the GI tract and brain. All BBS/GRP-like peptides share a similar C-terminal heptapeptide and are divided into three subfamilies, based on common amino acids in their C-terminal region: the bombesin-related peptides (BRP) family (BBS, ranatensin, and phyllolitorin), the NMB family and the GRP family (Volkoff et al., 2000). BBS/GRP-like peptides have been detected in GI tract and brain of a number of fishes, including teleosts (Bjenning et al., 1991) and elasmobranchs (Kojima et al., 1999).

Ghrelin is a recently discovered brain-gut peptide with two main physiological actions: it acts as a growth hormone secretagogue (GHS) (Nakazato et al., 2001) and stimulates food intake (Bednarek et al., 2000). In general, the first four amino acids are

"GSSF", with an octanoyl group located at the third residue (Serine), and are considered to be the active core of the peptide (Banks et al., 2002). This acylation (post-translational fatty acid modification) at the 3-Ser of the ghrelin molecule is thought to be essential for its transport across the blood-brain barrier and its binding to its receptor to stimulate both food intake and GH release (Matsuda et al., 2006a; Matsuda et al., 2006b). For example, in goldfish, administration of goldfish acylated ghrelin, but not des-acyl ghrelin, stimulates food intake and locomotor activity (Adams et al., 2002; Parker et al., 1993; Sherwood et al., 2000; Small and Nonneman, 2001).

2.2. Materials and Methods

2.2.1. Animals

Juvenile Atlantic cod (*Gadus morhua*) with an average body weight of 35 g were obtained from and kept at the Dr. Joe Brown Aquatic Research Building, Ocean Sciences Centre (Memorial University of Newfoundland, Canada). Fish were maintained in flow-through 1000 L tanks at 11 °C on a 16 hours light/ 8 hours dark photoperiod. Fish were fed once a day at the same time (12:00) with EWOS Marine Diet at a ration of 0.8% total body weight (BW). Fish were anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) before decapitation and dissection. Brain and stomach samples were collected in RNAlater (Qiagen, Mississauga, Ontario, Canada) and stored in -80 °C.

2.2.2. Isolation of precursor cDNAs from cod brain and gut

To clone PRP/PACAP, PPSS and OX precursor cDNA sequences, total RNA was isolated from hypothalamus with Tri-reagent (BioShop, Burlington, Ontario, Canada) according to the manufacturer's protocol. To clone ghrelin and GRP precursor sequences, total RNA was isolated from stomach with Tri-reagent. The mRNA was then extracted from 250 μg total RNA with an Oligotex mRNA mini kit (Qiagen, Mississauga, ON, Canada). The quantification and purity of mRNA were assessed using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA). Reverse transcription (RT) was performed at 42 °C for 1 hour in a total volume of 20 μl consisting of 5 μg total RNA, 1x M-MLV RT buffer, 0.5 mM each dNTP, 0.5 μg oligo-dT-AP (**Table 2.1**), and 200 U M-MLV Reverse Transcriptase (New England Biolabs, Pickering, Ontario, Canada).

Small fragments of the PRP/PACAP, PPSS and OX sequences were amplified using degenerate primer pairs PRP/PACAP1 and PRP/PACAP2, PPSS1 and PPSS2, and OX1 and OX2 respectively (**Table 2.1**), designed on the basis of regions of high identity among pufferfish, zebrafish, medaka and various other vertebrate sequences. The PCR reactions were carried out in a volume of 25 μl consisting of 1x PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 μM each primer, and 1 U of Taq polymerase (Sigma, St Louis, Missouri, USA). Once these short fragments were isolated and sequenced, gene-specific primers were designed for 3' and 5' rapid amplification of cDNA ends (RACE) (3RC-P1, 3RC-P2, 5RC-P1 and 5RC-P2 for PRP, and 3RC-S1, 3RC-S2, 5RC-S1 and 5RC-S2 for PPSS, and 3RC-OX1, 3RC-OX2, 5RC-OX1 and 5RC-OX2 for OX, **Table 2.1**). The 3' ends of cDNA were amplified by two rounds of PCR, with 3RC-(P, S or OX)1 and dT-AP, and 3RC-(P, S or OX)2 and AP (**Table 2.1**), respectively. To isolate

the 5' ends of cDNA, new cDNA was synthesized with primers 5RC-(P, S or OX)1, and unincorporated primers and dNTPs were removed from the cDNA using Montage PCR filter units (Millipore, Bedford, Massachusetts, USA). A polyA tail was then added to the 3' end of the cDNA using terminal transferase (TdT, Invitrogen, Burlington, Ontario, CA), and the reaction was purified using Montage PCR filter units. Two rounds of PCR were then performed by 5RC- (P, S or OX)1 and dT-AP, and 5RC- (P, S or OX)2 and AP, respectively.

For ghrelin and GRP, only very small regions of high identity and 3' ends were present, so that no small fragment could be generated and so only 3' ends were initially isolated. To isolate the 3' end of ghrelin from cod cDNA, two overlapping forward degenerate primers 3RC-GL1 and 3RC-GL2 were designed based on functional region GS(T)SFLSPS(T)QKP, which is conserved in all teleost ghrelins reported. To isolate the 3' end of GRP from cod cDNA, two overlapping forward degenerate primers 3RC-GP1 and 3RC-GP2 were designed based on C-terminal functional region KVYPRGNHWAVGHLM, which is conserved in all teleost GRPs reported. The 3' ends of cDNA were amplified by two rounds of PCR, with 3RC-(GL or GP)1 and dT-AP, and 3RC-(GL or GP)2 and AP (**Table 2.1**), respectively. To isolate the 5' end of cDNA, new primers (5RC-GL1 and 5RC-GL2 for ghrelin, 5RC-GP1 and 5RC-GP2 for GRP) were designed based on 3' RACE amplified sequences. cDNA was synthesized with primers 5RC-(GL or GP)1, and a polyA tail was then added to the 3' end of the cDNA as described for PRP/PACAP, PPSS and OX. Two rounds of PCR were then performed by 5RC-(GL or GP)2 and dT-AP, and 5RC-(GL or GP)3 and AP, respectively.

All amplified products were run on a 1.7% agarose gel (Bioshop, Burlington, ON, Canada), using a 100 bp ladder as a marker (Promega Corp., Madison, WI). Bands of expected sizes were excised from the gel and purified by QIAEX II Gel extraction Kit (Qiagen, Mississauga, ON, Canada). The fragments were then cloned using the pGEM-easy vector system (Promega Corp., Madison, WI) and sequenced at the Mobix lab of MacMaster University (Ontario, Canada).

Table 2.1. Sequences of primers used in the amplification of cDNA.

Primers	Sequences
Degenerate primers	
PRP/PACAP1	5`-GCAGACGGAATGTTTGATAAAGCCT-3`
PRP/PACAP2	5`-GCGTCCTTTGCTTCTATATCTCTGTC-3`
PPSS1	5`-GAVCTCGYGCMAGCMGAAAACGAGG -3`
PPSS2	5`-CANGANGTGAANGTTTTCCAGAAGAA -3`
OX1	5`-GCYGGCATCCTCACKCTGGG-3`
OX2	5`-ATWGTCAGGCTWCCGGCTGC-3`
3'RACE primers	
3RC-P1	5`- AACAGCGAGACAATATCTGCATTCTCTGATG-3`
3RC-P2	5`- ACAATACGTGTAGGCGAAGAGACTA-3`
3RC-S1	5`- TCGGGACGGATGATCAGGACGACGAG -3`
3RC-S2	5`-GAGCGCATGTATCTGGAGC -3`
3RC-OX1	5`-AGGCGGAGGAGCAGCACTTC-3`
3RC-OX2	5`-AGCAGCACTTCCACAGTCGG-3`

3RC-GL1	5`- GGCWCSAGCTTCCTCACYCC -3`
3RC-GL2	5`- CAGYCCHDCHCARAAACCWCAG -3`
3RC-GP1	5`- CCRCGCGGVARTCACTGGGC -3`
3RC-GP2	5`- CACTGGGCKGTNGGNCACTT -3`
5'RACE primers	
5RC-P1	5`-TCGATGGCGGCTGTAGCTGTCGGTGAATAT -3`
5RC-P2	5`-TTAGATAATGGTTCCGACTCGTCC -3`
5RC-S1	5`-CTCGAGGGGAGCCATCATTGGACCG -3`
5RC-S2	5`-GCTCCAGATCCATGCGCTCG -3`
5RC-OX1	5`-TTGCTGCGCCCATTGCATGA-3`
5RC-OX2	5`-AAGCTGGTGGAGCCGACTGT-3`
5RC-GL1	5`- GAAGGTCACTCTGTGGCAT-3`
5RC-GL2	5`- GTGCAGCGCCTGGTAGACCTGGAACT -3`
5RC-GL3	5`- TGAGGCCGACCTCGAAGGGT -3`
5RC-GP1	5`- CCCATTCTTCAGGTGTAAA-3`
5RC-GP2	5`- CCTTCCCTCGATCTCGTCCTCTCCAGA -3`
5RC-GP3	5`- CCGGTCTAGTTTGGAGGGGTA -3`
Adaptor primers	
dT-AP	5`- GGCCACGCGTCGACTAGTAC(T17) -3`
AP	5`- GGCCACGCGTCGACTAGTAC -3`

2.2.3. Cloning of ghrelin and gastrin-releasing peptide genomic sequences

Genomic DNA was extracted using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Oakville, ON, Canada) from whole blood cells. Gene specific primers were designed based on cod ghrelin and GRP cDNA sequences cloned above (**Table 2.2**). Partial genomic DNA fragments were amplified using 100 ng of genomic DNA as template, sequenced and assembled into full length genomic sequences.

Table 2.2. Sequences of primers used in the amplification of genomic sequences

Primers	sequences
GL1	5`- CTCCTTTGCTCTCTGGCCTT -3`
GL2	5`- GTTCTGCAGCAGCTGGTGCA -3`
GL3	5`- GCATGACAGGAGCTACGCTA -3`
GL4	5`- GTCCAGAGCTTTGAAGGTCA -3`
GP1	5`- ATGGGCGACGAGTGTGTAAG -3`
GP2	5`- GGTGTCTCCCTTCGTTCCAA -3`
GP3	5`- TAATGGGGAGGAAGAGCGTT -3`
GP4	5`- TGAACAGTGGGTTCATGTGG -3`

2.2.4. Sequence and structure analysis

DNA and deduced protein sequences were analyzed with the BLASTn and BLASTp programs available from the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The signal peptide was predicted with the program SignalP3.0 (www.cbs.dtu.dk/services/SignalP). Multiple alignments of amino

acid sequences were performed using ClustalW of MEGA3 and subsequent phylogenetic trees were generated using the neighbor-joining method (NJ) method with maximum composite likelihood model and bootstrap of 1000 replicates.

Secondary and tertiary protein structures for OX-A and OX-B were established using the ProModII program at the SWISS-MODEL automated protein modeling server (http://swissmodel.expasy.org/) based upon human OX-A (**1R02.pdb**) and OX-B (**1CQ0.pdb**) Protein Data Bank (PDB) structure files to compare structural similarities between human and cod orexins.

2.3. Results and discussion

2.3.1. Structure of PRP/PACAP and PPSS 1 in Atlantic cod

The 849 bp full length PRP/PACAP cDNA consists of a 75 bp 5'-untranslated region, a 525 bp open reading frame and a 249 bp 3'-untranslated region (**Figure 2.1**). The 155 aa protein includes a 25 aa signal peptide, a 45 aa PRP and a 38 aa PACAP. An alternative short transcript of PRP/PACAP cDNA was also cloned, lacking a partial sequence encoding (1-32) PRP as a result of alternative splicing. Exon skipping has been observed in the PRP/PACAP precursors of non-mammalian vertebrates, including fish (Alexandre et al., 2000), amphibians (Sherwood et al., 2000; Yoo et al., 2000) and birds (Lang et al., 2006).

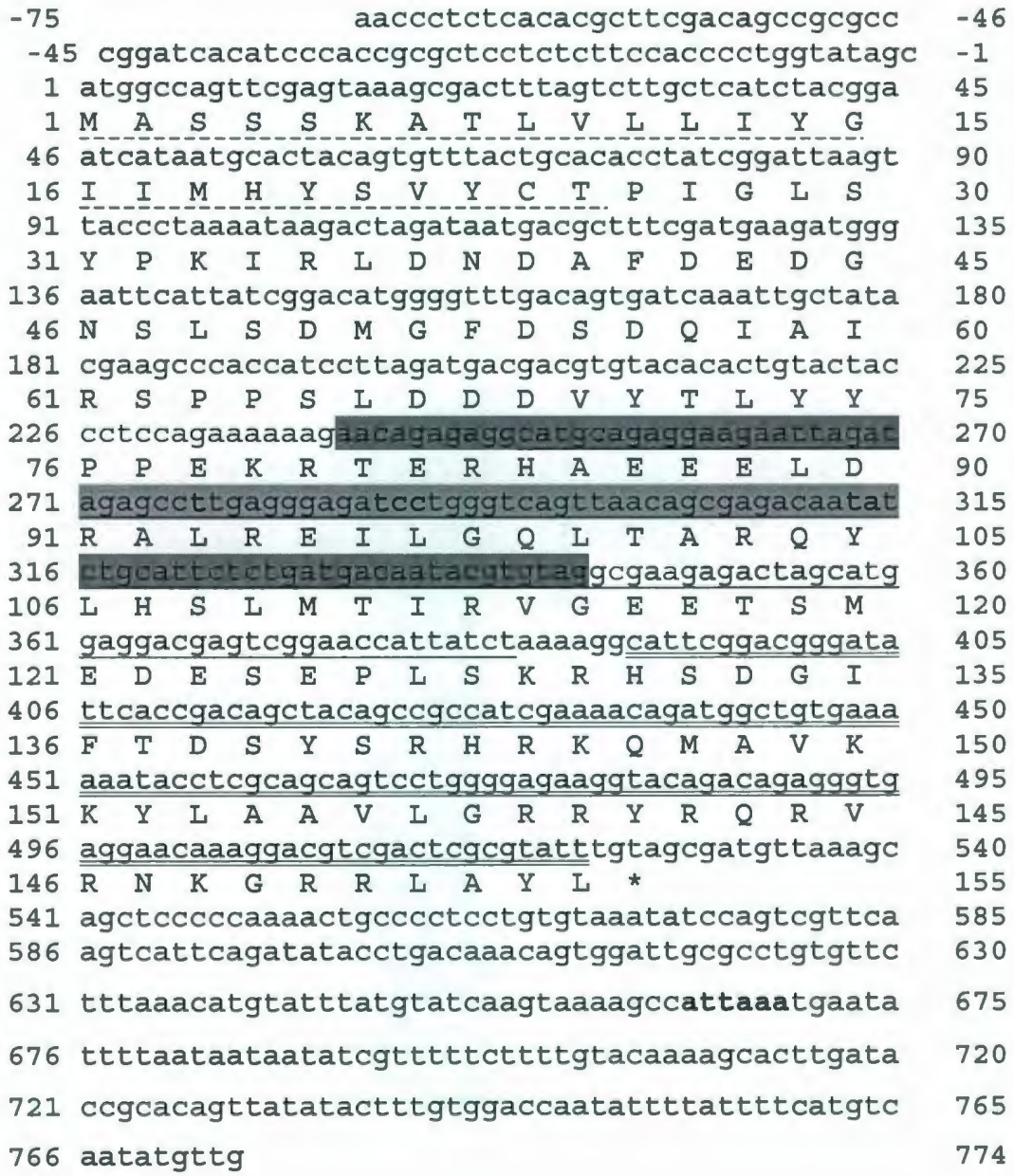
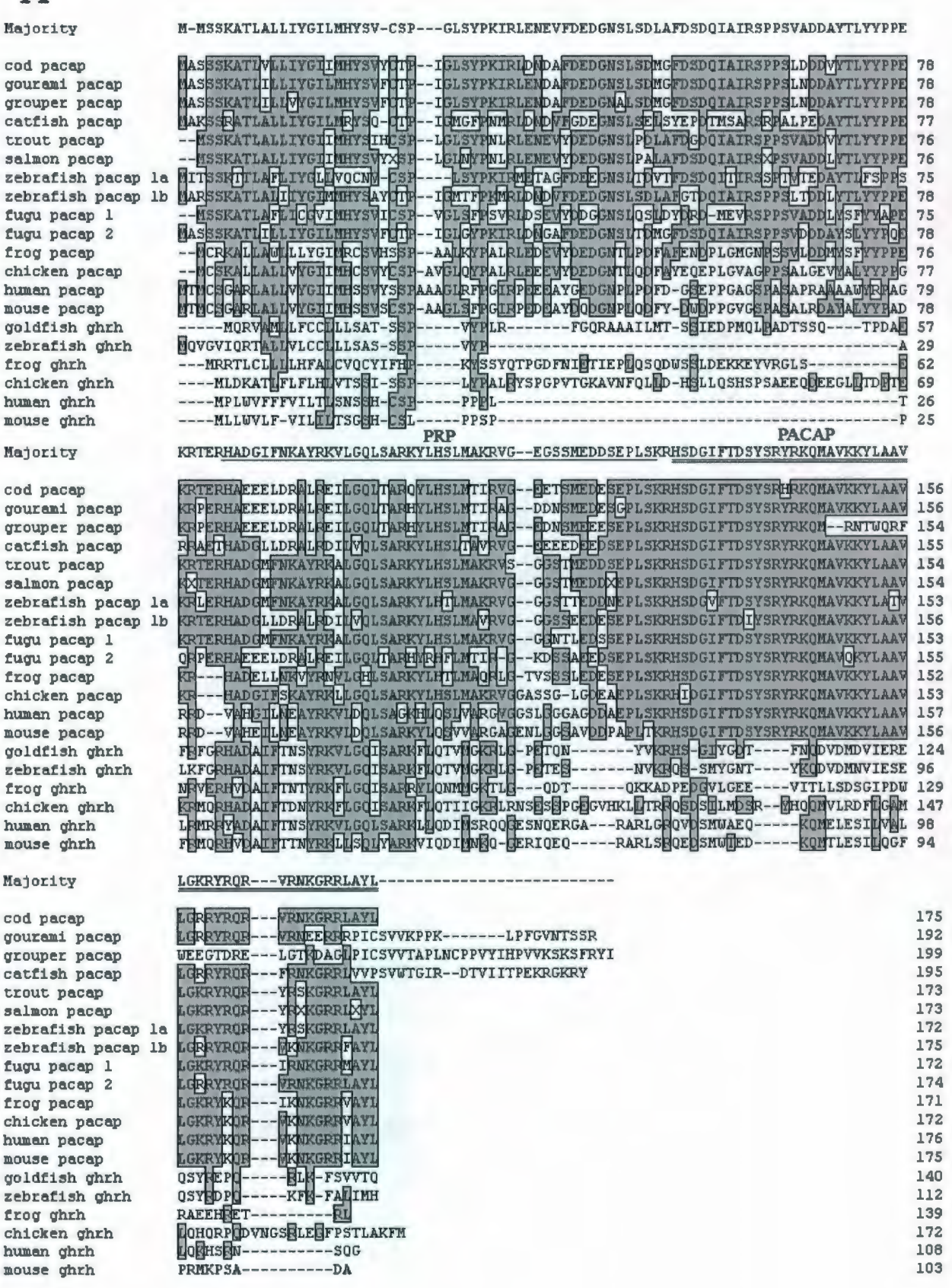


Figure 2.1. Nucleotide and deduced amino acid sequences of Atlantic cod PRP/PACAP cDNA (GenBank accession no. **DQ109988**). Signal peptides amino acid sequences are dash underlined. The mature peptide for PRP is single underlined and the mature peptide for PACAP is double underlined. The

alternatively spliced cDNA fragment is shaded. The poly-adenylation signal (ATTAAA) is in bold letters.

In order to analyze structural similarities at the protein level, multiple sequence alignments (**Figure 2.2 A**) as well as phylogenetic trees (**Figure 2.2 B**) were constructed based on the amino acid sequence of cod PRP/PACAP and PACAPs and GHRHs from other vertebrates. The PACAP precursor is highly conserved among species, with the highest conserved regions representing PRP and mature PACAP peptides. Cod PACAP preprohormone is clustered with other PACAP sequences in teleosts, amphibians, birds and mammals at the amino acid level. It shares highest identity levels to other teleosts PACAPs (ranging from 68.6% to catfish PACAP to 88.6% to gourami PACAP), and lower identity levels with amphibians (59.6% to frog PACAP), birds (62.2% to chicken PACAP), and mammals (54.3% human PACAP and 57.7% mouse PACAP). The cod PACAP preprohormone shows very low similarity with GHRH preprohormones, sharing 17.9% identity to zebrafish GHRH, 17.1% to goldfish GHRH, 19.8% to chicken GHRH, 20.1% to frog GHRH, 27.8% to human GHRH, 12.6% to mouse GHRH. GHRH displays relatively more variability than PACAP in amino acid composition among species. The recently discovered "true" GHRH in goldfish and zebrafish is clustered with amphibian, bird and mammalian GHRH sequences.

A


Majority M-MSSKATLALLIYGILMHYSV-CSP---GLSYPKIRLENEVFDEDGNSLSDLAFDSDQIAIRSPPSVADDAYTLYYPPE
cod pacap
gourami pacap
grouper pacap
catfish pacap
trout pacap
salmon pacap
zebrafish pacap 1a
zebrafish pacap 1b
fugu pacap 1
fugu pacap 2
frog pacap
chicken pacap
human pacap
mouse pacap
goldfish ghrh
zebrafish ghrh
frog ghrh
chicken ghrh
human ghrh
mouse ghrh
PRP
PACAP
Majority KRTERHADGIFNKAYRKVLGQLSARKYLHSLMAKRVG--EGSSMEDDSEPLSKRHSDGIFTDSYSRYRKQMAVKKYLAAV
cod pacap
gourami pacap
grouper pacap
catfish pacap
trout pacap
salmon pacap
zebrafish pacap 1a
zebrafish pacap 1b
fugu pacap 1
fugu pacap 2
frog pacap
chicken pacap
human pacap
mouse pacap
goldfish ghrh
zebrafish ghrh
frog ghrh
chicken ghrh
human ghrh
mouse ghrh
Majority LGKRYRQR---VRNKGRRLAYL----------
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gourami pacap
grouper pacap
catfish pacap
trout pacap
salmon pacap
zebrafish pacap 1a
zebrafish pacap 1b
fugu pacap 1
fugu pacap 2
frog pacap
chicken pacap
human pacap
mouse pacap
goldfish ghrh
zebrafish ghrh
frog ghrh
chicken ghrh
human ghrh
mouse ghrh

B

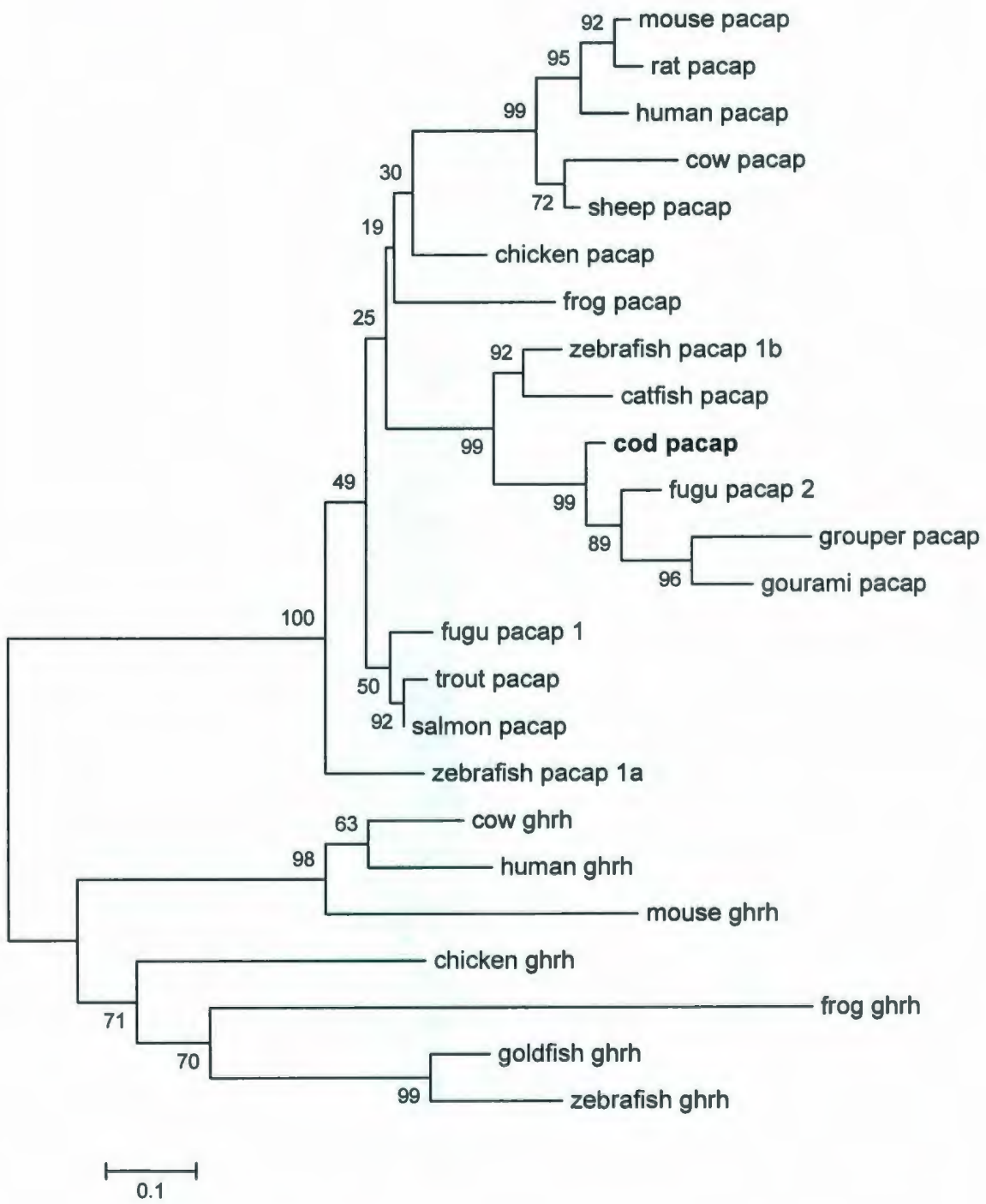

92
mouse pacap
95
rat pacap
99
human pacap
cow pacap
30
72
sheep pacap
19
chicken pacap
frog pacap
25
92
zebrafish pacap 1b
catfish pacap
99
cod pacap
49
99
fugu pacap 2
89
grouper pacap
96
gourami pacap
100
fugu pacap 1
50
trout pacap
92
salmon pacap
zebrafish pacap 1a
63
cow ghrh
98
human ghrh
mouse ghrh
chicken ghrh
frog ghrh
71
70
goldfish ghrh
99
zebrafish ghrh
0.1

Figure 2.2. Multiple alignment of amino acid sequences (A) and phylogenetic tree analysis (B) of cod PACAP preprohormone with sequences of PRP/PACAP and GHRH for other vertebrates. Residues that match the consensus sequence are shaded and boxed. GenBank accession nos. Grouper, *Epinephelus coioides* (**AY869693**); gourami, *Trichogaster trichopterus* (**ABW86799**); fugu, *Takifugu rubripes* (**DQ659331** and **DQ659332**); zebrafish, *Danio rerio* (**NM_152885**, **NM_214715** and **NM_001080092**); rainbow trout, *Oncorhynchus mykiss* (**AAK28557**); salmon, *Oncorhynchus nerka* (**X73233**); catfish, *Clarias macrocephalus* (**X79078**); goldfish, *Carassius auratus* (**DQ991243**); frog, *Xenopus laevis* (**AF187877** and **NM_001096728**); mouse, *Mus musculus* (**NM_009625** and **NM_010285**); rat, *Rattus norvegicus* (**NM_016989**); cow, *Bos taurus* (**AY924308** and **NM_178325**); sheep, *Ovis aries* (**NM_001009776**); chicken, *Gallus gallus* (**AY956323** and **NM_001040464**); human, *Homo sapiens* (**BC101803** and **NM_021081**). A scale bar showing the distance of 0.1 is at the lower left.

As teleost fishes have undergone an independent tetraploidization (called 3R, third round of genome doubling) after the two basal vertebrate (pre-gnathostome) tetraploidizations (2R), multiple copies of genes are often seen in fish but not in other vertebrates (Lang et al., 2006; Lang et al., 2004). Although only one form of PACAP has been cloned, it is very likely that other forms are present in cod.

The 603 bp full length PPSS 1 cDNA consists of a 45 bp 5'-untranslated region, a 321 bp open reading frame and a 237 bp 3'-untranslated region (**Figure 2.3**). The cloned

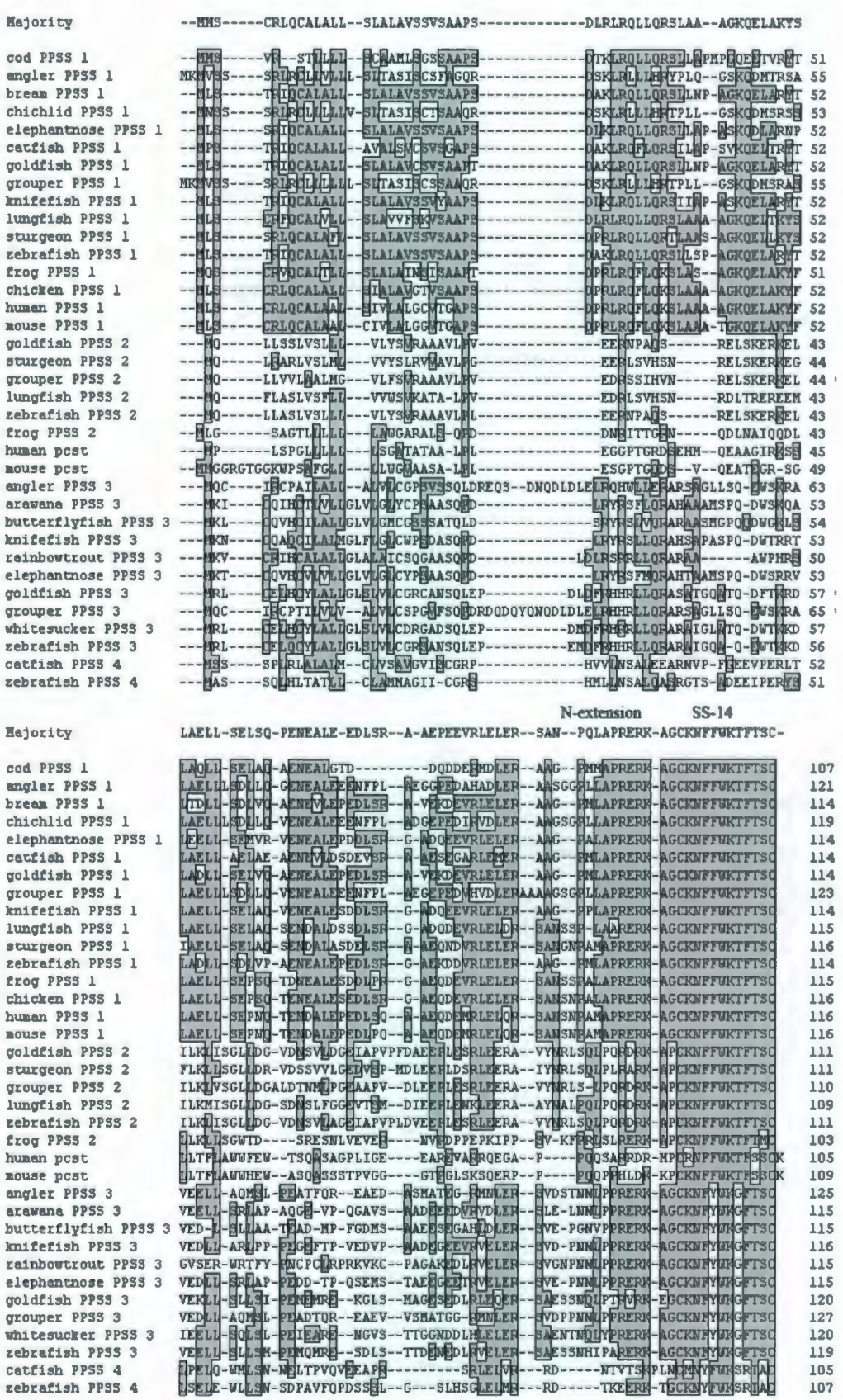
cod PPSS cDNA encodes 107 aa protein that displays a 23 aa signal peptide, a SS-14-1 and possible SS-26-1 with 12 aa extension at the N terminal end.

```
-45 gataaggagagttgaacgtcttcattaccgccctccaaacagaag  -1
  1 atgatgtcggtccgctccaccctgctgctgctctcctgtgccgcg  45
  1 M  M  S  V  R  S  T  L  L  L  L  S  C  A  A    15
 46 atgctcagcggctcctcggcagcgccgagcgacaccaagctgcgg  90
 16 M  L  S  G  S  S  A  A  P  S  D  T  K  L  R    30
 91 caactcctgcagagatccctgctggctccgatgccaggccaagag  135
 31 Q  L  L  Q  R  S  L  L  A  P  M  P  G  Q  E    45
136 gaaaccgttcgctacacgctcgcgcagctgttgtcggagctcgcg  180
 46 E  T  V  R  Y  T  L  A  Q  L  L  S  E  L  A    60
181 caggcggagaacgaagcgctcgggacggatgatcaggacgacgag  225
 61 Q  A  E  N  E  A  L  G  T  D  D  Q  D  D  E    75
226 cgcatggatctggagcgcgccgccggtccaatgatggctccccgc  270
 76 R  M  D  L  E  R  A  A  G  P  M  M  A  P  R    90
271 gagcgcaaagctggatgcaaaaacttcttctggaaaacttttaca  315
 91 E  R  K  A  G  C  K  N  F  F  W  K  T  F  T    105
316 tcgtgttgattgatgccttttttcattgtttcatgattgatatgt  360
106 S  C  *                                        107
361 taattgaatataatctcgtctttaaattggcatatccttcaatta  405
406 cccgtgcctccaacttccacaatttaatacgaaaaatgggattca  450
451 tccttcatatctcactttgaatgaaatgattgttttcaatcacac  495
496 ttttttgccgcccaagaatttctatttttaatttttggtttgaat  540
541 aaaatatgtttcagacct                            558
```

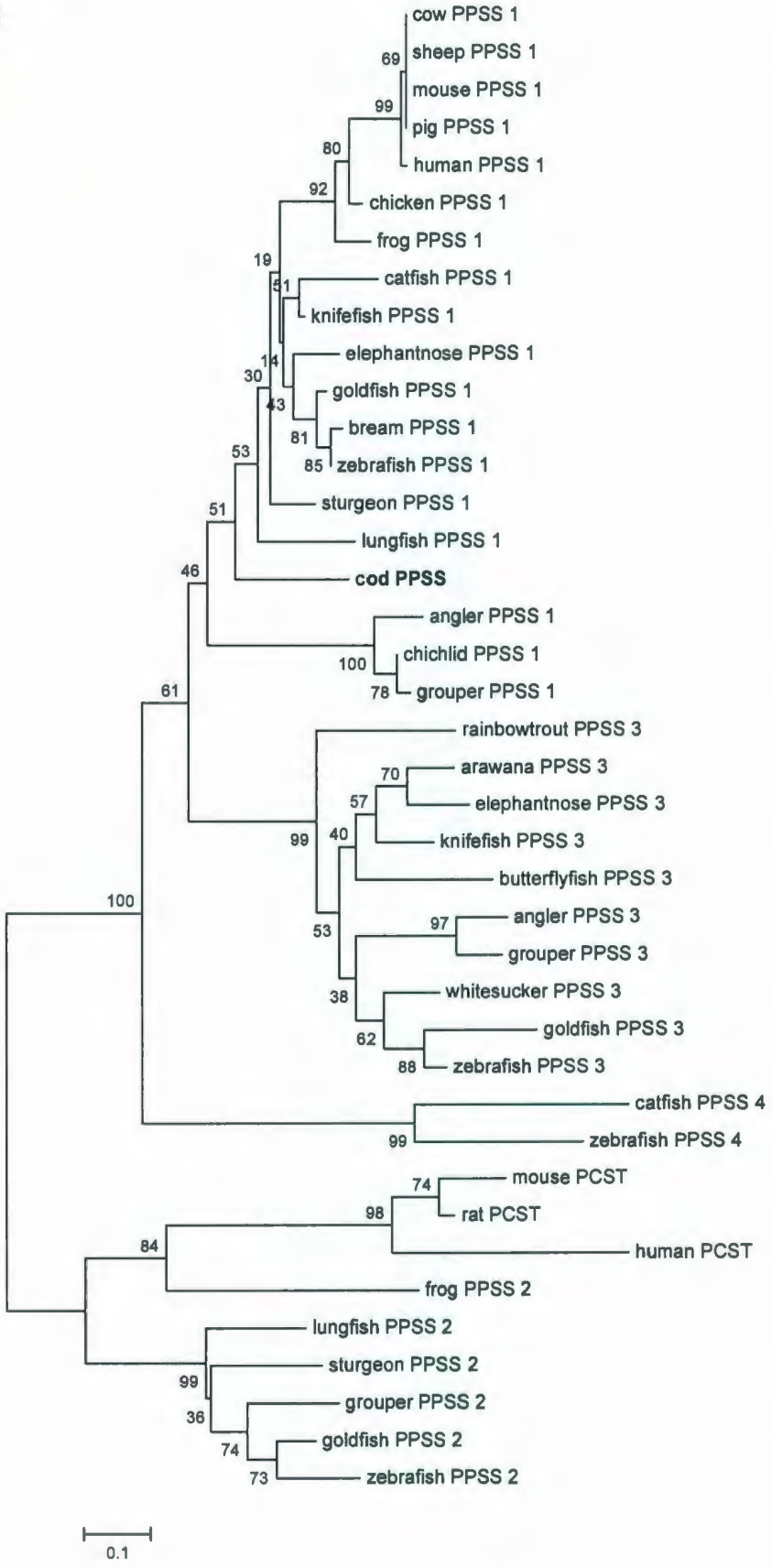
Figure 2.3. Nucleotide and deduced amino acid sequence of Atlantic cod PPSS I cDNA (GenBank accession no. **DQ109989**). Signal peptides amino acid sequences are dash underlined. The mature peptide for PPSS I is single underlined. The poly-adenylation signal (AATAAA) is in bold letters.

Multiple sequences alignments of PPSSs (**Figure 2.4 A**) from fish and PPSS and precortistatin (PCST) from mammals as well as phylogenetic analysis (**Figure 2.4 B**) show that the cloned cod PPSS is clustered with other PPSS 1 sequences from teleosts, amphibians, birds and mammals suggesting that it represents cod PPSS 1. In the phylogenical construction, PPSS 2 forms, seen in both teleosts and amphibians, are clustered with mammalians PCST, whereas PPSS 3 forms, only found in teleosts, are combined in a single cluster. Two atypical PPSS forms (PPSS 4) found in catfish and zebrafish are clustered together. In general, PPSS 1 is relatively more conserved among different vertebrate species than either PPSS 2, PPSS 3 or PPSS4. Cod PPSS 1 amino acid sequence displays similarities ranging from 46.7% (to angler PPSS 1) to 67.3% (to zebrafish PPSS 1) to fish PPSS 1, 57.0% to frog PPSS 1, 57.9% to chicken PPSS 1, 54.2% to human PPSS 1, and 54.2% to mouse PPSS 1. The highest degree of similarity occurs in the C-terminal region, corresponding to the mature SS-14-1 peptide, whereas the N terminal extension part displays more variability. Similar to PACAP, multiple PPSS genes have been demonstrated in fish and although only one form of PPSS has been cloned, it is possible that other forms are present in cod.

A



B


cow PPSS 1
sheep PPSS 1
mouse PPSS 1
pig PPSS 1
human PPSS 1
chicken PPSS 1
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catfish PPSS 1
knifefish PPSS 1
elephantnose PPSS 1
goldfish PPSS 1
bream PPSS 1
zebrafish PPSS 1
sturgeon PPSS 1
lungfish PPSS 1
cod PPSS
angler PPSS 1
chichlid PPSS 1
grouper PPSS 1
rainbowtrout PPSS 3
arawana PPSS 3
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zebrafish PPSS 4
mouse PCST
rat PCST
human PCST
frog PPSS 2
lungfish PPSS 2
sturgeon PPSS 2
grouper PPSS 2
goldfish PPSS 2
zebrafish PPSS 2
0.1

Figure 2.4. Multiple alignment of amino acid sequences (A) and phylogenetic analysis (B) of cod PPSS 1 preprohormone with sequences of PPSSs and PCST for other vertebrates. Residues that match the consensus sequence are shaded and boxed. GenBank accession nos. Grouper, *Epinephelus coioides* (**AY677120**, **AY677121** and **AY677122**); anglerfish, *Lophius americanus* (**V00640** and **V00641**); cichlid, *Astatotilapia burtoni* (**AY585720**); goldfish, *Carassius auratus* (**U40754**, **U60262** and **U72656**); rainbow trout, *Oncorhynchus mykiss* (**Kittilson et al., 1999**; **OMU32471**); lungfish, *Protopterus annectens* (**AF126243** and **AF126244**); sturgeon, *Acipenser transmontanus* (**AF395849** and **AF395850**); knifefish, *Chitala chitala* (**AF292653** and **AY785129**); whitesucker, *Catostomus commersoni* (**AF292654**); elephantnose, *Gnathonemus petersii* (**AF292652** and **AY785130**); butterflyfish, *Pantodon buchholzi* (**AF292651**); arawana, *Osteoglossum bicirrhosum* (**AF292650**); bream, *Megalobrama pellegrini* (**AY247267**); zebrafish, *Danio rerio* (**AF435965**, **NM_131727**, **AAI22389** and **XM_689051**); catfish, *Ictalurus punctatus* (**M25903** and **J00945**); frog, *Rana ridibunda* (**U68136** and **U68137**); human, *Homo sapiens* (**BC032625** and **AF013252**); mouse, *Mus musculus* (**NM_009215** and **NM_007745**); rat, *Rattus norvegicus* (**V01271** and **NM_012835**); sheep, *Ovis aries* (**AF031488**); bovine, *Bos taurus* (**NM_173960**); pig, *Sus scrofa* (**NM_001009583**); chicken, *Gallus gallus* (**X60191**). A scale bar showing the distance of 0.1 is at the lower left.

2.3.2. Structure of OX cDNAs in Atlantic cod

OX-A and B appear to be important neuropeptides for regulating feed intake and energy balance in vertebrates, including mammals and fish. To date, very few OXs have been identified in lower vertebrates, particularly in fish. The 652 bp full length Atlantic cod prepro-OX cDNA consists of a 40 bp 5'-untranslated region, a 432 bp open reading frame and a 180 bp 3'-untranslated region (**Figure 2.5**). The 143 aa prepro-OX protein includes a 38 aa signal peptide, a 50 aa OX-A and 29 aa OX-B.

Both cod orexins share a higher degree of identity with teleost orexins than tetrapod orexins. When tetrapods are considered, the protein sequence of the OX-A peptide seems more conserved than that of OX-B; whereas when both tetrapods and fish are taken into account, cod OX-B shares higher degree of identity than cod OX-A with other teleost fish orexins (**Figures 2.7A and 2.7B**). This discrepancy in similarities between tetrapods and teleosts can likely be explained by the presence of the highly variable insertion sequence in teleost OX-A. Compared to other species, cod OX-A also has an additional amino acid (an alanine, A) between Cys7 and Cys14 (7aa -SREPPRA- as opposed to 6aa). The GKR and GRR putative cleavage amino acid sites at the end of OX-A and OX-B peptides remain constant among vertebrates. The phylogenetic tree for amino acid sequences of prepro-OX among different vertebrate species shows that the deduced cod prepro-OX is evolutionarily related to teleost fish and distinct from mammals (**Figure 2.7C**).

-40 *ggtgagagcttctgaggaatcgagaaaaacaccggagaaa* 0
1 atgaagtggtcctccacagtgtcccaaacacctgctggggtggag 45
1 **M K W S S T V S Q T P A G V E** 15
46 aaatcagtgctcaagagaatccaagtcctcgtgttagtgctgctt 90
16 **K S V L K R I Q V L V L V L L** 30
91 gcgtctcacacgctctgcgacgctcacagcgtgtcggcgtcgtgc 135
31 **A S H T L C D A** H S V S A S C 45
136 tgcagcagagagccaccccgcgcctgccgcctctatgtgttgctg 180
46 C S R E P P R A C R L Y V L L 60
181 ctgtgtggcccggtaggcggtgccggccgggcgctaggcgggatg 225
61 L C G P V G G A G R A L G G M 75
226 catcttggcgaggacgcgtcagccggcatcctcactctagggaag 270
76 H L G E D A S A G I L T L G K 90
271 cgggaggcggaggagcagcacttccacagtcggctccaccagctt 315
76 R E A E E Q H F H S R L H Q L 105
316 ctccgcggtggcgcgcggaatcaggcagccgggatcttgactatg 360
106 L R G G A R N Q A A G I L T M 120
361 ggcaagcggtcagaggaggaagaggcggtcgggctgctcatgcaa 405
121 G K R S E E E E A V G L L M Q 135
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W A Q Q D F T A *
451 *gtttcaacaactgcctgttgctggggactttttttttaatgaga* 495
496 *tggagaggaagaggggtataactaaaagtccagtgtgttgccgtg* 540
541 *gtagcaggacatgtgcttagtggatgtgttggatgtaaagtgttt* 595
596 *ccaa**aataaa**ggcgcgacctaaagttc* 622

Figure 2.5. Nucleotide and deduced amino acid sequences of Atlantic cod prepro-OX cDNA (GenBank accession no. **DQ486137**). OX-A amino acid sequence is single underlined, and OX-B is double underlined. Cleavage sites for proteolysis are shaded. The poly-adenylation signal (AATAAA) is in bold italic letters. The signal peptide is in bold uppercase letters.

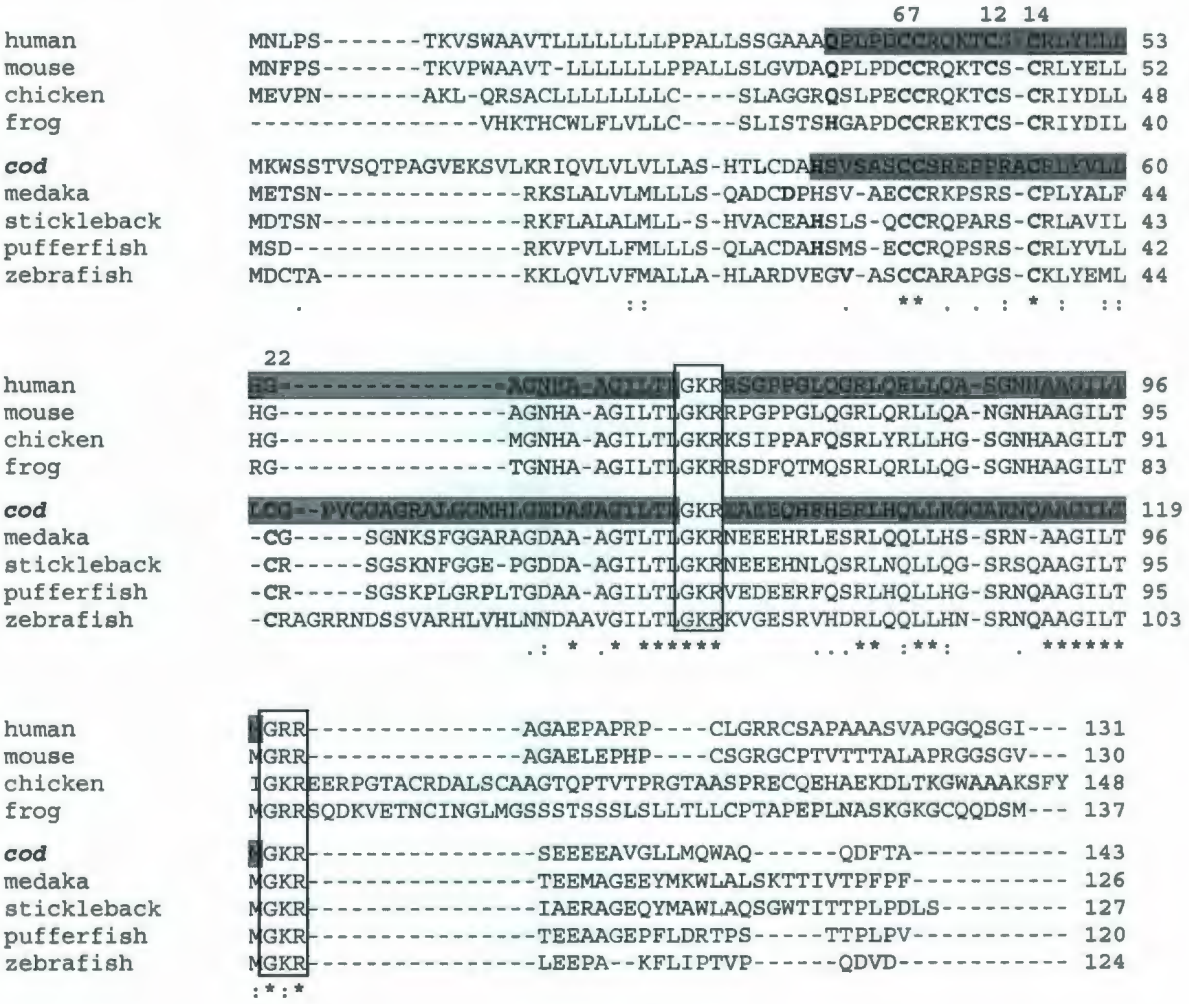


Figure 2.6. Comparison of amino acid sequences of prepro-OX from cod and other vertebrates. Cod OX (GenBank accession no. **DQ486137**) was compared to Human (GenBank accession no **NM_001524**), mouse (GenBank accession no **AF041242**), rat (GenBank accession no.**NM_013179**), chicken (GenBank accession no. **NM_204185**), frog [16], zebrafish (GenBank accession no. **DQ831346**), pufferfish (GenBank accession no. **CAG02825**) and sequences

available through genome databases: medaka (http://mbase.bioweb.ne.jp, **scaffold5097:24560-23509, GOLWno5793_117.gl:494-132**), stickleback (http://ensembl.genome.tugraz.at/Gasterosteus_aculeatus, **GENSCAN000000033466**, **Contig_4260:5838783-5839782**), GKR and GRR cleavage sites are boxed. Cys residues are labeled according to the position of Cys residues in human OX. Cys residues potentially involved in forming disulfide bridges and the first residues in each mature OX-A sequence - as predicted by signalP3.0 software - are indicated in bold. The mature peptide sequences of OX-A and OX-B are shaded in both human and cod sequences. The single- and double-underlined regions correspond to helix 1 and helix 2, respectively.

In the OX-A peptide of mammals, chicken and amphibians, four conserved cysteine residues are found to form two intra-chain disulfide bonds (Cys6-Cys12 and Cys7-Cys14). Only three of these four cysteine residues are conserved in fish, corresponding to the Cys6, Cys7 and Cys14 positions in the mammalian peptide (**Figure 2.6**). Whereas in tetrapods, a fourth Cys residue is found at position 12, the fourth Cys residue in fish OX-A corresponds to positions 23 for cod and medaka OX-A, 21 for stickleback and pufferfish OX-A and 19 for zebrafish OX-A (which corresponds to position 22 of the tetrapod OX-A in **Figure 2.6**). The distance between the four cysteine residues in fish is thus higher in fish (approximately 15 amino acids) than in tetrapods (9 amino acids). Three dimensional models suggest the existence of two disulfide bridges in human OX-A and only one (Cys7-Cys14) in cod OX-A (**Figure. 2.8A and 2.8C**).

The distance between Cys6 and Cys22 might not allow the formation of a second bridge, as the folding of protein by the Cys7-Cys14 might prevent Cys6 from reaching Cys22, which is located at the end of helix 1 in fish. It has been suggested that disulfide bonds are not required for OX-A binding to the OX receptors in mammals (Okumura et al., 2001). However, intact disulfide bonds in OX-A seem necessary for the stimulation of gastric acid secretion mediated by OX1 receptor activation in mammals (Alvarez and Sutcliffe, 2002; Kaslin et al., 2004). Although the absence of a second disulfide bridge might affect some biological activities of OX-A, a single bridge might be enough to maintain its 3D configuration - and thus its activity. The similarity between the human and cod OX-A models seem to indicate that this might be the case (**Figure 2.8**). However, it is also possible that cod OX-A is folded in a way different than that shown in the predicted model, which is based on comparisons with mammalian proteins. A different folding might bring Cys6 and Cys 22 close enough to form a second disulfide bond in fish.

Cod OX-A displays an additional amino acid sequence, called insertion sequence, which is also found in other fish OX-A peptides but not in other tetrapods (**Figure 2.6**). This sequence is clearly seen protruding as a β-sheet between the two helixes in the 3D model (**Figure 2.8**). Although this insertion sequence shows no or little homology among fish species, its location is highly conserved. It has been suggested that this insertion sequence has no true role in OX-A activity (Kineman et al., 2007). Three-dimensional (3D) structural modeling suggests that the tertiary structures of OX-A and OX-B are similar in cod and humans as both peptides are composed of two helixes (**Figure 2.8**). As

seen in the peptide alignment and 3D models, OX-B is less variable in structure and relatively well conserved among different species.

A

	Orexin A protein alignment								
	human	mouse	chicken	frog	zebrafish	medaka	stickleback	pufferfish	cod
human	100	100	92.5	83.0	34.0	43.4	52.8	52.8	34.0
mouse		100	92.5	83.0	34.0	43.4	52.8	52.8	34.0
chicken			100	84.9	30.2	45.3	52.8	54.7	34.0
frog				100	30.2	39.6	50.9	50.9	32.1
zebrafish					100	30.2	37.7	37.7	35.8
medaka						100	62.3	67.9	37.7
stickleback							100	77.4	43.4
pufferfish								100	45.3
cod									100

B

	Orexin B protein alignment								
	human	mouse	Chicken	frog	zebrafish	medaka	Stickleback	pufferfish	cod
human	100	92.9	89.3	75.0	53.6	51.9	53.6	50.0	42.9
mouse		100	96.4	67.9	50.0	48.1	50.0	46.4	42.9
chicken			100	64.3	46.4	44.4	46.4	42.9	39.3
frog				100	50.0	51.9	57.1	60.7	53.6
zebrafish					100	66.7	57.1	67.9	60.7
medaka						100	77.8	70.4	55.6
stickleback							100	71.4	64.3
pufferfish								100	71.4
cod									100

C

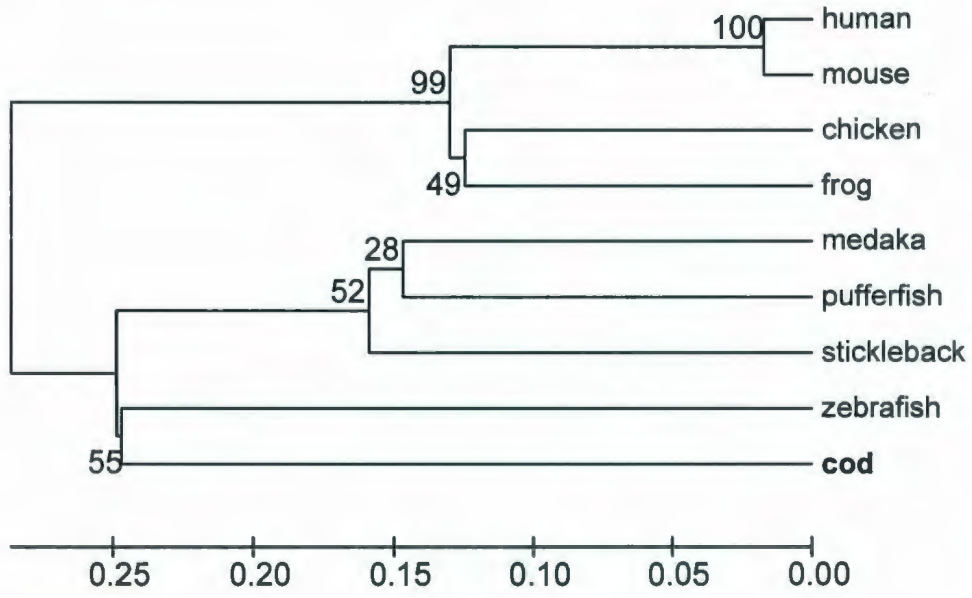


Figure 2.7. Homology comparison among OXs in several vertebrate groups. Percentage identities of OX-A (A) and OX-B (B) and phylogenetic tree (C) for vertebrate prepro-OXs. The identity analysis was performed using the neighbor-joining method in ClustalW and MEGA3. Numbers at nodes indicate the bootstrap values, as percentages, obtained for 1000 replicates. GenBank accession numbers are as in **Fig. 2.6.** A scale bar showing the distance is under the tree.

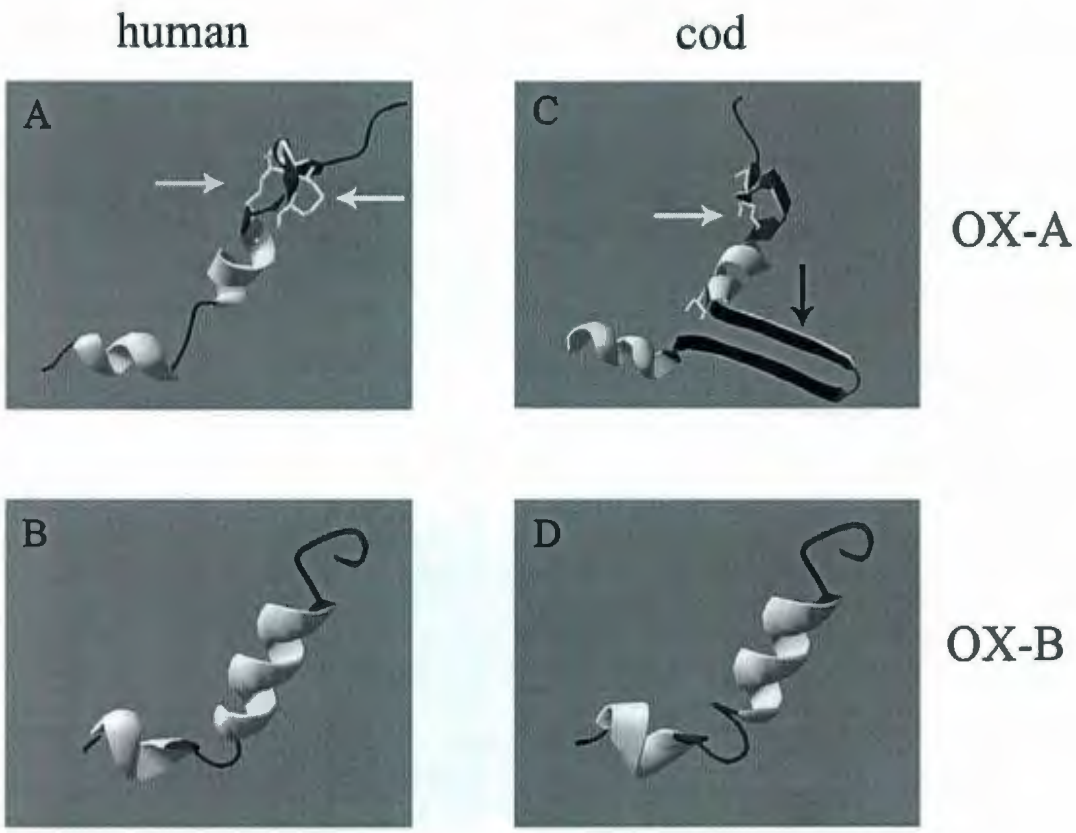


Figure 2.8. Ribbon diagrams showing the tertiary structures of human and cod OX-A and B. A, Human OX-A; B, Human OX-B; C, Cod OX-A; D, Cod OX-B. Secondary and tertiary protein structures were modeled using the ProModII program at the SWISS-MODEL automated protein modeling server, based upon human OX-A (**1R02.pdb**) and OX-B (**1CQ0.pdb**) Protein Data Bank structure file. The arrow in solid black indicates the insertion sequence in cod OX-A. White lines represent cysteine residues. The arrows in solid white indicates the disulfide bridges in human and cod OX-A.

2.3.3. Structure of cDNAs and genomic sequences for ghrelin and GRP in Atlantic cod

The cod ghrelin gene (accession number **EU128174**) is 904 bp in length and is comprised of 4 exons and 3 introns. This configuration is similar to that found in seabream and tilapia ghrelins, but different from rainbow trout and sea bass ghrelins, which, like mouse and rat ghrelins, have five exons and four introns. It seems that in cod, the second intron fragment can be alternatively kept in the transcript without affecting the downstream coding region. Indeed, two ghrelin transcripts, of 427 bp and 499 bp, were obtained from stomach mRNA, encoding a 99 aa and a 123 aa preprohormone respectively and are likely produced by alternative splicing of the same precursor RNA within the intron 2 region. This is the first report of alternative splicing in any teleost ghrelin. The functional significance of this splicing has yet to be determined. In mouse, an intron2-ghrelin variant mRNA has been reported and its expression levels found to be dependent on energy intake in the pituitary and hypothalamus, suggesting that this transcript may encode a peptide with an important role in coordinating the neuroendocrine response to metabolic stress (Bednarek et al., 2000).

The 530 bp full length ghrelin cDNA consists of a 35 bp 5'-untranslated region, a 369 bp open reading frame and a 81 bp 3'-untranslated region (**Figure 2.9**). The 143 aa ghrelin protein includes a 38 aa signal peptide and a 50 aa mature peptide. Based on multiple alignment (**Figure 2.10 A**) and phylogenetic trees (**Figure 2.10 B**) of the amino acid sequence of mature ghrelins for cod and other vertebrates, ghrelin amino acid sequences are relatively well-conserved among fish sequences and display variability compared to other vertebrate ghrelins. Cod ghrelin shares highest similarity levels with

other teleost ghrelins (ranging from 63.2% with halibut ghrelin to 50% with seabass ghrelin), and lower identity levels with amphibians (40% with frog ghrelin), reptiles (35% with turtle ghrelin), birds (from 40% with chicken ghrelin to 45% with turkey ghrelin), and mammals (40% with human ghrelin to 47.4% with sheep ghrelin). The lowest similarity levels occur with elasmobranchs (25% with shark ghrelin).

The deduced cod mature ghrelin is 'GVTFLSPAHKPLDKGKPRV', which appears to be a unique sequence when compared to other vertebrate ghrelins. In general, the first four amino acids consist of "GSSF", with an-octanoyl group located in the third residue (Serine), and are considered to be the active core of the peptide (Banks et al., 2002). This acylation (post-translational fatty acid modification) at the 3-Ser of the ghrelin molecule is thought to be essential for its transport across the blood brain barrier and its binding to its receptor to stimulate both food intake and GH release (Matsuda et al., 2006a; Matsuda et al., 2006b). For example, in goldfish, administration of goldfish acylated ghrelin, but not des-acyl ghrelin, stimulates food intake and locomotor activity (Kaiya et al., 2001). In cod ghrelin, the second serine is replaced by a valine, which is similar to shark ghrelin. Goldfish and zebrafish have a threonine at this position and bullfrog has a leucine. A threonine at amino acid position 3 (Thr(3)) in cod ghrelin differs from the serine present in other teleosts and mammalian ghrelins. Bullfrog ghrelin also displays a Thr(3), which can be acylated by either n-octanoic or n-decanoic acid (Fekete et al., 2007). It is likely that the Thr(3) in cod ghrelin is also acylated therefore providing ghrelin with its biological activity.

The cod GRP gene (accession numbers **EU096313**) is 904 bp in length and is comprised of three exons and two introns. GRP amino acid sequences share a similar C-

terminal sequence and are relatively variable at N terminal ends (**Figure 2.12 A**). The phylogenetic tree (**Figure 2.12 B**) shows that cod GRP is clustered with other vertebrates GRP sequences. Cod GRP amino acid sequence displays identities ranging from 54.5% (shark GRP) to 70% (zebrafish GRP) with fish GRPs, 72.7% with amphibian GRP, 68.2% with reptilian GRP, 63.6% with chicken GRP, and from 68.2% (human GRP) to 63.8% (mouse GRP) with mammalian GRPs.

The deduced cod mature GRP amino acid sequence is "SPPALLSLSYPRGNHWAVGHLM", which is clustered with other vertebrate GRP-related peptides. These peptides present a highly conserved C-terminal portion, the decapeptide GRP (GRP-10), which is crucial for biological activity, while their N-terminal regions show a higher degree of variability. Indeed, GRP-10, also termed neuromedin C, has been shown to decrease food intake when injected centrally or peripherally (Delfini and Diagne, 1985). The amino acid sequences of fish GRPs are shorter and show more variability than their mammalian counterparts. Interestingly, cod GRP is clustered with toad GRP. A highly conserved C-terminal portion and highly variable N-terminal regions are also found in BBS and NMB. GRP has "GN/SHWAVGHLM" at its C terminal region and BBS has "GNQWAVGHLM", while NMB has "GNQ/LWAT/IGHFM", as shown in the GRP sequence alignment. Similarly, a highly conserved C-terminal portion and highly variable N-terminal regions are found in BBS and NMB.

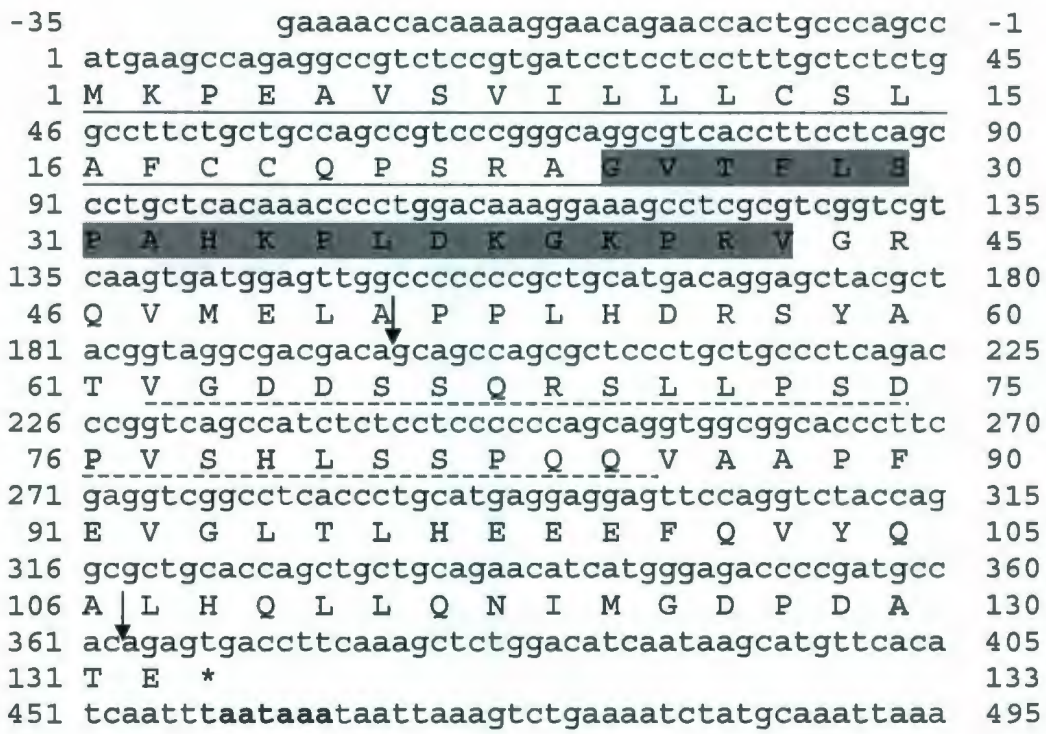


Figure 2.9. Nucleotide and deduced amino acid sequences of Atlantic cod ghrelin cDNA (GenBank accession no. **DQ109988**). The signal peptide is single underlined. The mature peptide for ghrelin is shaded and bolded. The dash-underlined sequence corresponds to the 24aa segment that is missing in a shorter cDNA encoding a second form of ghrelin. The putative poly-adenylation signal (AATAAA) is in bold letters. Positions of introns are indicated by arrows.

A

```
Cod             GVTFLSPAHKP----------LDKGK---PRV
Catfish         GSSFLSPTQKP----------QNRGDRKPPRV
Goldfish        GTSFLSPAQKP----------QGRR---PPRM
Zebrafish       GTSFLSPTQKP----------QGRR---PPRV
Seabream        GSSFLSPSQKP----------QNRGK--SSRV
Seabass         GSSFLSPSQKP----------QSRGK--SSRV
Tilapia         GSSFLSPSQKP----------QNKVK--SSRI
Trout1          GSSFLSPSQKPQV-------RQGKGK--PPRV
Trout2          GSSFLSPSQKP----------QGKGK--PPRV
Halibut         GSSFLSPSHKP-----------PKGK--PPRA
Eel             GSSFLSPSQRP----------QGKDK-KPPRV
Lewini          GVSFH-PRLKEKDDNSSGNSRKSNPK------
Melanopterus    GVSFH-PRLKEKDDNSSGNTRKFSPK------
Bullfrog        GLTFLSPADMQKIAERQSQNKLRHGNMN----
Turtle          GSSFLSPEYQNTQQRKDPKKHTKLN-------
Chicken         GSSFLSPTYKNIQQQKDTRKPTARLH------
Turkey          GSSFLSPAYKNIQQQKDTRKPTARLH------
Sheep           GSSFLSPEHQKLQ-RKEPKKPSGRLK---PR-
Goat            GSSFLSPEHQKLQ-RKEPKKPSGRLK---PR-
Cow             GSSFLSPEHQKLQ-RKEAKKPSGRLK---PR-
Pig             GSSFLSPEHQKVQQRKESKKPAAKLK---PR-
Dog             GSSFLSPEHQKLQQRKESKKPPAKLQ---PR-
Monkey          GSSFLSPEHQRAQQRKESKKPPAKLQ---PR-
Rat             GSSFLSPEHQKAQQRKESKKPPAKLQ---PR-
Mouse           GSSFLSPEHQKAQQRKESKKPPAKLQ---PR-
Human           GSSFLSPEHQRVQQRKESKKPPAKLQ---PR-
                *   *   *
```

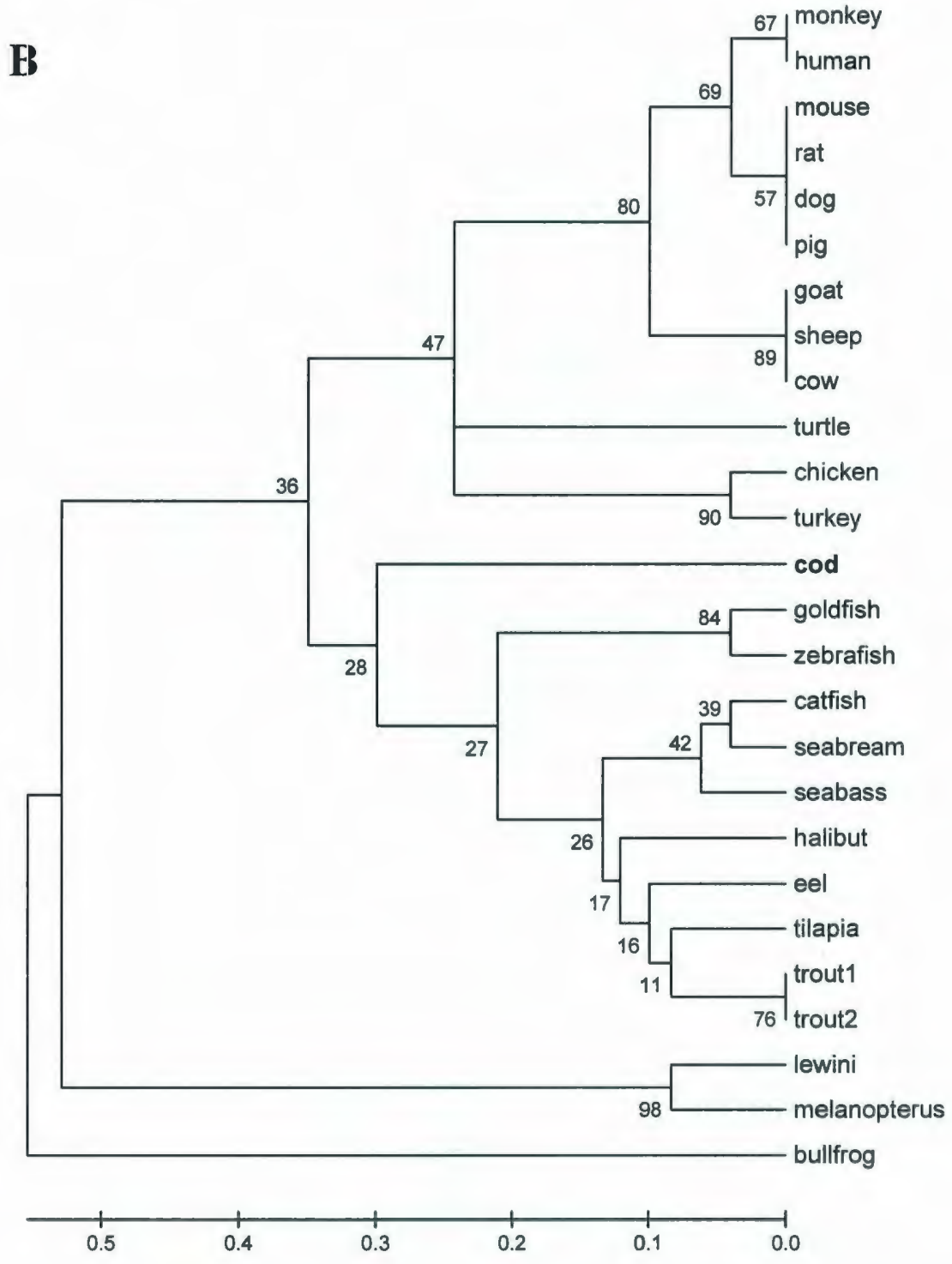


Figure 2.10. Multiple alignment of amino acid sequences (A) and phylogenetic analysis (B) of cod ghrelin mature peptides with sequences of ghrelins for other

vertebrates Identical amino acids were indicated with "*".Goldfish **AF454390**, zebrafish **AM055940**, channel catfish **AB196450**, rainbow trout 1 **AB096919**, rainbow trout 2 **AB101443**, black seabream **AY643809**, sea bass **DQ665912**, Nile tilapia **AB104860**, Atlantic halibut **EF493849**, Japanese eel **AB062427**, Carcharhinus melanopterus **AB254129**, Sphyrna lewini **AB254128**, bullfrog **AB058510**, turtle **BAD29730**, chicken **AB075215**, turkey **AY333783**, sheep **DQ294307**, goat **AB089200**, cow **NM_174067**, dog **NM_001003052**, pig **NM_213807**, mouse **NM_021488**, rat **AB029433**, monkey **AB365872** and human **AB035700**). Numbers at nodes indicate the bootstrap values, as percentages, obtained for 1000 replicates. A scale bar showing the distance is under the tree.

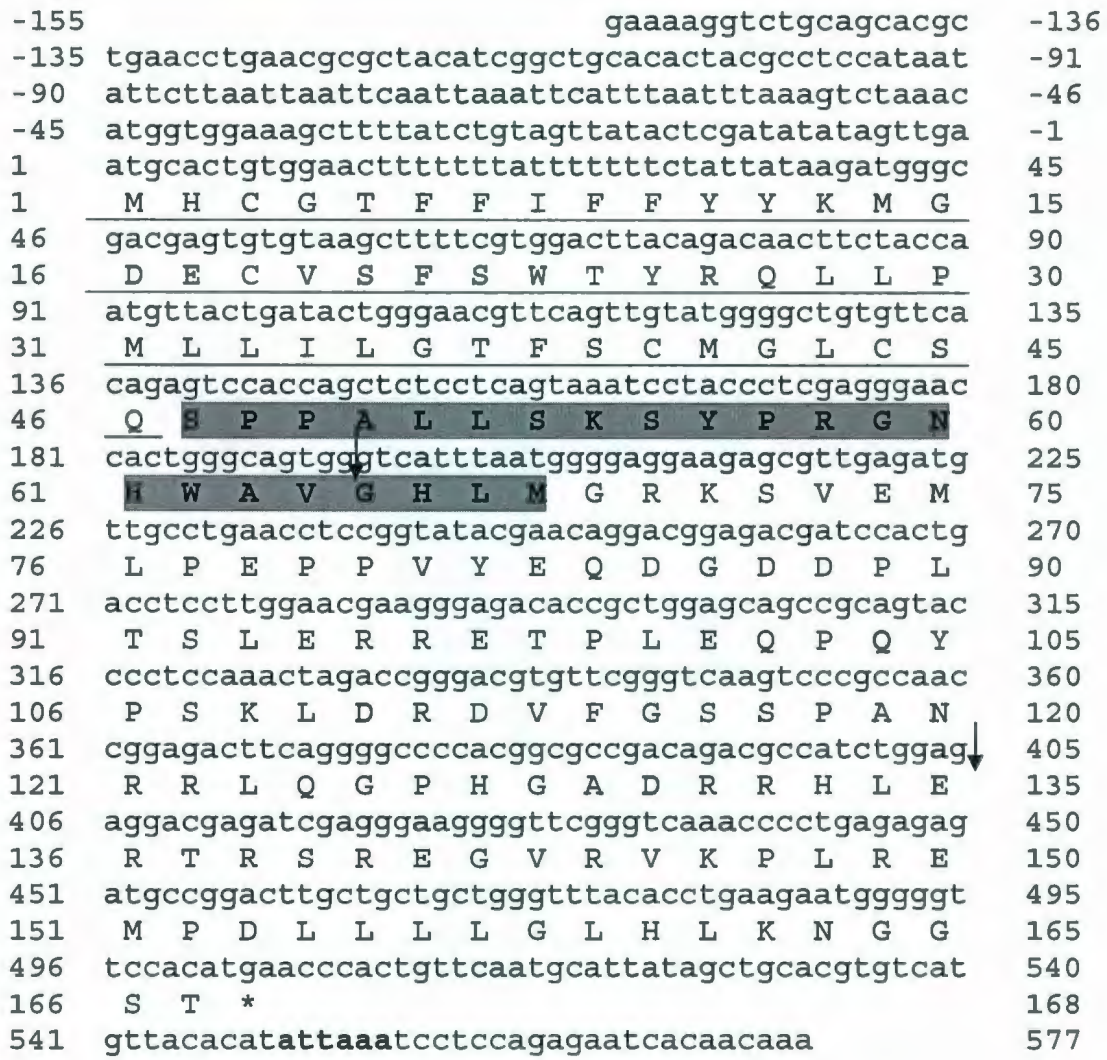


Figure 2.11. Nucleotide and deduced amino acid sequences of Atlantic cod GRP cDNA (GenBank accession no. **DQ109989**). The signal peptide sequence is single underlined. The mature peptide for GRP is shaded. The poly-adenylation signal (ATTAAA) is in bold letters. Positions of introns are indicated by arrows.

A

```
CodG            ---------------SPPA-LLSKSYPRGNHWAVGHLM
GoldfishG       ----------------SDAQPIGKVYPRGNHWAVGHLM
ZebrafishG      -----------------AQPIGKVYPRGNHWAVGHLM
TroutG          ---------------SENTGAIGKVFPRGNHWAVGHLM
DogfishG        -------------APVENQGSFPKMFPRGSHWAVGHLM
SharkG          -------------APVENQGSFPKMFPRGSHWAVGHLM
ToadG           ----------SPTSQQHNDAASLSKIYPRGSHWAVGHLM
AlligatorG      ----------APAPSGGGSAPLAKIYPRGSHWAVGHLM
ChickenG        ----------APLQPGG-SPALTKIYPRGSHWAVGHLM
CowG            ------APGIGRPVTAGRAGALAKMYTRGNHWAVGHLM
PigG            ------APVS-----VGGGTVLAKMYPRGNHWAVGHLM
DogG            ------APVP-----GGQGTVLDKMYPRGNHWAVGHLM
RatG            ------APVST---GAGGGTVLAKMYPRGSHWAVGHLM
MouseG          ------APVST---GAGGGTVLAKMYPRGSHWAVGHLM
HumanG          -----AVPLP-----AGGGTVLTKMYPRGNHWAVGHLM
FrogB           -------SCMEFVEDPNNQGRISLQQRLGNQWAVGHLM
ToadB           -------SCMEFVEDPNNQGGLNLQQRLGNQWAVGHLM
Ranatensin      ------SVCVEFAEDAGELDKSNAFRRQVPQWAVGHFM
Phyllolitorin STFVTLTVCKEVTEESDDLSKRNVLQRQL--WAVGSFM
ToadN           -SFIPLYFCMEFSEDARN----IEKIRRGNQWAIGHFM
RatN            ---------SWDLPEPRSRASKIRVHPRGNLWATGHFM
PigN            ------APLSWDLPEPRSRAGKIRVHPRGNLWATGHFM
MouseN          --------FNWDLPEPRSRASKIRVHPRGNLWATGHFM
HumanN          ---------SWDLPEPRSRASKIRVHSRGNLWATGHFM
                                                ** *  *
```

B

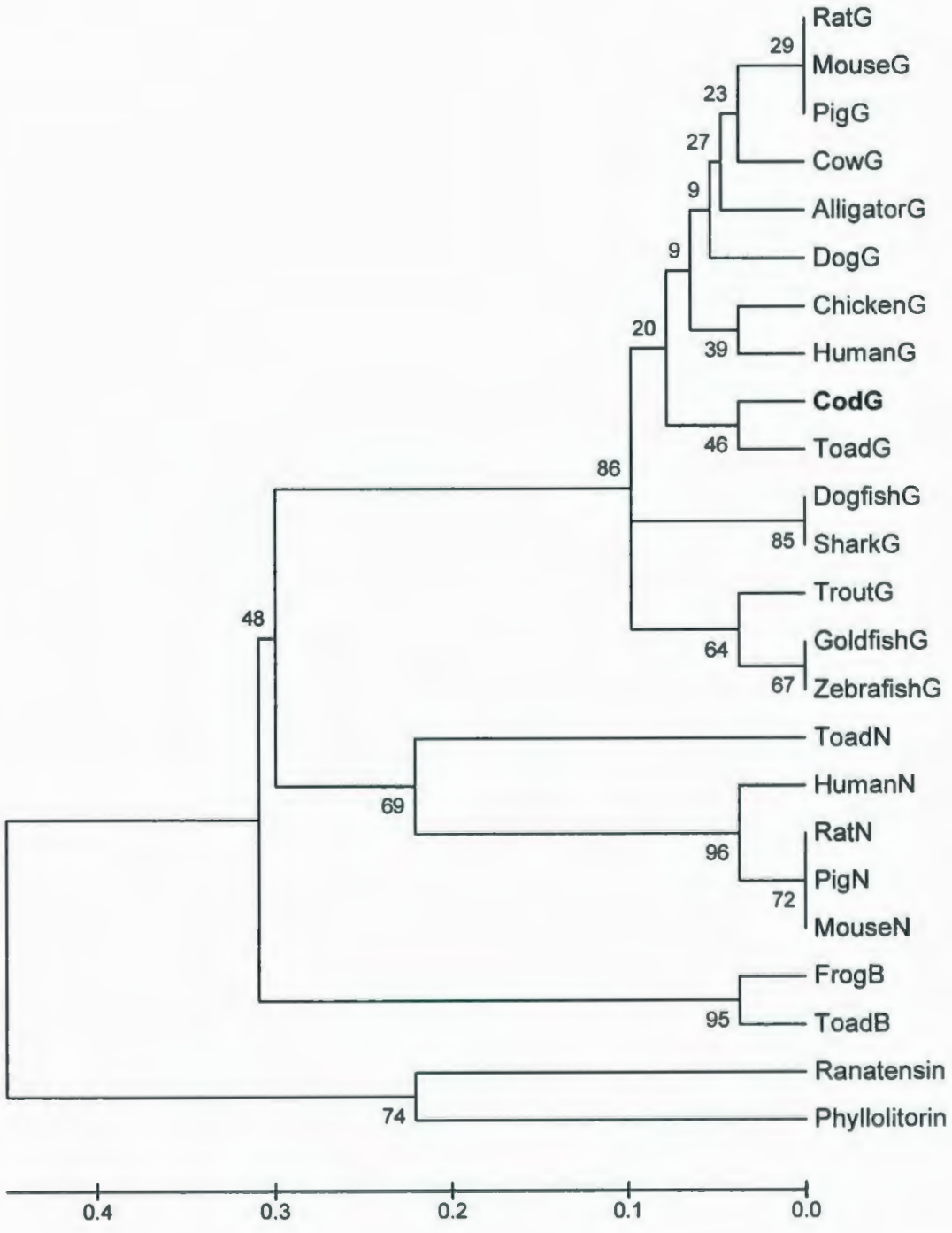

RatG
MouseG
PigG
CowG
AlligatorG
DogG
ChickenG
HumanG
CodG
ToadG
DogfishG
SharkG
TroutG
GoldfishG
ZebrafishG
ToadN
HumanN
RatN
PigN
MouseN
FrogB
ToadB
Ranatensin
Phyllolitorin
29
23
27
9
9
20
39
46
86
85
48
64
67
69
96
72
95
74
0.4
0.3
0.2
0.1
0.0

Figure 2.12. Multiple alignment of amino acid sequences (A) and phylogenetic analysis (B) of cod GRP mature peptides with sequences of GRPs for other vertebrates (G, GRP; B. bombesin BBS, N, neuromedin NMB; ranatensin; phyllolitorin). Identical amino acids were indicated with "*". Rainbow trout GRP **AAB24864**, goldfish GRP **AF111028**, zebrafish GRP **XM_683368**, dogfish GRP **P09472**, shark GRP, alligator GRP **AAB27520**, toad GRP **M83737**, cow GRP **NM_178319**, sheep GRP **NM_001009321**, dog GRP **P08989**, pig GRP **P63153**, chicken GRP **P01295**, rat GRP **NM_133570**, mouse GRP **NM_175012**, human GRP **BT006803**, frog BBS **2211320A**, toad BBS **M55255**, frog ranatensin **M21552**, frog phyllolitorin **AAB32787**, toad NMB **P43443**, pig NMB **EU375564**, rat NMB **BC158658**, mouse NMB **NM_026523**, human NMB **M21551**. A scale bar showing the distance is under the tree.

2.4 Summary and conclusion

In summary, full-length cDNAs of two growth-related hormones (PACAP and PPSS 1) and three appetite-related hormones (OX, ghrelin and GRP) were cloned from Atlantic cod. Among the cloned genes, GRP and OX are particularly interesting because they have only been identified very recently for only a few fish species. Multiple alignments and phylogenetic analyses show that the amino acid sequences and structures of all 5 peptides appear relatively conserved among fish, at least among teleosts. Cod ghrelin somewhat represents an exception as a threonine at amino acid position 3

differentiates it from other teleost ghrelins and places cod ghrelin in a single branch of the ghrelin phylogenetic tree, In addition, whereas alternative splicing transcripts due to exon skipping are not surprising in cod PRP/PACAP since they occur in most vertebrates, cod appears to be the only species to have an alternative splicing site in its ghrelin sequence. However, for all hormone sequences identified, major differences exist between mammals and fish, in the sense that fish show multiple isoforms (*e.g.* PPSS) and more prone to structural variations (*e.g.* spacer sequence in OX).

Chapter 3

Gene expression profiles for PACAP, PPSS I, OX, NPY, CART, ghrelin and GRP in tissues and brain regions, and at various developmental stages

3.1. Introduction

Following the identification of the encoding DNAs and the deduced peptide sequences for PACAP, PPSS I, OX, NPY, CART, ghrelin and GRP (**Chapter 2**), it was necessary to determine the expression profiles for these genes in order to localize their sites of production and further understand their biological function. In this investigation, the mRNA distributions of these peptides were examined throughout cod peripheral tissues and within the brain. Furthermore, as some of these genes might have a crucial role during early development and growth, this gene expression profiles in various developmental stages also were examined.

3.2. Materials and methods

The distribution of mRNA expression in different tissues and within the brain was studied by semi-quantitative RT-PCR using β-actin as a control gene. Total RNA from different peripheral (heart, liver, spleen, muscle, gill, stomach, intestine and kidney) and central (brain, pituitary) tissues, and from distinct brain regions (olfactory bulbs and tracts, telencephalon, optic-tectum, hypothalamus, cerebellum, spinal cord) were isolated as described in **Chapter 2**. Brain regions were dissected following the previously determined cod brain morphology (Hall et al., 2004).

The expression profiles of mRNAs were examined in cod embryos and larvae at different developmental stages, namely cleavage stage (15 hour post-fertilization, hpf), blastula period (40 hpf), gastrula stage (73 hpf), 30-somite stage (167 hpf), before hatching stage (250 hpf), newly hatched stage (304 hpf), 1day post-hatching stage (324 hpf), first-feeding stage 1 (346 hpf) (before first feeding), and first-feeding stage 2 (370 hpf) (after first feeding). Stages were determined by microscopic observations and according to previous classifications of Atlantic cod embryonic stages (Small and Nonneman, 2001). Approximately 10 eggs or 2 larvae were pooled for each stage. Total RNA extractions and RT reactions were conducted as described in **chapter 2**.

PCR amplification cycles were as follows: 95 °C 15 sec; 35 cycles of 95 °C for 15 sec, 55 °C for 30 sec, 72 °C for 1 min; final 72 °C for 5 min. PCR amplifications were conducted with pairs of gene-specific primers, namely P-F and P-R for PRP/PACAP, S-F and S-R for PPSS 1, OX-F and OX-R for OX, GL-F and GL-R for ghrelin, and GP-F and GP-R for GRP (**Table 3.1**), and β-actin was used as an internal control with primer pair, β-actin1 and β-actin2 (**Table 3.1**). Primers were designed based on Atlantic cod PRP/PACAP (**DQ109988**), PPSS 1 (**DQ109989**), OX (**DQ486137**), ghrelin (**EU128172, EU128173**) and GRP (**EU096314**) and on β-actin (**CO541508**). The PCR products were run at 1.7% agarose gel under 120V for 1 hour, and the 100bp DNA ladder (Promega Corp., Madison, WI) was used for indication of fragments size.

Table 3.1. Sequences of primers used in RT-PCR analysis

Primers for RT-PCR analysis	
P-F	5`-TTTACTGCACACCTATCGGAT -3`
P-R	5`-GCTGTAGCTGTCGGTGAATAT -3`
S-F	5`- CAGAAGATGTCGGTCCGC -3`
S-R	5`- TCGTCGTCCTGATCATCCGT-3`
NPY-F	5`-CAACTGAGAAACGGGGAAAA-3`
NPY-R	5`-CGGGTCATATCTGCTCTGTG-3`
CART-F	5`-GTCGTCCATGGAGCTGATCT-3`
CART-R	5`-GAACCACGTTCCCATTTCAC-3`
OX-F	5`-TCCAAGTCCTCGTGTTAGTGC-3`
OX-R	5`-AAGTCTTGCTGCGCCCATT-3`
GL-F	5`- GCATGACAGGAGCTACGCTA -3`
GL-R	5`- GAAGGTCACTCTGTGGCATC -3`
GP-F	5`- ATGGGCGACGAGTGTGTAAG -3`
GP-R	5`- AGTCCGGCATCTCTCTCAGG -3`
β-actin1	5`-TACAGCTTCACCACCACAGC-3`
β-actin2	5`-ATGCCACAAGACTCCATTCC-3`

3.3. Results and discussion

3.3.1. Distribution in tissues and brain regions

Peripheral tissue distribution analysis was performed for OX, GRP and ghrelin (**Fig 3.1**). Brain and pituitary distribution analysis was performed for PRP/PACAP, PPSS 1, OX, ghrelin and GRP (**Fig 3.2**).

RT-PCR amplifications for PRP/PACAP yielded two distinct bands in all brain regions (**Fig 3.2**) and at all developmental stages (**Fig 3.3**). PRP/PACAP mRNA is widely distributed in cod brain, as it was detected in all brain regions examined, *e.g.*, olfactory bulb, telencephalon, optic tectum, hypothalamus, medulla oblongata, cerebellum and spinal cord (**Figure 3.2**). Similar widespread brain PACAP mRNA expressions have been reported in channel catfish and goldfish (Wong et al., 2000). Although no PACAP expression has been detected in the pituitary gland of Atlantic cod (**Figure 3.2**), PACAP mRNA has been shown to be present in goldfish pituitary (Small and Nonneman, 2001) and at low levels in catfish pituitary (Xing et al., 2005). It is possible that, in the present study, PACAP mRNA expression in cod pituitary was too low to be detected.

PPSS 1 mRNA was detected in all brain regions examined as well as in the pituitary gland (**Figure 3.2**). Highest levels appeared to be present in telencephalon and optic tectum, with lowest levels in olfactory bulb and cerebellum, as seen by different intensities on the gel. This widespread distribution of PPSS 1 in brain and pituitary has been shown in other fish species such as orange-spotted grouper (Alexander et al., 2001), rainbow trout (Lin et al., 1999) and goldfish (Kaslin et al., 2004).

OX mRNA expression was detected in brain, gill, spleen, kidney and skin, and relatively weak signal in stomach and gut, but none in liver and heart (**Figure. 3.1**). In zebrafish, OX is expressed in heart and gill (Johren et al., 2001). In mammals, OX

mRNA expression has only been reported in brain and heart in rodents (Kirchgessner, 2002; Kirchgessner and Liu, 1999), but OX-A immunoreactivity has been shown in the enteric nervous system as well as endocrine cells of the gut and pancreas of guinea pigs (Kaslin et al., 2004).

Cod OX mRNA is also widely distributed in different brain regions and pituitary gland, with apparent highest levels in the hypothalamus, telencephalon and olfactory bulb, and low expression in optic tectum, cerebellum, spinal cord, and pituitary gland (**Figure 3.2**). High OX mRNA expression levels in the forebrain, especially in the hypothalamus - a region that contains important feeding-regulating centers in vertebrates, is consistent with previous results in vertebrates and with the function of OXs as appetite regulators in fish. In zebrafish, the OX system is widespread and occurs in forebrain and midbrain but not hindbrain. OX-immunoreactive (ir) fibers are present in hypothalamic cell clusters and widespread fiber projections in zebrafish (Huesa et al., 2005) and in goldfish hypothalamus (Suzuki et al., 2006). OX-A-ir fibers are also found in proximal part of the pars distalis of pituitary in Japanese seaperch (Date et al., 2000; Nambu et al., 1999; Taheri et al., 1999). In rat brain, although cell bodies of OX-ir neurons are confined to perifornical, lateral and dorsal hypothalami, these neurons have projections to neighbor hypothalamic nuclei, to several forebrain, midbrain, brainstem loci, as well as to pituitary gland (Galas et al., 2001; Shibahara et al., 1999; Singletary et al., 2005). Similarly, in amphibians, the distribution and density of OX-ir nerve fibers and terminals is wide, although most orexin neurons appear to be concentrated in the ventral hypothalamus or suprachiasmatic nucleus (Unniappan et al., 2002). This widespread

distribution of the orexin system in the brain suggests that these peptides are involved in the regulation of multiple physiological functions in all vertebrates including fish.

Cod ghrelin is present in almost all the peripheral tissues examined, including skin, spleen, kidney, heart, liver, stomach and gut (**Figure 3.1**). The highest expression levels appear to be found in stomach, indicating that, as in mammals and other fish, the stomach is the primary site of ghrelin production in cod. Ghrelin expression was also detected in several different brain regions (**Figure 3.2**), which indicates that ghrelin may act as a brain-gut hormone in cod, although the brain expression levels were very low. Similar results have been reported in goldfish (Kaiya et al., 2003b), Japanese eel (Kaiya et al., 2003a; Sakata et al., 2004), rainbow trout (Kaiya et al., 2003c) and tilapia (Conlon et al., 1987).

Cod GRP expression is present in different tissues, including brain, gill, skin, kidney, stomach and gut with relatively low expression (**Figure 3.1**). Within the brain, GRP has a widespread distribution but expression levels are extremely low (**Figure 3.2**). These results suggest that GRP may act as a brain-gut hormone in cod. Similarly, BBS/GRP-like peptides have been detected in both gastrointestinal tract and brain of several fish species, including dogfish (Volkoff et al., 2000), goldfish (Jensen and Conlon, 1992) and trout (Volkoff et al., 2000). Comparatively high GRP expression levels are seen in cod pituitary (**Figure 3.2**). Similar results have been reported for GRP in goldfish (Houben et al., 1993) and for GRP-related peptides, including GRP and NMB, in rat (Sherwood et al., 2000) , suggesting that GRP might act as a physiological regulator of pituitary function.

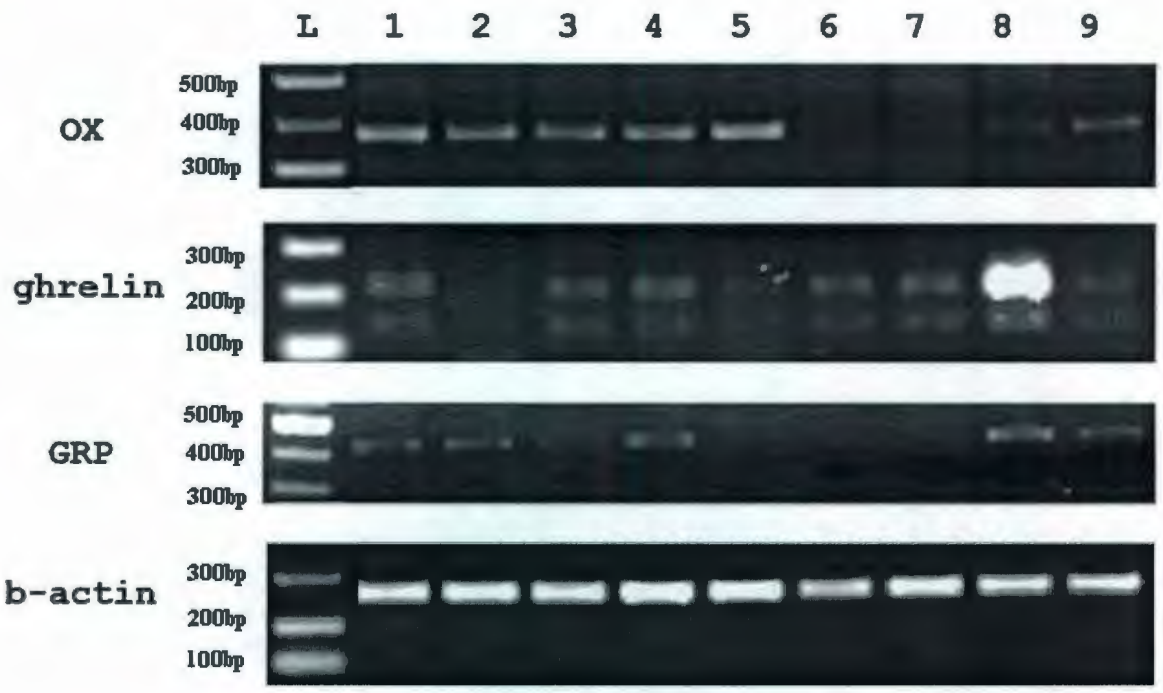


Figure 3.1. Expression of orexin, ghrelin and GRP transcripts in cod peripheral tissues, as detected by RT-PCR. Lane L, ladder; lane 1, brain; lane 2, gill; lane 3, skin; lane 4, kidney; lane 5, spleen; lane 6, heart; lane 7, liver; lane 8, stomach; lane 9, small intestine.

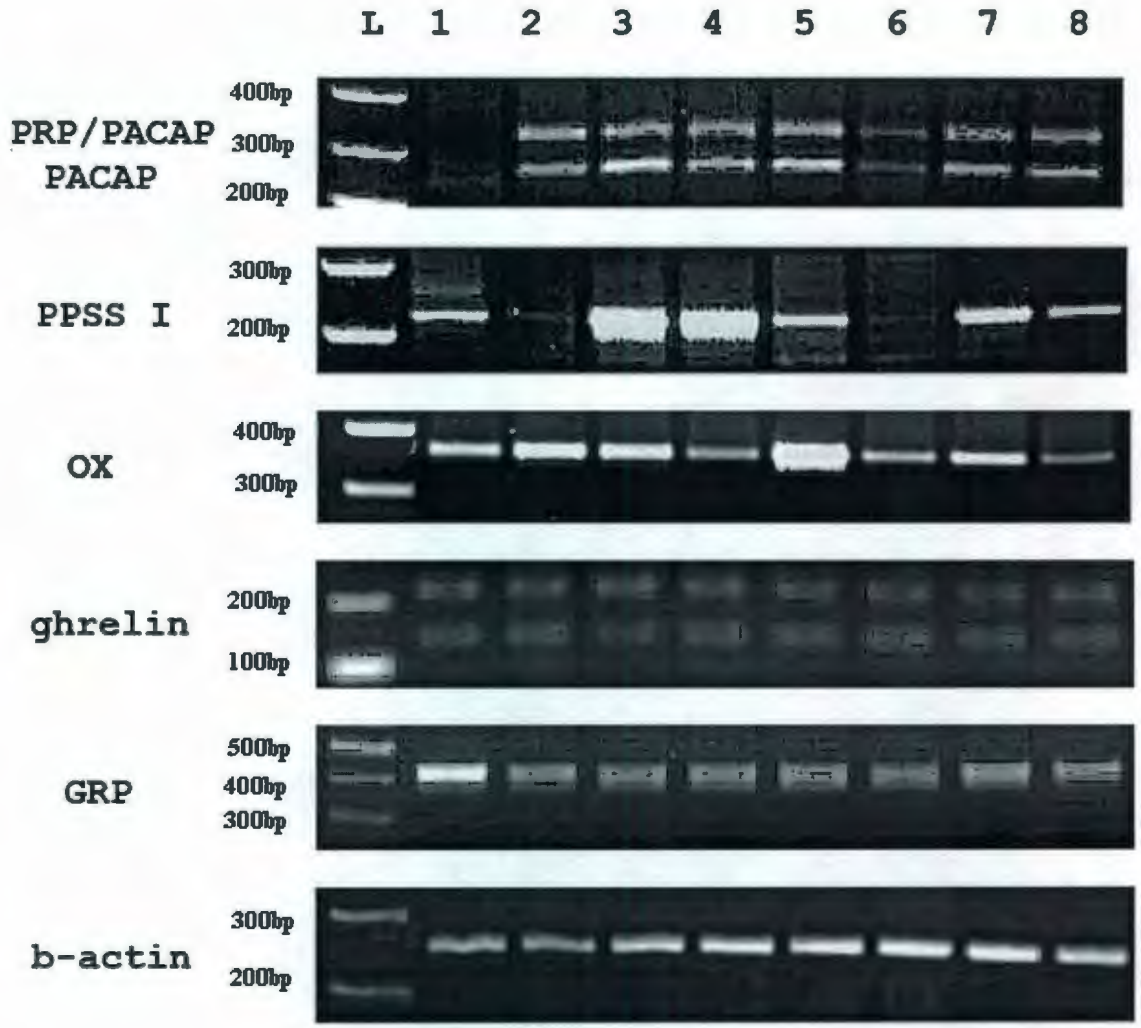


Figure 3.2. Distribution of PRP/PACAP, PPSS 1, OX, ghrelin and GRP transcripts in cod brain and pituitary, as detected by RT-PCR. PRP/PACAP yielded a long 359 bp and a short 254 bp band, corresponding to two differently processed mRNA transcripts, while PPSS 1 yielded a single 235 bp band. Lane L, ladder; lane 1, pituitary; lane 2, olfactory bulb; lane 3, telencephalon; lane 4, optic tectum; lane 5, hypothalamus; lane 6, cerebellum; lane 7, medulla oblongata; lane 8, spinal cord.

3.3.2 Expressions during development

Developmental profiles were conducted for PACAP, PPSS I, OX, NPY, CART, ghrelin and GRP (**Figure 3.3**).

Expression of PRP/PACAP mRNA was detected as early as the 30-somite stage with a relatively weak signal that seemed to gradually increase with the development of cod embryos and larvae (**Figure 3.3**). Previous studies show that PACP/PRP is generally expressed in fish during very early development stages, with slight differences between species. In zebrafish, PRP/PACAP mRNA is first expressed at 5-6 hours post fertilization, a transitional stage between blastula and gastrula stages (Sherwood et al., 2000), whereas in rainbow trout, PRP/PACAP mRNA is first detected at stage E4, corresponding to the late blastula stage (Allais et al., 2007). This early expression of PACAP/PRP is consistent with its crucial function in early brain development in embryonic and larval stages. Indeed, altered cerebellar development is observed in mice lacking a PACAP gene (Jarry et al., 1992) and morpholino-knockdown PACAP zebrafish embryos display morphological defects in their brains as early as 24 hpf (Xing et al., 2005).

Expression of PPSS 1 was first detected in the pre-hatching stage and seemed to gradually increase with the development of cod embryos and larvae. In orange-spotted grouper, PPSS 1 mRNA is detected from fertilized eggs to hatch-out stages with highest mRNA levels at the onset of hatching (Devos et al., 2002). In zebrafish, PPSS 1 mRNA expression is first detected at 19 hpf (23-somite stage) in the brain and 24 hpf in pancreas primordium (Biemar et al., 2001). This early expression of SS in fish seems to indicate that this peptide, like PRP/PACAP, might be essential for normal development, at least in

the brain and the pancreas (Faraco et al., 2006).

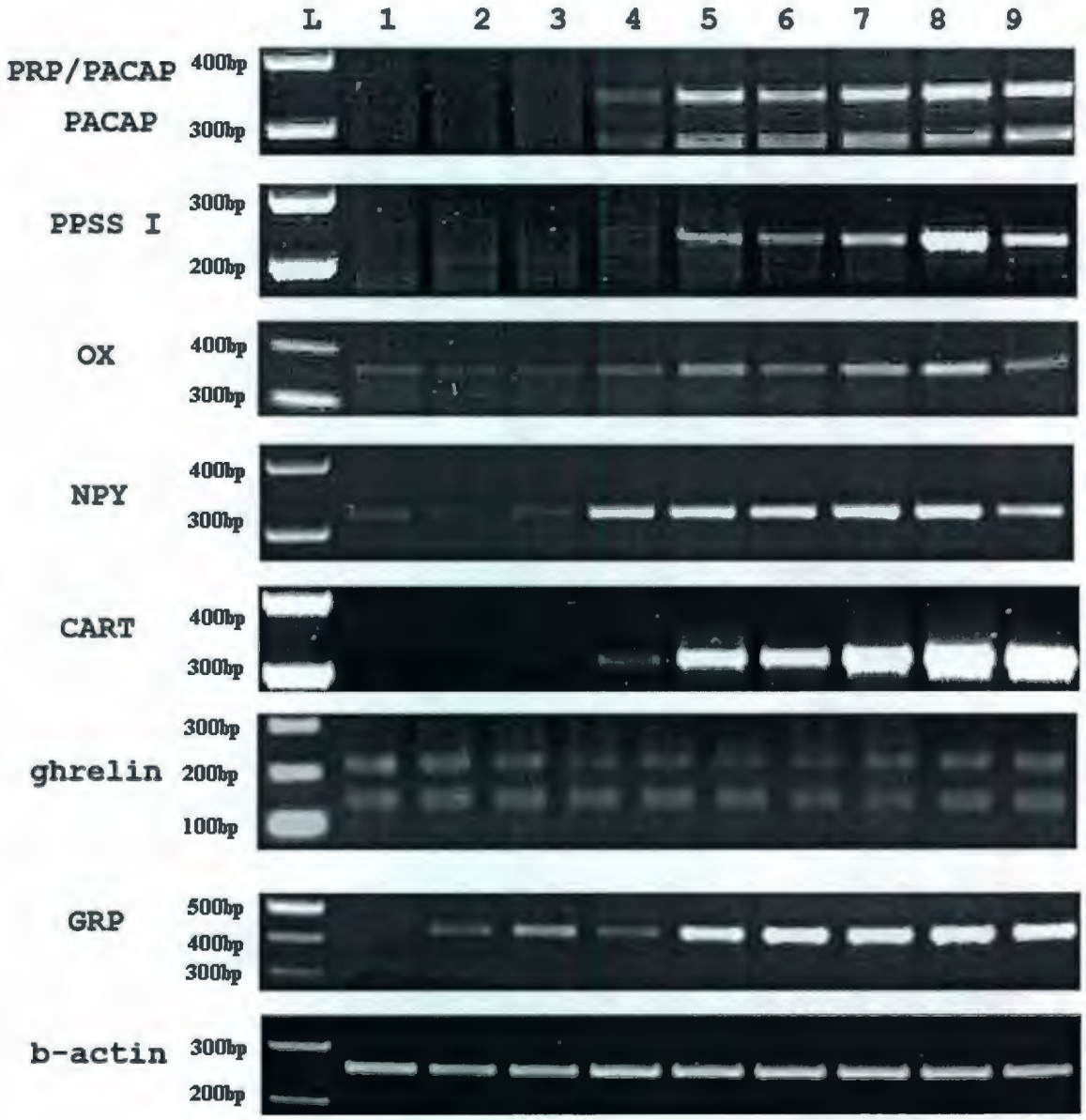


Figure 3.3. Expression of PACAP, PPSS I, OX, NPY, CART, ghrelin and GRP transcripts at different embryonic and larval stages. Lane L, ladder; lane 1, cleavage stage; lane 2, blastula stage; lane 3, gastrula stage; lane 4, 30-somite stage; lane 5, pre-hatching stage; lane 6, newly hatched stage; lane 7,

1day post-hatching stage; lane 8, first-feeding stage1 (before first feeding); lane 9, first-feeding stage2 (after first feeding).

During early larval stages, PPSS 1, but not PRP/PACAP expression levels, appeared to be affected by food availability, with an apparent decrease in expression in the first feeding stage larvae supplied with food compared to the same stage larvae without food supply (**Figure 3.3**). This apparent difference in PPSS 1 mRNA expression before and after first feeding probably indicates a regulation by nutrition status and a role of SS in the regulation of growth in cod larvae.

Expression of OX mRNA was detected as early as the cleavage period. The expression signal appeared relatively weak in the cleavage, blastula, and gastrula stages, and gradually increased with the development of cod embryos and larvae (**Figure 3.3**). From the segmentation stage on, time at which the cod embryo has a fully developed brain, expression levels become stronger and appear to gradually increase with the development of the embryos and larvae. In zebrafish, OX mRNA is first detected by *in situ* hybridization at 22hpf, corresponding to the segmentation stage (Amiot et al., 2005), which is later than the cleavage stage at which a signal was first detected in cod embryos but corresponds to the stage at which a strong signal was first detected. The discrepancy between zebrafish and cod in the stage at which OX expression is first detected might be due to differences in experimental procedures or in the sensitivity of the techniques used. In rats, OX mRNA and protein appear relatively early in development and both OX axon density and OX mRNA expression reach a peak at a developmental stage that coincides with the appearance of adult patterns of feeding and sleep-waking activity (de Pedro et

al., 2006; Neveu et al., 2002). In the present study, OX expression was the highest in the feeding larva before starting of the first feeding. After the first feeding, an apparent decrease in expression was observed (**Figure 3.3**), suggesting that feeding affects OX expression at very early stages of life.

NPY mRNA expression was first observed at low levels at the cleavage stage. The expression remained low in the blastula and gastrula stages and increased at the 30-somite stage, then gradually increased through the pre-hatching stage, newly hatched stage, 1day post-hatching stage, and first-feeding stage (**Figure 3.3**). The early expression of NPY suggests that it has a role in development and early feeding in cod. Similar results have been reported in rodents where the expressions of both NPY and its receptor as well as NPY ir are detected early in embryonic development and increase gradually until postnatal life (Chen et al., 2005). In orange spotted grouper, no NPY mRNA expression is observed from unfertilized eggs to morula stage, while low levels of expression are detected from blastula to neurula stages, and high expression levels are seen from the lens formation stage to the 52-day-old larval stage (Kurokawa et al., 2005). In Japanese flounder, NPY mRNA is expressed in the brain and retina during the larval stage (Chiba et al., 1995; Chiba et al., 2002). In cloudy dogfish, NPY ir is detected in cerebrospinal fluid-contacting neurons at the 34 mm stage (Mathieu et al., 2002). In zebrafish, NPY ir system gradually increases from eggs to juvenile stages and reaches a maximum in mature fish (Amiot et al., 2005; Wierup et al., 2004).

CART mRNA expression was first observed at the 30-somite stage with very low expression, followed by an abrupt increase at the pre-hatching stage, and a gradual increase in the newly hatched, 1day post-hatching, and first-feeding stages (**Figure 3.3**).

The results suggest that CART-peptides may play important roles in development. To my knowledge, the expression profile of CART during ontogeny has never been reported for any fish. In rodents, CART is expressed in some of the very first generated neurons in the embryonic brain (Abraham et al., 2007; Beloosesky et al., 2006). CART ir is first detected in early embryos (day 12 of pregnancy) and both CART ir and CART mRNA levels increase in the developing embryo and placenta throughout pregnancy (from day 14 to day 18 of pregnancy) (Kawamura et al., 2003).

During early larval stages, it appears that NPY and OX expression levels, but not CART, are affected by food availability, with an apparent decrease in expression in the first feeding stage larvae supplied with food compared to the same stage larvae without food supply (**Figure 3.3**), suggesting that NPY and OX, but not CART, might be involved in the regulation of feeding in early stages of life in teleosts.

The expression of ghrelin mRNA was first detected at the cleavage stage (**Figure 3.3**). Although detectable, the expression levels were comparatively weak in all the embryonic and larval stages observed, making it difficult to assess changes in expression during development using semi-quantitative methods. Studies in mammals have shown that ghrelin expression appears early during embryonic development (morula cells) (Sato et al., 2006), and that fetal neurogenesis is induced by ghrelin (Manning et al., 2008). In Atlantic halibut, notable increases in ghrelin expression are seen in metamorphosing larvae, particularly just prior to the post-metamorphic juvenile stage (Sherwood et al., 2000). The presence of ghrelin throughout development suggests that this peptide might have a role in organogenesis and larval development in cod.

The expression of GRP mRNA was detected as early as the blastula stage.

Expression levels were low at the blastula, gastrula and 30-somite stages, but increased at the pre-hatching embryo, newly hatched larva, 1 day post-hatching larva and first-feeding larva (**Figure 3.3**). This is the first report of GRP gene expression during development, for either mammals or fish. My results suggest that GRP might play a role in fish larval development, in particular around hatching and first feeding times.

Although expression was detected at very early developmental stages, neither ghrelin nor GRP appeared to be affected by food availability during early life stages. This may indicate that the digestive system is not yet well enough developed to fulfill feedback regulation of food intake of gut peptides at the first feeding stage.

3.4. Summary and conclusion

Gene expression studies revealed a widespread distribution of all genes examined in peripheral tissues and within the brain, indicating that these genes, at least for some hormones, may have a variety of physiological roles other than growth and appetite regulation. The typical expression patterns represent their functional regions and are highly similar to those of other teleosts reported so far, with high forebrain expression levels for brain peptides and high stomach expression levels for gut peptides. For all the peptides in the present study, an early appearance in development probably indicates a crucial role in development. The present study reports for the first time in any vertebrate the occurrence of GRP expression in very early developmental stages and suggests a role of GRP in the development of fish and perhaps in mammals. The apparent differences of expression for PPSS 1, OX and NPY before and after first larval feeding suggest that in

fish, growth and appetite are already under strict endocrine regulation at very early stages of life.

Chapter 4

Influence of nutritional status on growth rate and on pituitary adenylate cyclase activating polypeptide (PACAP)/ PACAP-related peptide (PRP) and somatostatin I (PPSS I) mRNA expression in juvenile Atlantic cod[1]

4.1. Introduction

In mammals, growth hormone-releasing hormone (GHRH) and somatostatin (SS) are two major hypothalamic hormones that regulate growth hormone (GH) secretion from the pituitary. Recent evidence suggests that other factors, such as pituitary adenylate cyclase-activating polypeptide (PACAP) may also be involved in regulating GH secretion (Miyata et al., 1989). PACAP was originally isolated from sheep hypothalamus on the basis of its ability to stimulate cAMP formation in anterior pituitary cells (Goth et al., 1992). The dose-dependent stimulating effects of PACAP on GH secretion were first reported in cultured rat anterior pituitary cells (Wong et al., 1998). PACAP was later found to be a potent stimulator of GH secretion in non-mammalian species as treatment with either form of PACAP induces a robust stimulation of GH release from goldfish (Olszewski et al., 2007; Wong et al., 2005), carp (Montero et al., 1998) and eel pituitary cells (Brazeau et al., 1973).

Somatostatin (SS) is a tetradecapeptide originally isolated from sheep hypothalamus that acts as an inhibitor of pituitary GH secretion (Canosa et al., 2007). Several *in vitro* and *in vivo* studies have shown that SS-14-1 regulates pituitary function in teleost fish, as it inhibits both basal GH secretion and GH secretion stimulated by

[1] some of the data presented in this chapter have been accepted in Peptides, 2009

factors such as gonadotropin-releasing hormone (GnRH), corticotrophin releasing hormone (CRH), dopamine (DA), and PACAP (Canosa et al., 2007; Marchant et al., 1989; Marchant et al., 1987; Yunker et al., 2003). The physiological effects of the other various fish SS isoforms on GH regulation remain unclear. It appears that SS-14-1, mammalian SS-28-1, goldfish brain SS-28-3, and SS-14-2 all inhibit GH release, whereas salmonid SS-25-3 and catfish SS-22 do not (Klein and Sheridan, 2007) .

SSs and PACAP have both been implicated in the regulation of feeding and metabolism of mammals and fish. The effects of SSs on food intake are not clearly defined as both orexigenic and anorexigenic effects have been reported (Eilertson and Sheridan, 1993). SSs seem to affect both lipid and carbohydrate metabolism by promoting lipid mobilization and hyperglycemia. Trout injected with either SS-14-1 or SS-25-3 display elevated plasma fatty acid levels in association with enhanced activity of the lipid mobilizing enzyme, triacylglycerol lipase, as well as elevated plasma glucose levels and reduced plasma glycogen concentration. Treatment with SS-25-3, but not SS-14-1, also reduces plasma insulin levels (Very et al., 2001). In addition, SS biosynthesis and secretion are both regulated by nutrients, such as lipids and glucose (Chance et al., 1995; Morley et al., 1992). In rodents, intracerebroventricular (ICV) injections of PACAP decrease food uptake (Morley et al., 1992) and antagonize the orexigenic effect of NPY (Matsuda et al., 2005b). In goldfish, centrally or peripherally injected PACAP suppresses food intake (Miura et al., 2007), and these actions might be mediated by the stimulation of pro-opiomelanocortin (POMC) and corticotropin-releasing hormone (CRH) pathways in the brain (Olsson and Holmgren, 2000). PACAP exerts a relaxant effect on intestinal smooth muscles in the Atlantic cod (Nakata and Yada, 2007),

suggesting that PACAP affects the digestive physiology of fish. In mammals, PACAP potentiates both the meal-induced release of insulin from pancreatic beta-cells and the lipid-storing actions of insulin on adipocytes (Hans-Joachim and Josep, 2003), suggesting that PACAP regulates glucose levels and energy metabolism in mammals. To date, there is no data available on the role of PACAP in the metabolic physiology of fish.

In order to characterize these peptides in cod and to better understand their role in feeding and metabolism in fish, I have cloned cDNAs encoding cod PACAP and PPSS 1, and examined their distribution in brain and pituitary and during larval development (see **chapters 2 and 3**). In the present study, I assessed the effects of starvation and re-feeding on their mRNA expression in juvenile fish.

4.2. Materials and methods

4.2.1. Experimental protocol

In order to study the effects of nutritional status on gene expression, 4 groups of approximately 100 fish each were placed in 4 different tanks and acclimated to the conditions described in **chapter 2** for one week. All fish were submitted to a scheduled feeding regimen and fed EWOS Marine Diet once daily at the same time (12:00) with a ration of 1.5% total body weight (BW). An average body weight was calculated weekly by averaging the weights of 4-5 fish picked randomly from each tank, and feed rations were adjusted in proportion to increasing body weights. Following the acclimation period, fish were divided into two groups (each consisting of duplicate tanks). The experimental group was submitted to starvation for 1 month (30 days) and subsequently re-fed for 10 days. The control group was kept under the same feeding regimen as during

the acclimation period. Tanks were duplicated for each experimental treatment in order to take into account any tank effect during the study. Brain tissues were sampled ten times during the experiment, namely 0 days, 2 days, 5 days, 9 days, 16 days, 23 days, 30 days of starvation and then after 2 days, 5 days, and 10 days of re-feeding. (4-5 fish per tank, in duplicate). Although in all experimental tanks a total of 40-50 fish were sampled (4-5 fish per sampling), an initial density of 100 fish per tank was used in order to avoid stress and abnormal behavior. Whole forebrains (including telencephalon, optic tectum, and hypothalamus) were dissected, kept in RNAlater (Qiagen) and stored at -80 °C until use. At each sampling time, growth was estimated by calculating the Fulton's condition factor (K) using the formula K = [total mass (g)/ fork length^3 (cm)] X100 (Lemieux et al., 2004). All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide and the UFAW Handbook on the care and management of laboratory animals published by Blackwell Science (www.tiny.cc/9y7sa) on the care and use of experimental animals.

4.2.2. mRNA expression analysis

Slot blots were used to examine the effects of food availability on cod PRP/PACAP and PPSS 1 mRNA expression. A 270 bp PRP/PACAP probe and a 370 bp PPSS 1 probe were generated by PCR using primer pairs, P-B1 and P-B2, and S-B1 and S-B2, respectively (**Table 4.1**). Primers were designed based on the PRP/PACAP and PPSS 1 sequences in Atlantic cod. A 380bp elongation factor 1α (EF1α) probe was amplified as internal control by PCR using primer pairs, EF-B1 and EF-B2 based on Atlantic cod EF1α sequence (accession no **CO541952**). EF1α was chosen as a reference

gene for slot blot analysis because although no variation in expression was seen for either EF1α or β-actin, EF1α produced a stronger and more stable hybridization signal than β-actin. The PCR products obtained for each primer pair were cloned by insertion into a plasmid and sequenced to confirm the target gene being amplified. Plasmid cDNAs were subsequently used as templates for PCR amplifications. PCR products were run in a 1.7% gel and purified with GenElute gel extraction kit (Sigma, St Louis, Missouri, USA). The purified DNA was quantified by spectrometry and used as a template for the synthesis of a biotinylated probe using the Bioprime DNA labeling kit (Invitrogen). The probes were purified with Montage PCR filter units (Millipore) to remove unincorporated primers and nucleotides.

Table 4.1. Sequences of primers used for slot blot analysis

Primer	Sequence
P-B1	5`-CCTCTCACACGCTTCGACAG -3`
P-B2	5`-CGTCCTTTGTTCCTCACCCT -3`
S-B1	5`-AGGAGAGTTGAACGTCTTCAG -3`
S-B2	5`-CATCAATCAACACGATGTAAA -3`
EF-B1	5`-ACKGTCTGCCTCATGTCACG-3`
EF-B2	5`-TGACAACGTYGGCTTCAACG-3`

Approximately 15 μg of total RNA from each fish brain (8 individual fish per group) were denatured in a ice-cold alkaline solution containing 10 mM NaOH and 1 mM

EDTA and blotted onto a Biodyne Membrane (Pierce, Rockford, Illinois, USA) by vacuum suction using a slot blot apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The membrane was then washed with 2XSSC and fixed by baking at 80°C for 1 hour. After a 30 min pre-hybridization, the membrane was hybridized overnight at 55 °C with a biotin-labeled PPSS 1 probe. The membrane was then washed and the hybridization signal detected using a North2South Chemiluminescent Hybridization and Detection Kit (Pierce, Thermo Fisher Scientific Inc., Rockford, USA). After signal detection, the membrane was stripped with 0.5% SDS at 60 °C for 1 hour, washed with 2x SSC buffer at room temperature and re-probed with biotin-labeled PRP/PACAP probe using the same procedure. The membrane was then stripped and re-probed with biotin-labeled EF1α probe. The normality and homogeneity of variances from data obtained with the reference gene EF1α were tested before any further analysis of the samples. No significant differences were found in EF1α expression levels between all experimental groups, thus validating EF1α as a housekeeping gene for the experiment. Before performing slot blots with experimental samples, serial dilutions of RNA were blotted onto a membrane and detected by each of the probes to verify the linearity of the signal and to determine the optimal amount of RNA to be used for quantification. In order to verify the specificity of the probes, PCR fragment from RT-PCRs were denatured by boiling and blotted on membranes. The membranes were then hybridized with either PACAP or PPSS 1 probes. This demonstrated that the PRP/PACAP probe hybridized with both long and short transcripts of PRP/PACAP, and that the PPSS 1 probe was specific to PPSS 1.

4.2.3. Data analysis and statistics

Hybridization signals were detected using an EpiChemi Darkroom (UVP, Upland, California, USA), and captured and quantified using LabWorks software (UVP). In expression studies, all samples are expressed as ratios of gene of interest to EF1α and all data are presented as mean ± SEM. To compare between different groups, a one-way ANOVA followed by a Student-Newman-Keuls multiple comparisons test was performed and significance was set at $p < 0.05$. Statistical analyses were performed using Instat 3 (Graphpad Software, San Diego, CA, USA).

4.3. Results

In order to study the effects of nutritional status on growth rate, and on PRP/PACAP and PPSS 1 mRNA expressions in juvenile cod, I compared two groups of juvenile cod, one under normal feeding condition (control group) and the other submitted to one month of starvation followed by 10 days of re-feeding (experimental group). In the control group, the growth condition factor (GCF) showed a slight but constant increase during the course of the experiment (**Figure 4.1**). The GCF of the experimental group was similar to that of the control group during the first 23 days of starvation, and subsequently showed a significant decrease from days 23 to 30. The GCF of the experimental group remained stable and lower than that of the control fed group for the first 5 days of re-feeding but displayed a significant increase from day 5 to day 10 and a return to levels similar to that of the control group by day 10 (**Figure 4.1**). Slot blot analyses were used to examine both PRP/PACAP and PPSS 1 mRNA expressions in juvenile cod under different nutritional status. In order to ensure specificity, cDNA probes were designed so they hybridized almost the entire encoding portion of the cloned

cDNAs. Southern Blot analyses revealed that the PRP/PACAP probe hybridized with both long and short transcripts of PRP/PACAP generated by RT-PCR, and that the PPSS 1 probe hybridized to the PPSS 1 PCR fragment generated by RT-PCR (data not shown). PRP/PACAP expression levels were similar between control and fasted fish throughout the 30 days of food deprivation (**Figure 4.2 A**). A significant increase in PRP/PACAP expression levels was observed at 10 days re-feeding in the experimental group as compared to the control group. Food deprivation led to increased PPSS 1 expression levels after about 10 days, and these levels in the experimental group remained significantly higher than that of control fish throughout the subsequent 20 days of starvation and 10 days of re-feeding (**Figure 4.2 B**).

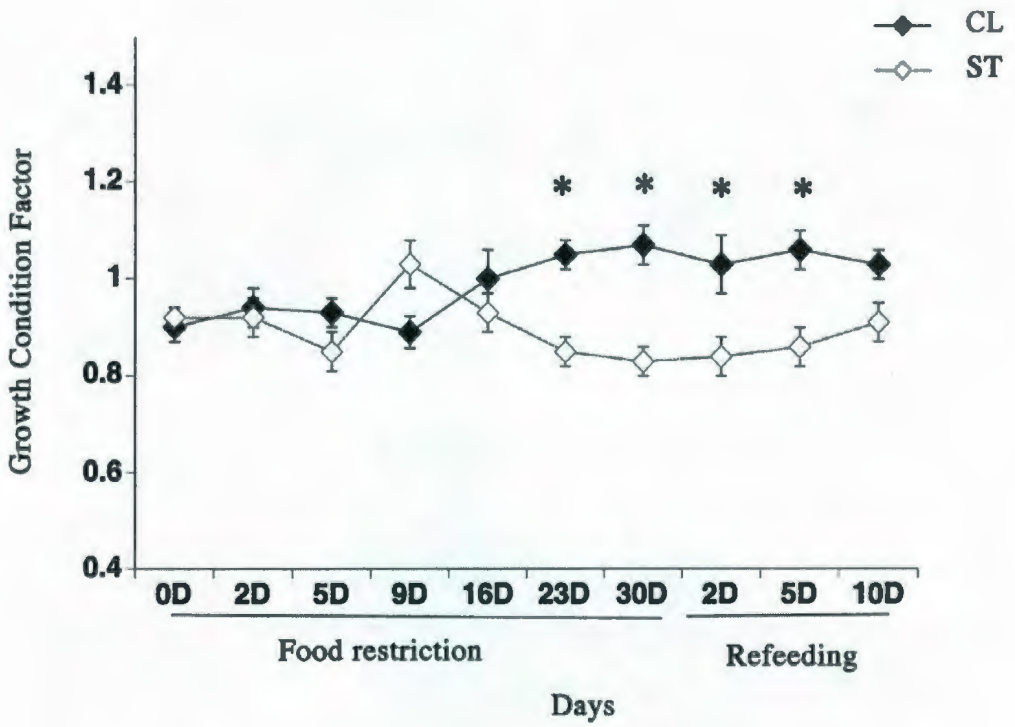


Figure 4.1. Growth condition factor of the control fed group (CL) (solid line and closed diamond) and the experimental group (ST) (dashed line and open diamond) submitted to 30 days starvation and 10 days re-feeding. Data are expressed as means ± SEM (n = 8-10 per group). Significant differences ($p < 0.05$) between groups were detected using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. A star indicates a significant difference between control and experimental group for a given day.

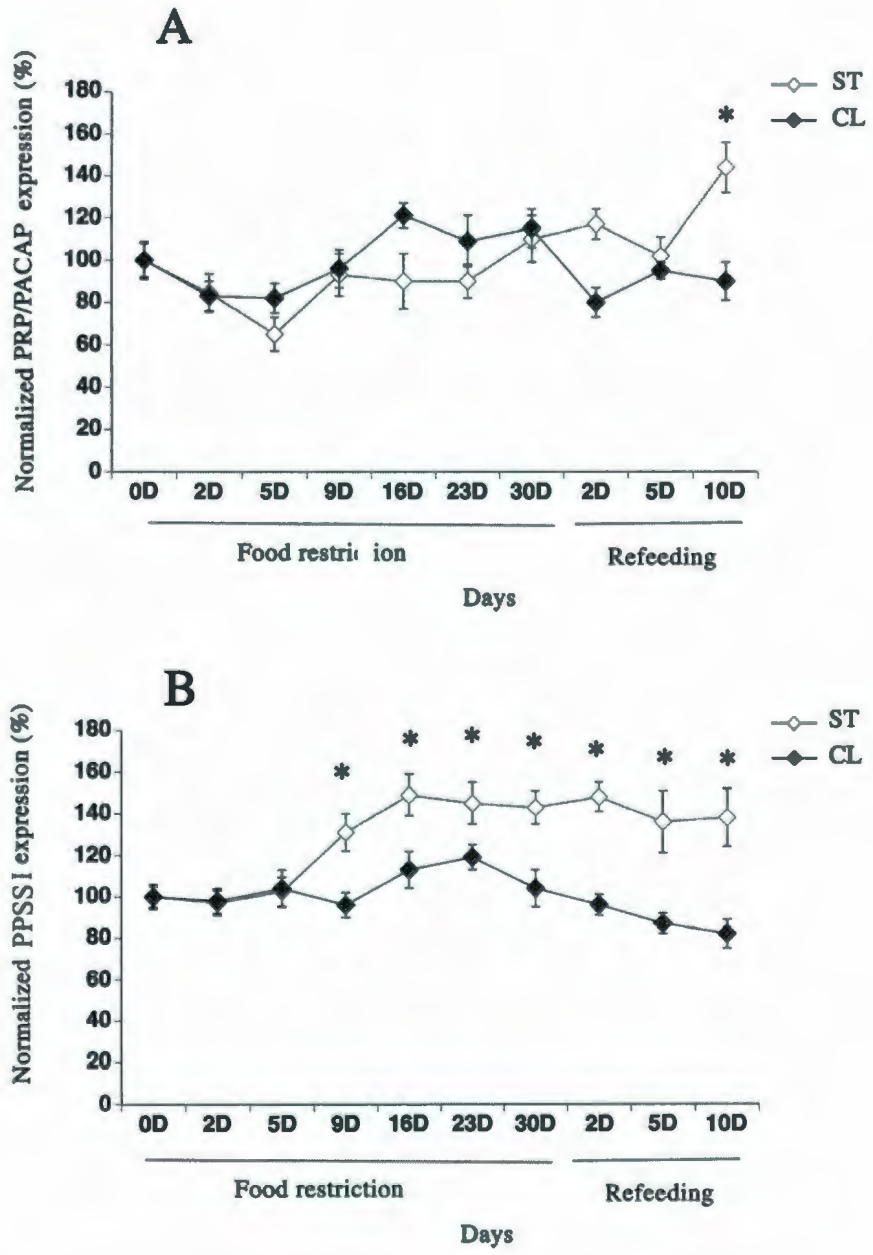


Figure 4.2. Slot blot analysis of forebrain PRP/PACAP (A) and PPSS 1 (B) mRNAs in control fed (CL) (solid line and closed diamond) and experimental group (ST) (dashed line and open diamond) submitted to 30 days starvation and 10 days re-feeding. Data are expressed as means ± SEM (n = 8 per group).

Significant differences ($p < 0.05$) between groups were detected using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. A star indicates a significant difference between control and experimental group for a given day.

4.4. Discussion

Nutritional status is a major factor affecting organic growth. Atlantic cod, like many other marine cold-water fish species, have the ability to survive long periods of food restriction. During starvation, growth rates decrease and muscle tissue exhibits signs of wasting (Ali et al., 2003; Schwalme and Chouinard, 1999). However, upon resumption of feeding, accelerated growth is often observed (Bandeen and Leatherland, 1997; Blier et al., 2007; de Pedro et al., 2006; Meton et al., 2003). In the present study, as expected, the control group showed a slow but constant growth during the whole experimental period. In fish submitted to food deprivation, growth initially displayed a plateau and became significantly lower than that of the control group from day 23 to day 30. A dramatic increase in growth was then observed during the re-feeding period, so that after 10 days of re-feeding, the growth of the experimental animals was similar to that of the control group. The hepatic somatic index (HSI=liver weight*100/body weight) is a good indicator of both metabolic energy demand and nutritional status. As cod use liver as an energy storage organ, low HSI values might indicate a state of negative energy balance. Not surprisingly, the HSI of cod decreased during starvation and was restored with re-feeding (Xu, personal observation). Results from the present study are consistent with previous studies in fish showing that long term food deprivation leads to significant

decreases in both condition factor and HSI, which are recovered during re-feeding (Blier et al., 2007; Hall et al., 2006; Jobling et al., 1994). This rapid restoration of the growth condition factor during re-feeding of fasted animals (compensatory growth stage) has previously been shown in cod (Montserrat et al., 2007) and other fish species such as rainbow trout (Barreto et al., 2003), Nile tilapia (Picha et al., 2006), and hybrid striped bass (Ehrman et al., 2002).

Nutritional status also affected the expression of two genes encoding two major GH regulating hormones, PPSS and PACAP. Increases in PPSS 1 expression levels were observed after 10 days of starvation and these levels remained high not only throughout starvation but also during the re-feeding period. Although nothing is known about the effects of nutritional status on brain PPSS 1 expression in fish, previous studies on rainbow trout have shown that fish fasted for 4-6 weeks display reduced growth and increased levels of pancreatic PPSS 1 mRNA compared to normal fed animals, both being restored during re-feeding to levels similar to those displayed by continuously fed fish (Ayson et al., 2007; Pedroso et al., 2006; Small et al., 2002; Sumpter et al., 1991). Interestingly, previous studies in fish have demonstrated that both pituitary GH mRNA and plasma GH levels increase significantly during starvation and are restored to basal normal levels when animals are re-fed (Pierce et al., 2005). For example, in chinook salmon, the GH response to fasting occurs in three phases: a rapid increase in GH blood levels (days 1-3) followed by a plateau of 10-20 ng/ml (days 4-12) and a dramatic increase (days 15-29) (Sumpter et al., 1991). It has been suggested that the major role of high GH during starvation is to aid in the mobilization of fatty acids and glycerol from adipose stores (Berelowitz et al., 1981; Chihara et al., 1981; Rogers et al., 1988;

Sheppard et al., 1978). As SSs inhibit GH release, one would expect increases in plasma GH levels to be accompanied by low PPSS mRNA levels. The present results showing high forebrain PPSS 1 mRNA levels during starvation might indicate that cod have lower GH levels in response to fasting. It is also possible that as in other fish, starvation in cod induces increases in GH levels, which may exert a positive feedback action on hypothalamic regulators of GH secretion, thus increasing hypothalamic PPSS expression. Indeed, such feedback mechanisms have been demonstrated in rodents, in which GH treatment induces dose-dependent increases in the synthesis and release of hypothalamic SS (Lanzi and Tannenbaum, 1992; Tannenbaum et al., 1989). Fasting results in enhanced plasma levels of both GH and SS (Caelers et al., 2005; Mori and Devlin, 1999). Although no direct evidence exists of such a feedback regulation in fish, reports may support it partially. For example, transgenic fish expressing GH ectopically display a regressed pituitary gland and low GH mRNA levels (Canosa et al., 2007; Canosa et al., 2002), and ICV administration of GH antibody significantly reduces mRNA levels of both PPSS 1 and PPSS 2 in goldfish forebrain (Parker et al., 1997). Future experiments measuring GH blood levels might help us establish the relationship between GH and PPSS during the fasting response in cod.

In contrast to PPSS 1, PRP/PACAP expression was not affected by a 30 days food deprivation but significantly increased during the 10 days re-feeding period compared to the control group. The increase in PRP/PACAP mRNA expression during re-feeding is likely related to physiological changes that occur during compensatory growth. In vertebrates, in particular bony fish, *e.g.* in rainbow trout (Wong et al., 1998), goldfish (Montero et al., 1998), European eel (Rousseau et al., 2001), turbot (Wong et al., 2005),

grass carp (Xiao et al., 2002) and common carp (Chance et al., 1995; Morley et al., 1992), PACAP acts as a potent secretagogue for GH release. In addition, PACAP also appears to be involved in the regulation of feeding as ICV injection of PACAP reduces food intake in rodents (Tachibana et al., 2003), chicken (Matsuda et al., 2005b) and goldfish (Matsuda et al., 2005a). In goldfish, the mRNA expressions of both PACAP and its receptor (PAC1) increases after 7 days of excessive feeding, but no changes in expression are seen after 7 days of starvation (Gehlert, 1999; Williams et al., 2004), suggesting that PACAP acts as a satiation signal in fish. In the present experiment the increase in PACAP expression during re-feeding is consistent with this hypothesis and might have been indicating the end of a hyperphagic state following long-term starvation.

It is noteworthy that, in order to ensure specificity in quantitative studies, both probes used in this study were designed to hybridize to most of the specific encoding portion of the cloned cDNAs. The nature of the probes thus decreased the risk of non-specific hybridization with other putative forms of PACAP or PPSS. If other PACAP and PPSS forms are present in cod, it is possible that they might have different functions and respond differently to changes in nutritional status.

4.5. Summary and conclusion

In summary, the results from this study show that both PPSS 1 and PRP/PACAP are affected by nutritional status in Atlantic cod. Changes in mRNA expression of these genes are accompanied by change in growth as reflected by variations in growth condition factor. The different expression patterns displayed by PPSS 1 and PRP/PACAP during fasting and re-feeding indicate that they might have distinct functions in the

regulation of feeding and metabolism of Atlantic cod. The increases in PPSS 1 expression levels during fasting and short-term re-feeding might occur through GH negative feedback control mechanisms and be the cause for decreased growth rates. As PACAP expression levels are not affected by starvation but increase during re-feeding, this peptide might be related to the inhibition of appetite at the end of hyperphagic states that occur after starvation.

Chapter 5

Effects of a meal, food rations, long-term starvation and re-feeding on the gene expression of neuropeptide Y (NPY), orexin (OX) and cocaine- and amphetamine-regulated transcript (CART) in Atlantic cod[2]

5.1. Introduction

In mammals, several brain areas, with their associated neurotransmitter populations and connectivity, participate in the regulation of feeding behavior by acting as energy sensing and modulating centers. The arcuate nucleus (ARC) is the chief hypothalamic area involved in the control of food intake. It produces key appetite-regulating factors that either increase [neuropeptide Y (NPY) and agouti-related peptide (AgRP)], or decrease [pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)] feeding. These factors affect other brain areas, such as the paraventricular nucleus (PVN), where the anorexigenic corticotropin-releasing hormone (CRH) and oxytocin are secreted, and the lateral hypothalamus area (LHA) where the orexigenic orexins (OX) are produced. When metabolic signals reach the ARC, anorexigenic peptides are released which activate a catabolic circuit. In contrast, the activation of anabolic pathways lead to the release of orexigenic peptides and occur when metabolic signal concentrations are low, thus indicating the urgency to replenish fuel stores. The detailed roles of these neuropeptides and their interaction with each other to regulate appetite in mammals are presented in **Chapter 1**.

As in many wild marine species, Atlantic cod (*Gadus morhua*) experience

[2] some of the data presented in this chapter have been published inMolecular Cellular Endocrinology, 2007

seasonal variations in energy storage and are able to survive for long periods without feeding. Although changes in energy metabolism and behavioral modifications have been well-described in cod submitted to prolonged fasting, information is lacking on the molecular mechanisms involved in these changes and the potential role of orexigenic and anorexigenic neuropeptides. In this study, I examined the short-term, periprandial changes in OX mRNA expression in juvenile cod fed at different rations as well as the gene expressions of both orexigenic (NPY and OX) and anorexigenic (CART) peptides in cod submitted to long-term fasting followed by re-feeding.

5.2. Materials and methods

5.2.1. Animals

To study periprandial changes in gene expression under different food supply conditions, 6 groups of approximately 100 fish each were placed in 6 different tanks and acclimated to the previously described conditions for 1.5 weeks. All fish were submitted to a scheduled feeding regimen and fed EWOS Marine Diet once daily at the same time (12:00) with a ration of 0.8% total body weight (BW). An average body weight was calculated weekly by averaging the weights of 4-5 fish picked randomly from each tank, and feed rations were adjusted in proportion to increasing body weight. Following the acclimation period, fish were divided into 3 groups (each consisting of two tanks) and fed with different food rations for 3 weeks. Tanks were duplicated for each experimental treatment in order to take into account any tank effect during the present study. One group was supplied with a low food ration of 0.2% BW, one group was kept at a medium food ration of 0.8% BW, and one group was supplied with a high food ration of 1.5%

BW (corresponding to satiation). After the 3-week experimental period, 8-10 fish were sampled 2 hours before feeding time (t= -2H), at feeding time (t= 0H), and 2 hours after feeding time (t= 2H) for each of the ration groups (4-5 fish per duplicate tank). Although in all experimental tanks 4-5 fish were sampled, an initial density of 100 fish per tank was necessary in order to avoid stress and abnormal behavior. Cod are social animals that show signs of stress and abnormal feeding behavior if kept at low density within a tank (Xu and Volkoff, personal observation). Fish sampled at mealtime were allowed to feed for 15 minutes and subsequently killed. Whole forebrains (including telencephalon, optic tectum, and hypothalamus) were dissected, kept in RNAlater and stored at -80 °C before use. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

For the starvation experiment, juvenile Atlantic cod with an average body weight of 35 g were used for 30 days of food deprivation and 10 days refeeding and detailed experimental treatment were the same as described in **4.2.1.** Brain tissues were sampled the first day of the experiment (day 0), after 10 days, 20 days, and 30 days of starvation and after 2 days, 5 days, and 10 days of re-feeding (8-10 fish per group). Fish were anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) before decapitation and dissection. Whole brain tissues were collected in RNAlater (Qiagen, Mississauga, ON, Canada) and stored in -80°C. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

5.2.2. Gene expression studies

Total RNA was isolated from hypothalamus with Tri-reagent (BioShop, Burlington, Ontario, Canada) according to the manufacturer's protocol. Slot blots were used to examine the effects of food availability on cod NPY, CART and OX mRNA expressions. A 332 bp NPY, a 319 bp CART and a 510 bp OX probe and a 380 bp EF1α probe were generated by PCR using primer pairs, NPY-B1 and NPY-B2, CART-B1 and CART-B2, OX-B1 and OX-B2, and EF-F and EF-R, respectively (**Table 5.1**). Primers were designed based on published cod NPY (Kehoe and Volkoff, 2007), CART (Xu and Volkoff, 2007), OX (Narnaware et al., 2000) sequences and on Atlantic cod EF1α (accession no **CO541952**). Biotinylated probe and slot blot analysis were conducted as described in **4.2.2.**

Table 5.1. Sequences of primers used in Slot blot analysis

Primer	Sequences
NPY-B1	5`-AGGCACAGAAGACAACTGAG-3`
NPY-B2	5`-AGAGCAGTTGTAGCACAAGC-3`
CART-B1	5`-GTCGTCCATGGAGCTGATCT-3`
CART-B2	5`-GAACCACGTTCCCATTTCAC-3`
OX-B1	5`-TGAGAGCTTCTGAGGAATCGAG-3`
OX-B2	5`-CCCCTCTTCCTCTCCATCTC-3`
EF-F	5`-ACKGTCTGCCTCATGTCACG-3`
EF-R	5`-TGACAACGTYGGCTTCAACG-3`

5.2.3. Data analysis and statistics

Hybridization signals were detected using an EpiChemi Darkroom (UVP, Upland, California, USA), and captured and quantified using LabWorks software (UVP, Upland, California, USA). In expression studies, all samples are expressed as ratios of target genes (NPY, CART or OX) to EF1α and all data are presented as mean ± SEM. To compare gene expression between days within the control or the experimental group, one-way ANOVAs, followed by Student-Newman-Keuls multiple comparisons tests were used. To compare gene expression between the control group and the experimental (fasted/re-fed) group at one given day, Student's t tests were used. Significance was at $p < 0.05$.

5.3. Results

5.3.1. Periprandial changes in prepro-OX mRNA expression in juvenile cod fed at different rations

To evaluate periprandial changes in OX expression levels, I compared expression levels in fish at times -2H, 0H and 2H for each of the three ration groups. Under both low (0.2% BW) and medium (0.8% BW) food ration conditions, but not under high food ration conditions (1.5% BW), OX mRNA expression levels within cod forebrain showed significant periprandial variations. Expression levels were significantly higher at mealtime (0H) than before (-2H) and after (2H) mealtime in fish submitted to the low food regimen (**Figure 5.1**). In fish submitted to a medium food ration, expression levels at 0H were significantly higher than levels at 2H, but similar to levels at -2H. In fish supplied with a high food ration, no significant differences were found among OX levels at either-2H, 0H or 2H.

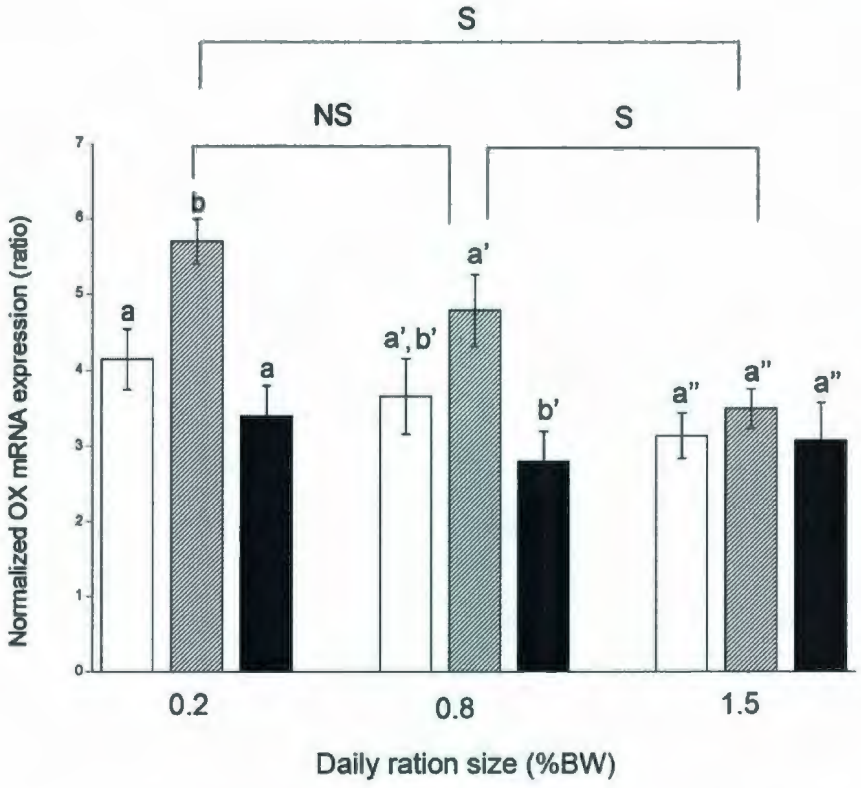


Figure 5.1. Periprandial changes in OX mRNA expression within the forebrain under different food regimens. Three groups of fish were submitted to a scheduled feeding regimen and fed once daily at the same time of the day (12:00) with low (0.2%BW), medium (0.8%BW) or high ration (1.5% BW, corresponding to satiation), for 3 weeks. For each group, OX expression was examined 2 hours before meal (-2H, white bars) at the scheduled mealtime (0H, hatched bars), and 2h after meal (2H, black bars). Total RNA was isolated and detected using slot blots using biotin-labeled probes. OX expression levels are expressed as a ratio between OX to elongation factor α hybridization signals. Data are expressed as means ± SEM (n = 8-10 per group). Significant differences ($p < 0.05$) between groups were detected

using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. Different letters above bars indicate significant differences between -2H, 0H and 2H within a ration group. Horizontal lines above the bars indicate differences between groups fed different rations at time 0H. S: significantly different, NS: not significantly different.

In order to assess the effects of ration on OX expression levels, I compared levels between fish submitted to different rations at the same time relative to mealtime. For fish sampled at mealtime, fish fed with a high food ration showed significantly lower mRNA expression level than fish fed with either a low or a medium food ration (**Figure 5.1**). At mealtime, expression levels were similar in fish fed medium and low rations. For fish sampled at either -2H or 2H, there were no significant differences in OX expression levels between groups fed different rations.

5.3.2. Effects of long-term fasting and re-feeding on NPY, CART and OX mRNA expression in juvenile cod

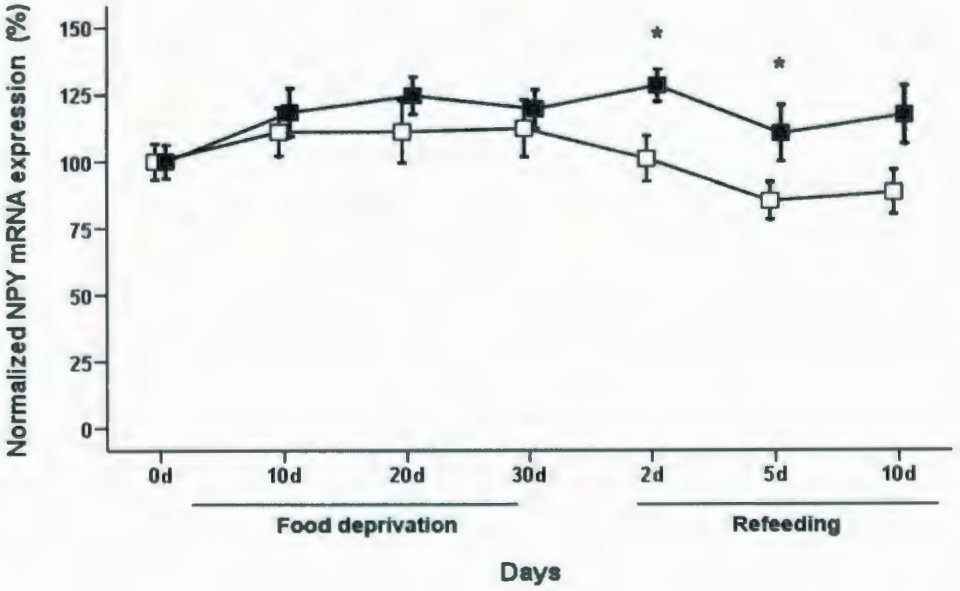
In order to study the effects of long-term fasting on NPY, CART and OX mRNA expression in juvenile cod, I compared two groups of juvenile cod, one under normal feeding condition (control group) and the other submitted to one month of starvation followed by 10 days of re-feeding (experimental group). Within either the control (continuously fed) group or the experimental (fasted and re-fed) group, there were no significant differences in NPY expression throughout the starvation period and the 10 days of re-feeding using an ANOVA analysis. NPY expression was significantly higher in the experimental group at days 2 and 5 of re-feeding when compared to the control

group using t tests (**Figure 5.2 A**).

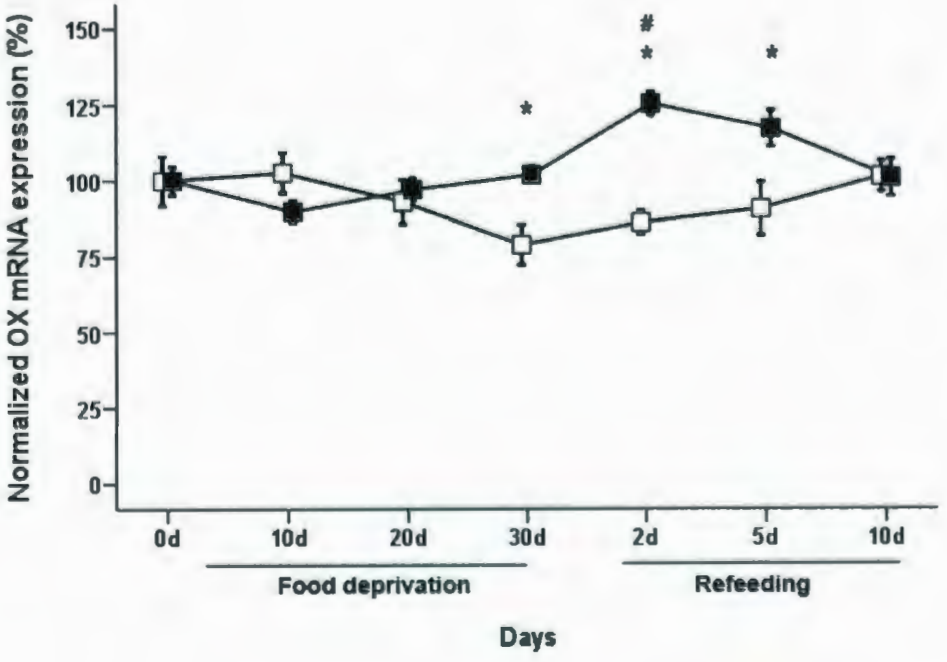
Within the control group, there were no significant differences in OX expression throughout the starvation period and the 10 days of re-feeding using an ANOVA analysis. When analyzed with an ANOVA, OX expression levels within the experimental group were not affected by 30 days of food deprivation, but significantly increased after 2 days re-feeding and subsequently returned to basic levels at day 5 and 10 re-feeding. When compared to levels in the control fed group (t test), OX expression levels were higher at day 30 of starvation and at days 2 and 5 of re-feeding (**Figure 5.2 B**).

Within the control group, there were no significant differences in CART expression throughout the starvation period and the 10 days of re-feeding using an ANOVA analysis. Within the experimental group, CART expression was significantly higher at day 20 of starvation and at day of 10 re-feeding than that at day 0. When compared to levels in the control fed group, CART expression levels were higher in the experimental group at all days except day 0 (**Figure 5.2 C**)

A


Normalized NPY mRNA expression (%)
150
125
100
75
50
25
0
*
*
0d
10d
20d
30d
2d
5d
10d
Food deprivation
Refeeding
Days

B


Normalized OX mRNA expression (%)
150
125
100
75
50
25
0
#
*
*
*
0d
10d
20d
30d
2d
5d
10d
Food deprivation
Refeeding
Days

C

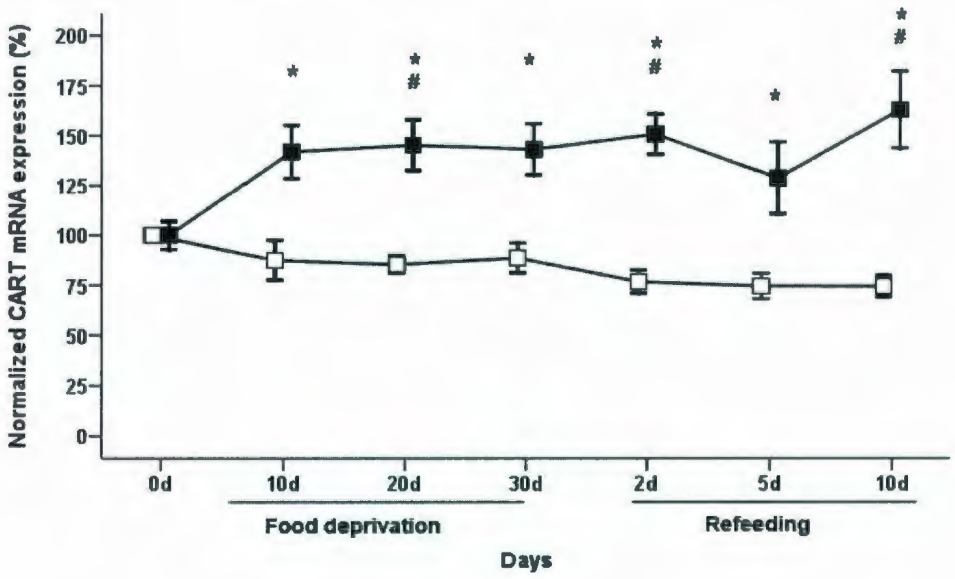


Figure 5.2. Slot blot analysis of forebrain NPY (A), OX (B) and CART (C) mRNA in control fed (CL) (open squares) and experimental starved group (ST) (closed squares) submitted to 30 days starvation and 10 days re-feeding. Data are expressed as means ± SEM (n = 8 per group). Significant differences ($p < 0.05$) between groups were detected using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. A'#' indicates a significant difference in experimental group compared to day 0. A '*' indicates a significant difference between control and experimental group for a given day.

5.4. Discussion

To further demonstrate the potential role of OX as an appetite regulator in juvenile Atlantic cod, the study was aimed at examining the periprandial changes of OX mRNA expression and the effects of different nutritional status on OX forebrain mRNA expression. Results show that forebrain OX expression displays pronounced periprandial changes in fish fed low (0.2%BW) and medium (0.8%BW) rations. In both fish groups, OX expression was significantly increased at 0H compared to -2H and 2H (**Figure 5.1**). Rapid increases in OX expression at mealtime suggest that OX is involved in the regulation of food intake in Atlantic cod by generating a hunger signal concomitant to the initiation of meals. Periprandial changes in expression have been shown for several appetite regulating peptides, such as neuropeptide Y (Narnaware et al., 2000), CART (Volkoff and Peter, 2001a), cholecystokinin (Peyon et al., 1999) or ghrelin (Unniappan et al., 2004). However, periprandial changes in OX mRNA expression had never been demonstrated in any vertebrate species. In this study, there are no significant differences between OX mRNA expression levels at -2H, 0H and 2H in fish fed high rations. The absence of detectable periprandial changes is likely due to low OX expression levels in these fish, as they were fed to satiation. Lower OX mRNA levels in well-fed, satiated fish would be consistent with the role of OX as an appetite stimulator in cod. Indeed, these results also show that at feeding time, fish fed a high ration had significantly lower OX mRNA expression levels than fish fed at either low or medium high rations, suggesting that different food regimens have an effect on OX mRNA expression level in cod brain. Differences in OX expression could only be detected in animals sampled at feeding time,

when OX levels are the highest. Lower OX expression levels at -2H and 2H might have made it more difficult to detect expression changes.

Although the effects of different food regimens on OX mRNA expression have never been studied in fish, short-term food deprivation has been shown to increase OX mRNA levels in both goldfish (Nakamachi et al., 2006) and zebrafish (Novak et al., 2005) brain and to increase the number of OX-like immunoreactive cells in goldfish brain (Nakamachi et al., 2006). In rats, although severe food restriction or starvation increases hypothalamic OX expression (Cai et al., 1999) and induces the activation of hypothalamic OX-containing neurons (Kurose et al., 2002), a moderate food restriction to 50% of satiating levels does not affect hypothalamic OX expression (Cai et al., 1999). Similarly, in sheep, a food restriction to 40% of satiating levels does not affect hypothalamic OX mRNA expression (Iqbal et al., 2003). These results suggest that OX might not play an important role in the regulation of long-term chronic food deprivation in mammals. In the present study, both moderate (fish fed with a medium ration of 53% of satiation) and severe (fish fed a low ration of 13% of satiation) food restriction induced significant increases in forebrain OX expression at meal time, suggesting that in cod, orexins might have a crucial role in chronic adaptation to fasting and to fluctuations in food availability to which the animals are submitted in their natural environment.

Interestingly, in the second study, OX expression levels appeared little or not affected by 30 days of food deprivation but significantly increased at day 2 and day 5 of re-feeding and returned to normal level after 10 days of re-feeding (**Figure 5.2.B**). In contrast with data from this study, starvation has been shown to affect the OX system in a number of fish. Fasting induces an increase in OX-like ir in the hypothalamus of goldfish

(Nakamachi et al., 2006) and OX mRNA levels have been found to increase during short-term food deprivation (4 days) in goldfish (Nakamachi et al., 2006) and relative long-term food deprivation (14 days) in zebrafish (Novak et al., 2005). The fact that cod submitted to either moderate (ration of 53% of satiation) or severe (ration of 13% of satiation) long-term (3 weeks) food restriction display increases in forebrain OX expression suggest that partial food deprivation might affect OX expression more than complete starvation, which might be indicative of an adaptation of cod to "famine" by "shutting down" the OX system. It is noteworthy that when comparing expression levels in fed and fasted fish for a given day (t-test), OX levels appeared higher in fasted fish than fed fish after 30 days of starvation. However, the considerable variance in OX expression between samples, in particular in the control group (for which OX levels inexplicably decreased at day 30), does not allow us to definitely conclude that fasting affects OX expression.

Juvenile cod NPY expression levels were not affected by 30 days of food deprivation (**Figure 5.2.A**). This is consistent with previous studies showing that NPY expression is not affected by 1-week food deprivation in cod (Kehoe and Volkoff, 2007). In mammals, hypothalamic NPY mRNA levels are increased under conditions of either acute or chronic food restriction, and return to normal after re-feeding (Beck, 2006; Beck et al., 1990). Similarly, food deprivation leads to marked increases in hypothalamic NPY mRNA and peptide levels in chick (Kameda et al., 2001). NPY appears to regulate feeding in fish, as both central and peripheral injections of mammalian or fish NPY dose-dependently increase food intake in goldfish (Lopez-Patino et al., 1999; Narnaware et al., 2000), catfish (Silverstein and Plisetskaya, 2000) and rainbow trout (Aldegunde and

Mancebo, 2006). Fasting induces an increase in NPY hypothalamic expression in goldfish (Narnaware and Peter, 2001) and salmon (Silverstein et al., 1998) and re-feeding food-deprived goldfish reverses these effects (Narnaware and Peter, 2001). The fact that NPY forebrain expression is not affected by either medium-term (1 week, Kehoe and Volkoff 2007) or long-term (one month, this study) fasting but is affected by re-feeding (**Fig 5.1A**) and displays periprandial changes (Kehoe and Volkoff, 2007) might indicate that NPY acts as a short-term, meal-related hunger peptide in cod.

Food deprivation led to significantly higher levels of CART expression, and these levels remained higher than that of control fish throughout the subsequent 30 days of starvation and 10 days of re-feeding (**Figure 5.2C**). ICV injections of human CART inhibit feeding in goldfish (Volkoff and Peter, 2000) and brain CART mRNA levels have been shown to decline following short-term fasting in both goldfish (Volkoff and Peter, 2001a) and cod (Kehoe and Volkoff, 2007), suggesting that CART act as an anorexigenic factor in fishes. In channel catfish, 30 days of fasting decreases CART mRNA brain expression, while re-feeding for 15 days restores its amount to a level similar to the fed control and CART mRNA expression is lower in fish that consume more food (Kobayashi et al., 2008). The apparent discrepancy between a putative anorexigenic role of CART and the results showing increases in CART expression levels during long-term starvation might represent an adaptive mechanism evolved in cod to cope with long-term food deprivation periods. As interactions between CART and NPY and OX have previously been shown in fish (Volkoff and Peter, 2001a), it is possible that high CART expression levels maintain normal NPY and OX expression levels, and thus counterbalance possible noxious effects caused by orexigenic factors. My results showing

little or no effect of starvation on NPY and OX expression are consistent with this hypothesis. The apparent discrepancy between higher CART expression levels during starvation and the anorexigenic effects of CART could also be explained by recent finding in rats. Whereas ICV injections of CART have anorexigenic effects, direct injections of CART into different hypothalamic areas significantly increase food intake of 24-hour fasted rats (Abbott et al., 2001), suggesting that CART may have a dual effect on feeding. Recent studies have reported contradictory results with regards to changes in CART expression after food deprivation, with either only slight attenuation of CART gene expression (Germano et al., 2007) or significantly increased CART expression (Johansson et al., 2008). I studied expression levels of CART in the entire forebrain, rather than in a specific brain region (*e.g.* the hypothalamus), and it is possible that differential effects of food deprivation on CART levels in different neuron populations during fasting might account for an increase in CART expression in the present study. The results might suggest that CART has a dual effect on eating behavior in cod, having appetite-inhibiting and appetite-stimulating effects in different brain regions.

5.5. Summary and conclusion

In summary, it appears that NPY, CART and OX are involved in the regulation of feeding of juvenile cod. OX and NPY both display periprandial changes and appear to be more affected by re-feeding than by a long fasting periods, suggesting that in cod, these peptides might act more as short-term hunger signals. This is in contrast to the current hypothesis of long-term regulators for NPY and OX in mammals (and perhaps other fish). As for the CART, it appears to display little periprandial changes, but its expression

was decreased by 1-week fasting (Kehoe and Volkoff, 2007). In this study, long-term food deprivation induced an increase in CART expression, which seems go against a role of CART as a satiation signal and might indicate that CART has a dual effect on eating behavior in cod but it is also possible that high CART expression levels maintain normal NPY and OX expression levels, and thus counterbalance possible noxious effects caused by orexigenic factors during long-term food deprivation.

Chapter 6

Effects of nutritional status on ghrelin and gastrin-releasing peptide (GRP) mRNA expression in juvenile Atlantic cod[3]

6.1. Introduction

In fish, feeding and growth appear to be regulated by complex mechanisms involving elaborate interactions between the central nervous system (CNS) and peripheral signals. Receptors located within the gastrointestinal (GI) tract can sense the presence of food and convey signals to the brain via both neural and endocrine mechanisms to regulate short-term appetite and satiety. This short-term regulation, on a meal-to-meal basis, is ensured by several hormones produced by the endocrine intestinal cells, including cholecystokinin (CCK) and gastrin-related peptide (GRP), which inhibit the expression and release of central appetite-stimulating factors and further stimulate central appetite-inhibiting factors (Naslund and Hellstrom, 2007). In contrast, during fasting, the GI tract releases ghrelin, an orexigenic factor which exerts its actions through the regulation of both orexigenic and anorexigenic central neuropeptides (Anastasi, 1971).

GRP and bombesin (BBS) are structurally related peptides that have similar biological actions. BBS is a tetradecapeptide originally purified from the skin of the European frog (*Bombina bombina*) (Volkoff et al., 2000). Two BBS-like peptides, GRP and neuromedin B (NMB) were later purified from mammalian tissues and found to be widely distributed in the GI tract and brain. BBS/GRP-like peptides have been detected in GI tract and brain of a number of fishes, including teleosts (Bjenning et al., 1991) and

[3] some of the data presented in this chapter have been accepted in General and Comparative Endocrinology, 2009

elasmobranchs (Yamauchi et al., 1998). BBS/GRP-like peptides modulate a wide variety of biological functions, including feeding behavior. In rodents, intracerebroventricular (ICV) injections of BBS (Rushing et al., 1996) or vena cava infusion of either BBS or GRP (Fekete et al., 2002; Fekete et al., 2007) or injections of GRP into the amygdala (McColl and el-Omar, 1995; Walsh et al., 1988) dose-dependently decreased food intake. BBS/GRP-like peptides also stimulate gastric acid secretion and gastrointestinal motility, and inhibit gastric emptying (Beach et al., 1988). BBS/GRP appears to mediate satiety in fish, as BBS decreases feeding following intraperitoneal (IP) injections in carp (Himick and Peter, 1994a) and following both IP and ICV injections in goldfish (Ladenheim et al., 2002; Ohki-Hamazaki et al., 1999; Ohki-Hamazaki et al., 1997). In mammals, GRP-related peptides have different selectivity for their receptors: BBS/GRP-like peptide receptor subfamilies include BBS receptors subtype-3, GRP-preferring receptors and NMB-preferring receptors, the former two, types being involved in mediating the effects of BBS/GRP-like peptides on feeding (Kojima et al., 1999). GRP-like receptors have to date not been identified in fish.

Ghrelin is a recently discovered brain-gut peptide with two main physiological actions: it acts as a growth hormone secretagogue (GHS) (Nakazato et al., 2001) and stimulates food intake (Banks et al., 2002; Shuto et al., 2002). Ghrelin is primarily synthesized by the stomach and released into the general circulation. Ghrelin stimulates feeding and pituitary GH release through either a direct pathway, by crossing the blood brain barrier and binding to GHS receptors in the hypothalamus (Date et al., 2002) or an indirect pathway via gastric vagal afferents (Nakazato et al., 2001). Both peripheral and central injections of ghrelin induce feeding, as ICV, intravenous or subcutaneous

administration of ghrelin stimulates food intake and decreases energy expenditure in rats (Schmid et al., 2005) and intravenous (IV) ghrelin administration stimulates food intake in humans (Hewson and Dickson, 2000). In rodents, systemic administration of ghrelin increases the number of neurons expressing transcription factors such as c-Fos and Egr-1 protein, in the arcuate nucleus (ARC) (Shuto et al., 2002), suggesting that ARC has a key role in mediating the orexigenic effects of ghrelin. Furthermore, animals with damaged ARC neurons (Shuto et al., 2002) or a selective disruption of GHS receptors in the ARC by antisense GHS receptor mRNA (Nakazato et al., 2001) do not exhibit increases in appetite following ghrelin ICV injection. As the ARC nucleus contains populations of neurons producing feeding-regulating peptides, it has been suggested that ghrelin might exert its feeding stimulating actions by mediating neuropeptides in CNS, including orexigenic pathways such as neuropeptide Y (NPY) (Toshinai et al., 2003) and orexin (Mollet et al., 2004) and anorexigenic pathways such as α-MSH (Solomon et al., 2005) and CART (Levin et al., 2006; Masuda et al., 2000). Ghrelin is also believed to be a strong gastrokinetic agent, and to increase gastric motility, gastric acid secretion and gastric emptying (Muccioli et al., 2001). Ghrelin secretion is mainly regulated by metabolic signals: it is inhibited by feeding and high glucose and insulin levels, and stimulated by energy restriction (Manning et al., 2008; Terova et al., 2008). Ghrelin has been isolated from several teleosts (Kawakoshi et al., 2007) and elasmobranchs (Kaiya et al., 2005). In fish as in mammals, ghrelin stimulates GH release, as IP injection of ghrelin increases both plasma GH levels and pituitary GH mRNA in the catfish (Matsuda et al., 2006b; Unniappan et al., 2004). To date, the role of ghrelin in the regulation of feeding and metabolism of fish remains unclear. Food intake is stimulated by both IP and ICV

administration of ghrelin in goldfish (Miura et al., 2006; Miura et al., 2007), and this effect is mediated by the NPY and OX pathways (Riley et al., 2005). In tilapia, long-term ghrelin treatment increases food consumption (Shepherd et al., 2007). In trout, IP injections of rat ghrelin increase feeding (Jonsson et al., 2007), whereas IP injection of trout ghrelin does not affect voluntary food intake (Unniappan et al., 2004).

In order to assess the effects of nutritional status on these peptides in Atlantic cod, I examined the effects of different food rations and of long-term starvation (one month) and re-feeding on ghrelin and GRP mRNA expressions in the stomach, and assessed their periprandial variations.

6.2. Materials and methods

6.2.1. Animals

To study periprandial changes in gene expression under different food supply conditions, 6 groups of approximately 100 fish each were placed in 6 different tanks and acclimated to the previously described conditions for 1.5 weeks. All fish were submitted to the treatment of food ration the same as described in **5.2.1**. In order to assess the effects of ration on gene expression, after the 3-week experimental period, 8-10 fish were sampled at feeding time (t=0H) from tanks fed low food ration, medium food ration and high food ration. In addition, to assess periprandial changes in expression, 8-10 fish were sampled 2 hours before feeding time (t = -2H), at feeding time (t = 0H), and 2 hours after feeding time (t = 2H) from the group fed medium ration (0.8% BW). Fish sampled at mealtime were allowed to feed for 15 minutes and subsequently killed. Stomach tissue samples close to the pyloric portion were dissected, kept in RNAlater and stored at -80°C

until use.

To study the effects of food deprivation on ghrelin expression, 4 groups of approximately 100 fish each were placed in 4 different tanks and acclimated to the previously described conditions for 1 week, except that fish were fed a ration of 1.5% total body weight (BW). All fish were submitted to the treatment of food deprivation or satiation as described in **5.2.1**. Fish were sampled after 10 days and 30 days of starvation and after 2 days and 5 days of re-feeding (4-5 fish per duplicate tank). Stomach tissues were sampled as described above.

All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

6.2.2. Gene expression studies

Total RNA from stomach was extracted (n= 4-5 fish per tank) using TRIreagent (Molecular Research Center, Cincinatti, OH) and quantified using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA). 1 μg of total RNA was submitted to genomic DNA removal with Dnase I and used for cDNA synthesis using a QuantiTech Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. The synthesized cDNA was then diluted 5 times and 2 μl of diluted cDNA was used as template in 10 μl PCR reactions.

Gene expression was determined by real-time quantitative PCR using a Mastercycler® ep realplex 2S system (Eppendorf, Mississauga, ON, Canada) with ABsolute™ Blue QPCR SYBR Green Kit (Thermal scientific, Odessa, TX, USA).

Reaction mixtures were prepared in accordance with the manufacturer's protocol. All samples and reaction mixtures were pipetted and transfered to 96-well PCR plates using an Epmotion5070 automated pipetting system (Eppendorf). Initial validation experiments were conducted for all primer pairs to determine optimal primer annealing temperature and verify high PCR efficiencies (E>0.96). Standard curves showed linear relationships between cycle threshold values (R^2>0.98) and the logarithm of input gene copy number. Relative quantification experiments were conducted under the following conditions: 95°C for 15 min followed by 40 cycles of 95°C for 15 sec, 61°C for 15 sec and 68°C for 20 sec. Real-time PCRs for ghrelin and GRP were conducted with specific primer pairs, GL-1 and GL-2, and GP-1 and GP-2 (**Table 6.1**), respectively. Elongation factor was used as an internal control with the primer pair, EF-1 and EF-2 (**Table 6.1**). Primers were designed based on the ghrelin (GenBank accession no. **EU128172, EU128173** and **EU128174**) and GRP (GenBank accession no. **EU096314** and **EU096313**) cDNA and genomic sequences and on Atlantic cod EF1α sequence (GenBank accession no **CO541952**). For all primer pairs, at least one primer lies across an exon/exon boundary, so that primer pairs only amplify cDNA and not genomic DNA. The primers were designed to have similar melting temperatures and to give similar amplicon sizes. All real-time PCR amplifications were performed in duplicate and negative controls were set with each primer pair. All specific quantities were normalized as ΔΔCt against housekeeping genes (EF1α) compared with calibrator sample from the control group. EF1α was used as a reference gene as its expression did not differ among treatments (as seen by similar Ct values).

Table 6.1. Sequences of primers used for real-time PCR analysis

Primer	Sequence
GL-1	5`- CATGAGGAGGAGTTCCAGGT -3`
GL-2	5`- TGAAGGTCACTCTGTGGCAT -3`
GP-1	5`- CTGGGCAGTGGGTCATTTA -3`
GP-2	5`- AACACGTCCCGGTCTAGTTT -3`
EF-1	5`- CAACGCCCAGGTCATCATCC -3`
EF-2	5`- ACGCTCTTGGGCAGATCCTC -3`

6.2.3. Statistics

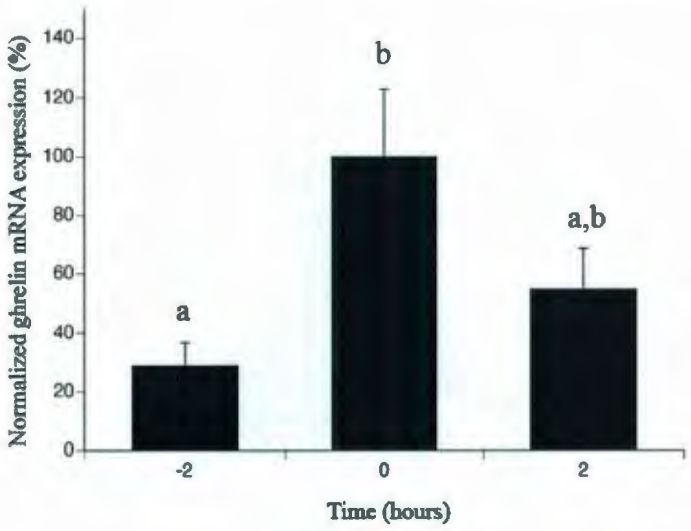
All samples are expressed as ratios of specific target gene to EF1α and normalized as a percentage of mRNA levels of fish in the control group. All data are expressed as the mean± SEM (n= 7-8). For periprandial changes, food ration experiments and fasting/re-feeding experiments, group comparisons were performed using a one-way ANOVA followed by a Student-Newman-Keuls multiple comparisons test, after checking for data normality and homogeneity of variances. In fasting/re-feeding experiments, expression levels between experimental and control groups at a given day were compared using Student's t tests. Differences were considered significant if $P < 0.05$. Statistical analyses were performed using Instat 3 (Graphpad Software, San Diego, CA, USA).

6.3. Results

To evaluate periprandial changes in ghrelin and GRP gut mRNA expression

levels, I compared expression levels in fish at times -2H, 0H and 2H for the group fed medium food rations (0.8% BW), which is the usual meal size used to maintain cod in aquaculture conditions. Ghrelin expression levels at 0H were significantly higher than levels at -2H but similar to those at 2H (**Figure 6.1.A**). GRP did not show significant periprandial changes (**Figure 6.1.B**).

A



B

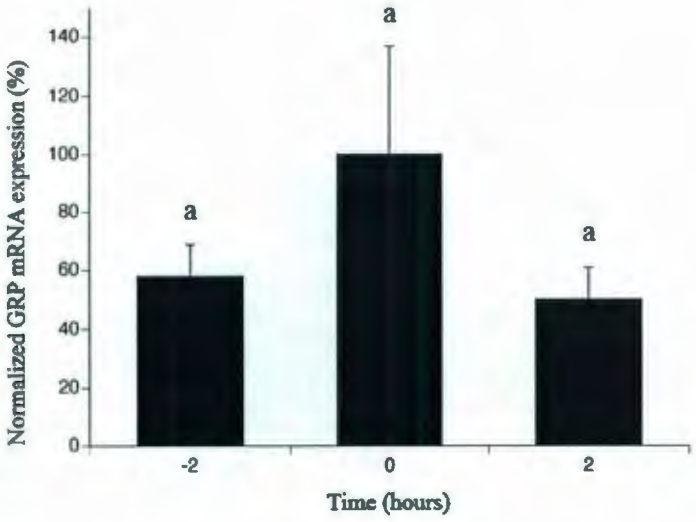


Figure 6.1. QPCR analysis of periprandial changes in ghrelin (A) and GRP (B) mRNA expressions within the stomach under medium food ration regimens. Significant differences ($p < 0.05$) between groups were detected using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. All samples were expressed as a percentage relative to the control group (0 time), which was set at 100%.

I also examined the expressions of ghrelin and GRP in animals fed different meal sizes. Samplings were performed in fed animals at (or shortly after) mealtime, as higher variation levels of expression of appetite-related hormones could be expected at that time. At mealtime (0H), ghrelin expression levels did not show significant differences between fish fed different rations (**Figure 6.2.A**). GRP mRNA levels were significantly higher in fish fed high food rations compared to fish fed a low ration (**Figure 6.2.B**).

In order to study the effects of nutritional status on ghrelin and GRP gut mRNA expressions, I compared two groups of juvenile cod, one under normal feeding condition (control group) and the other submitted to one month of starvation followed by 5 days of re-feeding (experimental group). When all groups were compared using an ANOVA, neither ghrelin nor GRP stomach mRNA expressions were affected by 30 days of starvation or 5 days of refeeding (**Figure 6.3 A and B**). When comparing fed and fasted groups using t tests, no significant differences in ghrelin stomach mRNA expression could be detected. GRP stomach mRNA expression levels were significantly lower in the fasted group at day 30 of starvation compared with the control fed group.

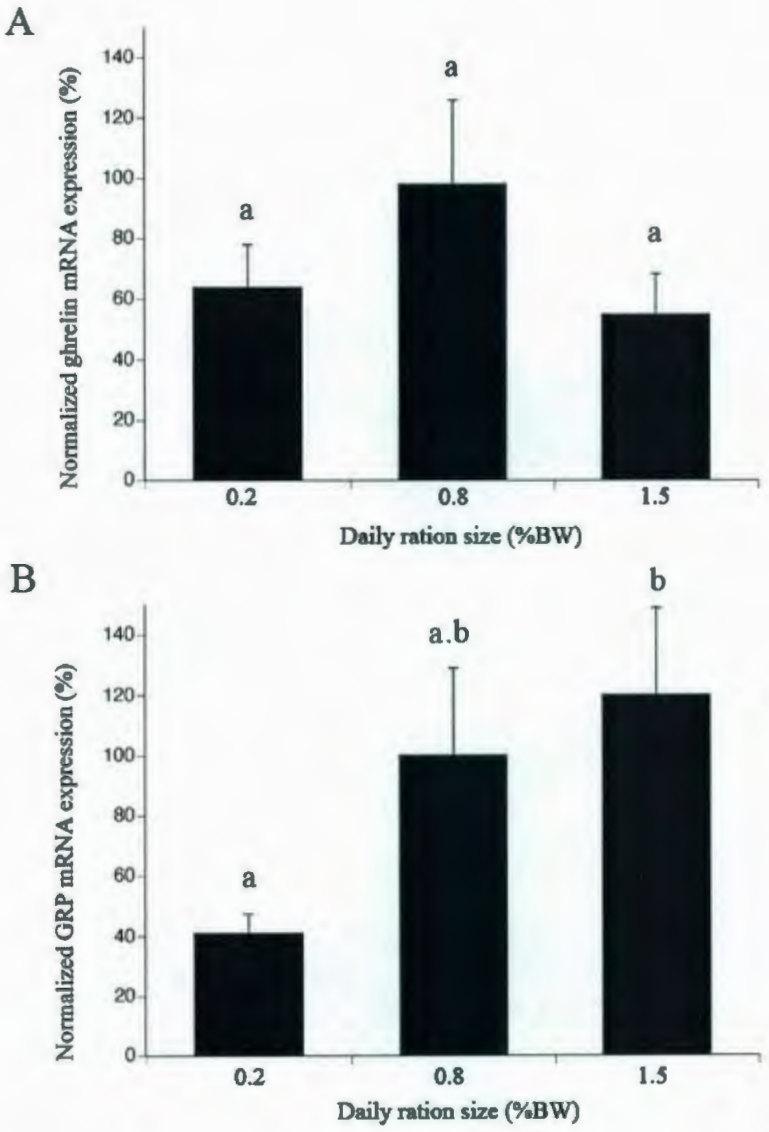


Figure 6.2. QPCR analysis of the effects of different food rations in ghrelin (A) and GRP (B) mRNA expressions within the stomach at mealtime. Significant differences ($p < 0.05$) between groups were detected using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. Different letters above bars indicate significant differences. All samples were expressed

as a percentage relative to the control group (medium ration), which was set at 100%.

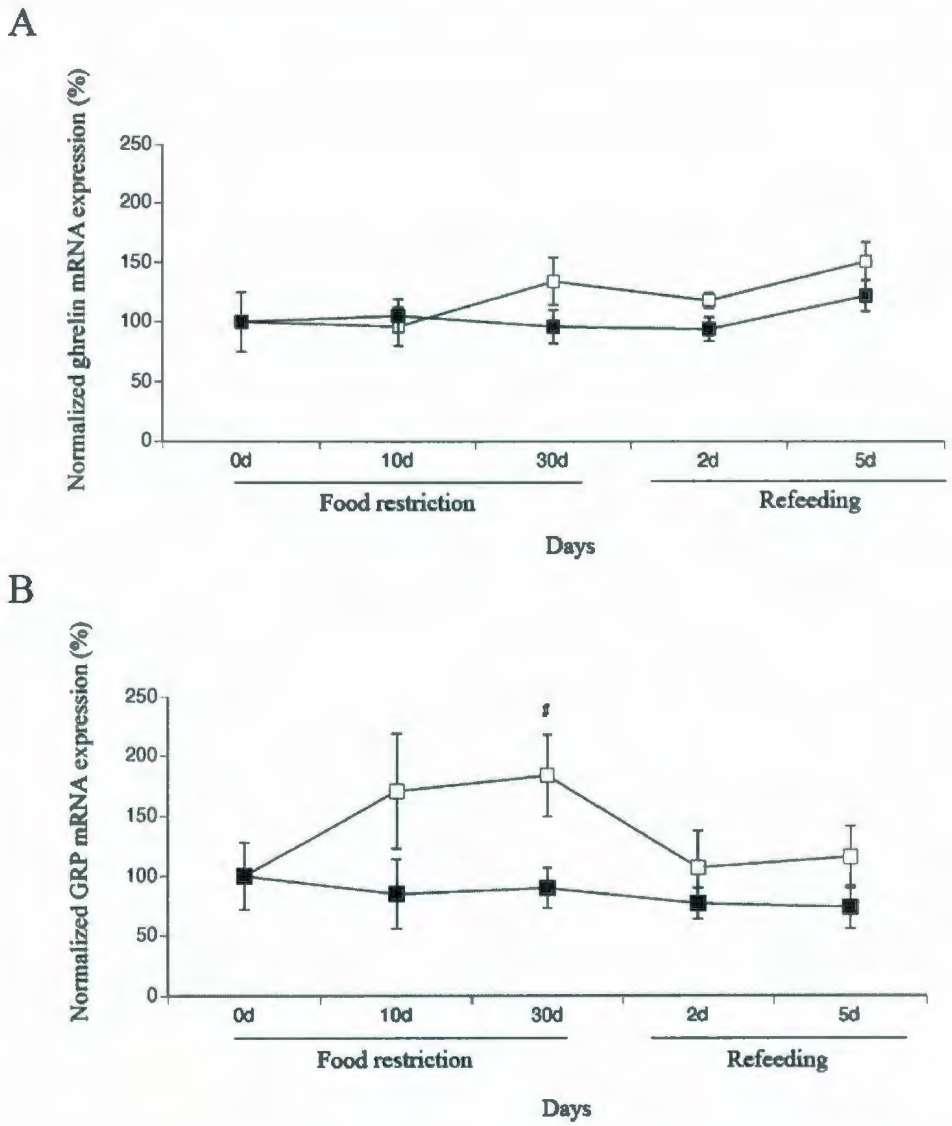


Figure 6.3. QPCR analysis of ghrelin (A) and GRP (B) mRNA within the stomach in control fed (CL) (open squares) and experimental group (ST) (closed squares) submitted to 30 days starvation and 10 days re-feeding. Data between groups were analyzed using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. Expression levels

between experimental and control groups at a given day were compared using Student's t tests. Significant differences were at $p < 0.05$ and presented with "#". All samples were expressed as a percentage relative to the control group (day 0) which was set at 100%.

6.4. Discussion

I examined the effects of different food rations on ghrelin and GRP stomach mRNA expressions, as well as possible periprandial changes in expression. To my knowledge, this is the first report on the effects of meal size on ghrelin expression. My results show that in cod, ghrelin expression levels are not affected by ration size. However, I detected periprandial changes in ghrelin expression in fish fed medium (maintenance) ration, with higher expression levels at mealtime (0H) compared to 2 hours before feeding. Although not significant, expression levels tended to decrease at 2h after a meal. In humans, plasma ghrelin levels increase shortly before meal time and show a postprandial fall, indicating that ghrelin plays a physiological role in meal initiation (Jonsson et al., 2007). In fish, changes in expression levels of ghrelin in relation to meal time appear to be species specific, which may be related to different feeding habits or physiologies. Whereas rainbow trout display no postprandial changes in plasma ghrelin levels (Beach et al., 1988), in goldfish, preproghrelin mRNA expression in both hypothalamus and gut as well as serum ghrelin levels display significant postprandial decreases (Himick and Peter, 1994a). The presence of periprandial variations in stomach preproghrelin mRNA expression in cod suggest that ghrelin might act as a short-term hunger factor in this species.

GRP stomach mRNA expression was affected by rations, as significant increases were found in fish fed high compared to fish fed low rations, suggesting that GRP might act as a long term satiety factor in cod. These results are consistent with the anorexigenic actions of BBS reported in goldfish (Beach et al., 1988) and carp (Jonsson et al., 2007). However, in rainbow trout, plasma GRP levels are very low and are not influenced by meal size (Jonsson et al., 2007). I found no significant periprandial changes in GRP mRNA expression levels, which is consistent with studies in rainbow trout, showing no postprandial changes in GRP plasma levels (Kateb and Merali, 1992) but contrast with studies in rats showing postprandial increases in both gut and brain BBS/GRP-like peptide levels (Porreca et al., 1985; Weigert et al., 1996). In mammals, BBS/GRP-like peptides regulate meal-related gastric functions and stimulate acid secretion and gastric motility while inhibiting gastric emptying (Holmgren and Jonsson, 1988; Holstein and Humphrey, 1980). In cod, early studies show that BBS-like ir is present in endocrine cells and nerves throughout the gut and that both gastric acid secretion and motility are stimulated by both BBS and GRP (De Vriese and Delporte, 2008), suggesting that GRP regulates gastric function in fish. The results showing no meal-related changes in GRP expression in the stomach of cod appears to go against this hypothesis. It is very possible that, in cod, changes in stomach GRP mRNA expression and peptide blood levels occur soon following a meal and that a more refined sampling time frame (encompassing the first 2 hours post feeding) might be necessary to detect any periprandial change of GRP expression levels in the gut.

A 30 days starvation followed by 5 days re-feeding had no effect on stomach mRNA expression of ghrelin. In mammals, plasma ghrelin levels are high in starved

animals and decrease after re-feeding in response to an increase in glycemia. In addition, plasma ghrelin levels are negatively correlated to the body mass index (BMI), so that plasma ghrelin levels are high in anorexia nervosa and cachexia, and low in obesity (Unniappan et al., 2004). The effects of fasting on ghrelin peptide and mRNA levels in fish are not consistent. In goldfish, starvation increases ghrelin mRNA expression in the hypothalamus and gut on day 7 and serum ghrelin levels on days 3 and 5 (Terova et al., 2008). In sea bass, stomach ghrelin mRNA levels are up-regulated during starvation, and down-regulated during re-feeding (Jonsson et al., 2007). Rainbow trout fasted for 1-3 weeks have significantly lower plasma ghrelin levels than fed fish (Nieminen et al., 2003) and in burbot, fasting for 2 weeks decreases plasma ghrelin-ir peptides levels (Parhar et al., 2003). However, in tilapia, there are no significant differences in gastric ghrelin mRNA expression after 7 days of food deprivation and following 7 days of re-feeding (Bowden et al., 2007). The differences in ghrelin responses to fasting between species may reflect species-specific differences in digestive physiology, energy metabolism and resistance to starvation. The results suggest that ghrelin might act more as a short-term hunger signals than a long-term feeding regulator in cod.

Stomach GRP mRNA expression did not appear to be affected by either a 30 days starvation or 5 days re-feeding. When comparing expression levels in fed and fasted fish for a given day (t test), GRP levels appeared to be lower in fasted fish than fed fish after 30 days of starvation. This would be in line with the findings that GRP expression levels are lower in fish fed low rations than in fish fed to satiety. However, the considerable variance in GRP expression between samples, in particular in the control group, does not allow to me conclude that fasting affects GRP expression. A conclusion is even more

difficult to draw as nothing is known about the effects of fasting on GRP gene expression in either fish or mammals.

6.5. Summary and conclusion

In summary, both GRP and ghrelin appear to have a role in the regulation of feeding in Atlantic cod. Ghrelin displays periprandial changes in expression but is not affected by fasting, suggesting that ghrelin might act more as a short-term hunger signal than a long-term feeding regulator in cod. GRP did not display periprandial changes but was affected by food ration, suggesting that it acts as a long-term satiety factor in cod when food is present. A 30 days starvation followed by 5 days re-feeding had no effect on stomach mRNA expression of ghrelin or GRP, which might be indicative of an adaptation of cod to "famine" by "desensitization" the gut system, including ghrelin and GRP.

Chapter 7

Effects of photoperiod on feeding and on the mRNA expression of appetite-regulating hormones in juvenile Atlantic cod

7.1. Introduction

Many teleost fish species living in temperate and cold climates are exposed to changing environmental factors, such as temperature, photoperiod and food availability, and usually display profound seasonal cycles in food intake and body weight (Skjæraasen et al., 2004) that are often correlated with reproductive cycles. Reduced or negative weight gain is often observed during the spawning season and is partly explained by a loss of appetite and by an increased energy allocated to gametogenesis and spawning (Biswas and Takeuchi, 2003; Kohbara et al., 2000; Petit et al., 2003; Turker et al., 2005). Although photoperiod has been shown to affect feeding and growth in a number of fish species (Iqbal et al., 2003), the endocrine mechanisms behind these changes are unclear.

The regulation of food intake is under the control of a number of neuronal systems within the brain, and long-term changes in nutrition affect the gene expression of many different appetite-regulating peptides (Zhao and Wang, 2006).

In mammals, a number of appetite-related hormones [*e.g.* leptin, ghrelin, neuropeptide Y (NPY), Agouti-related peptide (AgRP), cocaine- and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC)] have been shown to be affected by photoperiod. In sheep, photoperiod-induced changes in food intake and body weight are mediated by the mediobasal hypothalamic region, which includes the arcuate

nucleus (ARC), a brain area that produces important appetite-related hormones. Voles exposed to short days (SD) have lower serum leptin levels than animals exposed to long days (LD) (Mercer et al., 2003; Mercer et al., 2000). When compared to animals kept at LDs, Siberian hamsters exposed to SDs display higher CART and AgRP mRNA levels and lower POMC and leptin receptor (OB-Rb) mRNA levels throughout the ARC (Jacob et al., 1998) as well as a significant increase in the number of NPY mRNA-containing neurons in the intergeniculate leaflet, which is the homolog of the primate pregeniculate nucleus for modulating circadian rhythms (Harrison et al., 2008). Photoperiod can also influence the response of an animal to hypothalamic appetite-related peptides. For example, in rodents, ghrelin-induced increases in food intake and growth hormone (GH) release are more or less pronounced depending on whether the animals are placed in LD or SD (Reddy and Leatherland, 2003b).

To date, there is no evidence that photoperiod-induced or seasonal changes in food intake are correlated to changes in the gene expression of appetite-regulating peptides in fish. However, studies show that other metabolic hormones might be involved. Plasma GH, thyroid hormone and cortisol concentrations are affected by light regimens in rainbow trout (Nordgarden et al., 2007) and Atlantic salmon respond to an increased photoperiod by elevating plasma GH (Davis and McEntire, 2006). In contrast, photoperiod does not affect circulating insulin-like growth factor-1 (IGF-I) levels in sunshine bass (Lopez-Olmeda et al., 2006a; Lopez-Olmeda et al., 2006b).

Melatonin, a hormonal output signal of vertebrate circadian clocks, contributes to synchronizing behaviors and neuroendocrine regulations with variations in photoperiod. Light is the most important synchronizer of melatonin rhythms in fish. Melatonin

administration diminishes locomotor activity and food intake in both diurnal and nocturnal fish (Brown et al., 2003).

Recent worldwide decreases in wild stocks of cod have led to an increased interest in developing a sustainable aquaculture for this species (Brown et al., 2003). Photoperiod manipulation, in particular the use of 24 hour of illumination, is becoming a viable way to prevent early maturation in cod aquaculture (Davie et al., 2003; Taranger et al., 2006) and to achieve maximum growth potential from cultured stock. However, to date, nothing is known about the effects of photoperiod on feeding in cod.

The aim of the present study was to examine the effects of photoperiod on food intake and growth and to quantify the levels of expression of genes encoding for appetite-regulating peptides in the hypothalamus (NPY, OX and CART) and in the stomach (ghrelin and GRP) of juvenile cod exposed to different light regimens.

7.2. Materials and methods

7.2.1. Animals

Juvenile Atlantic cod (*Gadus morhua*) with an average body weight of 50g were obtained from and kept at the Dr. Joe Brown Aquatic Research Building, Ocean Sciences Centre (Memorial University of Newfoundland, Canada). Fish were maintained in flow-through 1000 L tanks with seawater at 11°C on a 16h light/ 8h dark photoperiod. Juvenile cod were fed EWOS Marine Diet once daily at the same time (10:00) to satiation. To study the changes in gene expression under different photoperiod conditions, 8 tanks were set up and duplicate tanks were assigned to different photoperiod [24L:0D (continuous light, CL), 16L:8D (long light, LL), 8L:16D (short light, SL), 0L:24D

(continuous dark, CD)]. Tanks were duplicated for each experimental treatment in order to take into account any tank effect during the study. Approximately 50 fish were placed in each tank. During treatment, fish were fed to satiation once daily (10:00). Food amounts delivered each day in each tank were measured (food intake = initial food offered to the fish minusfood left after feeding). Fish in complete darkness were fed with the help of a red lamp so that fish were not exposed to daylight. After 4 weeks, 4-5 fish were sampled from each tank at feeding time and anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) before decapitation and dissection. Brain and stomach samples were collected in RNAlater (Qiagen, Mississauga, ON, Canada) and stored at -80°C. At sampling time, growth was estimated by calculating the Fulton's condition factor (K) using the formula K = [total mass (g)/ total length^3 (cm)] X 100 (Bray, 2000). All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

7.2.2. Isolation of total RNA and reverse transcription

Total RNA was isolated from hypothalamus and stomach using a Trizol/chloroform extraction method with TRIreagent (BioShop, Burlington, Ontario, Canada) according to the protocol of the manufacturer. The quantification and purity of RNAs were measured with a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Rockland, DE) and 1 µg of total RNA was used for cDNA synthesis. Reverse transcription (RT) was performed at 37°C for 1.5 hour in a total volume of 20 µl consisting of 5 µg total RNA, 1x M-MLV RT buffer, 0.5 mM each dNTP, 0.5 µg oligo-

dT-AP, and 200 U M-MLV Reverse Transcriptase (New England Biolabs, Pickering, Ontario, Canada). The synthesized cDNAs were then diluted 1:5 and 2 µl of diluted cDNA were used as template in 10 µl qPCR reactions.

7.2.3. Real-time PCR quantitative PCR analysis of gene expression

Gene expression was determined by real-time quantitative PCR using a Realplex Mastercycler system (Eppendorf) with ABsolute™ Blue QPCR SYBR Green Kit (Thermal scientific, Odessa, TX, USA). Reaction mixtures were prepared according to the manufacturer's protocol. All samples and reaction mixtures were pipetted and transfered to 96-well PCR plate using an Epmotion 5070 automated pipetting system (Eppendorf). Initial validation experiments were conducted to determine optimal primer annealing temperature and high PCR efficiencies (R_2>0.95). The standard curve showed a linear relationship between cycle threshold values and the logarithm of input gene copy number. Real-time PCR for NPY, CART, OX, ghrelin, GRP were conducted separately with specific primer pairs, NPY-1/NPY-2, CART-1/CART-2, OX-1/OX-2, GLN-1/ GLN-2, and GRP-1/GRP-2 (**Table 7.1**). Relative quantification experiments were conducted on 96-well plates under the following conditions: 95°C for 15 min followed by 40 cycles of 95°C for 15 sec, 58-61°C (depending on specific primers) for 15 sec and 68°C for 20 sec. Elongation factor 1αwas used as a reference gene with the primer pair, EF-1/ EF-2 (**Table 7.1**). Primers were designed based on cod NPY (GenBank accession no. **AY822596** and **DQ256082**), CART (GenBank accession no. **DQ167209** and **DQ167210**), orexin (GenBank accession no. **DQ486137** and **EU096315**), ghrelin (GenBank accession no. **EU128172, EU128173 and EU128174**) and GRP (GenBank

accession no. **EU096314, EU096313**) cDNA and genomic sequences and on Atlantic cod EF1α sequence (GenBank accession no **CO541952**). For all primer pairs, at least one primer lies across an intron/exon boundary, so that primer pairs only amplify cDNA and not genomic DNA. The primers were designed to have similar melting temperatures and to give similar amplicon sizes. All real-time PCR amplifications were performed in duplicates and negative controls were set with each primer pair. All specific quantities were normalized as ΔΔCt against housekeeping genes (EF1α) compared with calibrator sample from the control group (LL group).

Table 7.1. Sequences of primers used for real-time PCR analysis

Primer	Sequence
NPY-1	5`- GACAAAGGTACGGGAAGAGG -3`
NPY-2	5`- CAATGACGGGTCATATCTGC -3`
CART-1	5`- CCAACGTGTGATATCGGAGA -3`
CART-2	5`- CTACATTCGAACCACGTTCC -3`
OX-1	5`- TCAGTGCTCAAGAGAATCCAA -3`
OX-2	5`- TTCCCTAGAGTGAGGATGCC -3`
GL-1	5`- CATGAGGAGGAGTTCCAGGT -3`
GL-2	5`- TGAAGGTCACTCTGTGGCAT -3`
GP-1	5`- CTGGGCAGTGGGTCATTTA -3`
GP-2	5`- AACACGTCCCGGTCTAGTTT -3`
EF-1	5`- CAACGCCCAGGTCATCATCC -3`
EF-2	5`- ACGCTCTTGGGCAGATCCTC -3`

7.2.4. Data analysis and statistics

All specific quantities were normalized as ΔΔCt against the reference gene, EF1α. All samples were then expressed as a percentage relative to the control group (LL) which was set at 100%. All data are expressed as the mean ± SEM. Group comparisons were performed using one-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. Significance was considered at $p<0.05$. All statistical tests were performed using InStat 3.0 (GraphPad Software, San Diego, CA).

7.3. Results

There were no significant differences in either food intake or body length or growth condition factor between the four groups, CD, CL, SL or LL (**Figures. 7.1, 7.2 A and 7.2 B**). Body mass was significantly higher under the CL compared to the SL (**Figure 7.2 C**).

Both NPY and CART (**Figures. 7.3 A and 7.3 B**) expressions in the hypothalamus showed increased expression in the CD compared with the CL group.

There were no significant variations in either hypothalamic OX expression (**Figure 7.3 C**) or stomach ghrelin or GRP mRNA expressions (**Figures. 7.4 A and 7.4 B**) between the four groups.

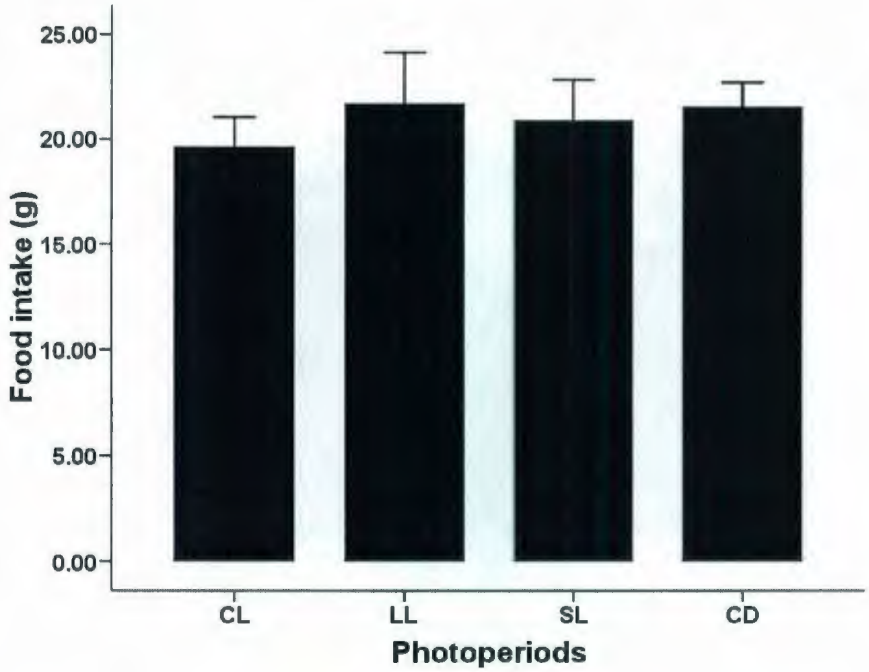
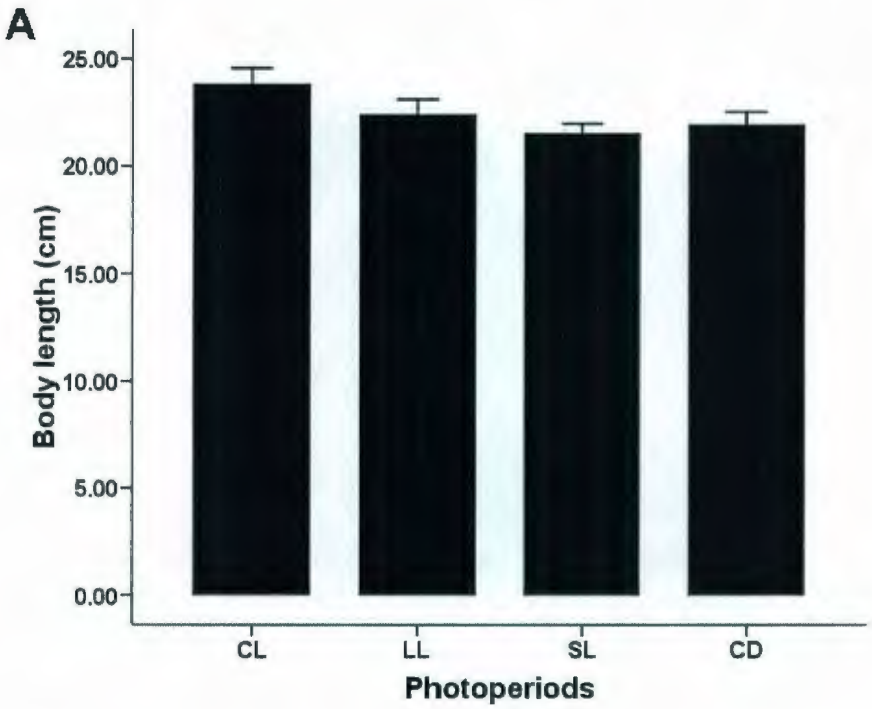
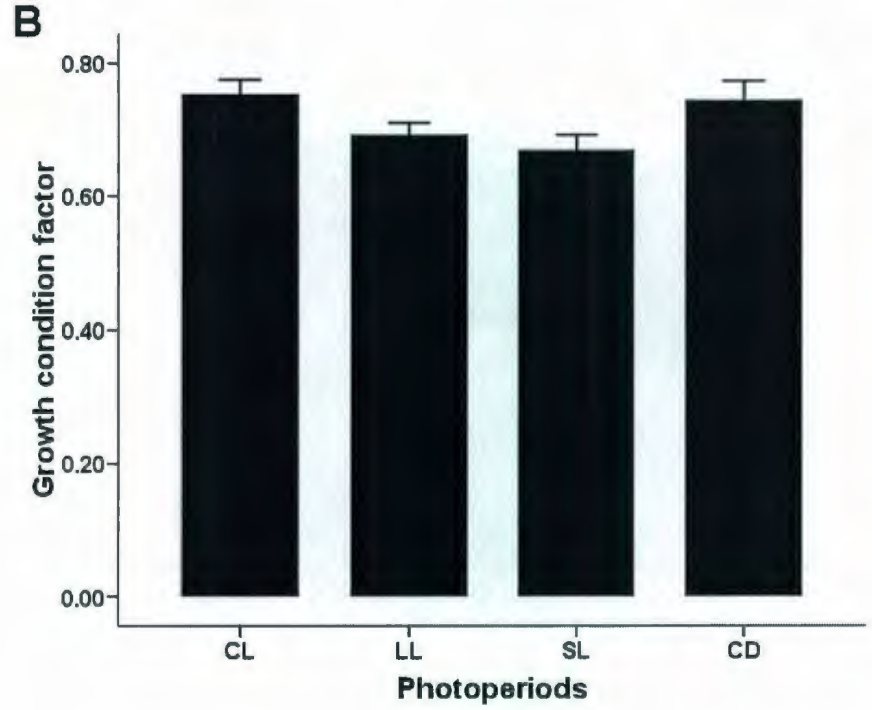


Figure 7.1. Food intake of each photoperiod group during the last week of the experiment. Statistical analyses were performed using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. Significant differences were set at $p < 0.05$.


A
25.00
20.00
15.00
10.00
5.00
0.00
Body length (cm)
CL
LL
SL
CD
Photoperiods


B
0.80
0.60
0.40
0.20
0.00
Growth condition factor
CL
LL
SL
CD
Photoperiods

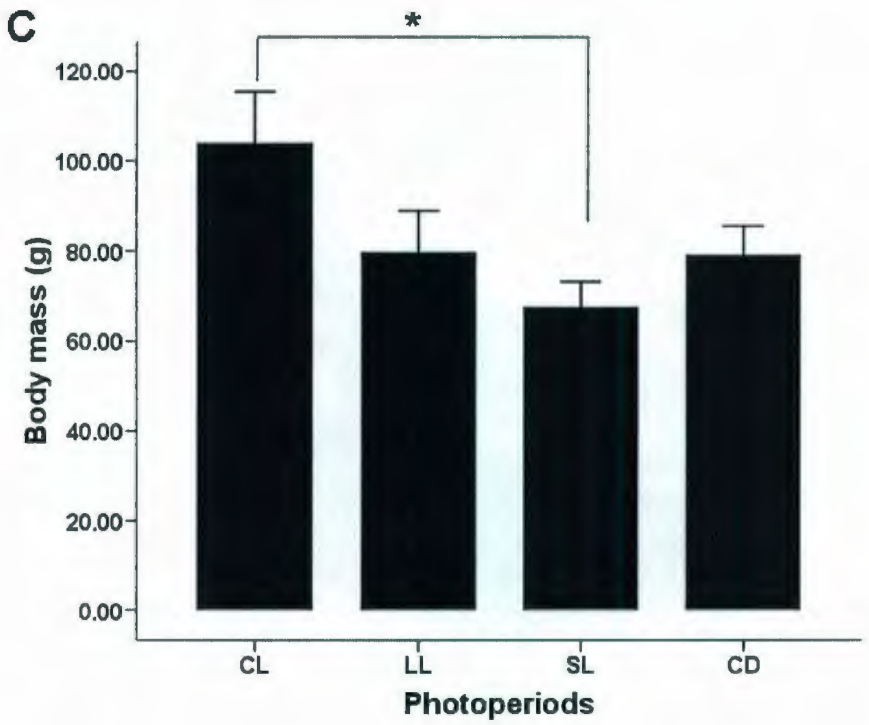
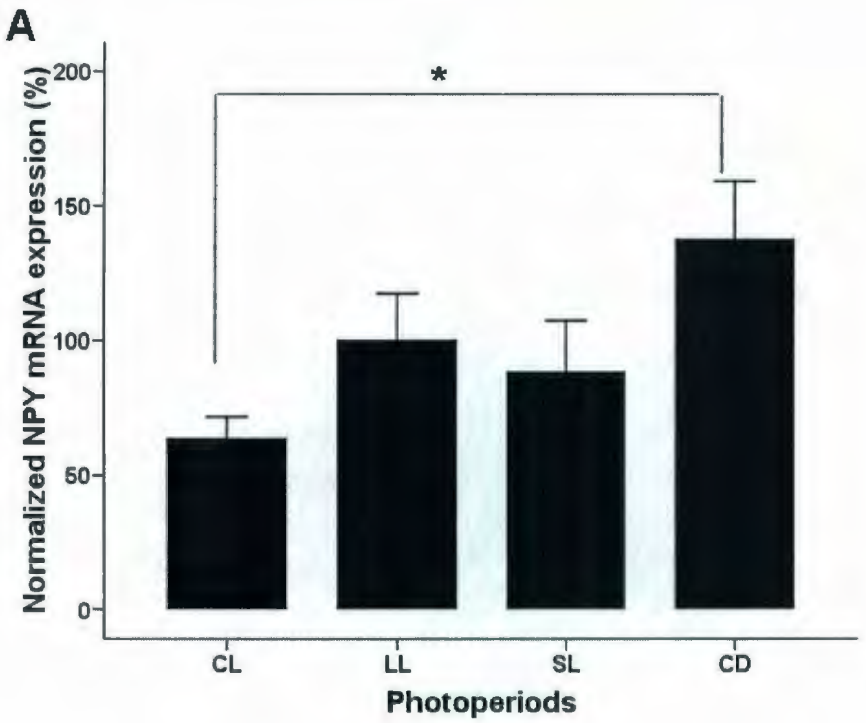
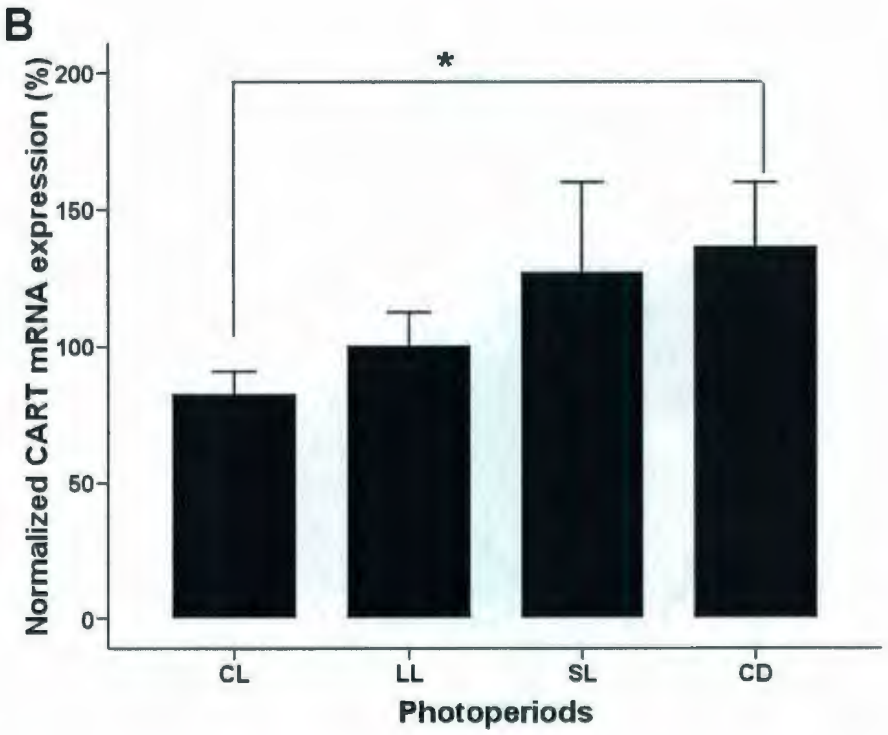


Figure 7.2. Body length (A), growth condition factor (B) and body mass (C), of each photoperiod group during the last week of the experiment. Statistical analyses were performed using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. Significant differences were set at $p < 0.05$ and labeled with "*".


A
Normalized NPY mRNA expression (%)
200
150
100
50
0
*
CL
LL
SL
CD
Photoperiods


B
Normalized CART mRNA expression (%)
200
150
100
50
0
*
CL
LL
SL
CD
Photoperiods

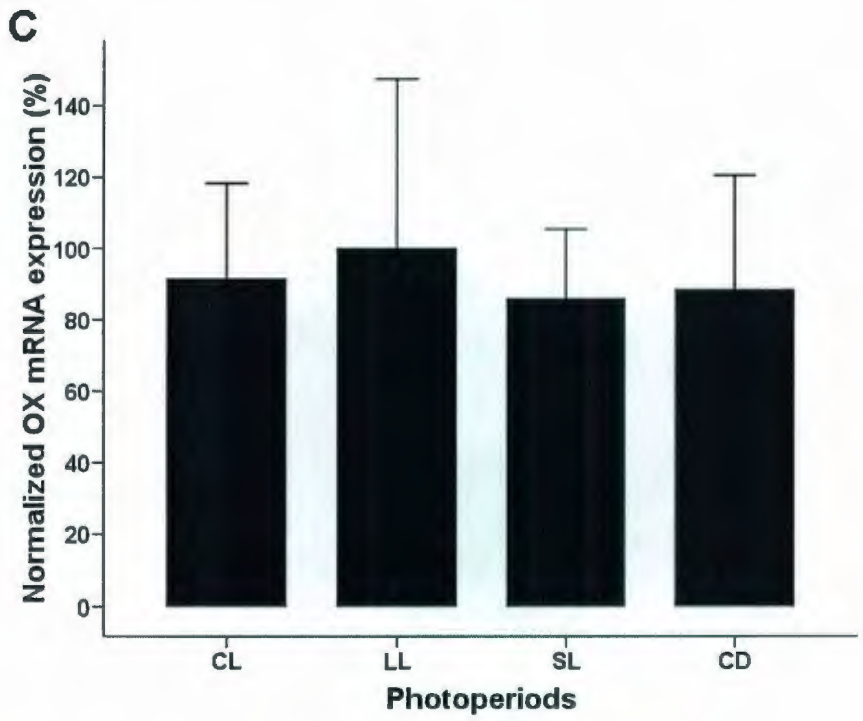


Figure 7.3. Q-RT-PCR analysis of the effects of photoperiod on NPY (A), CART (B) and OX (C) mRNA expression levels in the hypothalamus. Data were analyzed using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test. Significant differences were set at $p < 0.05$ and labeled with "*". All samples were expressed as a percentage relative to the control group (LL) which was set at 100%.

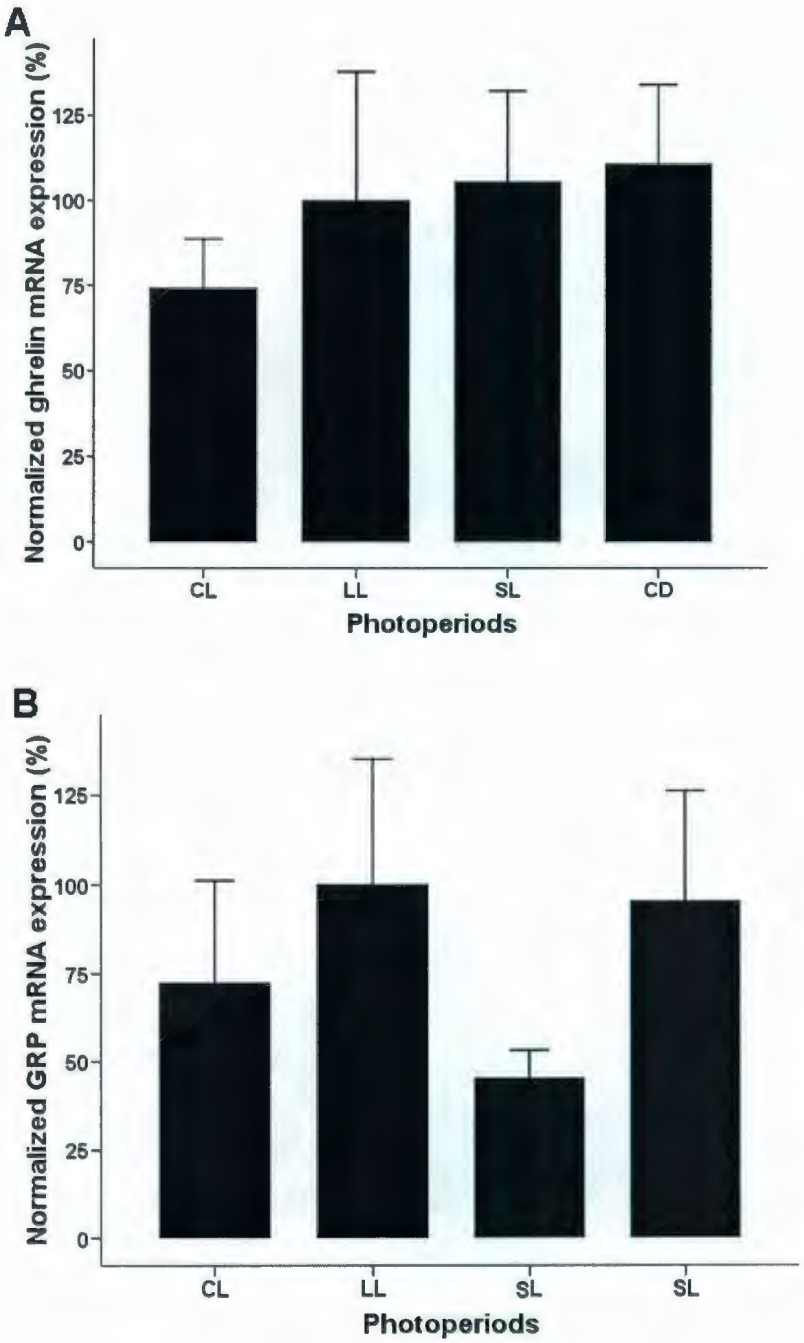


Figure 7.4. Q-RT-PCR analysis of the effects of photoperiod on ghrelin (A) and GRP (B) mRNA expression levels in the stomach. Data were analyzed using a 1-way ANOVA, followed by a Student-Newman-Keuls multiple comparisons test.

Significant differences were set at $p < 0.05$. All samples were expressed as a percentage relative to the control group (LL) which was set at 100%.

7.4. Discussion

There were no significant differences in either food intake, body length or growth condition factors between fish submitted to different photoperiods. Cod have a diurnal rhythm of swimming activity and search more actively for food during the day (Okkeborg, 1998), so a lower food intake (and thus growth) might have been expected in fish held in the dark. However, studies suggest that olfaction (chemically-mediated food searching), and not vision, is the major sense used by cod in feeding (Okkeborg, 1998). My results are consistent with a recent study showing that juvenile cod held under the same photoperiod regimen feed and grow equally well when fed either only during light hours or only during dark hours (Siikavuopio et al., 2008), indicating that light conditions alone might not affect the feeding response in juvenile Atlantic cod. It is also possible that exposures to different photoperiods longer than one month might induce changes in food intake and growth in Atlantic cod, perhaps by modulating other endocrine systems, such as the reproductive system (Skjæraasen et al., 2004) or the pineal/ melanocotin system (Porter et al., 2000). The significant increase in body mass in the group held in continuous light (CL) compared to group held in 8h light (SL), suggests that photostimulation may affect juvenile cod growth, perhaps through an increase in food conversion efficiency, as is the case in green sunfish (Boeuf and Le Bail, 1999), rather than through the stimulation of food intake. Previous studies in juvenile cod have shown

that specific growth rates (based on body weight gain) can be increased under a 1-3 month CL regimen compared to a natural light regimen, although this increase also depends on temperature and genotypes (Imslanda et al., 2005).

Despite no visible effect of photoperiod on feeding, I examined the expression of several orexigenic and anorexigenic peptides. This was done to assess whether photoperiod indeed did not affect gene expression or whether counterbalancing changes occurred (*e.g.* increases in both orexigenic and anorexigenic peptide expression) that would prevent changes in feeding. My results show that the hypothalamic expressions of both NPY, an appetite stimulator, and CART, a feeding inhibitor, increase in cod exposed to complete darkness (CD) compared to fish in constant light. Similar results have been reported in Siberian hamsters. When compared to animals in long days, animals submitted to short day exposure appear to display elevated levels of both anorexigenic peptides, as seen in increases in ARC CART, POMC and leptin receptor (OB-Rb) mRNA levels (Mercer et al., 2003; Mercer et al., 2000) and orexigenic peptides, as seen by high numbers of NPY mRNA containing neurons in the intergeniculate leaflet (Jacob et al., 1998) and high ARC AgRP mRNA levels (Mercer et al., 2000). An increase in both NPY and CART suggests that one month photoperiod regimen treatments might perturb appetite-related neuroendocrine systems but changes might not be strong enough to lead to variations in food intake.

There were no significant effects of photoperiod on the expression levels of either hypothalaminc OX or gut ghrelin and GRP. My results are consistent with studies in Siberian hamsters, showing that photoperiod does not affect either OX-A- or OX-B imunoreactivity levels in the lateral hypothalamus (Helwig et al., 2006). Sheep submitted

to short days have low appetite and display increases in hypothalamic OX gene expression but interestingly, this expression is not affected by food restriction (Archer et al., 2005). This data suggests that the major role of OXs in seasonal animals might not be the regulation of food intake but perhaps controlling other functions such as the reproductive axis. Recent evidence in goldfish shows a role of orexin in reproduction, via an interaction with gonadotropin releasing hormone (Hoskins et al., 2008). Also, the distribution of OX fibers and cells is similar in nocturnal and diurnal mammals, suggesting that OX networks and functions are conserved (Nixon and Smale, 2007). To my knowledge no study has ever examined a possible relationship between ghrelin and GRP expression levels and photoperiod. The results on gene expression are consistent with the finding that no differences in food intake exist between groups.

7.5. Summary and conclusion

In summary, one month exposure to different photoperiods did not affect food intake or body length or growth condition factor in juvenile cod. Neither OX in the hypothalamus nor ghrelin and GRP in the gut were affected by photoperiod changes. Hypothalamic mRNA expression levels of both the orexigenic factor NPY and the anorexigenic factor CART increased in the CD compared to the CL group. The fact that olfaction (chemically-mediated food searching), and not vision, is the major sense used by cod in feeding might explain the modest impact of photoperiod on food intake and on the expression of appetite-related peptide in this species. However, significant increases in body mass under CL compared to SL support the application of 24 hour illumination to achieve maximum growth in cod.

Chapter 8

General discussion and conclusions

In vertebrates, including fish, the regulation of food intake and growth is tightly controlled by intricate networks of internal factors produced by both the central nervous system and peripheral tissues, and also by environmental factors such as temperature, photoperiod, salinity, oxygen levels, ammonia concentration and pH. Periods of reduced food availability are common in the life cycles of wild fishes and lead to considerable fluctuations in energetic status. In response to sustained decreases in food availability, fish may decrease locomotor and metabolic activities (Beaulieu and Guderley, 1998; Black and Love, 1986), modify tissue metabolic capacities and degrade their muscle protein supplies (Beaulieu and Guderley, 1998). To date, little is known about the involvement of growth- and appetite-related hormones in these physiological adaptive changes.

The main goal of this research was to identify and characterize genes encoding growth-related factors (PACAP and SS) and appetite-related factors (OX, ghrelin and GRP) in Atlantic cod (*Gadus morhua*), a marine cold-water fish species off the coast of Newfoundland. As a result of a decrease in food availability during the prolonged winter periods, Atlantic cod has developed a resistance to starvation and has been shown to survive up to 16 weeks of fasting (Jonsson et al., 2007). The molecular mechanisms regulating this adaptation as well as the role of growth- and appetite-related factors are not known. Following cloning of the above named factors, studies were undertaken to investigate their potential roles in the regulation of growth and food intake and to assess

variations in mRNA expression under different nutritional status and environmental conditions (*i.e.* changes in photoperiod). Two previously cloned central appetite-related factors, NPY and CART, were also examined.

Molecular structures of DNAs, RNAs and deduced peptides were analyzed by multiple sequence alignments and phylogeny reconstruction **(Chapter 2)**. Expression profiles throughout cod brain, peripheral tissues and developmental stages were obtained by semi-quantitative reverse transcriptase polymerase chain reaction **(Chapter 3)**. These steps allowed for the identification of the structure of the genes and for a further understanding of their biological function. All genes examined showed a widespread distribution in peripheral tissues and brain regions, indicating that some of these hormones might have a variety of physiological roles other than growth and appetite regulation. All genes also appeared very early in development, suggesting a crucial role in embryogenesis. The mRNA expressions for PPSS 1, OX and NPY showed apparent differences before and after first larval feeding, indicating that these hormones might be implicated in the regulation of feeding and growth in early stages of life.

In order to further understand the roles of these hormones in food intake and growth in juvenile cod, further study was aimed at fasting experiments, as well as periprandial changes and food rations experiments.

Previous studies have examined the effects of fasting on growth. As a characteristic feature, fasting and malnourished fish usually have slower growth and show a decrease in hepatic growth hormone (GH)-binding and circulating IGF-I levels, which increases pituitary GH release due to a lack of negative feedback inhibition by IGF-I on the pituitary somatotrophs. Although the importance of GH and IGF-1 in the

regulation of growth and metabolism in fish has been well-established, relatively few studies have been undertaken to investigate the role of GH regulators (stimulating PACAP and inhibiting SS). In the present study (**Chapter 4**), PPSS I expression levels increased and remained significantly higher throughout 30 days of starvation accompanied by decreased GCF growth in juvenile cod, indicating that SS might be important in feedback regulation of GH secretion during food deprivation in Atlantic cod. In contrast, PRP/PACAP expression levels were not affected by 30 days of food deprivation, but a significant increase in expression levels was observed during the 10 days re-feeding period following recovery of growth in GCF in juvenile cod. This significant increase in PRP/PACAP expression following compensatory growth indicates that PACAP might be important in counterbalancing feeding and growth after abrupt nutrition status changes. The results suggest that both PRP/PACAP and PPSS I are involved in the complex regulation of growth, feeding and metabolism mechanisms during nutritional status changes in Atlantic cod, with brain SS specificly functional for food deprivation period and brain PACAP specificly effective for re-feeding period.

In fish, as in all vertebrates, the brain is the primary center of regulation of food intake. Afferent signals continuously inform the central nervous system about changes in energy homeostasis. The brain interprets and integrates these signals and responds with efferent signals that help maintain a constant energy balance. Neuropeptides that originate from the hypothalamus regulate food intake either by stimulating (orexigenic factors) or inhibiting (anorexigenic factors) appetite. Three central appetite-related factors (the orexigenic OX and NPY, and the anorexigenic CART) were studied with regards to their relationships to nutritional status in Atlantic cod. OX expression levels showed

periprandial changes, with higher expression levels at meal time, and were also affected by meal sizes, being higher in fish fed low and medium rations than in fish fed high rations. The results (**Chapter 5**) suggest that orexins might be involved in both short- and long-term regulation of feeding in Atlantic cod. As previous studies show that NPY and CART mRNA also both undergo periprandial changes in expression (Narnaware and Peter, 2001), so it is necessary to assess whether these peptides (NPY, CART and OX) were involved in the long-term regulation of feeding and starvation in Atlantic cod (**Chapter 5**). Previously, it has been reported that food deprivation increases NPY (Nakamachi et al., 2006) and OX (Volkoff and Peter, 2001a) expression and suppresses CART (Davie et al., 2003; Taranger et al., 2006) expression in goldfish. Surprisingly in this study NPY and OX mRNA expressions seemed to be not or only modestly affected by a one month of food deprivation, but both expressions increased significantly after 2 days of re-feeding and returned back to normal levels after 5 and 10 days of re-feeding. As opposed to other fish where fasting decreases CART brain expression levels, CART expression levels were significantly higher in fasted cod than in fed fish throughout one month of food deprivation and the subsequent 10 days of re-feeding. This unique expression pattern of appetite-related peptides might be related to the ability of Atlantic cod to endure long-term food deprivation period during harsh winter months.

The gastrointestinal tract represents a major peripheral organ for regulating short-term appetite and satiety. It contains mechano- and chemo-receptors that sense the presence of food and convey signals to the brain either via the vagus nerve or through endocrine mechanisms. This short-term regulation, on a meal-to-meal basis, is performed by several hormones from the endocrine gastrointestinal cells. The balance and

interactions between anorexigenic and orexigenic factors originating from the gastrointestinal tract appears to play an important role in the short-term regulation of food intake. Two gastrointestinal appetite-related factors (the orexigenic ghrelin and the anorexigenic GRP) were studied with regards to their relation to nutritional status in Atlantic cod (**Chapter 6**). Juvenile cod fed medium rations displayed periprandial changes in ghrelin, but not GRP, expression, with higher expression levels at meal time compared to 2 hours before feeding time. Ghrelin gut mRNA expression was not affected by rations whereas GRP gut mRNA expression was higher in fish fed medium and high rations as compared to fish fed low rations. The results suggest that both ghrelin and GRP have a role in the regulation of feeding in cod. However, there were no changes in ghrelin or GRP mRNA expressions during fasting and re-feeding, which seem to indicate that different mechanisms may exist in cod for the regulation of feeding and metabolism in order to adapt to long-term food deprivation in the wild environment.

The expression patterns of both growth-related peptides and appetite-related peptides might be related to the ability of Atlantic cod to preserve energy and to endure long-term food deprivation periods during harsh winters. By which mechanism and to what extent these peptides are involved in the adapted resistance to extreme harsh conditions in the wild habitat will need further investigation. These findings highlight the need for a greater understanding of the mechanisms responsible for controlling feeding and growth in teleosts as a whole.

Photoperiod manipulation, in particular the use of 24 hour of illumination, is becoming a viable way to prevent early maturation in Atlantic cod aquaculture (Okkeborg, 1998) and to achieve maximum growth potential from cultured stocks.

However, the endocrinal mechanisms underlying this manipulation remain unknown. I have undertaken preliminary studies to examine the effects of photoperiod on food intake and growth and on the gene expression of appetite-related peptides in juvenile cod (**Chapter 7**). When submitted to different light regimens [24L:0D (CL), 16L:8D (LL), 8L:16D (SL), 0L:24D (CD)] for one month, juvenile fish showed significantly higher body mass in the CL group compared to SL but neither overall food intake nor body length or growth condition factors varied significantly among groups. The hypothalamic gene expression studies revealed that both orexigenic NPY and anorexigenic CART appeared to be higher in the CD group compared to the CL group and no variations were seen in either OX expression or ghrelin and GRP stomach expression among groups, which further support the fact food intake was not affected under one month photoperiod regimens treatment. The modest impact of photoperiod on food intake and on the expression of appetite-related peptide in this species might be explained by the fact that olfaction (chemically-mediated food searching), and not vision, is the major sense used by cod in feeding (Boeuf and Le Bail, 1999). The increased body mass under CL compared to SL might occur via an increase in food conversion rate rather than an increase in food intake, as is the case in green sunfish (Skjæraasen et al., 2004). To better understand how different photoperiods might affect growth and food intake and the reproductive system (e.g. the process of maturation, (Porter et al., 2000)) in cod, as well as potential interactions with other endocrine systems (e.g. the pineal/ melanocotin system), exposure times longer than one month might be needed.

To conclude, the work presented in this thesis represents novel research which explores the endocrine regulation of feeding and growth in Atlantic cod. The fundamental

study might help to optimize nutrition level and support the use of 24 hours illumination to obtain maximized growth in Atlantic cod aquaculture. However, it also demonstrates that substantial further research remains to be performed to more clearly define the mechanisms that regulate feeding and growth in one of the most commercially significant marine teleosts of the Northern Atlantic.

References:

Abbott, C. R., Rossi, M., Wren, A. M., Murphy, K. G., Kennedy, A. R., Stanley, S. A., Zollner, A. N., Morgan, D. G., Morgan, I., Ghatei, M. A., Small, C. J., and Bloom, S. R. (2001). Evidence of an orexigenic role for cocaine- and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. *Endocrinology* **142,** 3457-63.

Abraham, H., Orsi, G., and Seress, L. (2007). Ontogeny of cocaine- and amphetamine-regulated transcript (CART) peptide and calbindin immunoreactivity in granule cells of the dentate gyrus in the rat. *Int J Dev Neurosci* **25,** 265-74.

Adams, B. A., Lescheid, D. W., Vickers, E. D., Crim, L. W., and Sherwood, N. M. (2002). Pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in sturgeon, whitefish, grayling, flounder and halibut: cDNA sequence, exon skipping and evolution. *Regulatory Peptides* **109,** 27-37.

Ahima, R. S. (2008). Revisiting leptin's role in obesity and weight loss. *J Clin Invest* **118,** 2380-3.

Aja, S., Sahandy, S., Ladenheim, E. E., Schwartz, G. J., and Moran, T. H. (2001). Intracerebroventricular CART peptide reduces food intake and alters motor behavior at a hindbrain site. *Am J Physiol Regul Integr Comp Physiol* **281,** R1862-7.

Aldegunde, M., and Mancebo, M. (2006). Effects of neuropeptide Y on food intake and brain biogenic amines in the rainbow trout (Oncorhynchus mykiss). *Peptides* **27,** 719-27.

Alexander, L., Knutson, D., Kittilson, J. D., and Sheridan, M. A. (2001). Localization of somatostatin mRNAs in the brain and pancreas of rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **129,** 221-8.

Alexandre, D., Vaudry, H., Jegou, S., and Anouar, Y. (2000). Structure and distribution of the mRNAs encoding pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in the frog, *Rana ridibunda*. *The Journal of Comparative Neurology* **421,** 234-46.

Ali, M., Nicieza, A., and Wootton, R. (2003). Compensaoty growth in fishes: a response to growth depression. *Fish and Fisheries* **4,** 147-190.

Allais, A., Burel, D., Isaac, E. R., Gray, S. L., Basille, M., Ravni, A., Sherwood, N. M., Vaudry, H., and Gonzalez, B. J. (2007). Altered cerebellar development in mice lacking pituitary adenylate cyclase-activating polypeptide. *The European Journal of Neuroscience* **25,** 2604-18.

Alvarez, C. E., and Sutcliffe, J. G. (2002). Hypocretin is an early member of the incretin gene family. *Neurosci Lett* **324,** 169-72.

Amiot, C., Brischoux, F., Colard, C., La Roche, A., Fellmann, D., and Risold, P. Y. (2005). Hypocretin/orexin-containing neurons are produced in one sharp peak in the developing ventral diencephalon. *Eur J Neurosci* **22,** 531-4.

Anastasi, A. (1971). Alytesin and bombesin, two peptide analogs from amphibian skin. *Naunyn Schmiedebergs Arch Pharmakol* **269,** 135-9.

Archer, Z. A., Rhind, S. M., Findlay, P. A., Kyle, C. E., Barber, M. C., and Adam, C. L. (2005). Hypothalamic responses to peripheral glucose infusion in food-restricted sheep are influenced by photoperiod. *J Endocrinol* **184,** 515-25.

Asnicar, M. A., Smith, D. P., Yang, D. D., Heiman, M. L., Fox, N., Chen, Y. F., Hsiung, H. M., and Koster, A. (2001). Absence of cocaine- and amphetamine-regulated transcript results in obesity in mice fed a high caloric diet. *Endocrinology* **142,** 4394-400.

Ayson, F. G., de Jesus-Ayson, E. G., and Takemura, A. (2007). mRNA expression patterns for GH, PRL, SL, IGF-I and IGF-II during altered feeding status in rabbitfish, *Siganus guttatus*. *General and Comparative Endocrinology* **150,** 196-204.

Baker, D. M., Larsen, D. A., Swanson, P., and Dickhoff, W. W. (2000). Long-term peripheral treatment of immature coho salmon (Oncorhynchus kisutch) with human leptin has no clear physiologic effect. *Gen Comp Endocrinol* **118,** 134-8.

Baker, J., Liu, J. P., Robertson, E. J., and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **75,** 73-82.

Bandeen, J., and Leatherland, J. F. (1997). Changes in the proximate composition of juvenile white suckers following re-feeding after a prolonged fast *Aquaculture International* **5,** 327-337.

Banks, W. A., Tschop, M., Robinson, S. M., and Heiman, M. L. (2002). Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther* **302,** 822-7.

Barreto, R. E., Moreira, P. S., and Carvalho, R. F. (2003). Sex-specific compensatory growth in food-deprived Nile tilapia. *Brazilian Journal of Medical and Biological Research* **36,** 477-83.

Beach, M. A., McVean, A., Roberts, M. G., and Thorndyke, M. C. (1988). The effects of bombesin on the feeding of fish. *Neurosci. Lett.* **32,** 46.

Beaulieu, M. A., and Guderley, H. (1998). Changes in qualitative composition of white muscle with nutritional status of Atlantic cod, Gadus morhua. *Comp Biochem Physiol A Mol Integr Physiol* **121,** 135-41.

Beck, B. (2006). Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. *Philos Trans R Soc Lond B Biol Sci* **361,** 1159-85.

Beck, B., Jhanwar-Uniyal, M., Burlet, A., Chapleur-Chateau, M., Leibowitz, S. F., and Burlet, C. (1990). Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status. *Brain Res* **528,** 245-9.

Beck, B., Richy, S., Dimitrov, T., and Stricker-Krongrad, A. (2001). Opposite regulation of hypothalamic orexin and neuropeptide Y receptors and peptide expressions in obese Zucker rats. *Biochem Biophys Res Commun* **286,** 518-23.

Bednarek, M. A., Feighner, S. D., Pong, S. S., McKee, K. K., Hreniuk, D. L., Silva, M. V., Warren, V. A., Howard, A. D., Van Der Ploeg, L. H., and Heck, J. V. (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. *J Med Chem* **43,** 4370-6.

Beloosesky, R., Gayle, D. A., Amidi, F., Ahanya, S. N., Desai, M., and Ross, M. G. (2006). Ontogenic expression of putative feeding peptides in the rat fetal brain and placenta. *Nutr Neurosci* **9,** 33-40.

Berelowitz, M., Firestone, S. L., and Frohman, L. A. (1981). Effects of growth hormone excess and deficiency on hypothalamic somatostatin content and release and on tissue somatostatin distribution. *Endocrinology* **109,** 714-9.

Bi, S., Scott, K. A., Kopin, A. S., and Moran, T. H. (2004). Differential roles for cholecystokinin a receptors in energy balance in rats and mice. *Endocrinology* **145,** 3873-80.

Biemar, F., Argenton, F., Schmidtke, R., Epperlein, S., Peers, B., and Driever, W. (2001). Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. *Developmental Biology* **230,** 189-203.

Biswas, A., and Takeuchi, T. (2003). Effects of photoperiod and feeding interval on food intake and growth rate of Nile tilapia (*Oreochromis niloticus* L.). *Fisheries Science* **69,** 1010-1016.

Bjenning, C., Farrell, A., and Holmgren, S. (1991). Bombesin-like immunoreactivity in skates and the in vitro effect of bombesin on coronary vessels from the longnose skate, Raja rhina. *Regul Pept* **35,** 207-19.

Black, D., and Love, R. M. (1986). The sequential mobilization and restoration of energy reserves in tissues of Atlantic cod during starvation and refeeding. . *J. Comp. Physiol. B* **156,** 469-479.

Blier, P. U., Dutil, J.-D., Lemieux, H., Belanger, F., and Bitetera, L. (2007). Phenotypic flexibility of digestive system in Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **146,** 174-179.

Boeuf, G., and Le Bail, P. Y. (1999). Does light have an influence on fish growth? *Aquaculture* **177,** 129-152.

Bona, G., and Bellone, S. (2003). Central and peripheral activities of ghrelin, a ligand of the growth hormone secretagogue receptor. *Panminerva Med* **45,** 197-201.

Bowden, T. J., Thompson, K. D., Morgan, A. L., Gratacap, R. M., and Nikoskelainen, S. (2007). Seasonal variation and the immune response: a fish perspective. *Fish Shellfish Immunol* **22,** 695-706.

Bray, G. A. (2000). Afferent signals regulating food intake. *The Proceedings of the Nutrition Society* **59,** 373-384.

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., and Guillemin, R. (1973). Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179,** 77-9.

Brown, J. A., Minkoffb, G., and Puvanendran, V. (2003). Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols and problems *Aquaculture* **227,** 357-372

Burlet, S., Tyler, C. J., and Leonard, C. S. (2002). Direct and indirect excitation of laterodorsal tegmental neurons by Hypocretin/Orexin peptides: implications for wakefulness and narcolepsy. *J Neurosci* **22,** 2862-72.

Caelers, A., Maclean, N., Hwang, G., Eppler, E., and Reinecke, M. (2005). Expression of endogenous and exogenous growth hormone (GH) messenger (m) RNA in a GH-transgenic tilapia (*Oreochromis niloticus*). *Transgenic Research* **14,** 95-104.

Cai, X. J., Widdowson, P. S., Harrold, J., Wilson, S., Buckingham, R. E., Arch, J. R., Tadayyon, M., Clapham, J. C., Wilding, J., and Williams, G. (1999).

Hypothalamic orexin expression: modulation by blood glucose and feeding. *Diabetes* **48,** 2132-7.

Canavate, J. P., Zerolo, R., and Fernandez-Diaz, C. (2006). Feeding and development of Senegal sole (Solea senegalensis) larvae reared in different photoperiods. *Aquaculture* **258,** 368-377.

Canosa, L. F., Cerda-Reverter, J. M., and Peter, R. E. (2004). Brain mapping of three somatostatin encoding genes in the goldfish. *J Comp Neurol* **474,** 43-57.

Canosa, L. F., Chang, J. P., and Peter, R. E. (2007). Neuroendocrine control of growth hormone in fish. *General and Comparative Endocrinology* **151,** 1-26.

Canosa, L. F., Lin, X., and Peter, R. E. (2002). Regulation of expression of somatostatin genes by sex steroid hormones in goldfish forebrain. *Neuroendocrinology* **76,** 8-17.

Canosa, L. F., Unniappan, S., and Peter, R. E. (2005). Periprandial changes in growth hormone release in goldfish: role of somatostatin, ghrelin, and gastrin-releasing peptide. *Am J Physiol Regul Integr Comp Physiol* **289,** R125-33.

Carro, E., Senaris, R., Considine, R. V., Casanueva, F. F., and Dieguez, C. (1997). Regulation of in vivo growth hormone secretion by leptin. *Endocrinology* **138,** 2203-6.

Cerda-Reverter, J. M., and Peter, R. E. (2003). Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology* **144,** 4552-61.

Cerda-Reverter, J. M., Ringholm, A., Schioth, H. B., and Peter, R. E. (2003a). Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. *Endocrinology* **144,** 2336-49.

Cerda-Reverter, J. M., Schioth, H. B., and Peter, R. E. (2003b). The central melanocortin system regulates food intake in goldfish. *Regul Pept* **115,** 101-13.

Chamorro, S., Della-Zuana, O., Fauchere, J. L., Feletou, M., Galizzi, J. P., and Levens, N. (2002). Appetite suppression based on selective inhibition of NPY receptors. *Int J Obes Relat Metab Disord* **26,** 281-98.

Chan, C. B., Fung, C. K., Fung, W., Tse, M. C., and Cheng, C. H. (2004). Stimulation of growth hormone secretion from seabream pituitary cells in primary culture by growth hormone secretagogues is independent of growth hormone transcription. *Comp Biochem Physiol C Toxicol Pharmacol* **139,** 77-85.

Chance, W. T., Thompson, H., Thomas, I., and Fischer, J. E. (1995). Anorectic and neurochemical effects of pituitary adenylate cyclase activating polypeptide in rats. *Peptides* **16,** 1511-6.

Chemelli, R. M., Willie, J. T., Sinton, C. M., Elmquist, J. K., Scammell, T., Lee, C., Richardson, J. A., Williams, S. C., Xiong, Y., Kisanuki, Y., Fitch, T. E., Nakazato, M., Hammer, R. E., Saper, C. B., and Yanagisawa, M. (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98,** 437-51.

Chen, J., Scott, K. A., Zhao, Z., Moran, T. H., and Bi, S. (2007). Characterization of the feeding inhibition and neural activation produced by dorsomedial hypothalamic cholecystokinin administration. *Neuroscience*.

Chen, R., Li, W., and Lin, H. (2005). cDNA cloning and mRNA expression of neuropeptide Y in orange spotted grouper, Epinephelus coioides. *Comp Biochem Physiol B Biochem Mol Biol* **142,** 79-89.

Chiba, A., Honma, Y., and Oka, S. (1995). Ontogenetic development of neuropeptide Y-like-immunoreactive cells in the gastroenteropancreatic endocrine system of the dogfish. *Cell Tissue Res* **282,** 33-40.

Chiba, A., Oka, S., and Saitoh, E. (2002). Ontogenetic changes in neuropeptide Y-immunoreactive cerebrospinal fluid-contacting neurons in the hypothalamus of the cloudy dogfish, Scyliorhinus torazame (Elasmobranchii). *Neurosci Lett* **329,** 301-4.

Chihara, K., Minamitani, N., Kaji, H., Arimura, A., and Fujita, T. (1981). Intraventricularly injected growth hormone stimulates somatostatin release into rat hypophysial portal blood. *Endocrinology* **109,** 2279-81.

Clark, J. T., Kalra, P. S., Crowley, W. R., and Kalra, S. P. (1984). Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. *Endocrinology* **115,** 427-9.

Cocchi, D., De Gennaro Colonna, V., Bagnasco, M., Bonacci, D., and Muller, E. E. (1999). Leptin regulates GH secretion in the rat by acting on GHRH and somatostatinergic functions. *J Endocrinol* **162,** 95-9.

Cone, R. D. (2005). Anatomy and regulation of the central melanocortin system. *Nat Neurosci* **8,** 571-8.

Conlon, J. M., Henderson, I. W., and Thim, L. (1987). Gastrin-releasing peptide from the intestine of the elasmobranch fish, Scyliorhinus canicula (common dogfish). *Gen Comp Endocrinol* **68,** 415-20.

Couceyro, P. R., and Fritz, T. (2003). Production of recombinant CART peptides in *Escherichia coli* with agonist and antagonist effects on food intake in rats. *Protein Expr Purif* **32,** 185-93.

Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdan, M. G., Diano, S., Horvath, T. L., Cone, R. D., and Low, M. J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* **411,** 480-4.

Cummings, D. E., Purnell, J. Q., Frayo, R. S., Schmidova, K., Wisse, B. E., and Weigle, D. S. (2001). A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* **50,** 1714-9.

Date, Y., Mondal, M. S., Matsukura, S., Ueta, Y., Yamashita, H., Kaiya, H., Kangawa, K., and Nakazato, M. (2000). Distribution of orexin/hypocretin in the rat median eminence and pituitary. *Brain Res Mol Brain Res* **76,** 1-6.

Date, Y., Murakami, N., Toshinai, K., Matsukura, S., Niijima, A., Matsuo, H., Kangawa, K., and Nakazato, M. (2002). The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* **123,** 1120-8.

Davie, A., Porter, M. J. R., and Bromage, N. R. (2003). Photoperiod manipulation of maturation and growth of Atlantic cod (*Gadus morhua*) *Fish Physiology and Biochemistry* **28,** 399-401.

Davis, K., and McEntire, M. (2006). Effect of photoperiod on feeding, intraperitoneal fat, and insulin-like growth factor-I in sunshine bass. *Journal of the World Aquaculture Society* **37,** 431-436.

de Beaurepaire, R., and Suaudeau, C. (1988). Anorectic effect of calcitonin, neurotensin and bombesin infused in the area of the rostral part of the nucleus of the tractus solitarius in the rat. *Peptides* **9,** 729-33.

de Lecea, L. (2005). Cortistatin: a natural somatostatin analog. *Journal of Endocrinological Investigation* **28,** 10-4.

de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L., Gautvik, V. T., Bartlett, F. S., 2nd, Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., and Sutcliffe, J. G. (1998). The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* **95,** 322-7.

de Pedro, N., Martinez-Alvarez, R., and Delgado, M. J. (2006). Acute and chronic leptin reduces food intake and body weight in goldfish (Carassius auratus). *J Endocrinol* **188,** 513-20.

De Vriese, C., and Delporte, C. (2008). Ghrelin: A new peptide regulating growth hormone release and food intake. *Int J Biochem Cell Biol* **40,** 1420-4.

del Giudice, E. M., Santoro, N., Cirillo, G., D'Urso, L., Di Toro, R., and Perrone, L. (2001). Mutational screening of the CART gene in obese children: identifying a mutation (Leu34Phe) associated with reduced resting energy expenditure and cosegregating with obesity phenotype in a large family. *Diabetes* **50,** 2157-60.

Delfini, C., and Diagne, M. (1985). [Brain of the cod (Gadus morhua morhua, Linne 1758) (Pisces, Paracanthopterygii). Qualitative and quantitative analysis of major subdivisions]. *J Hirnforsch* **26,** 439-49.

Devos, N., Deflorian, G., Biemar, F., Bortolussi, M., Martial, J. A., Peers, B., and Argenton, F. (2002). Differential expression of two somatostatin genes during zebrafish embryonic development. *Mechanisms of Development* **115,** 133-7.

Dube, M. G., Kalra, S. P., and Kalra, P. S. (1999). Food intake elicited by central administration of orexins/hypocretins: identification of hypothalamic sites of action. *Brain Res* **842,** 473-7.

Ebihara, K., Ogawa, Y., Katsuura, G., Numata, Y., Masuzaki, H., Satoh, N., Tamaki, M., Yoshioka, T., Hayase, M., Matsuoka, N., Aizawa-Abe, M., Yoshimasa, Y., and Nakao, K. (1999). Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* **48,** 2028-33.

Edwards, C. M., Abbott, C. R., Sunter, D., Kim, M., Dakin, C. L., Murphy, K. G., Abusnana, S., Taheri, S., Rossi, M., and Bloom, S. R. (2000). Cocaine- and amphetamine-regulated transcript, glucagon-like peptide-1 and corticotrophin releasing factor inhibit feeding via agouti-related protein independent pathways in the rat. *Brain Res* **866,** 128-34.

Ehrman, M., Melroe, G., Moore, C., JD., K., and Sheridan, M. (2002). Nutritional regulation of somatostatin expression in rainbow trout, *Oncorhynchus mykiss Fish Physiology and Biochemistry* **26,** 309-314.

Eilertson, C. D., and Sheridan, M. A. (1993). Differential effects of somatostatin-14 and somatostatin-25 on carbohydrate and lipid metabolism in rainbow trout *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **92,** 62-70.

Faraco, J. H., Appelbaum, L., Marin, W., Gaus, S. E., Mourrain, P., and Mignot, E. (2006). Regulation of hypocretin (OREXIN) expression in embryonic zebrafish. *J Biol Chem.*

Fekete, E., Vigh, J., Bagi, E. E., and Lenard, L. (2002). Gastrin-releasing peptide microinjected into the amygdala inhibits feeding. *Brain Res* **955,** 55-63.

Fekete, E. M., Bagi, E. E., Toth, K., and Lenard, L. (2007). Neuromedin C microinjected into the amygdala inhibits feeding. *Brain Res Bull* **71,** 386-92.

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

Friedman, J. M., and Halaas, J. L. (1998). Leptin and the regulation of body weight in mammals. *Nature* **395,** 763-70.

Galas, L., Vaudry, H., Braun, B., Van Den Pol, A. N., De Lecea, L., Sutcliffe, J. G., and Chartrel, N. (2001). Immunohistochemical localization and biochemical characterization of hypocretin/orexin-related peptides in the central nervous system of the frog Rana ridibunda. *J Comp Neurol* **429,** 242-52.

Gallmann, E., Arsenijevic, D., Williams, G., Langhans, W., and Spengler, M. (2006). Effect of intraperitoneal CCK-8 on food intake and brain orexin-A after 48 h of fasting in the rat. *Regul Pept* **133,** 139-46.

Gehlert, D. R. (1999). Role of hypothalamic neuropeptide Y in feeding and obesity. *Neuropeptides* **33,** 329-38.

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

Germano, C. M., de Castro, M., Rorato, R., Laguna, M. T., Antunes-Rodrigues, J., Elias, C. F., and Elias, L. L. (2007). Time course effects of adrenalectomy and food intake on cocaine- and amphetamine-regulated transcript expression in the hypothalamus. *Brain Res* **1166,** 55-64.

Gibbs, J., Fauser, D. J., Rowe, E. A., Rolls, B. J., Rolls, E. T., and Maddison, S. P. (1979). Bombesin suppresses feeding in rats. *Nature* **282,** 208-10.

Giustina, A., and Veldhuis, J. D. (1998). Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. *Endocr Rev* **19,** 717-97.

Goth, M. I., Lyons, C. E., Canny, B. J., and Thorner, M. O. (1992). Pituitary adenylate cyclase activating polypeptide, growth hormone (GH)-releasing peptide and GH-releasing hormone stimulate GH release through distinct pituitary receptors. *Endocrinology* **130,** 939-44.

Gottero, C., Prodam, F., Destefanis, S., Benso, A., Gauna, C., Me, E., Filtri, L., Riganti, F., Van Der Lely, A. J., Ghigo, E., and Broglio, F. (2004). Cortistatin-17 and -14 exert the same endocrine activities as somatostatin in humans. *Growth Hormone & IGF Research* **14,** 382-7.

Griffond, B., Risold, P. Y., Jacquemard, C., Colard, C., and Fellmann, D. (1999). Insulin-induced hypoglycemia increases preprohypocretin (orexin) mRNA in the rat lateral hypothalamic area. *Neurosci Lett* **262,** 77-80.

Grill, H. J., Ginsberg, A. B., Seeley, R. J., and Kaplan, J. M. (1998). Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. *J Neurosci* **18,** 10128-35.

Hagan, M. M., Rushing, P. A., Pritchard, L. M., Schwartz, M. W., Strack, A. M., Van Der Ploeg, L. H., Woods, S. C., and Seeley, R. J. (2000). Long-term orexigenic effects of AgRP-(83---132) involve mechanisms other than melanocortin receptor blockade. *Am J Physiol Regul Integr Comp Physiol* **279,** R47-52.

Hall, J. R., Short, C. E., and Driedzic, W. R. (2006). Sequence of Atlantic cod (*Gadus morhua*) GLUT4, GLUT2 and GPDH: Developmental stage expression, tissue expression and relationship to starvation-induced changes in blood glucose. *The Journal of Experimental Biology* **209,** 4490-502.

Hall, T. E., Smith, P., and Johnston, I. A. (2004). Stages of embryonic development in the Atlantic cod Gadus morhua. *J Morphol* **259,** 255-70.

Hans-Joachim, R., and Josep, L. (2003). Variation in fish condition between Atlantic cod (*Gadus morhua*) stocks, the effect of their productivity and management implications. *Fisheries Research* **60,** 369-380.

Hara, J., Beuckmann, C. T., Nambu, T., Willie, J. T., Chemelli, R. M., Sinton, C. M., Sugiyama, F., Yagami, K., Goto, K., Yanagisawa, M., and Sakurai, T. (2001). Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* **30,** 345-54.

Harrison, J. L., Miller, D. W., Findlay, P. A., and Adam, C. L. (2008). Photoperiod influences the central effects of ghrelin on food intake, GH and LH secretion in sheep. *Neuroendocrinology* **87,** 182-92.

Haynes, A. C., Jackson, B., Overend, P., Buckingham, R. E., Wilson, S., Tadayyon, M., and Arch, J. R. (1999). Effects of single and chronic intracerebroventricular administration of the orexins on feeding in the rat. *Peptides* **20,** 1099-105.

Hewson, A. K., and Dickson, S. L. (2000). Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J Neuroendocrinol* **12,** 1047-9.

Himick, B. A., and Peter, R. E. (1994a). Bombesin acts to suppress feeding behavior and alter serum growth hormone in goldfish. *Physiol Behav* **55,** 65-72.

Himick, B. A., and Peter, R. E. (1994b). CCK/gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish. *Am J Physiol* **267,** R841-51.

Himick, B. A., Vigna, S. R., and Peter, R. E. (1995). Characterization and distribution of bombesin binding sites in the goldfish hypothalamic feeding center and pituitary. *Regul Pept* **60,** 167-76.

Holmgren, S., and Jonsson, A. C. (1988). Occurrence and effects on motility of bombesin related peptides in the gastrointestinal tract of the Atlantic cod, Gadus morhua. *Comp Biochem Physiol C* **89,** 249-56.

Holstein, B., and Humphrey, C. S. (1980). Stimulation of gastric acid secretion and suppression of VIP-like immunoreactivity by bombesin in the Atlantic codfish, Gadus morhua. *Acta Physiol Scand* **109,** 217-23.

Hoskins, L. J., Xu, M., and Volkoff, H. (2008). Interactions between gonadotropin-releasing hormone (GnRH) and orexin in the regulation of feeding and reproduction in goldfish (Carassius auratus). *Horm Behav* **54,** 379-85.

Houben, H., Vandenbroucke, A. T., Verheyden, A. M., and Denef, C. (1993). Expression of the genes encoding bombesin-related peptides and their receptors in anterior pituitary tissue. *Mol Cell Endocrinol* **97,** 159-64.

Huesa, G., van den Pol, A. N., and Finger, T. E. (2005). Differential distribution of hypocretin (orexin) and melanin-concentrating hormone in the goldfish brain. *J Comp Neurol* **488,** 476-91.

Huising, M. O., Geven, E. J., Kruiswijk, C. P., Nabuurs, S. B., Stolte, E. H., Spanings, F. A., Verburg-van Kemenade, B. M., and Flik, G. (2006). Increased leptin expression in common Carp (Cyprinus carpio) after food intake but not after fasting or feeding to satiation. *Endocrinology* **147,** 5786-97.

Imslanda, A. K., Fossb, A., Folkvordc, A., Stefanssonc, S. O., and Jonassend, T. M. (2005). Genotypic response to photoperiod treatment in Atlantic cod (*Gadus Morhua*) *Aquaculture* **250,** 525-532

Iqbal, J., Henry, B. A., Pompolo, S., Rao, A., and Clarke, I. J. (2003). Long-term alteration in bodyweight and food restriction does not affect the gene expression of either preproorexin or prodynorphin in the sheep. *Neuroscience* **118,** 217-26.

Isaksson, O. G., Lindahl, A., Nilsson, A., and Isgaard, J. (1987). Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. *Endocr Rev* **8,** 426-38.

Ishii, Y., Blundell, J. E., Halford, J. C., Upton, N., Porter, R., Johns, A., Jeffrey, P., Summerfield, S., and Rodgers, R. J. (2005). Anorexia and weight loss in male rats 24 h following single dose treatment with orexin-1 receptor antagonist SB-334867. *Behav Brain Res* **157,** 331-41.

Jacob, N., Vuillez, P., Moller, M., and Pevet, P. (1998). Photoperiodic dependent changes in the number of neurons containing mRNA encoding neuropeptide Y in the intergeniculate leaflet of the Syrian hamster. *Brain Res* **813,** 160-166.

Jarry, H., Leonhardt, S., Schmidt, W. E., Creutzfeldt, W., and Wuttke, W. (1992). Contrasting effects of pituitary adenylate cyclase activating polypeptide (PACAP) on in vivo and in vitro prolactin and growth hormone release in male rats. *Life Sci* **51,** 823-30.

Jensen, J., and Conlon, J. M. (1992). Isolation and primary structure of gastrin-releasing peptide from a teleost fish, the trout (Oncorhynchus mykiss). *Peptides* **13,** 995-9.

Jobling, M., Meloy, O., Santos, J. d., and Christiansen, B. (1994). The compensatory growth response of the Atlantic cod : effects of nutritional history. *Acquaculture International* **2,** 75-90.

Johansson, A., Fredriksson, R., Winnergren, S., Hulting, A. L., Schioth, H. B., and Lindblom, J. (2008). The relative impact of chronic food restriction and acute food deprivation on plasma hormone levels and hypothalamic neuropeptide expression. *Peptides*.

Johren, O., Neidert, S. J., Kummer, M., Dendorfer, A., and Dominiak, P. (2001). Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. *Endocrinology* **142,** 3324-31.

Jonsson, E., Forsman, A., Einarsdottir, I. E., Kaiya, H., Ruohonen, K., and Bjornsson, B. T. (2007). Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. *Comp Biochem Physiol A Mol Integr Physiol* **147,** 1116-24.

Kaiya, H., Kojima, M., Hosoda, H., Koda, A., Yamamoto, K., Kitajima, Y., Matsumoto, M., Minamitake, Y., Kikuyama, S., and Kangawa, K. (2001). Bullfrog ghrelin is

modified by n-octanoic acid at its third threonine residue. *J Biol Chem* **276,** 40441-8.

Kaiya, H., Kojima, M., Hosoda, H., Moriyama, S., Takahashi, A., Kawauchi, H., and Kangawa, K. (2003a). Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. *Endocrinology* **144,** 5215-26.

Kaiya, H., Kojima, M., Hosoda, H., Riley, L. G., Hirano, T., Grau, E. G., and Kangawa, K. (2003b). Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. *J Endocrinol* **176,** 415-23.

Kaiya, H., Kojima, M., Hosoda, H., Riley, L. G., Hirano, T., Grau, E. G., and Kangawa, K. (2003c). Identification of tilapia ghrelin and its effects on growth hormone and prolactin release in the tilapia, Oreochromis mossambicus. *Comp Biochem Physiol B Biochem Mol Biol* **135,** 421-9.

Kaiya, H., Small, B. C., Bilodeau, A. L., Shepherd, B. S., Kojima, M., Hosoda, H., and Kangawa, K. (2005). Purification, cDNA cloning, and characterization of ghrelin in channel catfish, Ictalurus punctatus. *Gen Comp Endocrinol* **143,** 201-10.

Kameda, Y., Miura, M., and Nishimaki, T. (2001). Localization of neuropeptide Y mRNA and peptide in the chicken hypothalamus and their alterations after food deprivation, dehydration, and castration. *J Comp Neurol* **436,** 376-88.

Kamegai, J., Tamura, H., Shimizu, T., Ishii, S., Sugihara, H., and Wakabayashi, I. (2001). Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. *Diabetes* **50,** 2438-43.

Kamegai, J., Tamura, H., Shimizu, T., Ishii, S., Tatsuguchi, A., Sugihara, H., Oikawa, S., and Kineman, R. D. (2004). The role of pituitary ghrelin in growth hormone (GH) secretion: GH-releasing hormone-dependent regulation of pituitary ghrelin gene expression and peptide content. *Endocrinology* **145,** 3731-8.

Kask, A., Pahkla, R., Irs, A., Rago, L., Wikberg, J. E., and Schioth, H. B. (1999). Long-term administration of MC4 receptor antagonist HS014 causes hyperphagia and obesity in rats. *Neuroreport* **10,** 707-11.

Kaslin, J., Nystedt, J. M., Ostergard, M., Peitsaro, N., and Panula, P. (2004). The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. *J Neurosci* **24,** 2678-89.

Kateb, C. C., and Merali, Z. (1992). A single meal elicits regional changes in bombesin-like peptide levels in the gut and brain. *Brain Res* **596,** 10-6.

Kawakoshi, A., Kaiya, H., Riley, L. G., Hirano, T., Grau, E. G., Miyazato, M., Hosoda, H., and Kangawa, K. (2007). Identification of a ghrelin-like peptide in two species of shark, Sphyrna lewini and Carcharhinus melanopterus. *Gen Comp Endocrinol* **151,** 259-68.

Kawamura, K., Sato, N., Fukuda, J., Kodama, H., Kumagai, J., Tanikawa, H., Nakamura, A., Honda, Y., Sato, T., and Tanaka, T. (2003). Ghrelin inhibits the development of mouse preimplantation embryos in vitro. *Endocrinology* **144,** 2623-33.

Kehoe, A. S., and Volkoff, H. (2007). Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (Gadus morhua). *Comp Biochem Physiol A Mol Integr Physiol* **146,** 451-61.

Kineman, R. D., Gahete, M. D., and Luque, R. M. (2007). Identification of a mouse ghrelin gene transcript that contains intron 2 and is regulated in the pituitary and hypothalamus in response to metabolic stress. *J Mol Endocrinol* **38,** 511-21.

Kineman, R. D., Kamegai, J., and Frohman, L. A. (1999). Growth hormone (GH)-releasing hormone (GHRH) and the GH secretagogue (GHS), L692,585, differentially modulate rat pituitary GHS receptor and GHRH receptor messenger ribonucleic acid levels. *Endocrinology* **140,** 3581-6.

Kirchgessner, A. L. (2002). Orexins in the brain-gut axis. *Endocr Rev* **23,** 1-15.

Kirchgessner, A. L., and Liu, M. (1999). Orexin synthesis and response in the gut. *Neuron* **24,** 941-51.

Klein, S. E., and Sheridan, M. A. (2007). Somatostatin signaling and the regulation of growth and metabolism in fish. *Molecular and Cellular Endocrinology.*

Kobayashi, Y., Peterson, B. C., and Waldbieser, G. C. (2008). Association of cocaine- and amphetamine-regulated transcript (CART) messenger RNA level, food intake, and growth in channel catfish. *Comp Biochem Physiol A Mol Integr Physiol* **151,** 219-25.

Kohbara, J., Hidaka, I., Kuriyama, I., Yamashita, M., Ichikawa, M., Furukawa, K., Aida, K., Sanchez-Vazquez, F. J., and Tabata, M. (2000). Nocturnal/diurnal demand-feeding pattern of yellowtail Seriola quinqueradiata under different keeping conditions. *Fisheries Science* **66,** 955-962.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402,** 656-60.

Kong, W. M., Stanley, S., Gardiner, J., Abbott, C., Murphy, K., Seth, A., Connoley, I., Ghatei, M., Stephens, D., and Bloom, S. (2003). A role for arcuate cocaine and amphetamine-regulated transcript in hyperphagia, thermogenesis, and cold adaptation. *Faseb J* **17,** 1688-90.

Korbonits, M., Little, J. A., Forsling, M. L., Tringali, G., Costa, A., Navarra, P., Trainer, P. J., and Grossman, A. B. (1999). The effect of growth hormone secretagogues and neuropeptide Y on hypothalamic hormone release from acute rat hypothalamic explants. *J Neuroendocrinol* **11,** 521-8.

Kristensen, P., Judge, M. E., Thim, L., Ribel, U., Christjansen, K. N., Wulff, B. S., Clausen, J. T., Jensen, P. B., Madsen, O. D., Vrang, N., Larsen, P. J., and Hastrup, S. (1998). Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* **393,** 72-6.

Kurokawa, T., Uji, S., and Suzuki, T. (2005). Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, Takifugu rubripes. *Peptides* **26,** 745-50.

Kurose, T., Ueta, Y., Yamamoto, Y., Serino, R., Ozaki, Y., Saito, J., Nagata, S., and Yamashita, H. (2002). Effects of restricted feeding on the activity of hypothalamic Orexin (OX)-A containing neurons and OX2 receptor mRNA level in the paraventricular nucleus of rats. *Regul Pept* **104,** 145-51.

Kyrkouli, S. E., Stanley, B. G., and Leibowitz, S. F. (1987). Bombesin-induced anorexia: sites of action in the rat brain. *Peptides* **8,** 237-41.

Ladenheim, E. E., Hampton, L. L., Whitney, A. C., White, W. O., Battey, J. F., and Moran, T. H. (2002). Disruptions in feeding and body weight control in gastrin-releasing peptide receptor deficient mice. *J Endocrinol* **174,** 273-81.

Lambert, P. D., Couceyro, P. R., McGirr, K. M., Dall Vechia, S. E., Smith, Y., and Kuhar, M. J. (1998). CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse* **29,** 293-8.

Lang, M., Bufe, B., De Pol, S., Reiser, O., Meyerhof, W., and Beck-Sickinger, A. G. (2006). Structural properties of orexins for activation of their receptors. *J Pept Sci* **12,** 258-66.

Lang, M., Soll, R. M., Durrenberger, F., Dautzenberg, F. M., and Beck-Sickinger, A. G. (2004). Structure-activity studies of orexin a and orexin B at the human orexin 1 and orexin 2 receptors led to orexin 2 receptor selective and orexin 1 receptor preferring ligands. *J Med Chem* **47,** 1153-60.

Lanzi, R., and Tannenbaum, G. S. (1992). Time-dependent reduction and potentiation of growth hormone (GH) responsiveness to GH-releasing factor induced by exogenous GH: role for somatostatin. *Endocrinology* **130,** 1822-8.

Larhammar, D., and Salaneck, E. (2004). Molecular evolution of NPY receptor subtypes. *Neuropeptides* **38,** 141-51.

Lee, L. T., Siu, F. K., Tam, J. K., Lau, I. T., Wong, A. O., Lin, M. C., Vaudry, H., and Chow, B. K. (2007). Discovery of growth hormone-releasing hormones and receptors in nonmammalian vertebrates. *Proceedings of the National Academy of Sciences of the United States of America* **104,** 2133-8.

Lemieux, H., Dutil, J., Guderley, H., and Larocque, R. (2004). Growth, starvation and enzyme activity in white muscle of Atlantic cod: at what point do muscle metabolic capacities change? *Marine and Freshwater Behaviour and Physiology* **37,** 287-294.

Leshin, L. S., Barb, C. R., Kiser, T. E., Rampacek, G. B., and Kraeling, R. R. (1994). Growth hormone-releasing hormone and somatostatin neurons within the porcine and bovine hypothalamus. *Neuroendocrinology* **59,** 251-64.

Levin, F., Edholm, T., Schmidt, P. T., Gryback, P., Jacobsson, H., Degerblad, M., Hoybye, C., Holst, J. J., Rehfeld, J. F., Hellstrom, P. M., and Naslund, E. (2006). Ghrelin stimulates gastric emptying and hunger in normal-weight humans. *J Clin Endocrinol Metab* **91,** 3296-302.

Levine, A. S., and Morley, J. E. (1982). Peripherally administered somatostatin reduces feeding by a vagal mediated mechanism. *Pharmacol Biochem Behav* **16,** 897-902.

Lin, X., Otto, C. J., and Peter, R. E. (1999). Expression of three distinct somatostatin messenger ribonucleic acids (mRNAs) in goldfish brain: characterization of the complementary deoxyribonucleic acids, distribution and seasonal variation of the mRNAs, and action of a somatostatin-14 variant. *Endocrinology* **140,** 2089-99.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., and Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* **75,** 59-72.

Lo, C. M., Samuelson, L. C., Chambers, J. B., King, A., Heiman, J., Jandacek, R. J., Sakai, R. R., Benoit, S. C., Raybould, H. E., Woods, S. C., and Tso, P. (2008). Characterization of mice lacking the gene for cholecystokinin. *Am J Physiol Regul Integr Comp Physiol* **294,** R803-10.

Lopez-Olmeda, J. F., Bayarri, M. J., Rol de Lama, M. A., Madrid, J. A., and Sanchez-Vazquez, F. J. (2006a). Effects of melatonin administration on oxidative stress and daily locomotor activity patterns in goldfish. *J Physiol Biochem* **62,** 17-25.

Lopez-Olmeda, J. F., Madrid, J. A., and Sanchez-Vazquez, F. J. (2006b). Melatonin effects on food intake and activity rhythms in two fish species with different activity patterns: Diurnal (goldfish) and nocturnal (tench). *Comp Biochem Physiol A Mol Integr Physiol* **144,** 180-187.

Lopez-Patino, M. A., Guijarro, A. I., Isorna, E., Delgado, M. J., Alonso-Bedate, M., and de Pedro, N. (1999). Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (Carassius auratus). *Eur J Pharmacol* **377,** 147-53.

Lopez, M., Lage, R., Tung, Y. C., Challis, B. G., Varela, L., Virtue, S., O'Rahilly, S., Vidal-Puig, A., Dieguez, C., and Coll, A. P. (2007). Orexin expression is regulated by alpha-melanocyte-stimulating hormone. *J Neuroendocrinol* **19,** 703-7.

Lopez, M., Seoane, L., Garcia, M. C., Lago, F., Casanueva, F. F., Senaris, R., and Dieguez, C. (2000). Leptin regulation of prepro-orexin and orexin receptor mRNA levels in the hypothalamus. *Biochem Biophys Res Commun* **269,** 41-5.

Lotter, E. C., Krinsky, R., McKay, J. M., Treneer, C. M., Porter, D., Jr., and Woods, S. C. (1981). Somatostatin decreases food intake of rats and baboons. *J Comp Physiol Psychol* **95,** 278-87.

Ludwig, D. S., Mountjoy, K. G., Tatro, J. B., Gillette, J. A., Frederich, R. C., Flier, J. S., and Maratos-Flier, E. (1998). Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. *Am J Physiol* **274,** E627-33.

Lutz, T. A. (2005). Pancreatic amylin as a centrally acting satiating hormone. *Curr Drug Targets* **6,** 181-9.

Manning, A. J., Murray, H. M., Gallant, J. W., Matsuoka, M. P., Radford, E., and Douglas, S. E. (2008). Ontogenetic and tissue-specific expression of preproghrelin in the Atlantic halibut, Hippoglossus hippoglossus L. *J Endocrinol* **196,** 181-92.

Marchant, T. A., Dulka, J. G., and Peter, R. E. (1989). Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, Carassius auratus L. *General and Comparative Endocrinology* **73,** 458-68.

Marchant, T. A., Fraser, R. A., Andrews, P. C., and Peter, R. E. (1987). The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (Carassius auratus L.) pituitary fragments in vitro. *Regulatory Peptides* **17,** 41-52.

Masuda, Y., Tanaka, T., Inomata, N., Ohnuma, N., Tanaka, S., Itoh, Z., Hosoda, H., Kojima, M., and Kangawa, K. (2000). Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* **276,** 905-8.

Mathieu, M., Tagliafierro, G., Bruzzone, F., and Vallarino, M. (2002). Neuropeptide tyrosine-like immunoreactive system in the brain, olfactory organ and retina of the zebrafish, Danio rerio, during development. *Brain Res Dev Brain Res* **139,** 255-65.

Matsuda, K., Maruyama, K., Miura, T., Uchiyama, M., and Shioda, S. (2005a). Anorexigenic action of pituitary adenylate cyclase-activating polypeptide

(PACAP) in the goldfish: feeding-induced changes in the expression of mRNAs for PACAP and its receptors in the brain, and locomotor response to central injection. *Neuroscience Letters* **386,** 9-13.

Matsuda, K., Maruyama, K., Nakamachi, T., Miura, T., Uchiyama, M., and Shioda, S. (2005b). Inhibitory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) on food intake in the goldfish, Carassius auratus. *Peptides* **26,** 1611-6.

Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Shimakura, S., Uchiyama, M., Kangawa, K., and Shioda, S. (2006a). Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. *Peptides* **27,** 2321-5.

Matsuda, K., Miura, T., Kaiya, H., Maruyama, K., Uchiyama, M., Kangawa, K., and Shioda, S. (2006b). Stimulatory effect of n-octanoylated ghrelin on locomotor activity in the goldfish, Carassius auratus. *Peptides* **27,** 1335-40.

Matsumoto, M., Hosoda, H., Kitajima, Y., Morozumi, N., Minamitake, Y., Tanaka, S., Matsuo, H., Kojima, M., Hayashi, Y., and Kangawa, K. (2001). Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. *Biochem Biophys Res Commun* **287,** 142-6.

McAlister, E. D., and Van Vugt, D. A. (2004). Effect of leptin administration versus re-feeding on hypothalamic neuropeptide gene expression in fasted male rats. *Can J Physiol Pharm* **82,** 1128-34.

McColl, K. E., and el-Omar, E. (1995). Review article: gastrin releasing peptide and its value in assessing gastric secretory function. *Aliment Pharmacol Ther* **9,** 341-7.

McMinn, J. E., Wilkinson, C. W., Havel, P. J., Woods, S. C., and Schwartz, M. W. (2000). Effect of intracerebroventricular alpha-MSH on food intake, adiposity, c-Fos induction, and neuropeptide expression. *Am J Physiol Regul Integr Comp Physiol* **279,** R695-703.

Menyhert, J., Wittmann, G., Lechan, R. M., Keller, E., Liposits, Z., and Fekete, C. (2007). Cocaine- and amphetamine-regulated transcript (CART) is colocalized with the orexigenic neuropeptide Y and agouti-related protein and absent from the anorexigenic alpha-melanocyte-stimulating hormone neurons in the infundibular nucleus of the human hypothalamus. *Endocrinology* **148,** 4276-81.

Mercer, J. G., Ellis, C., Moar, K. M., Logie, T. J., Morgan, P. J., and Adam, C. L. (2003). Early regulation of hypothalamic arcuate nucleus CART gene expression by short photoperiod in the Siberian hamster. *Regul Pept* **111,** 129-136.

Mercer, J. G., Moar, K. M., Rayner, D. V., Trayhurn, P., and Hoggard, N. (1997). Regulation of leptin receptor and NPY gene expression in hypothalamus of leptin-treated obese (ob/ob) and cold-exposed lean mice. *FEBS Lett* **402,** 185-8.

Mercer, J. G., Moar, K. M., Ross, A. W., and Morgan, P. J. (2000). Regulation of leptin receptor, POMC and AGRP gene expression by photoperiod and food deprivation in the hypothalamic arcuate nucleus of the male Siberian hamster (Phodopus sungorus). *Appetite* **34,** 109-11.

Meton, I., F.Fernandez, and Baanante, I. V. (2003). Short- and long-term effects of refeeding on key enzyme activities in glycolysis-gluconeogenesis in the liver of gilthead seabream (*Sparus aurata*)
Aquaculture **225,** 99-107.

Miura, T., Maruyama, K., Shimakura, S., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S., and Matsuda, K. (2006). Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, Carassius auratus. *Neurosci Lett* **407,** 279-83.

Miura, T., Maruyama, K., Shimakura, S., Kaiya, H., Uchiyama, M., Kangawa, K., Shioda, S., and Matsuda, K. (2007). Regulation of food intake in the goldfish by interaction between ghrelin and orexin. *Peptides* **28,** 1207-13.

Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uehara, A., Jiang, L., Culler, M. D., and Coy, D. H. (1989). Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* **164,** 567-74.

Mollet, A., Gilg, S., Riediger, T., and Lutz, T. A. (2004). Infusion of the amylin antagonist AC 187 into the area postrema increases food intake in rats. *Physiol Behav* **81,** 149-55.

Montero, M., Yon, L., Rousseau, K., Arimura, A., Fournier, A., Dufour, S., and Vaudry, H. (1998). Distribution, characterization, and growth hormone-releasing activity of pituitary adenylate cyclase-activating polypeptide in the European eel, Anguilla anguilla. *Endocrinology* **139,** 4300-10.

Montserrat, N., Gabillard, J. C., Capilla, E., Navarro, M. I., and Gutierrez, J. (2007). Role of insulin, insulin-like growth factors, and muscle regulatory factors in the compensatory growth of the trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **150,** 462-72.

Moran, T. H., Ameglio, P. J., Schwartz, G. J., and McHugh, P. R. (1992). Blockade of type A, not type B, CCK receptors attenuates satiety actions of exogenous and endogenous CCK. *Am J Physiol* **262,** R46-50.

Moran, T. H., Katz, L. F., Plata-Salaman, C. R., and Schwartz, G. J. (1998). Disordered food intake and obesity in rats lacking cholecystokinin A receptors. *Am J Physiol* **274,** R618-25.

Mori, T., and Devlin, R. H. (1999). Transgene and host growth hormone gene expression in pituitary and nonpituitary tissues of normal and growth hormone transgenic salmon. *Molecular and Cellular Endocrinology* **149,** 129-39.

Morley, J. E., Horowitz, M., Morley, P. M., and Flood, J. F. (1992). Pituitary adenylate cyclase activating polypeptide (PACAP) reduces food intake in mice. *Peptides* **13,** 1133-5.

Muccioli, G., Papotti, M., Locatelli, V., Ghigo, E., and Deghenghi, R. (2001). Binding of 125I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. *J Endocrinol Invest* **24,** RC7-9.

Mullett, M. A., Billington, C. J., Levine, A. S., and Kotz, C. M. (2000). Hypocretin I in the lateral hypothalamus activates key feeding-regulatory brain sites. *Neuroreport* **11,** 103-8.

Murakami, Y., Koshimura, K., and Kato, Y. (1996). Effect of PACAP on ion channels involved in stimulating growth hormone secretion from the somatotrophs. *Ann N Y Acad Sci* **805,** 666-9.

Murashita, K., Fukada, H., Hosokawa, H., and Masumoto, T. (2006). Cholecystokinin and peptide Y in yellowtail (Seriola quinqueradiata): molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting. *Gen Comp Endocrinol* **145,** 287-97.

Nagalla, S. R., Barry, B. J., Falick, A. M., Gibson, B. W., Taylor, J. E., Dong, J. Z., and Spindel, E. R. (1996). There are three distinct forms of bombesin. Identification of [Leu13]bombesin, [Phe13]bombesin, and [Ser3,Arg10,Phe13]bombesin in the frog Bombina orientalis. *J Biol Chem* **271,** 7731-7.

Nakamachi, T., Matsuda, K., Maruyama, K., Miura, T., Uchiyama, M., Funahashi, H., Sakurai, T., and Shioda, S. (2006). Regulation by orexin of feeding behaviour and locomotor activity in the goldfish. *J Neuroendocrinol* **18,** 290-7.

Nakata, M., and Yada, T. (2007). PACAP in the glucose and energy homeostasis: physiological role and therapeutic potential. *Current Pharmaceutical Design* **13,** 1105-12.

Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001). A role for ghrelin in the central regulation of feeding. *Nature* **409,** 194-8.

Nambu, T., Sakurai, T., Mizukami, K., Hosoya, Y., Yanagisawa, M., and Goto, K. (1999). Distribution of orexin neurons in the adult rat brain. *Brain Res* **827,** 243-60.

Narnaware, Y. K., and Peter, R. E. (2001). Effects of food deprivation and refeeding on neuropeptide Y (NPY) mRNA levels in goldfish. *Comp Biochem Physiol B Biochem Mol Biol* **129,** 633-7.

Narnaware, Y. K., Peyon, P. P., Lin, X., and Peter, R. E. (2000). Regulation of food intake by neuropeptide Y in goldfish. *Am J Physiol Regul Integr Comp Physiol* **279,** R1025-34.

Naslund, E., and Hellstrom, P. M. (2007). Appetite signaling: from gut peptides and enteric nerves to brain. *Physiol Behav* **92,** 256-62.

Neveu, I., Remy, S., and Naveilhan, P. (2002). The neuropeptide Y receptors, Y1 and Y2, are transiently and differentially expressed in the developing cerebellum. *Neuroscience* **113,** 767-77.

Nieminen, P., Mustonen, A. M., and Hyvarinen, H. (2003). Fasting reduces plasma leptin-and ghrelin-immunoreactive peptide concentrations of the burbot (Lota lota) at 2 degrees C but not at 10 degrees C. *Zoolog Sci* **20,** 1109-15.

Nixon, J. P., and Smale, L. (2007). A comparative analysis of the distribution of immunoreactive orexin A and B in the brains of nocturnal and diurnal rodents. *Behav. Brain Funct.* **3,** 1-28.

Noble, C., Mizusawa, K., and Tabata, M. (2005). Does light intensity affect self-feeding and food wastage in group-held rainbow trout and white-spotted charr? *Journal of Fish Biology* **66,** 1387-1399.

Nordgarden, U., Bjornsson, B. T., and Hansen, T. (2007). Developmental stage of Atlantic salmon parr regulates pituitary GH secretion and parr-smolt transformation. *Aquaculture* **264,** 441-448.

Novak, C. M., Jiang, X., Wang, C., Teske, J. A., Kotz, C. M., and Levine, J. A. (2005). Caloric restriction and physical activity in zebrafish (Danio rerio). *Neurosci Lett* **383,** 99-104.

Ohki-Hamazaki, H., Sakai, Y., Kamata, K., Ogura, H., Okuyama, S., Watase, K., Yamada, K., and Wada, K. (1999). Functional properties of two bombesin-like peptide receptors revealed by the analysis of mice lacking neuromedin B receptor. *J Neurosci* **19,** 948-54.

Ohki-Hamazaki, H., Watase, K., Yamamoto, K., Ogura, H., Yamano, M., Yamada, K., Maeno, H., Imaki, J., Kikuyama, S., Wada, E., and Wada, K. (1997). Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature* **390,** 165-9.

Ohkubo, T., Boswell, T., and Lumineau, S. (2002). Molecular cloning of chicken prepro-orexin cDNA and preferential expression in the chicken hypothalamus. *Biochim Biophys Acta* **1577,** 476-80.

Okkeborg, S. (1998). Feeding behaviour of cod, Gadus morhua: activity rhythm and chemically mediated food search. *Anim Behav* **56,** 371-378.

Okumura, T., Takeuchi, S., Motomura, W., Yamada, H., Egashira Si, S., Asahi, S., Kanatani, A., Ihara, M., and Kohgo, Y. (2001). Requirement of intact disulfide bonds in orexin-A-induced stimulation of gastric acid secretion that is mediated by OX1 receptor activation. *Biochem Biophys Res Commun* **280,** 976-81.

Olsson, C., and Holmgren, S. (2000). PACAP and nitric oxide inhibit contractions in the proximal intestine of the atlantic cod, Gadus morhua. *The Journal of Experimental Biology* **203,** 575-83.

Olszewski, P. K., Bomberg, E. M., Grace, M. K., and Levine, A. S. (2007). Alpha-melanocyte stimulating hormone and ghrelin: central interaction in feeding control. *Peptides* **28,** 2084-9.

Parhar, I. S., Sato, H., and Sakuma, Y. (2003). Ghrelin gene in cichlid fish is modulated by sex and development. *Biochem Biophys Res Commun* **305,** 169-75.

Parker, D. B., Coe, I. R., Dixon, G. H., and Sherwood, N. M. (1993). Two salmon neuropeptides encoded by one brain cDNA are structurally related to members of the glucagon superfamily. *European Journal of Biochemistry* **215,** 439-48.

Parker, D. B., Power, M. E., Swanson, P., Rivier, J., and Sherwood, N. M. (1997). Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. *Endocrinology* **138,** 414-23.

Patel, Y. C. (1999). Somatostatin and its receptor family. *Frontiers in Neuroendocrinology* **20,** 157-98.

Pedroso, F. L., de Jesus-Ayson, E. G., Cortado, H. H., Hyodo, S., and Ayson, F. G. (2006). Changes in mRNA expression of grouper (*Epinephelus coioides*) growth hormone and insulin-like growth factor I in response to nutritional status. *General and Comparative Endocrinology* **145,** 237-46.

Peng, C., Gallin, W., Peter, R. E., Blomqvist, A. G., and Larhammar, D. (1994). Neuropeptide-Y gene expression in the goldfish brain: distribution and regulation by ovarian steroids. *Endocrinology* **134,** 1095-103.

Petit, G., Beauchaud, M., Attia, J., and Buisson, B. (2003). Food intake and growth of largemouth bass (*Micropterus salmoides*) held under alternated light/dark cycle (12L:12D) or exposed to continuous light *Aquaculture* **228,** 397-401

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

Peyon, P., Saied, H., Lin, X., and Peter, R. E. (1999). Postprandial, seasonal and sexual variations in cholecystokinin gene expression in goldfish brain. *Brain Res Mol Brain Res* **74,** 190-6.

Picha, M. E., Silverstein, J. T., and Borski, R. J. (2006). Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (*Morone chrysops* x *Morone saxatilis*). *General and Comparative Endocrinology* **147,** 196-205.

Pierce, A. L., Shimizu, M., Beckman, B. R., Baker, D. M., and Dickhoff, W. W. (2005). Time course of the GH/IGF axis response to fasting and increased ration in chinook salmon (Oncorhynchus tshawytscha). *General and Comparative Endocrinology* **140,** 192-202.

Pinto, S., Roseberry, A. G., Liu, H., Diano, S., Shanabrough, M., Cai, X., Friedman, J. M., and Horvath, T. L. (2004). Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science* **304,** 110-5.

Porreca, F., Burks, T. F., and Koslo, R. J. (1985). Centrally-mediated bombesin effects on gastrointestinal motility. *Life Sci* **37,** 125-34.

Porter, M. R., Stefansson, S. O., Nyhammer, G., Karlsen, Ø., Norberg, B., and Bromage, N. R. (2000). Environmental influences on melatonin secretion in Atlantic cod (*Gadus morhua* L.) and their relevance to commercial culture. *Fish Physiology and Biochemistry* **23,** 191–200.

Preston, E., Jonsson, A. C., McManus, C. D., Conlon, J. M., and Courtice, G. P. (1998). Comparative vascular responses in elasmobranchs to different structures of neuropeptide Y and peptide YY. *Regul Pept* **78,** 57-67.

Ran, X., Li, W., and Lin, H. (2004). Rat ghrelin stimulates GH release and mRNA expression in the pituitary of orange-spotted grouper, Epinephelus coioides. *Fish Physiol. Biochem* **30,** 95-102.

Raposinho, P. D., Castillo, E., d'Alleves, V., Broqua, P., Pralong, F. P., and Aubert, M. L. (2000). Chronic blockade of the melanocortin 4 receptor subtype leads to obesity independently of neuropeptide Y action, with no adverse effects on the gonadotropic and somatotropic axes. *Endocrinology* **141,** 4419-27.

Reddy, P. K., and Leatherland, J. F. (2003a). Influences of photoperiod and alternate days of feeding on plasma growth hormone and thyroid hormone levels in juvenile rainbow trout. *Journal of Fish Biology* **63,** 197-212.

Reddy, P. K., and Leatherland, J. F. (2003b). Influences of photoperiod and alternate days of feeding on plasma growth hormone and thyroid hormone levels in juvenile rainbow trout *Journal of Fish Biology* **63,** 197-212.

Riley, L. G., Fox, B. K., Breves, J. P., Kaiya, H., Dorough, C. P., Hirano, T., and Grau, E. G. (2008). Absence of Effects of Short-Term Fasting on Plasma Ghrelin and Brain Expression of Ghrelin Receptors in the Tilapia, Oreochromis mossambicus. *Zoolog Sci* **25,** 821-7.

Riley, L. G., Fox, B. K., Kaiya, H., Hirano, T., and Grau, E. G. (2005). Long-term treatment of ghrelin stimulates feeding, fat deposition, and alters the GH/IGF-I axis in the tilapia, Oreochromis mossambicus. *Gen Comp Endocrinol* **142,** 234-40.

Rodgers, R. J., Halford, J. C., Nunes de Souza, R. L., Canto de Souza, A. L., Piper, D. C., Arch, J. R., Upton, N., Porter, R. A., Johns, A., and Blundell, J. E. (2001). SB-334867, a selective orexin-1 receptor antagonist, enhances behavioural satiety and blocks the hyperphagic effect of orexin-A in rats. *Eur J Neurosci* **13,** 1444-52.

Rogers, K. V., Vician, L., Steiner, R. A., and Clifton, D. K. (1988). The effect of hypophysectomy and growth hormone administration on pre-prosomatostatin messenger ribonucleic acid in the periventricular nucleus of the rat hypothalamus. *Endocrinology* **122,** 586-91.

Rohner-Jeanrenaud, F., Craft, L. S., Bridwell, J., Suter, T. M., Tinsley, F. C., Smiley, D. L., Burkhart, D. R., Statnick, M. A., Heiman, M. L., Ravussin, E., and Caro, J. F. (2002). Chronic central infusion of cocaine- and amphetamine-regulated transcript (CART 55-102): effects on body weight homeostasis in lean and high-fat-fed obese rats. *Int J Obes Relat Metab Disord* **26,** 143-9.

Rossi, M., Kim, M. S., Morgan, D. G., Small, C. J., Edwards, C. M., Sunter, D., Abusnana, S., Goldstone, A. P., Russell, S. H., Stanley, S. A., Smith, D. M., Yagaloff, K., Ghatei, M. A., and Bloom, S. R. (1998). A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. *Endocrinology* **139,** 4428-31.

Rousseau, K., and Dufour, S. (2007). Comparative aspects of GH and metabolic regulation in lower vertebrates. *Neuroendocrinology* **86,** 165-74.

Rousseau, K., Le Belle, N., Pichavant, K., Marchelidon, J., Chow, B. K., Boeuf, G., and Dufour, S. (2001). Pituitary growth hormone secretion in the turbot, a phylogenetically recent teleost, is regulated by a species-specific pattern of neuropeptides. *Neuroendocrinology* **74,** 375-85.

Rushing, P. A., Gibbs, J., and Geary, N. (1996). Brief, meal-contingent infusions of gastrin-releasing peptide1-27 and neuromedin B-10 inhibit spontaneous feeding in rats. *Physiol Behav* **60,** 1501-4.

Rushing, P. A., Hagan, M. M., Seeley, R. J., Lutz, T. A., D'Alessio, D. A., Air, E. L., and Woods, S. C. (2001). Inhibition of central amylin signaling increases food intake and body adiposity in rats. *Endocrinology* **142,** 5035.

Sakata, I., Mori, T., Kaiya, H., Yamazaki, M., Kangawa, K., Inoue, K., and Sakai, T. (2004). Localization of ghrelin-producing cells in the stomach of the rainbow trout (Oncorhynchus mykiss). *Zoolog Sci* **21,** 757-62.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski, G. P., Wilson, S., Arch, J. R., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu, W. S., Terrett, J. A., Elshourbagy, N. A., Bergsma, D. J., and Yanagisawa, M. (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* **92,** 573-85.

Sato, M., Nakahara, K., Goto, S., Kaiya, H., Miyazato, M., Date, Y., Nakazato, M., Kangawa, K., and Murakami, N. (2006). Effects of ghrelin and des-acyl ghrelin on neurogenesis of the rat fetal spinal cord. *Biochem Biophys Res Commun* **350,** 598-603.

Schmid, D. A., Held, K., Ising, M., Uhr, M., Weikel, J. C., and Steiger, A. (2005). Ghrelin stimulates appetite, imagination of food, GH, ACTH, and cortisol, but does not affect leptin in normal controls. *Neuropsychopharmacology* **30,** 1187-92.

Schwalme, K., and Chouinard, G. A. (1999). Seasonal dynamics in feeding, organ weights, and reproductive maturation of Atlantic cod (*Gadus morhua*) in the southern Gulf of St Lawrence. *ICES Journal of Marine Science* **56,** 303-319.

Schwartz, M. W., Baskin, D. G., Kaiyala, K. J., and Woods, S. C. (1999). Model for the regulation of energy balance and adiposity by the central nervous system. *Am J Clin Nutr* **69,** 584-96.

Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J., and Baskin, D. G. (2000). Central nervous system control of food intake. *Nature* **404,** 661-71.

Seoane, L. M., Al-Massadi, O., Lage, M., Dieguez, C., and Casanueva, F. F. (2004). Ghrelin: from a GH-secretagogue to the regulation of food intake, sleep and anxiety. *Pediatr Endocrinol Rev* **1 Suppl 3,** 432-7.

Seoane, L. M., Lopez, M., Tovar, S., Casanueva, F. F., Senaris, R., and Dieguez, C. (2003). Agouti-related peptide, neuropeptide Y, and somatostatin-producing neurons are targets for ghrelin actions in the rat hypothalamus. *Endocrinology* **144,** 544-51.

Shahbazi, F., Holmgren, S., Larhammar, D., and Jensen, J. (2002). Neuropeptide Y effects on vasorelaxation and intestinal contraction in the Atlantic cod Gadus morhua. *Am J Physiol Regul Integr Comp Physiol* **282,** R1414-21.

Shepherd, B. S., Johnson, J. K., Silverstein, J. T., Parhar, I. S., Vijayan, M. M., McGuire, A., and Weber, G. M. (2007). Endocrine and orexigenic actions of growth hormone secretagogues in rainbow trout (Oncorhynchus mykiss). *Comp Biochem Physiol A Mol Integr Physiol* **146,** 390-9.

Sheppard, M. C., Kronheim, S., and Pimstone, B. L. (1978). Stimulation by growth hormone of somatostatin release from the rat hypothalamus in vitro. *Clinical Endocrinology* **9,** 583-6.

Sherwood, N. M., Krueckl, S. L., and McRory, J. E. (2000). The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev* **21,** 619-70.

Shibahara, M., Sakurai, T., Nambu, T., Takenouchi, T., Iwaasa, H., Egashira, S. I., Ihara, M., and Goto, K. (1999). Structure, tissue distribution, and pharmacological characterization of Xenopus orexins. *Peptides* **20,** 1169-76.

Shuto, Y., Shibasaki, T., Otagiri, A., Kuriyama, H., Ohata, H., Tamura, H., Kamegai, J., Sugihara, H., Oikawa, S., and Wakabayashi, I. (2002). Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. *J Clin Invest* **109,** 1429-36.

Siikavuopio, S. I., Sæthera, B. S., and Aasa, K. (2008). Growth in juvenile Atlantic cod (*Gadus morhua* L.) fed continuously or during light or dark hours *Aquaculture* **278,** 192-194.

Silverstein, J. T., Breininger, J., Baskin, D. G., and Plisetskaya, E. M. (1998). Neuropeptide Y-like gene expression in the salmon brain increases with fasting. *Gen Comp Endocrinol* **110,** 157-65.

Silverstein, J. T., and Plisetskaya, E. M. (2000). The effects of NPY and insulin on food intake regulation in fish. *Am. Zool* **40,** 296-308.

Sindelar, D. K., Palmiter, R. D., Woods, S. C., and Schwartz, M. W. (2005). Attenuated feeding responses to circadian and palatability cues in mice lacking neuropeptide Y. *Peptides* **26,** 2597-602.

Singletary, K. G., Delville, Y., Farrell, W. J., and Wilczynski, W. (2005). Distribution of orexin/hypocretin immunoreactivity in the nervous system of the green Treefrog, Hyla cinerea. *Brain Res* **1041,** 231-6.

Skjæraasen, J. E., Salvanes, A. G. V., Karlsen, Ø., Dahle, R., Nilsen, T., and Norberg, B. (2004). The Effect of Photoperiod on Sexual Maturation, Appetite and Growth in Wild Atlantic Cod (*Gadus morhua* L.) *Fish Physiology and Biochemistry* **30,** 163-174.

Small, B., H., J., Soures, J., Curry Woods III, L., and GE., G. (2002). Effect of fasting on pituitary growth hormone expression and circulating growth hormone levels in striped bass. *North American Journal of Acquaculture* **64,** 278-283.

Small, B. C., and Nonneman, D. (2001). Sequence and expression of a cDNA encoding both pituitary adenylate cyclase activating polypeptide and growth hormone-releasing hormone-like peptide in channel catfish (*Ictalurus punctatus*). *General and Comparative Endocrinology* **122,** 354-63.

Solomon, A., De Fanti, B. A., and Martinez, J. A. (2005). Peripheral ghrelin participates in glucostatic feeding mechanisms and in the anorexigenic signalling mediated by CART and CRF neurons. *Nutr Neurosci* **8,** 287-95.

Song, Y., Golling, G., Thacker, T. L., and Cone, R. D. (2003). Agouti-related protein (AGRP) is conserved and regulated by metabolic state in the zebrafish, Danio rerio. *Endocrine* **22,** 257-65.

Stuckey, J. A., and Gibbs, J. (1982). Lateral hypothalamic injection of bombesin decreases food intake in rats. *Brain Res Bull* **8,** 617-21.

Sumpter, J. P., Le Bail, P. Y., Pickering, A. D., Pottinger, T. G., and Carragher, J. F. (1991). The effect of starvation on growth and plasma growth hormone concentrations of rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **83,** 94-102.

Sun, Y., Ahmed, S., and Smith, R. G. (2003). Deletion of ghrelin impairs neither growth nor appetite. *Mol Cell Biol* **23,** 7973-81.

Sun, Y., Wang, P., Zheng, H., and Smith, R. G. (2004). Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci U S A* **101,** 4679-84.

Sunuma, T., Amano, M., Yamanome, T., Furukawa, K., and Yamamori, K. (2007). Self-feeding activity of a pleuronectiform fish, the barfin flounder. *Aquaculture* **270,** 566-569.

Suzuki, H., Miyoshi, Y., and Yamamoto, T. (2006). Orexin-A (hypocretin 1)-like immunoreactivity in growth hormone-containing cells of the Japanese seaperch (Lateolabrax japonicus) pituitary. *Gen Comp Endocrinol.*

Suzuki, H., Miyoshi, Y., and Yamamoto, T. (2007). Orexin-A (hypocretin 1)-like immunoreactivity in growth hormone-containing cells of the Japanese seaperch (Lateolabrax japonicus) pituitary. *Gen Comp Endocrinol* **150,** 205-11.

Tachibana, T., Saito, S., Tomonaga, S., Takagi, T., Saito, E. S., Boswell, T., and Furuse, M. (2003). Intracerebroventricular injection of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibits feeding in chicks. *Neuroscience Letters* **339,** 203-6.

Taheri, S., Mahmoodi, M., Opacka-Juffry, J., Ghatei, M. A., and Bloom, S. R. (1999). Distribution and quantification of immunoreactive orexin A in rat tissues. *FEBS Lett* **457,** 157-61.

Takahashi, N., Okumura, T., Yamada, H., and Kohgo, Y. (1999). Stimulation of gastric acid secretion by centrally administered orexin-A in conscious rats. *Biochem Biophys Res Commun* **254,** 623-7.

Tam, J. K., Lee, L. T., and Chow, B. K. (2007). PACAP-related peptide (PRP)--molecular evolution and potential functions. *Peptides* **28,** 1920-9.

Tannenbaum, G. S., Epelbaum, J., and Bowers, C. Y. (2003). Interrelationship between the novel peptide ghrelin and somatostatin/growth hormone-releasing hormone in regulation of pulsatile growth hormone secretion. *Endocrinology* **144,** 967-74.

Tannenbaum, G. S., Gurd, W., and Lapointe, M. (1998). Leptin is a potent stimulator of spontaneous pulsatile growth hormone (GH) secretion and the GH response to GH-releasing hormone. *Endocrinology* **139,** 3871-5.

Tannenbaum, G. S., Painson, J. C., Lengyel, A. M., and Brazeau, P. (1989). Paradoxical enhancement of pituitary growth hormone (GH) responsiveness to GH-releasing factor in the face of high somatostatin tone. *Endocrinology* **124,** 1380-8.

Taranger, G. L., Aardal, L., Hansen, T., and Kjesbu, O. S. (2006). Continuous light delays sexual maturation and increases growth of Atlantic cod (*Gadus morhua* L.) in sea cages. *ICES Journal of Marine Science* **63,** 365-375.

Tatemoto, K., Carlquist, M., and Mutt, V. (1982). Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* **296,** 659-60.

Terova, G., Rimoldi, S., Bernardini, G., Gornati, R., and Saroglia, M. (2008). Sea bass ghrelin: molecular cloning and mRNA quantification during fasting and refeeding. *Gen Comp Endocrinol* **155,** 341-51.

Toshinai, K., Date, Y., Murakami, N., Shimada, M., Mondal, M. S., Shimbara, T., Guan, J. L., Wang, Q. P., Funahashi, H., Sakurai, T., Shioda, S., Matsukura, S., Kangawa, K., and Nakazato, M. (2003). Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology* **144,** 1506-12.

Tostivint, H., Joly, L., Lihrmann, I., Ekker, M., and Vaudry, H. (2004). Chromosomal localization of three somatostatin genes in zebrafish. Evidence that the [Pro2]-somatostatin-14 isoform and cortistatin are encoded by orthologous genes. *Journal of Molecular Endocrinology* **33,** R1-8.

Tostivint, H., Lihrmann, I., and Vaudry, H. (2008). New insight into the molecular evolution of the somatostatin family. *Mol Cell Endocrinol* **286,** 5-17.

Tucker, B. J., Booth, M. A., Allan, G. L., Booth, D., and Fielder, D. S. (2006). Effects of photoperiod and feeding frequency on performance of newly weaned Australian snapper Pagrus auratus. *Aquaculture* **258,** 514-520.

Turker, A., Yigit, M., and Ergun, S. (2005). Growth and Feed Utilization in Juvenile Black Sea Turbot (*Psetta maeotica*) under Different Photoperiod Regimes. *Turkish Journal of Veterinary and Animal Society* **29,** 1203-1208.

Unniappan, S., Canosa, L. F., and Peter, R. E. (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,** 100-8.

Unniappan, S., Lin, X., Cervini, L., Rivier, J., Kaiya, H., Kangawa, K., and Peter, R. E. (2002). Goldfish ghrelin: molecular characterization of the complementary

deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. *Endocrinology* **143,** 4143-6.

Unniappan, S., and Peter, R. E. (2004). In vitro and in vivo effects of ghrelin on luteinizing hormone and growth hormone release in goldfish. *Am J Physiol Regul Integr Comp Physiol* **286,** R1093-101.

Very, N. M., Knutson, D., Kittilson, J. D., and Sheridan, M. A. (2001). Somatostatin inhibits growth of rainbow trout. *J. Fish Biol* **59,** 157-165.

Vettor, R., Fabris, R., Pagano, C., and Federspil, G. (2002). Neuroendocrine regulation of eating behavior. *J Endocrinol Invest* **25,** 836-54.

Vicentic, A., Lakatos, A., Hunter, R., Philpot, K., Dominguez, G., and Kuhar, M. J. (2005). CART peptide diurnal rhythm in brain and effect of fasting. *Brain Res* **1032,** 111-5.

Volkoff, H., Bjorklund, J. M., and Peter, R. E. (1999). Stimulation of feeding behavior and food consumption in the goldfish, Carassius auratus, by orexin-A and orexin-B. *Brain Res* **846,** 204-9.

Volkoff, H., Canosa, L. F., Unniappan, S., Cerda-Reverter, J. M., Bernier, N. J., Kelly, S. P., and Peter, R. E. (2005). Neuropeptides and the control of food intake in fish. *Gen Comp Endocrinol* **142,** 3-19.

Volkoff, H., Eykelbosh, A. J., and Peter, R. E. (2003). 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 Res* **972,** 90-109.

Volkoff, H., and Peter, R. E. (2000). Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, Carassius auratus: actions on neuropeptide Y- and orexin A-induced feeding. *Brain Res* **887,** 125-33.

Volkoff, H., and Peter, R. E. (2001a). Characterization of two forms of cocaine- and amphetamine-regulated transcript (CART) peptide precursors in goldfish: molecular cloning and distribution, modulation of expression by nutritional status, and interactions with leptin. *Endocrinology* **142,** 5076-88.

Volkoff, H., and Peter, R. E. (2001b). Interactions between orexin A, NPY and galanin in the control of food intake of the goldfish, Carassius auratus. *Regul Pept* **101,** 59-72.

Volkoff, H., and Peter, R. E. (2004). Effects of lipopolysaccharide treatment on feeding of goldfish: role of appetite-regulating peptides. *Brain Res* **998,** 139-47.

Volkoff, H., Peyon, P., Lin, X., and Peter, R. E. (2000). Molecular cloning and expression of cDNA encoding a brain bombesin/gastrin-releasing peptide-like peptide in goldfish. *Peptides* **21,** 639-48.

Wagner, C., Caplan, S. R., and Tannenbaum, G. S. (1998). Genesis of the ultradian rhythm of GH secretion: a new model unifying experimental observations in rats. *Am J Physiol* **275,** E1046-54.

Walsh, J. H., Kovacs, T. O., Maxwell, V., and Cuttitta, F. (1988). Bombesin-like peptides as regulators of gastric function. *Ann N Y Acad Sci* **547,** 217-24.

Wank, S. A. (1995). Cholecystokinin receptors. *Am J Physiol* **269,** G628-46.

Weigert, N., Li, Y. Y., Lippl, F., Coy, D. H., Classen, M., and Schusdziarra, V. (1996). Role of endogenous bombesin-peptides during vagal stimulation of gastric acid secretion in the rat. *Neuropeptides* **30,** 521-7.

Wierup, N., Kuhar, M., Nilsson, B. O., Mulder, H., Ekblad, E., and Sundler, F. (2004). Cocaine- and amphetamine-regulated transcript (CART) is expressed in several islet cell types during rat development. *J Histochem Cytochem* **52,** 169-77.

Williams, G., Cai, X. J., Elliott, J. C., and Harrold, J. A. (2004). Anabolic neuropeptides. *Physiol Behav* **81,** 211-22.

Wong, A. O., Leung, M. Y., Shea, W. L., Tse, L. Y., Chang, J. P., and Chow, B. K. (1998). Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. *Endocrinology* **139,** 3465-79.

Wong, A. O., Li, W., Leung, C. Y., Huo, L., and Zhou, H. (2005). Pituitary adenylate cyclase-activating polypeptide (PACAP) as a growth hormone (GH)-releasing factor in grass carp. I. Functional coupling of cyclic adenosine 3',5'-monophosphate and Ca2+/calmodulin-dependent signaling pathways in PACAP-induced GH secretion and GH gene expression in grass carp pituitary cells. *Endocrinology* **146,** 5407-24.

Wong, A. O., Li, W. S., Lee, E. K., Leung, M. Y., Tse, L. Y., Chow, B. K., Lin, H. R., and Chang, J. P. (2000). Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. *Biochemistry and Cell Biology* **78,** 329-43.

Woods, S. C., Porte, D., Jr., Bobbioni, E., Ionescu, E., Sauter, J. F., Rohner-Jeanrenaud, F., and Jeanrenaud, B. (1985). Insulin: its relationship to the central nervous system and to the control of food intake and body weight. *Am J Clin Nutr* **42,** 1063-71.

Wortley, K. E., Anderson, K. D., Garcia, K., Murray, J. D., Malinova, L., Liu, R., Moncrieffe, M., Thabet, K., Cox, H. J., Yancopoulos, G. D., Wiegand, S. J., and Sleeman, M. W. (2004). Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. *Proc Natl Acad Sci U S A* **101,** 8227-32.

Wren, A. M., Small, C. J., Fribbens, C. V., Neary, N. M., Ward, H. L., Seal, L. J., Ghatei, M. A., and Bloom, S. R. (2002). The hypothalamic mechanisms of the hypophysiotropic action of ghrelin. *Neuroendocrinology* **76,** 316-24.

Xiao, D., Chu, M. M., Lee, E. K., Lin, H. R., and Wong, A. O. (2002). Regulation of growth hormone release in common carp pituitary cells by pituitary adenylate cyclase-activating polypeptide: signal transduction involves cAMP- and calcium-dependent mechanisms. *Neuroendocrinology* **76,** 325-38.

Xing, Y., Wensheng, L., and Haoran, L. (2005). Polygenic expression of somatostatin in orange-spotted grouper (*Epinephelus coioides*): molecular cloning and distribution of the mRNAs encoding three somatostatin precursors. *Molecular and Cellular Endocrinology* **241,** 62-72.

Xu, M., and Volkoff, H. (2007). Molecular characterization of prepro-orexin in Atlantic cod (Gadus morhua): cloning, localization, developmental profile and role in food intake regulation. *Mol Cell Endocrinol* **271,** 28-37.

Yamada, H., Okumura, T., Motomura, W., Kobayashi, Y., and Kohgo, Y. (2000). Inhibition of food intake by central injection of anti-orexin antibody in fasted rats. *Biochem Biophys Res Commun* **267,** 527-31.

Yamamoto, T., Suzuki, H., Uemura, H., Yamamoto, K., and Kikuyama, S. (2004). Localization of orexin-A-like immunoreactivity in prolactin cells in the bullfrog (Rana catesbeiana) pituitary. *Gen Comp Endocrinol* **135,** 186-92.

Yamauchi, T., Kaburagi, Y., Ueki, K., Tsuji, Y., Stark, G. R., Kerr, I. M., Tsushima, T., Akanuma, Y., Komuro, I., Tobe, K., Yazaki, Y., and Kadowaki, T. (1998). Growth hormone and prolactin stimulate tyrosine phosphorylation of insulin receptor substrate-1, -2, and -3, their association with p85 phosphatidylinositol 3-kinase (PI3-kinase), and concomitantly PI3-kinase activation via JAK2 kinase. *J Biol Chem* **273,** 15719-26.

Yamazaki, M., Nakamura, K., Kobayashi, H., Matsubara, M., Hayashi, Y., Kangawa, K., and Sakai, T. (2002). Regulational effect of ghrelin on growth hormone secretion from perifused rat anterior pituitary cells. *J Neuroendocrinol* **14,** 156-62.

Yegen, B. C., Gurbuz, V., Coskun, T., Bozkurt, A., Kurtel, H., Alican, I., and Dockray, G. J. (1996). Inhibitory effects of gastrin releasing peptide on gastric emptying in rats. *Regul Pept* **61,** 175-80.

Yoo, S. J., You, S., Kim, H., Kim, S. C., Choi, Y. J., El Halawani, M., Farris, J., and Foster, D. N. (2000). Molecular cloning and characterization of alternatively spliced transcripts of the turkey pituitary adenylate cyclase-activating polypeptide. *General and Comparative Endocrinology* **120,** 326-35.

Yunker, W. K., Smith, S., Graves, C., Davis, P. J., Unniappan, S., Rivier, J. E., Peter, R. E., and Chang, J. P. (2003). Endogenous hypothalamic somatostatins differentially regulate growth hormone secretion from goldfish pituitary somatotropes in vitro. *Endocrinology* **144,** 4031-41.

Zhang, J. V., Ren, P. G., Avsian-Kretchmer, O., Luo, C. W., Rauch, R., Klein, C., and Hsueh, A. J. (2005). Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* **310,** 996-9.

Zhao, Z. J., and Wang, D. H. (2006). Short photoperiod influences energy intake and serum leptin level in Brandt's voles (Microtus brandtii). *Horm Behav* **49,** 463-9.

Zhu, Y., Yamanaka, A., Kunii, K., Tsujino, N., Goto, K., and Sakurai, T. (2002). Orexin-mediated feeding behavior involves both leptin-sensitive and -insensitive pathways. *Physiol Behav* **77,** 251-7.