NPY, CART AND CCK IDENTIFICATION IN WINTER FLOUNDER (PLEURONECTES AMERICANUS) AND WINTER SKATE (RAJA OCELLATA): MOLECULAR CLONING, TISSUE DISTRIBUTION AND RESPONSE TO SEASON AND FASTING

ERIN E. MacDONALD

NPY, CART AND CCK IDENTIFICATION IN WINTER FLOUNDER (*PLEURONECTES AMERICANUS*) AND WINTER SKATE (*RAJA OCELLATA*): MOLECULAR CLONING, TISSUE DISTRIBUTION AND RESPONSE TO SEASON AND FASTING

by

Erin E. MacDonald

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

Department of Biology

Memorial University of Newfoundland

August 2008

St. John's Newfoundland and Labrador

Abstract

In fish, as in all vertebrates, feeding is regulated by complex interactions between brain and peripheral hormonal signals. Brain signals include neuropeptide Y (NPY) and cocaine-amphetamine-regulated transcript (CART), which induces and decreases food intake, respectively. Cholecystokinin (CCK) is a peripheral hormone produced by the intestine that inhibits appetite. The winter flounder, *Pleuronectes americanus* (Teleostei) and the winter skate, *Raja ocellata* (Elasmobranchii) are two bottom-dwelling marine fish species inhabiting the coasts of Newfoundland. Both species undergo seasonal cycles in feeding and growth. Nothing is known about the structure or function of NPY, CART and CCK in these species or about the role that these peptides might have in mediating seasonal feeding patterns. In order to characterize NPY, CART and CCK in winter flounder and winter skate, I have identified cDNAs encoding these hormones in both species using RT-PCR and rapid amplification of cDNA ends (RACE). Tissue distribution studies using RT-PCR show that all three peptides are expressed in brain and peripheral tissues, including gut. Gene expression quantification using real-time RT-PCR indicates that these peptides might be involved in nutritional and seasonal feeding adaptations in these two species.

Acknowledgments

I would like to sincerely thank my supervisor Dr. Hélène Volkoff for her endless words of encouragement, especially when I had trouble with my cloning. I'd like to thank my lab mates Leah Hoskins and Meiyu Xu for answering my endless questions. I thank my supervisory committee Dr. Brian Staveley and Dr. Dawn Marshall for all their support and Dr. Lang's lab for saving me hours of quantification by letting me use their NanoDrop. This work would not have been possible without funding from NSERC and Memorial University. Last but not least, many thanks to my parents who pretended to know what I was talking about when I called to complain that none of my PCRs were working.

Table of Contents

List of Tables

List of Figures

Introduction

In vertebrates, as in all living organisms, there is a constant struggle to achieve homeostasis. Homeostasis occurs when the internal environment is maintained within endurable limits. This is achieved through the dynamic equilibrium between the nervous system and the endocrine system. Information about changes that occur in the periphery of the body is relayed via afferent signals (both nervous and endocrine) to the central nervous system. The brain deciphers and interprets these peripheral messages and initiates the appropriate response to maintain homeostasis. In fish, as in all vertebrates, the brain region referred to as the hypothalamus links the nervous system to the endocrine system. Termed the "master gland", the hypothalamus produces and secretes releasing hormones that control the secretion of other hormones from the pituitary gland. Together these two glands control the animal's major metabolic processes.

In order for energy homeostasis to be established, an animal needs to balance its energy intake with its energy output. Feeding satisfies energy intake and is regulated by signals to the brain (Volkoff 2006). The balance between signals to the brain to increase and decrease appetite is intimately controlled by a number of hypothalamic appetite regulating hormones, including neuropeptide Y (NPY), orexin, cocaine-amphetamine-regulated transcript (CART) and cholecystokinin (CCK). Orexigenic factors, such as NPY and orexin, stimulate appetite and anorexigenic factors, such as CART and CCK, suppress appetite. Appetite regulation is controlled by a number of complex interactions between these neuropeptide systems. For example, NPY and orexin act synergistically to cause an increase in food intake and, in turn they are both regulated by CART (Volkoff *et*

al. 2001). These central systems are also regulated by peripheral endocrine factors. CCK produced by endocrine cells in the intestine and insulin produced by the pancreas are peripheral hormones that inhibit food intake by acting in part on brain feeding centers (Volkoff *et al.* 2003) .

The brain receives both hormonal (endocrine) and metabolic signals from the periphery of the body to initiate or to stop feeding (Volkoff 2006). These signals can travel via the circulatory system, enter the brain and bind to central receptors or bind to peripheral receptors and transmit their information via the vagus nerve. The hypothalamus processes these signals and releases its own endocrine signals that travel to other areas of the brain or body and elicit the appropriate response. Many of these signals have been characterized using molecular cloning paired with gene expression studies and by peptide injections followed by measurement of food intake and feeding behaviour.

NPY was the first orexigenic neuropeptide discovered over 20 years ago, and is considered the most potent orexigenic factor in vertebrates, including fish (Tatemoto 1982). It has been extensively studied in mammals and characterized in a number of fish species. CCK and CART have only been discovered in recent years so little information is known on the structure and function of these hormones.

Research on appetite regulating hormones in fish is important to establish a model of hormone function and interactions in vertebrates; especially where so little is currently known for some of the most recently discovered hormones. Studying the neuroendocrine regulation of food intake in fish will contribute to the overall understanding of feeding

behaviour in animals. Structural comparison of hormones and hormone receptors can provide information on how these structures evolved in fish and mammals. A fish model may be developed and used to explain human health problems related to the dysregulation of homeostasis like diabetes and obesity. Fish represent good experimental model for this type of study because they have various important advantages over mammals. They display a wide range of feeding behaviours, diets, and feeding adaptations making them good candidates to study the evolution of appetite regulating systems. Fish are also easier to maintain and submit to repeated sampling without affecting behaviour or feeding. Information on appetite regulating mechanisms in fish should provide insights on how to manage the feeding and growth of fish for the aquaculture industry.

Studying appetite regulating hormones and feeding

Feeding is a complex behaviour regulated by a number of factors. These include for example, how the animal obtains its food, modes of feeding and food detection and frequency of feeding (Volkoff *et al.* 2006). In fish, the study of feeding behavior is a complex topic since feeding can be altered by environmental conditions, physiological conditions and genetics. Environmental conditions include photoperiod, temperature, water salinity and rearing conditions. For example, some fish species feed less in colder temperatures, or stop feeding altogether in the winter. Some fish species in captivity have increased food intake compared to those in the wild and a longer photoperiod can

cause an increase in food intake (Imsland *et al.* 2001). Clearly, there are many factors to consider when studying appetite regulation.

Information on the neural control of feeding has recently become more available in fish. Early studies used either electrical stimulation of the brain, or brain lesions. In rodents, lesions in the ventromedial hypothalamus (VMH) increased food intake and appetite, whereas lesions in the lateral hypothalamic area (LHA) decreased food intake (Horvath *et al.* 1999). Lesions between the VMH and the LHA or other brain regions caused an increase in feeding but not to the same extent as complete VMH lesions (Tokunaga *et al.* 1986). The results of these studies firmly support the idea that appetite is regulated by different areas of the brain. In both teleost and elasmobranch fish, stimulation of either the inferior lobes of the hypothalamus or the telencephalon has been shown to induce feeding (Demski *et al.* 1971; Demski 1973; Roberts *et al.* 1978). These early studies using electrical brain stimulation and brain lesions have been followed by more recent studies focused on the molecular and functional characterization of neuropeptides produced by feeding centers within the brain.

Neurohormones and the hypothalamus

Specific regions can be distinguished within the vertebrate hypothalamus (Williams *et al.* 2001). The arcuate nucleus (ARC), which occupies roughly half of the hypothalamus, displays two distinct but interacting neuron populations: the neuropeptide Y and agouti gene-related protein (NPY/AGRP) neurons and the pro-opiomelanocortin and cocaine-and-amphetamine-regulated transcript (POMC/CART) neurons. NPY and

AgRP are both appetite stimulating hormones whereas CART and POMC are both appetite suppressing hormones. The ARC has elaborate connections with other hypothalamic areas such as the paraventricular nucleus (PVN), the dorsomedial hypothalamic nucleus (DMH), the ventromedial hypothalamic nucleus (VHN) and the lateral hypothalamus (LH). The ARC is one area of seven areas of the brain that lack the blood brain barrier. Therefore it is accessed by peripheral hormonal signals from the body, such as leptin, insulin and glucose.

The PVN is stimulated by neuronal axons projecting from the NPY/AGRP and POMC/CART ARC nucleus regions (Williams *et al.* 2001). The VMH is one of the largest hypothalamic nuclei and functions as the appetite inhibiting center. Leptin receptors have been found in abundance in this area, so blood-circulating leptin may be a good target for this brain area.

Physiologically, appetite is regulated by hormone levels in the blood and neural inputs that converge on the brain. These stimuli are processed by the brain, and the brain responds by secreting orexigenic or anorexigenic regulatory hormones. By studying appetite regulating peptides, the mechanisms that regulate homeostasis can be better understood.

Neuropeptide Y

Neuropeptide Y is a member of the peptide family that also includes pancreatic polypeptide and peptide YY. Each of these peptides has a specific amino acid sequence that allows it to take the necessary functional three-dimensional structure called the

pancreatic polypeptide fold (Cerda-Reverter *et al.* 2000). NPY is a 36 amino acid peptide which was originally discovered in the porcine brain (Tatemoto 1982). Since its discovery, it has become known as one of the most potent orexigenic neuropeptides discovered to date. NPY is a complex hormone because of its diverse physiological effects and because it has a number of different receptor subtypes known as the Y receptors. Both NPY mRNA and Y receptors have been identified in numerous mammals, birds, amphibians and fish (Cerda-Reverter *et al.* 2000). The various receptors differ in their peptide selectivity.

In mammals, NPY is present in both brain and peripheral tissues. Within the brain, NPY neurons are found in the ARC nucleus of the hypothalamus (Fetissov *et al.* 2004) where they co-localize with agouti related peptide (Agrp) (Akabayashi *et al.* 1994). NPY is widely expressed in the peripheral tissues including the gut, intestine and cardiovascular tissue (Bjenning *et al.* 1993; Preston *et al.* 1998). The wide localization of NPY throughout the body might explain why it has been implicated in the regulation of numerous physiological processes including body temperature, blood pressure and reproduction (Gehlert 1999). However it remains most effective as an appetite regulator.

NPY was first isolated from a non-mammalian brain in goldfish, *Carassius auratus* (Peng *et al.* 1994). NPY has been isolated and characterized in a number of species, including fish such as sea bass, *Dicentrarcus labrax* (Cerda-Reverter *et al.* 2000), halibut, *Paralichthys olivaceus* (GenBank accession number AB055211), perch, *Siniperca chuatsi* (Liang *et al.* 2007), trout, *Oncorhynchus mykiss* (Doyon *et al.* 2003), cod, *Gadus morhua* (Kehoe *et al.* 2007), goldfish (Blomqvist *et al.* 1992), catfish,

Ictalurus punctatus (GenBank accession number AF267164), dogfish, *Scyliorhinus canicula* (Conlon *et al.* 1992), ray, *Torpedo marmorata* (Blomqvist *et al.* 1992) and lampreys (*Petromyzon marinus*, *Icthyomyzon gagei*) (Montpetit *et al.* 2005). NPY shows good conservation among species. Both the human and lower vertebrate NPY gene is located next to the homeobox (hox) gene cluster A (copy A in zebrafish) (Soderberg *et al.* 2000). The Hox clusters have been inherited nearly intact for 400 million years. By mapping NPY gene beside the homobox gene supports the theory that they arose from a common ancestral gene.

NPY is a ligand for the G-protein coupled NPY-family receptors that consist of Y1, Y2, Y4, Y5 and Y6 (Volkoff 2006). The Y1 subfamily is the main subfamily and includes the Y1, Y4, and Y6 receptor subtypes. Of the 5 identified receptors, Y1 and Y5 appear to be the most biologically important because they regulate the orexigenic effects of the NPY family of peptides in mammals.

NPY receptors have been identified in fish, and the majority of them appear to be members of the Y1 family receptors. Three different NPY receptors have been identified in zebrafish, (*Danio rerio*), which were named zYa, zYb and zYc (Arvidsson *et al.* 1998). The Yb was cloned from Atlantic cod and rainbow trout (Arvidsson *et al.* 1998). Recently, Y2 and Y7 have also been cloned from rainbow trout (Larsson *et al.* 2006). The Y4 subtype was cloned in river lamprey (*Lampetra fluviatilis*) (Salaneck *et al.* 2001), and Y1, Y4 and Y6 were all identified in the spiny dogfish (*Squalus acanthias*) (Salaneck *et al.* 2003). Receptors Y5 and Y6 were identified in two coelacanth species, *Latimeria chalumnae* and *Latimeria menadoensis*, which are lobed finned fish (Larsson *et al.* 2007).

The presence of the Y receptors in the lobed finned fish indicates that there is an early vertebrate origin for the NPY receptors. At the same time, the diversity of receptors found among species indicates there may have been some differential loss of genes in each vertebrate class. If receptors are found evolutionarily before and after a particular species, this species must have undergone its own loss of that receptor encoding gene.

Several studies provide evidence that NPY action is regulated through the Y receptor family. In rodents, NPY injections increase food intake whereas treatment with NPY Y5 antagonists decreases feeding (Yokosuka *et al.* 2001). In rodents, ARC NPY mRNA expression has been shown to decrease after starvation (Davies *et al.* 1994). Rats deprived of food for a 48 hour period and then fed display a drastic increase in NPY expression in the hypothalamus especially in the PVN and to an even greater extent in the ARC nucleus (Beck *et al.* 1990). In regularly fed rats, when antisense NPY RNA is expressed in the brain there is an overall decrease in feeding and weight gain (Gardiner *et al.* 2005). Similarily Akabayashi and colleagues (Akabayashi *et al.* 1994) found that antisense oligodeoxynucleotides (ODNs) injected in rat brains resulted in a decrease in feeding behaviour compared to untreated rats (Akabayashi *et al.* 1994). NPY must have a role in appetite regulation.

Y1 receptor-deficient rats have no major abnormalities in body weight or feeding early in their lives. But they do display obesity when they are older, suggesting that the Y1 receptor might not be the only receptor controlling food intake (Kushi *et al.* 1998). Schaffhauser and colleagues (Schaffhauser *et al.* 1997) injected Y5 antagonists or Y5 antisense ODNs into rats which blocked the NPY-induced feeding, suggesting an

important role for Y5 in feeding regulation. Futhermore, an NPY analog specifically designed to bind to Y5 caused an increase in food uptake (Hwa *et al.* 1999). The Y family receptors have shown roles in the regulation of feeding yet the exact function of each receptor is not well defined.

NPY appears to be an orexigenic factor in fish. Intracerebroventricular (ICV) injections in channel catfish (*Ictalurus punctatus*) (Silverstein *et al.* 2001) and goldfish (Lopez-Patino *et al.* 1999) cause an increase in food intake. In addition, ICV injections of NPY antagonist in starved goldfish decrease feeding (Lopez-Patino *et al.* 1999). Studies that use ICV injections of NPY are a good indication that NPY is orexigenic.

In pacific salmon, *Oncorhyncus* sp., fasted fish display higher NPY mRNA expression levels in the brain than fed fish (Silverstein *et al.* 1998). Not only does lack of food increase NPY expression, but low nutrient diets also cause an increase in NPY expression in the brain of goldfish (Narnaware *et al.* 2002). In goldfish, evidence suggests that NPY exerts its orexigenic actions through Y1 and Y5 receptors because central injections of Y1 or Y5 agonists cause dose-dependent feeding (Narnaware *et al.* 2001). When fish were given ICV injections of both agonists together, they displayed a synergistic effect and feeding rate increased to higher than the levels reached by one agonist injection alone (Narnaware *et al.* 2001). NPY might have other physiological roles in fish. In dogfish a Y1 agonist elicited a response in vasoconstriction over the Y2 receptor (Bjenning *et al.* 1993). Desensitizing one receptor did not influence the reaction from the other. It is unclear precisely which receptors regulate feeding in fish due to evidence in recent experiments that many Y receptors are missing from teleosts.

NPY appears to interact with other appetite regulating hormones including orexin, CART, leptin, and insulin. In rats, injections of a NPY Y5 receptor antagonist fifteen minutes before injecting orexin A into the brain significantly decrease orexin-induced feeding, suggesting that NPY mediates the orexigenic actions of orexins (Dube *et al.* 2000). Similar interactions have been demonstrated in fish, as co-injections of NPY and orexin A into goldfish brain cause an increase in feeding higher than that induced by either peptide alone, suggesting a synergy between the two peptides (Volkoff *et al.* 2001). In addition, treatment with a NPY receptor antagonist caused an inhibition of orexin-induced feeding and blockade of orexin receptors results in an inhibition of NPY induced feeding (Volkoff *et al.* 2001), suggesting a close interaction between the two systems

When co-injected with NPY and orexin A or NPY and leptin, goldfish have a lower feeding rate than that of fish injected with either orexin A or NPY alone (Volkoff *et al.* 2003). In mammals, leptin receptors have been identified in the ARC nucleus (Elias *et al.* 1999) , suggesting that leptin closely regulates NPY synthesis and release. Indeed, mice with mutated leptin receptors are obese and have elevated NPY levels (Korner *et al.* 2001). NPY and insulin seem to have a similar dynamic as leptin and NPY. Insulin, injections into the brain of rats cause a decrease in the secretion of NPY (Sahu *et al.* 1995). These interactions among different appetite peptides demonstrate the complexity of the physiological systems surrounding feeding.

Cocaine-and amphetamine-regulated transcript

Cocaine-and amphetamine-regulated transcript (CART) was first discovered in rats (Douglass *et al.* 1995) as the transcript of a brain mRNA up-regulated following administration of two stimulants, cocaine and amphetamine (Douglass *et al.* 1995). CART has been shown to be involved in a number of physiological processes such as appetite regulation, endocrine functions and the stress response (Volkoff 2006).

To date, CART has been cloned from mammals (Douglass *et al.* 1995; Douglass *et al.* 1996; Adams *et al.* 1999), amphibians (Lazar *et al.* 2004) and fish, including goldfish (Volkoff *et al.* 2001), zebrafish (GenBank accession number BQ480503), spotted green pufferfish, (*Tetraodon fluviatilis*), (GenBank accession number CR688746), Japanese pufferfish (Fugu genome project, gene SINFRUT00000129073) and Atlantic cod (Kehoe *et al.* 2007). CART was mapped to chromosome 5 in humans and to chromosome 13 in the mouse (Adams *et al.* 1999). The CART gene is composed of three exons and two introns (Douglass *et al.* 1996). The mammalian prepro-CART mRNA can be cleaved to produce two forms of CART (Kuhar *et al.* 2002). CART mRNA is further cleaved into several biologically active forms, including CART (42-89) and CART (55-102), which are tissue specific (Thim *et al.* 1999). In goldfish, two different genes express two different CART isoforms (Volkoff *et al.* 2001); there is a 70% amino acid identity between the two preproCART forms and 76% identity between the two mature peptides. When examining CART in a new system its important to keep in mind that there can be different forms coded by different genes, or due to different splicing.

CART mRNA is expressed in a number of regions of the central nervous system including the hypothalamus. Within the hypothalamus, CART mRNA is expressed in many feeding areas including the PVN, the LHA and the ARC. The latter region displays the highest CART expression levels and co-localization of CART with POMC neurons (Broberger *et al.* 1999; Elias *et al.* 1999; Vrang *et al.* 1999; Larsen *et al.* 2003), suggesting a role for CART in feeding regulation. If the ARC nucleus is disrupted, there is a decrease in the immunoreactivity of CART peptides suggesting CART peptides originate in the ARC (Broberger *et al.* 1999). In mammals, in addition to the central nervous system, CART mRNA and peptides have been identified in a number of peripheral tissues such as the gastrointestinal tract (Ekblad *et al.* 2003). CART peptides have been identified in the blood of rats and monkeys, with CART (55-102) as the predominant form (Vicentic *et al.* 2004). In fish, CART mRNA is expressed in brain and peripheral tissues including gonads and kidney (Volkoff *et al.* 2001; Kehoe *et al.* 2007). CART immunoreactivity has also been detected throughout brain and pituitary of catfish (Singru *et al.* 2007) and in the venom gland of niquim, (*Thalassophryne nattereri*), (Magalhaes *et al.* 2006). The location of CART mRNA has been documented throughout the body of mammals and fish, yet to date, no CART receptors have been identified in either mammals or fish.

CART has been shown to be an anorexigenic peptide. In rodents, intracerebroventricular (ICV) injections of either CART (42-89) or CART (55-102) dose-dependently inhibited food intake for three hours (Vrang *et al.* 1999; Zheng *et al.* 2001). PVN-injected CART (55-102) also decreased feeding in rats up to four hours after

injection (Wang *et al.* 2000). Okumura and colleagues (Okumura *et al.* 2000) showed that injections of CART (55-102) peptide into the cerebrospinal fluid of rats food deprived for 24 hours not only inhibited food intake but also suppressed gastric acid secretion and gastric emptying (Okumura *et al.* 2000), suggesting multiple targets for the CART peptide. CART knockout mice display higher feeding rates than wild type mice (Asnicar *et al.* 2001). CART has been shown to potentially effect the function of the anterior pituitary (Larsen *et al.* 2003). CART peptides produced in *E. coli* proved effective at decreasing food intake when injected into rat brains (Couceyro *et al.* 2003). When CART is expressed in yeast, several cleaved forms of CART are produced and many of these cause decreased feeding when administered in the brain of rats, with CART (55-102) having greater effect than CART (62-76). Fasting causes a decrease in CART mRNA in goldfish (Volkoff *et al.* 2001) and cod (Kehoe *et al.* 2007) compared to fed animals.

CART peptides have been shown to interact with other appetite-regulating peptides. Anatomical studies show that CART immunoreactivity is co-localized with NPY immunoreactive nerve terminals in the ARC nucleus and the amygdala (Broberger *et al.* 1999). In rodents, ICV or PVN injection of CART blocks NPY-induced feeding, suggesting that CART interacts with the NPY system and that CART may have a dominant role over NPY when both peptides are present (Lambert *et al.* 1998; Wang *et al.* 2000). CART is affected by circulating hormone levels in rodents. Leptin, whose plasma level is kept proportional to the body fat, enters the brain and binds to leptin receptors in the ARC nucleus (Elias *et al.* 1999) therefore activating the POMC/CART

nucleus and causing a decrease in food intake (Kristensen *et al.* 1998). In fish, CART interacts with other peptides. In goldfish, ICV injection of CART blocks not only NPY-induced feeding (Volkoff *et al.* 2000), but the orexigenic actions of orexin A (Volkoff *et al.* 2001). Leptin injections have been shown to decrease food intake in goldfish by increasing CART mRNA expression in the brain (Volkoff *et al.* 2001). The complex interactions CART has with other peptides makes it difficult to study its precise role in appetite regulation.

Cholecystokinin

CCK is a linear peptide that is synthesized as a preprohormone which is later proteolytically cleaved to produce a family of peptides that share the carboxy-terminal ends. Pro-CCK cleavage into smaller peptides is strictly regulated and is tissue specific (Vishnuvardhan *et al.* 2002). In some species, there are several biologically active forms, but CCK-8 is the most abundant form in mammals (Moran *et al.* 2004). Pro-CCK has three sulphated tyrosine residues which are important for the interaction of CCK with its receptors (Beinfeld 2003). Studies have shown that the amount of CCK excreted by a cell can be decreased by changing which residue is sulphated. The change in sulphation causes a decrease in CCK expression which demonstrates the importance of the peptide-specific sulphated residue (Vishnuvardhan *et al.* 2000). The post translational modifications on CCK are vital to the function of CCK.

CCK/gastrin-like immunoreactivity has been shown in the nervous system and gut of several fish species including Atlantic cod (Jonsson *et al.* 1987), starry ray, (*Raja*

radiate), and spiny dogfish, (*Squalus acanthias*), (Jonsson 1991), bowfin, (*Amica calva*), and bluegill, (*Lepomis macrochirus*), (Rajjo *et al.* 1988), rainbow trout (Barrenechea *et al.* 1994), goldfish (Himick *et al.* 1994), herring (Kamisaka *et al.* 2005), turbot (Bermudez *et al.* 2007) brown trout (Bosi *et al.* 2004) and halibut, (*Hippoglossus hippoglossus*), (Kamisaka *et al.* 2001). mRNA sequences have also been determined for a number of fish species including goldfish (Peyon *et al.* 1998), rainbow trout (Jensen *et al.* 2001), dogfish (Johnsen *et al.* 1997), catfish (GenBank accession number BE574232), yellowtail, (*Seriola quinqueradiata*), (Murashita *et al.* 2006), pufferfish (Kurokawa *et al.* 2003) and Japanese flounder, (*Paralichthys olivaceus*), (Kurokawa *et al.* 2003). Various forms of CCK, including CCK-8, are present in fish. In fish, the structure of CCK-8 displays species-specific variation, differing at the amino acid on the sixth residue from the C-terminus. Atlantic herring (Kamisaka *et al.* 2005) have a methionine; goldfish (Peyon *et al.* 1998) and Japanese flounder (Kurokawa *et al.* 2003) have a leucine; rainbow trout (Jensen *et al.* 2001) have either a leucine, asparagine or a threonine and spotted river puffer (Kurokawa *et al.* 2003) have either a leucine or valine. CCK appears to have slightly different sequences depending on the species.

CCK, and the related peptide gastrin, bind to two different receptors; CCK-A (CCK-1), mainly located in the gastrointestinal tract, and CCK-B (CCK-2) localized to the brain (Moran *et al.* 2004). Research performed on Otsuka Long-Evans Tokushima fatty (OLETIF) rats has shown that they have a hereditary deficiency in CCK-A receptors which leads them to become obese and hyperphagic (Kawano *et al.* 1992; Moran *et al.*

1998). These results suggest that lack of CCK-A receptor results in a satiety deficit causing the rats to consume larger quantities of food.

There appears to be only one type of CCK cell receptor in fish (Himick *et al.* 1996). CCK binding sites have been found in the brain and gastrointestinal tract of different fish species such as goldfish (Himick *et al.* 1996), sea bass (Moons *et al.* 1992), and shark, (*Isurus oxyrinchus*), (Oliver *et al.* 1996). The presence of only one receptor in the more basal vertebrates, elasmobranches and teleosts, suggests that CCK-A and CCK-B of mamals arose later through evolution.

While cholecystokinin (CCK) in vertebrates is primarily secreted from the endocrine cells in the small intestine, it is also synthesized in the brain. CCK has a key role in the feedback control of gastrointestinal function such as short term inhibition of gastric emptying, acid secretion, stimulation of pancreas, stimulation of the gallbladder and inhibition of food intake (Moran *et al.* 2004). Thus CCK plays a major role in the management of food entry into the small intestine and in nutrient absorption. CCK can act as a short-term inhibitor of food intake because it slows the movement of food through the digestive tract.

In fish, as in mammals, CCK influences digestion and appetite. In teleosts, food entering the small intestine causes the release of CCK, which in turn induce contractions of the gall bladder (Aldman *et al.* 1995). This effect can be mimicked by intra-arterial CCK injections (Aldman *et al.* 1995). In salmon, vascular injections of CCK cause a decrease in gastric emptying (Olsson *et al.* 1999) and an increase in gut motility (Forgan *et al.* 2007). CCK also influences appetite regulation in fish. In goldfish, both central

and peripheral injections of CCK cause a decrease in food intake (Himick *et al.* 1994; Volkoff *et al.* 2003). Oral administration of CCK in a gelatin capsule causes a decrease in food intake in sea bass (Rubio *et al.* 2008). Whereas oral administration of a CCK antagonist causes an increase in food consumption in both trout and sea bass (Gelineau *et al.* 2001; Rubio *et al.* 2008). Peyon and colleagues showed an increase of CCK mRNA levels in brain regions such as the hypothalamus and telencephalon two hours after feeding (Peyon *et al.* 1999). A similar time dependent increase was shown in yellowtail where CCK mRNA levels increased in the pyloric caeca reaching a maximum level between one and a half to three hours after feeding (Murashita *et al.* 2007).

CCK has been shown to interact with leptin, NPY and orexin in fish. Goldfish co-injected with leptin and CCK at doses ineffective by themselves show a decreased food intake, suggesting a synergistic action of leptin and CCK (Volkoff *et al.* 2003). In goldfish, CCK mRNA expression in the brain increases following central injection of leptin and blockade of CCK brain receptors inhibits leptin-induced decrease in food intake, suggesting that the actions of leptin are in part mediated by CCK (Volkoff *et al.* 2003). CCK appears to act with other anorexigenic peptides to decrease food intake.

Winter flounder (*Pleuronectes americanus)*

Winter flounder (*Pleuronectes americanus*) is a right-handed flatfish in the family *Pleuronectidae*. Winter flounder are bottom-dwelling fish that spawn in late winter or early spring in shallow waters. Their eggs are unique because they sink to the bottom of the water and remain stuck in clusters (Scott *et al.* 1988). Larvae are born with a laterally

compressed body with one eye on each side of their head. They undergo metamorphosis around three months after hatching when their body becomes flattened and their left eye migrates to the right side of their heads (de Montgolfier *et al.* 2005). The left side of their body remains near the ocean floor and is usually white, while the right sides colour varies depending on the colour of the sediment on which they lie. Mature flounder are typically 18 inches in length and two pounds in weight (Litvak 1999; Mercier *et al.* 2004). Winter flounder is a euryhaline species and survives in a wide range of salinities and is eurythermic and survives in below 0°C water (de Montgolfier *et al.* 2005).

Winter flounder undergo an onshore offshore movement throughout the year, depending on their geographic location (Hanson *et al.* 1996). Off the coast of Newfoundland, they tend to move inshore during the winter and offshore during the summer. This movement is opposite to that seen in other areas of the world and is thought to be due mainly to the presence of antifreeze proteins in the winter flounder surrounding Newfoundland areas (Gauthier *et al.* 2005). Antifreeze proteins have been isolated in winter flounder which help them survive the cold waters (Litvak 1999; Fredette *et al.* 2000; Murray *et al.* 2002).

Winter flounder is becoming increasingly attractive to consumers and to the aquaculture industry. In various parts of the world, especially Korea, winter flounder has been gaining popularity for commercial fisheries due to its high quality meat, it can withstand various salinities and survive at low temperatures (Mercier *et al.* 2004; Cho 2005). In recent years, increased winter flounder research has been conducted in an attempt to learn how to optimize living conditions for commercial use (de Montgolfier *et*

al. 2005). To date, studies on winter flounder research have consequently focused on life cycle, feeding strategies and juvenile rearing methods.

Winter flounder are well adapted to harsh and changing environments which makes the species a good candidate as a cold water aquaculture species (Plante *et al.* 2003; de Montgolfier *et al.* 2005). In addition, winter flounder do not suffer from chronic stress when in captivity (Plante *et al.* 2003), which permits rapid growth and prevents unnecessary and premature death. In order to effectively utilize winter flounder in aquaculture, the establishment of a successful feeding regime is crucial. An understanding of the regulation of feeding and the development of cost effective diets is essential for the development of a successful aquaculture not only for maintaining adults but also for larval growth and metamorphosis (Ben Khemis *et al.* 2003; Hebb *et al.* 2003; Cho 2005; de Montgolfier *et al.* 2005). To date, very few studies have examined the appetite-controlling mechanisms in this species and the appetite regulating neurohormones have never been examined in the winter flounder.

Winter flounder presents an additional challenge for aquaculture as these fish enter a dormant-like state during the colder winter months, when feeding and activity are minimal. Winter flounder spend twice as much time actively swimming at 4.4°C compared to the time spent at -1.2°C (He 2003). They decrease feeding in colder temperatures (Martell *et al.* 1994; Stoner *et al.* 1999; Meise *et al.* 2003), resulting in weight loss. Interestingly, winter flounder spawn immediately following winter (Scott *et al.* 1988), which seems surprising as starvation and weight loss might induce a state of negative energy balance that would be detrimental to the energetically-demanding

activity of reproduction. The physiological and endocrine mechanisms regulating this inactive state are to date unknown. Understanding the regulation of this yearly feeding pattern is crucial for the development of a successful year-round aquaculture.

Winter skate (*Raja ocellata)*

The winter skate is an oviparous elasmobranch benthic species from the family *Rajidae* that can live up to depths of 371m, but prefer depths of 36.6 to 90m (Scott *et al.* 1988). Adults have a depressed body with a long slender tail and usually grow to a length of 30 inches. The winter skate's range extends from the Gulf of St. Lawrence to the south coast of Newfoundland and to the Scotian Shelf and Bay of Fundy (Scott *et al.* 1988). Mating usually occurs in late winter or spring. Skates lay eggs in tough cases which are often referred to as mermaid's purses due to their rectangular shape with two hooks coming out from each end. Skates play a role in regulating the trophodynamics of Western Atlantic ecosystems because they prey largely on the benthic fauna (Scott *et al.* 1988).

Until recently, skates have not been the object of a specific fishery. However, large numbers of skates are accidentally caught every year by fisheries using otter trawls, traps and weirs and these by-catches have led to a decline in skate populations (Scott *et al.* 1988). Skates have recently become the object of specific fisheries as their mild-tasting, low in cholesterol meat is sought by consumers (Frisk *et al.* 2006). In the past, winter skate has received little attention from the scientific community, although they have been used in biochemical and physiology experiments. Past research on skates has

mostly focused on osmoregulation, as they are well-adapted to exposure to different salinities (Sulikowski *et al.* 2004; Treberg *et al.* 2006). Given the recent interest in these fish, it is crucial that we gain a better understanding of their life cycle and physiology, in particular feeding physiology, in order to develop sustainable fisheries and possibly aquaculture. Currently, there is no information on appetite regulating hormones in winter skate.

Objective of this study

Our understanding of the regulation of appetite in fish is limited as the majority of such studies have been performed on mammals. Fish provide valuable experimental models for the study of feeding regulation for a number of reasons. Fish species display seasonal feeding behaviours which are influenced by water temperature and photoperiod. This makes them ideal models with which to study the evolution of appetite regulating systems. Fish are usually easier to maintain in laboratory settings which facilitates repeated sampling. Fish are also great models because they are realistic surrogates for mammals and humans. Winter flounder and winter skates were chosen because they are both benthic species which allowed us to compare appetite regulation between the two. As well, winter flounder in particular was chosen because it displays seasonal variation in feeding patterns which could be linked to the regulation of appetite hormones.

The goals of this study have been to characterize at the molecular level the appetite-regulating hormones NPY, CART and CCK in two species of fish, the winter flounder and winter skate. The cDNA sequences for each hormone were determined

using RT-PCR and RACE and then central and peripheral tissue mRNA distributions were established for each of these hormones. The effects of starvation on gene expression of brain NPY and CART and gut CCK were assessed using real time quantitative PCR. In order to assess the influence of season on the feeding physiology of flounder, gene expression of the winter flounder genes were examined under both summer and winter seasons.

Materials and Methods

Animals

Wild winter flounder (*Pleuronectes americanus*) and winter skates (*Raja ocellata*) were collected by divers off the shore of St. John's, a city in the province of Newfoundland and Labrador, and kept in flow through tanks (2x2m tanks for flounder and 4x4m tanks for skates) at the Ocean Sciences Centre (Memorial University of Newfoundland, St. John's, NL, Canada). Fish were kept under natural photoperiod and temperature conditions (see below). Fish consisted of both males and females and were fed frozen herring twice a week at the same time of the day (10:00 NST). Prior to the starvation experiments, three to four acclimated fed fish were sampled for cloning purposes (see below). During all samplings, the weights of fish were measured. The sex and sexual maturity was noted for all fish.

Experimental Protocol

Flounder-winter experiment

Sixty flounder (average weight of 355.7g) were divided into four tanks (15 fish per tank), and acclimated for two weeks in flow through water tanks at an average temperature of 0°C. The experiment was conducted from the 21st of March 2007, to the 2nd of May, 2007. The fish were fed as described above. Once acclimated, two tanks were food deprived for six weeks and two tanks continued to be fed at the above-described conditions. Five flounder were sampled from each tank two, four and six weeks after the start of the experiment (for a total of 20 animals per sampling).

Flounder-summer experiment

Thirty-six flounder (average weight of 446.9g) were divided among four tanks (eight fish per tank). The experiment ran from the 1st of August 2007, to the 29th of August 2007. The average water temperature was 11.9°C. As fish are more active than in the winter, they were fed three times a week as contrasted to twice for the winter experiment. Once acclimated, two tanks were food deprived for four weeks and two tanks were continued to be fed as above. Two to five flounder were sampled from each tank two and four weeks after the start of the experiment, for a total of 24 animals.

Skate-summer experiment

Twenty skates (average weight of 1.86kg) were divided into four tanks and acclimated for two weeks in flow through water tanks at an average temperature of 11.4°C. The experiment ran from the 19th of September 2007 to the 3rd of October 2007. Skates were fed chopped frozen herring three times a week to satiety at the same time each day (10:00 NST). Following the acclimation period, two tanks were food deprived and two tanks were maintained on the regular feeding schedule. Samples were collected two weeks after the start of the experiment.

RNA preparation

For cloning and tissue distribution studies three to four fed fish of each species were dissected to obtain samples of brain and peripheral tissues (gill, heart, stomach, gut, spleen, liver, kidney, muscle and gonad). Fish were anesthetized by immersion in 0.05%

tricaine methanesulfonate (Syndel Laboratories, Vancouver, British Columbia, Canada) and killed by spinal section. Tissues were dissected and immediately placed on ice in RNA*later* (Qiagen Inc., Mississauga, Ontario, Canada) and stored at -20°C until RNA extractions were performed.

For brain tissue distribution, individual brains were further dissected and total RNA was isolated from hypothalamus, telencephalon, optic tectum, and cerebellum. For gene expression studies, total RNA was isolated from hypothalamus, telencephalon and gut in both flounder and skates. Total RNA was isolated using a trizol/chloroform extraction with Tri-reagent (BioShop, Mississauga, Ontario, Canada) following the manufacturers' protocol. Final RNA concentrations were determined by optical density reading at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). The quality of RNA samples was assessed by measuring the ratio of sample absorbance at 260 and 280 nm. RNA samples with a ratio between 1.8 and 2.1 were used.

Cloning of cDNA

Two micrograms of total RNA were subjected to reverse transcription into cDNA with a dT-adapter primer (Table 1 and 2) using M-MLV Reverse Transcriptase (New England Biolabs, Pickering, Ontario, Canada). Fragments of the unknown sequences were initially obtained by performing PCR amplifications using degenerate forward and reverse primers designed in regions of high identity among fish and various vertebrate sequences and the above cDNA. Zero point five micrograms of cDNA were used for

each PCR. The annealing temperature was optimized for each primer set. 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). PCR conditions were: 45 s at 94°C; 29 cycles of 30 s at 94 °C, 30 s at 30 °C, 60 s at 72 °C; 2 min at 72 °C. A negative control was included for each primer set by omitting cDNA from the PCR reaction. The PCR products were electrophoresed in a 1% agarose gel in TAE buffer (Tris-acetate-EDTA). Bands of predicted size were isolated and purified with the GenElute Gel Extraction Kit (Sigma, Oakville, Ontario, Canada), ligated into the pGEM easy vector using the pGEMeasy vector system (Promega, Madison, Wisconsin, USA) and sequenced by the MOBIX Lab (McMaster University, Ontario, Canada).

Table 1. Primers used in the cDNA cloning, tissue distribution and qPCR analysis in winter flounder (*Pleuronectes americanus*).

Primer	Sequence[1]
NPY	
Degenerate primers	
dNPY-F	5' AARCCNGARAAYCCNGGNGA 3'
dNPY-R	5' GTRATNARRTTRATRTARTG 3'
Primers for 3' and 5' RACE	
3'RC-NPY1	5' GAGGATCTGGCGAAATACTA 3'
3'RC-NPY2	5, CTACTCAGCCCTGAGACAACT 3'
5'RC-NPY1	5' ATGTGGATTCAACTTTGATG 3'
5'RC-NPY2	5' CTCGTGATGAGGTTGATGTAG 3'
5'RC-NPY3	5' TGTAGTGTCTCAGGGCTGAG 3'
Specific primers for RT-PCR	
NPYF	5' ATGCATCCTAACTTGGTGAG 3'
NPYR	5' CCACAATGATGGGTCATATC 3'
CART	
Primers for 3' and 5' RACE	
3'R-CART 1	5' CATTHTGGGARAAGAARTTYGG 3'
3'R-CART 2	5' TACGTGYGAYFTBGGRGAGC 3'
5'R-CART 1	5' CAGGAAGAAGTTGCAGAACG 3'
5'R-CART 2	5' CTCGGGGACAGTCGCACATC 3'
5'R-CART 3	5' CATCTTCCCAATCCGAGCTC 3'

Specific primers for RT-PCR	
CART qF	5' GAGAGTTCCGAGGAGCTGAG 3'
CART qR	5' TTTCGACTGAAGCTTCTCCA 3'
CCK	
Degenerate primers	
dCCK-F	5' TGGCDGCYCTBTCCACCAGC 3'
dCCK-R	5' CCAKCCCARGTARTCTCTGTC 3'
Primers for 3' and 5' RACE	
3'RC-CCK 1	5' CCTCTCCTCTCAGCACCTAG 3'
3'RC-CCK 2	5' CTCCGACAGCGCCGCTCTGC 3'
5'RC-CCK 1	5' CAGCCACAGGAAGAGCATTC 3'
5'R-CCK 2	5' TGGGGTATCAGCCTCGAGGA 3'
5'R-CCK 3	5' GCTGAGAGGAGAGGGGGTGC 3'
Specific primers for RT-PCR	
CCK qF	5' TTCCTGTGGCTGAGGAGAAT 3'
CCK qR	5' GCACAGAACCTTTCCTGGAG 3'
Adaptor primers	
dT-AP	5' GGCCACGCGTCGACTAGTAC(T17) 3'
AP	5' GGCCACGCGTCGACTAGTAC 3'
Primers for internal control of RT-PCR	
EF1	5' CCTGGACACAGGGACTTCAT 3'
EF2	5' CGGTGTTGTCCATCTTGTTG 3'

Primers for qPCR

NPY qF	5' CACGAGACAGAGGTATGGGA 3'
NPY qR	5' GACTGTGGAAGTGTGTCCGT 3'
CART qF	5' GAGAGTTCCGAGGAGCTGAG 3'
CART qR	5' TTTCGACTGAAGCTTCTCCA 3'
CCK qF	5' TTCCTGTGGCTGAGGAGAAT 3'
CCK qR	5' GCACAGAACCTTTCCTGGAG 3'
EF qF	5' CGCTCTGTGGAAGTTTGAGA 3'
EF qR	5' CAGTCAGCCTGAGAGGTTCC 3'
BA qF	5' TCCTGACCCTGAAGTATCCC 3'
BA qR	5' TGTGATGCCAGATCTTCTCC 3'

1 A=adenine
T=thymine
C=cytosine
G=guanine
B=T/C/G
K=T/G
H=A/T/C
N=A/T/C/G
R=A/G
Y=C/T
D=A/T/G

Table 2. Primers used in the cDNA cloning, tissue distribution and qPCR analysis in winter skate (*Raja ocellata*).

Primer	Sequence[1]
NPY	
Primers for 3' and 5' RACE	
3'R-NPY1	5' GAGATTTGGCCAAGTATTAYTC 3'
3'R-NPY2	5' TACAAGGCAGAGGTATGG 3'
5'R-NPY1	5' TCACATTAAAGAAACTGCAG 3'
5'R-NPY2	5'ATCTCTCAGCATCAGTTCAG 3'
5'R-NPY3	5' TAGTGCTTCGGGGTTGGATC 3'
Specific primers for RT-PCR	
NPYF	5' AACATGAAGTCTTGGCTGGG 3'
NPYR	5' CCACATGGAAGGTTCATCAT 3'
CART	
Primers for 3' and 5' RACE	
3'R-CART 1	5' CTCGGGGCTTTACATGANGT 3'
3'R-CART 2	5' GANGTTCTGGAGAAACTGCA 3'
5'R-CART 1	5' GGGTCCTTTTCTCACTGCAC 3'
5'R-CART 2	5' TCCTCCAAATCCTGGGTCCT 3'
5'R-CART 3	5' TCAGGCAGTTACAGGTCCTC 3'
Specific primers for RT-PCR	
CART qF	5' GCAGCGAGAAGGAACTGCT 3'
CART qR	5' GCACACATGTCTCGGATGTT 3'

CCK

Degenerate primers

dCCK-F	5' GTGGGATCTGTGTGTGYGT 3'
dCCK-R	5' CGTCGGCCRAARTCCATCCA 3'

Primers for 3' and 5' RACE

3'RC-CCK 1	5' CAGGCTGAACAGTGAGCAG 3'
3'RC-CCK 2	5' AGCAGGGACCCGGCCTAGTG 3'
5'RC-CCK 1	5' GTAGTAAGGTGCTTCTCTC 3'
5'R-CCK 2	5' GCTGGTGCAGGGGTCCGTGC 3'
5'R-CCK 3	5' TCCCTCTCGGTCCGTCCGTC 3'

Specific primers for RT-PCR

CCK qF	5' CACCTACCTGCACAAAGACAA 3'
CCK qR	5' CCATGTAGTCCCTGTTGGTG 3'

Adaptor primers

dT-AP	5' GGCCACGCGTCGACTAGTAC(T17) 3'
AP	5' GGCCACGCGTCGACTAGTAC 3'

Primers for internal control of RT-PCR

EF1	5' AAGGAAGCTGCTGAGATGGG 3'
EF2	5' CAGCTTCAAACTCACCCACA 3'

Primers for qPCR

NPY qF	5' CCCGAAGCACTAATGATGAC 3'
NPY qR	5' CATGGAAGGTTCATCATACCTAA 3'
CART qF	5' GCAGCGAGAAGGAACTGCT 3'

CART qR	5' GCACACATGTCTCGGATGTT 3'
CCK qF	5' CACCTACCTGCACAAAGACAA 3'
CCK qR	5' CCATGTAGTCCCTGTTGGTG 3'
EF qF	5' GAACATGATTACCGGCACCT 3'
EF qR	5' TTCAAACTCACCCACACCAG 3'

1 A=adenine
T=thymine
C=cytosine
G=guanine
B=T/C/G
K=T/G
H=A/T/C
N=A/T/C/G
R=A/G
Y=C/T
D=A/T/G

Cloning winter flounder neuropeptide Y

A small fragment of the unknown sequence was isolated by performing PCR amplifications using degenerate forward and reverse primers (dNPY-F, and -R, Table 1). Once the short fragments were isolated and sequenced (see above), gene-specific primers were designed for both 5' and 3' end amplification. The 3' ends of cDNA were amplified by the technique of 3' Rapid Amplification of CDNA Ends (3'RACE). Briefly, brain mRNA was subjected to reverse transcription as described above and the cDNA submitted to two rounds of nested PCR, firstly 3'RC-NPY1 and secondly with dT-AP, and 3'RC-NPY2 and AP (Table 1). The PCR products were electrophoresed on a gel, and the bands of expected size were isolated, purified, cloned, and sequenced as described above. To isolate the 5' portion of the cDNA, 5'RACE was used. The first strand of cDNA was generated from mRNA with reverse transcription reaction with the gene specific primer 5'RC-NPY1 based on the sequence cloned from the 3'RACE then the cDNA was purified to remove primers and unincorporated dNTPs using a Montage PCR Millipore kit (Bedford, MA, USA). A polyA tail was added to the 3' end of the cDNA using Terminal Deoxynucleotidyl Transferase (Invitrogen, Burlington, Ontario, Canada). The product was then amplified using two rounds of nested PCR using first 5'RC-NPY2 and dT-AP and then 5'RC-NPY3 and AP. The product was electrophoresed on a gel, isolated, purified, cloned and sequenced as described previously.

Cloning winter flounder CART

In order to isolate flounder CART, a protocol similar to the above was used except that the initial fragment was obtained using 3'RACE and the degenerate primers (dT-AP and 3'R-CART1 and AP and 3'R-CART2) in two rounds of nested PCR.

Cloning winter flounder CCK

Flounder CCK was isolated as described for winter flounder NPY (with CCK specific primers, Table 1).

Cloning winter skate NPY

Skate NPY was isolated as described for winter flounder CART above (with skate NPY specific primers, Table 2).

Cloning winter skate CART

Skate CART was isolated as described for winter flounder CART above (with skate CART specific primers, Table 2).

Cloning winter skate CCK

Skate CCK was isolated as described for winter flounder NPY above (with skate CCK specific primers, Table 2).

Sequence analysis

DNA and deduced amino acid sequences were examined by the Basic Local Alignment Search Tool (BLAST) available from the National Center for Biotechnology Information (NCBI) website (*www.ncbi.nlm.nih.gov*). Multiple alignments of amino acid sequences were performed using ClustalW software (*www.ebi.ac.uk/clustalw/*).

Assessment of brain and tissue distribution by RT-PCR

Total RNA from brain, gills, heart, gut, liver, spleen, kidney, muscle, skin and gonads and from distinct brain regions (olfactory bulbs, telencephalon, optic tectum-thalamus, hypothalamus, cerebellum, posterior brain and spinal cord) were isolated as described above. The first strand of cDNA was generated from two micrograms of RNA subjected to reverse transcription with dT-adapter primer using M-MLV Reverse Transcriptase (New England Biolabs). NPY, CART and CCK were amplified using gene specific primers (Table 1, 2) that were designed based on our cloned sequences. The PCR cycling conditions for all reactions were 30 cycles, 94°C for 30 s, annealing temperature for 45 s, and 72°C for 60 s. The annealing temperature was optimized for each primer set. PCR products were electrophoresed on a 1% agarose gel with TAE buffer and visualized using the Epichemi Darkroom BioImaging System (UVP, Upland, CA, USA) equipped with a 12-bit cooled camera. Image processing and analysis were performed using LabWorks 4.0 software (UVP). Elongation factor-1 alpha (EF-1α) was used as a control gene. Primers were designed based on winter flounder and little skate

(*Raja erinacea*) EF-1α (GenBank accession number AW013637, E988144 respectively) (Table 1 and 2). Bands amplified with EF-1α were cloned and sequenced in order to verify their nucleotide sequence.

Quantification of gene expression of NPY, CART and CCK by qPCR

Total RNA from hypothalamus, telencephalon and gut were extracted using TRI-reagent. Total RNA was quantified using a Nanodrop ND-100 spectrophotometer. Total RNA was then subjected to reverse transcription into cDNA, using a QuantiTect Reverse Transcription kit (QIAGEN), according to the manufacturers protocol. Briefly, 1 ug of template total RNA were submitted to genomic DNA removal and reverse transcribed using an optimized mix of oligo-dT and random primers, Quantiscript RT buffer, and reverse transcriptase. Reverse transcription products were then diluted 1:3 in water and subjected to a PCR using specific primers (Tables 1 and 2). For all primer pairs, at least one primer was designed to lie across an exon/exon boundary, so that primer pairs can amplify only from mRNA but not from genomic DNA. The primers were designed to have similar melting temperatures and to give similar amplicon sizes. All PCR reactions were prepared using an epMotion® 5070 automated pipetting system (Eppendorf) in a final volume of 10 µl containing 2 µl of cDNA, 1µM of each sense and antisense primer, and 5 µl of the QuantiFast SYBR Green PCR Kit master mix (Qiagen). SYBR Green real-time quantitative RT-PCR amplifications were performed using the Mastercycler® ep realplex 2S system (Eppendorf). Reactions were conducted in 96-well plates. Samples were analyzed in duplicate. In all cases, a "no template" negative control in

which cDNAs were replaced by water was included. In addition a melting curve was conducted at the end of each qPCR experiment to ensure amplification of only one product. Initial validation experiments were conducted to determine optimal primer annealing temperatures and to ensure that PCRs were reproducible ($0.98>R^2>1.02$) and that all primer pairs had equivalent PCR efficiencies. The reference gene EF1-α was tested to verify that starvation did not affect its expression level, as demonstrated by similar Ct (cycle threshold) values.

Data Analysis of real time PCR

The gene of interest was normalized to the reference gene (EF1-α) and expression levels were compared using the relative Ct (ΔΔCT) method. Amplification, dissociation curves and gene expression analysis were performed using the Realplex1.5 software (Eppendorf). Each expression level was then expressed as a percentage relative to the control group which was set at 100%. By doing this, samples that were run in separate qPCR experiments could be compared to each other. Finally, gene expression levels were compared using student t-tests in the GraphPad Instat software program (GraphPad Software Inc.).

Results

Cloning

Winter flounder NPY, CART and CCK

The winter flounder NPY sequence of the open reading frame is 300 bp long (GenBank Accession number EU684053) with a 338 base pairs (bp) 5' untranslated (UTR) region and a 60 bp 3'UTR (Figure 1). The open reading frame has 99 amino acids which encode for preproNPY. Based on comparisons with other fish NPY sequences, winter flounder NPY likely has four putative exons that are separated by three introns located after nucleotides 60, 251, and 337.

Winter flounder CART has a 396 bp (unpublished) coding sequence with an 82 bp 5'UTR and a 40 bp 3'UTR (Figure 2). The open reading frame encodes the 92 preproCART. Based on comparisons with other CART sequences, flounder CART likely has three exons that are separated by two introns located after nucleotides 121 and 200.

Winter flounder CCK has a length of 469 bp (GenBank Accession number EU684055) that includes an 18 bp 5'UTR and a 56 bp 3'UTR (Figure 3). The open reading frame has 92 amino acids which codes for preproCCK. Based on comparisons with other CCK sequences, flounder CCK has two putative exons that are divided by one intron located after nucleotide 323. The sizes of each mature peptide are indicated in the figures.

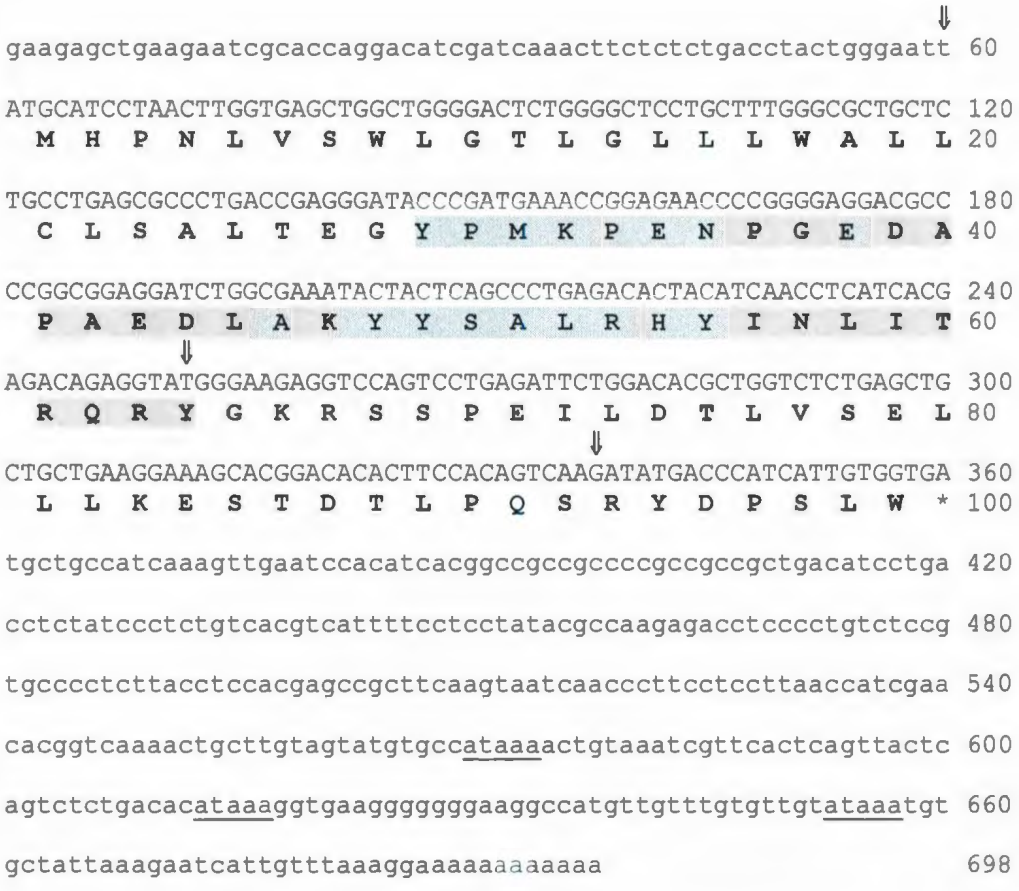


Figure 1. Predicted amino acid sequence for winter flounder NPY. Untranslated regions are in small case letters, the positions of putative introns are indicated by arrows and the amino acids for the NPY precursor sequence are in bold. Amino acids that code for the translated mature peptide are shaded in grey. The stop codon is indicated by a star (*). Potential polyadenylation sites are underlined.

Figure 2. Predicted amino acid sequence for winter flounder CART. Untranslated regions are in small case letters, putative introns are indicated by arrows and the amino acids for the CART precursor are in bold. Amino acids that code for the predicted translated mature peptide are shaded in grey. The stop codon is indicated by a star (*).

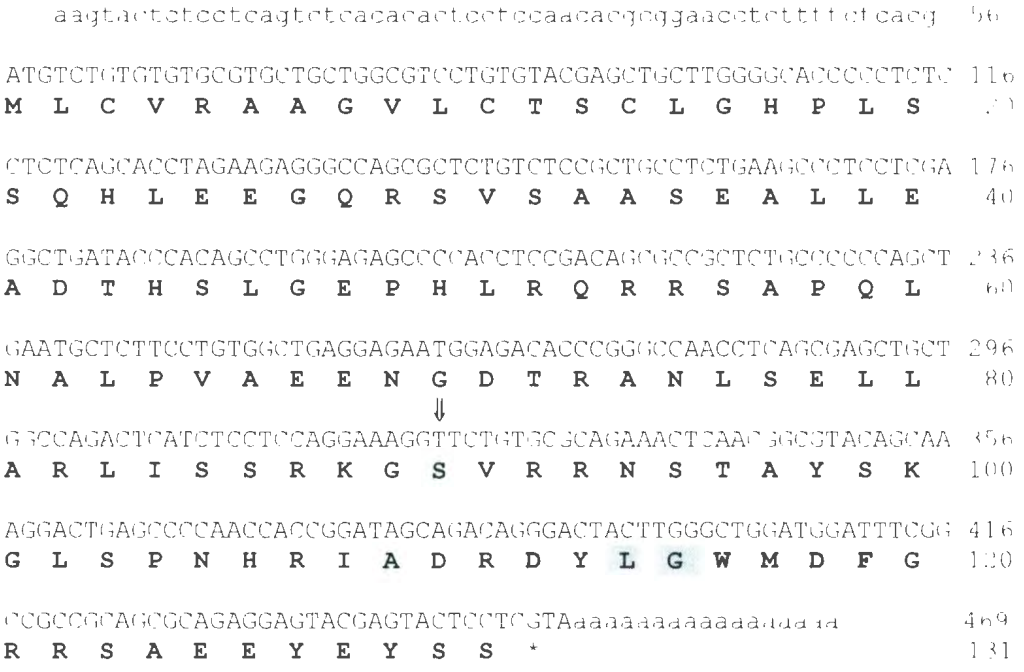


Figure 3. Predicted amino acid sequence for winter flounder CCK. Untranslated regions are in small case letters, putative introns are indicated by arrows and amino acids are in bold. Amino acids that code for the translated mature CCK-8 peptide are shaded in grey. The stop codon is indicated by a star (*).

Winter skate NPY, CART and CCK

Winter skate NPY is a 695 bp sequence (GenBank Accession number EU684052) that includes a 76 bp 5'UTR and a 325 bp 3'UTR (Figure 4). The open reading frame contains 98 amino acids which encode for preproNPY. The NPY precursor sequence has three putative exons that are divided by two introns located after nucleotides 264 and 348.

A partial sequence was obtained for winter skate CART, which displays a 549 bp sequence (unpublished) with a 345 bp 3'UTR. The 5'UTR and the start codon have not yet been identified (Figure 5). The open reading frame has 95 amino acids. The CART precursor sequence has three putative exons that are divided by two introns located after nucleotides 96 and 177.

Winter skate CCK is a 536 bp sequence (GenBank Accession number EU684054) with a 78 bp 5'UTR and a 107 bp 3'UTR (Figure 6). The open reading frame has 115 amino acids which encode for preproCCK. CCK has two putative exons that are divided by one intron located after amino acid 302 bp.

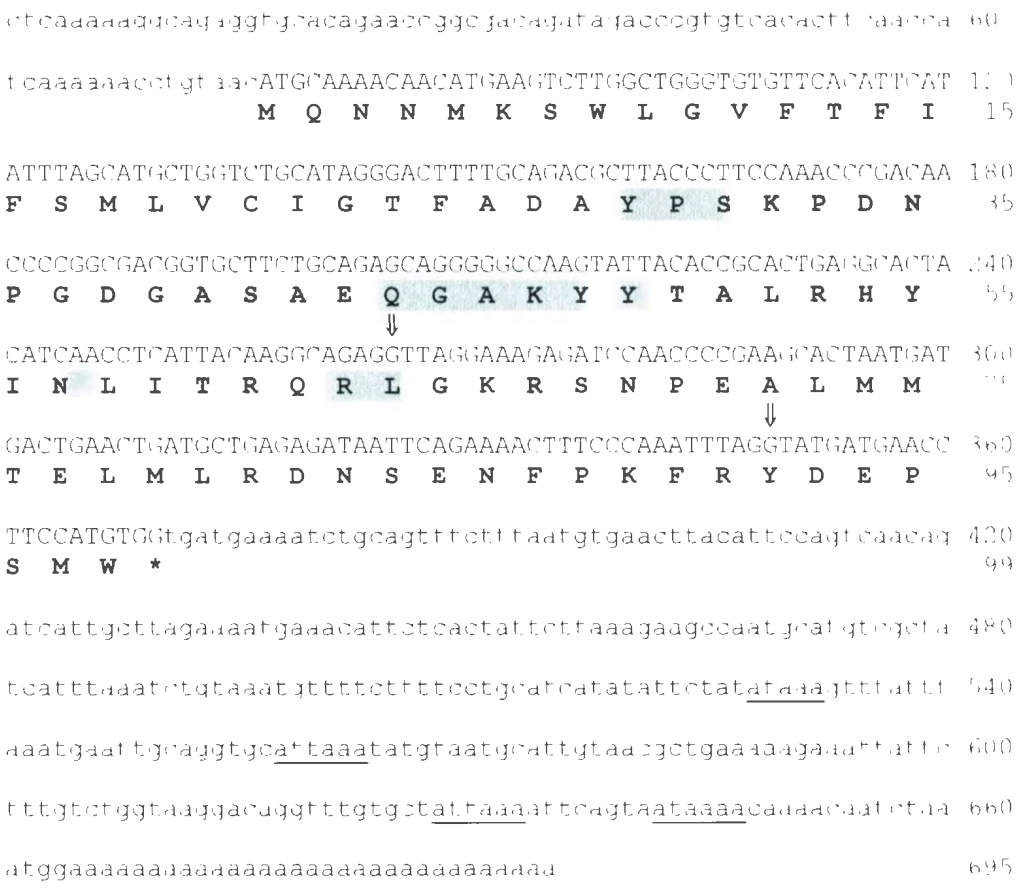


Figure 4. Predicted amino acid sequence for winter skate NPY. Untranslated regions are in small case letters, putative introns are indicated by arrows and amino acids are in bold. Amino acids that encode for the translated mature peptide are shaded in grey. The top codon is indicated by a star (*). Potential polyadenylation sites are underlined.

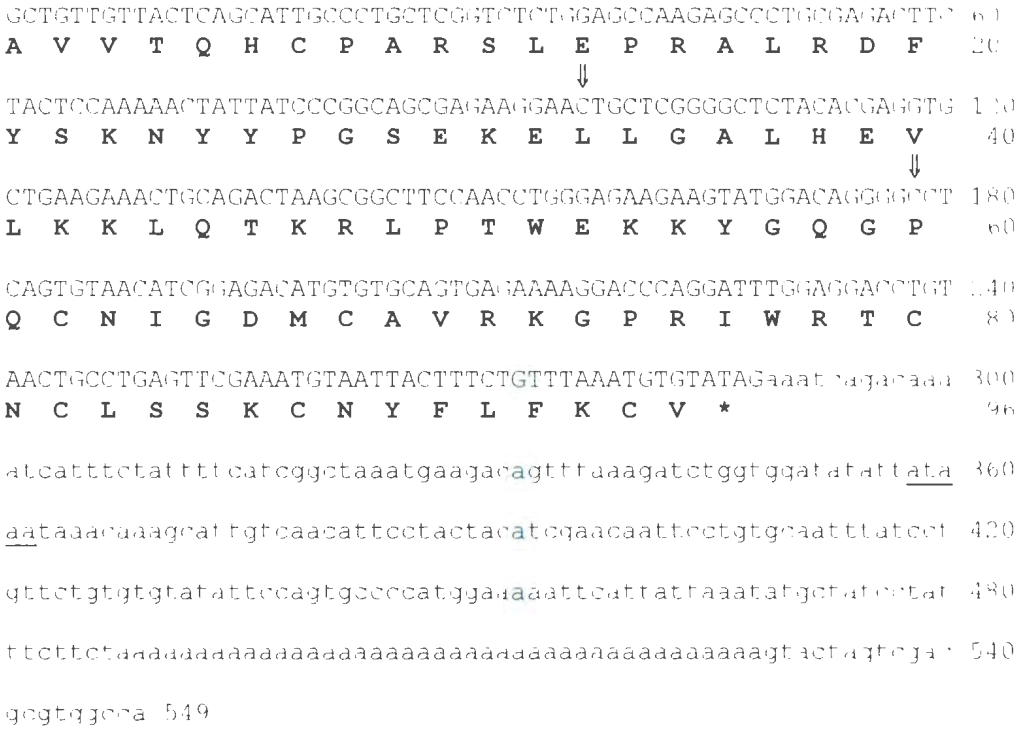


Figure 5. Predicted partial amino acid sequence for winter skate CART. The 3' untranslated regions are in small case letters, putative introns are indicated by arrows and the amino acids are in bold. The stop codon is indicated by a star (*). Polyadenylation sites are underlined. The start codon and the 5' UTR have not been identified.

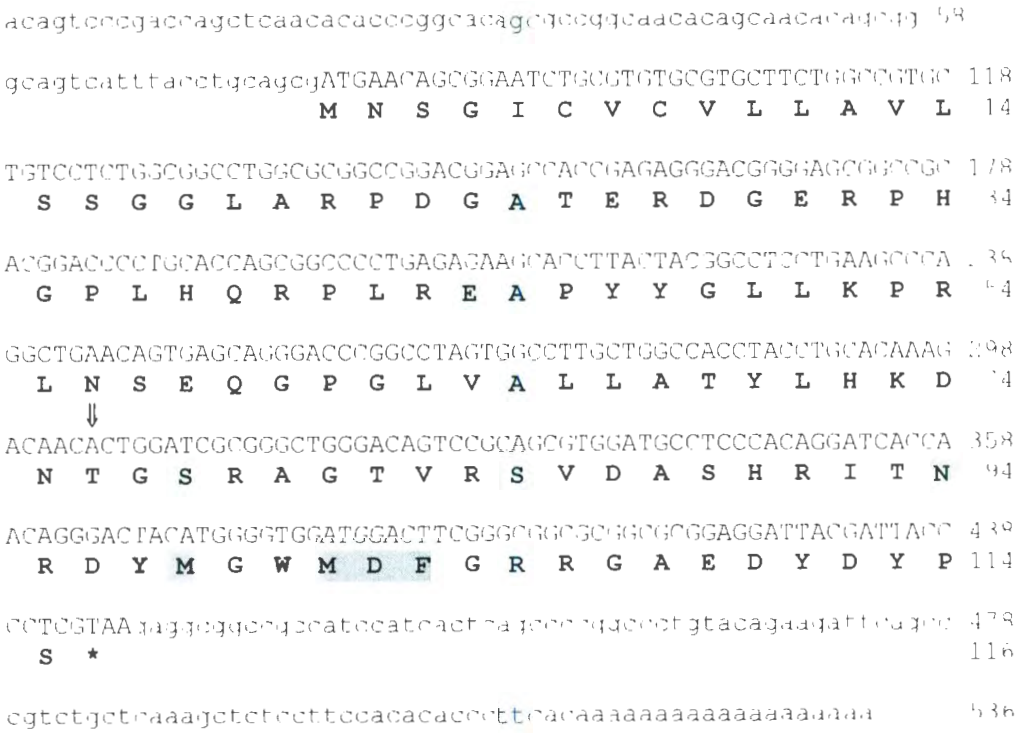


Figure 6. Predicted amino acid sequence for winter skate CCK. Untranslated regions are in small case letters, putative introns are indicated by arrows and the amino acids are in bold. Amino acids that code for the mature peptide CCK-8 are shaded in grey. The stop codon is indicated by a star (*).

Sequence analyses

NPY

The amino acid sequences of winter flounder NPY and winter skate NPY were aligned with sequences from other fish species and with one mammal sequence (Figure 7). Winter flounder NPY has 53 to 96% amino acid similarity to NPY from other fish species, with highest similarity (96%) to the bastard halibut and orange spotted grouper. The winter skate NPY has low sequence similarity to teleost fish and mammalian sequence (around 50%), but shared a relatively high degree of similarity with the electric ray NPY (81%) (Figure 7).

CART

Amino acid sequences of winter flounder CART and winter skate CART were aligned with other fish species and a mammalian sequence (Figure 8). Winter flounder CART has 65 to 84% amino acid similarity to other fish species, with the greatest to Atlantic cod. Winter skate CART displayed 47 to 53% amino acid similarity, with goldfish CART having the highest similarity (Figure 8).

CCK

Amino acid sequences of winter flounder CCK and winter skate CCK were aligned with sequences from other fish species and one mammalian species (Figure 9). Winter flounder CCK showed an amino acid similarity ranging from 38% to 91%, with

the highest similarity to halibut CCK. Winter skate CCK displayed 34 to 46% amino acid similarity, with the highest similarity to dogfish CCK (Figure 9).

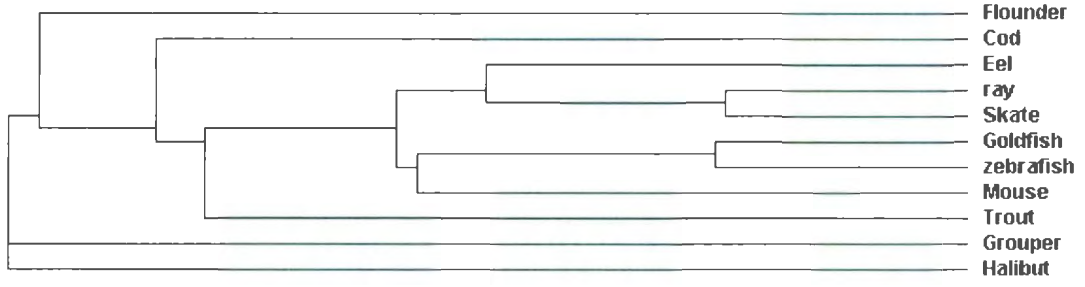
Figure 7. A. NPY amino acid sequence alignment acquired with Clustal W of winter flounder (GenBank accession number EU684053) and winter skate (GenBank accession number EU684052), with Atlantic cod, *Gadus morhua* (GenBank accession number AY822596); Japanese eel, *Anguilla japonica* (GenBank Accession number AB109557); Orange-spotted grouper, *Epinephelus coioides* (GenBank accession number AB055211); Goldfish, *Carassius auratus* (GenBank accession number M87297); Bastard halibut, *Paralichthys olivaceus* (GenBank accession number AB055211); Southern brook lamprey, *Ichthyomyzon gagei* (GenBank accession number AY823509); Electric ray, *Torpedo marmorata* (GenBank accession number M87296); Mouse, *Mus musculus* (GenBank accession number BC043012); Rainbow trout, *Oncorhynchus mykiss* (GenBank accession number AF203902); Zebrafish, *Danio rerio* (GenBank accession number BC162071). B. The phylogenetic tree of NPY. (*) indicates identical amino acids; (:) indicates similar amino acids; (-) indicates gaps introduced by the Clustal W algorithm when aligning the sequences.

A

```
Flounder        MHPNLVSWLGTLGLLLWALLCLSALTEGYPMKPENPGEDAPAEDLAKYYSALRHYINLIT 60
Cod             MHSNLATWLGALGFLLCALICLGTLTEGYPIKPENPGEDAPADELAKYYSALRHYINLIT 60
Eel             MAVVLKPWTVLVALVLCLLVCLGTFVDAYPPKPENPGEDASPEEQAKYYTALRHYINLIT 60
Grouper         MHPNLVSWLGTLGLLLWALLCLSALTEGYPVKPENPGDDAPAEELAKYYSALRHYINLIT 60
Goldfish        MHPNMKMWTGWAACAFLLFVCLGTLTEGYPTKPDNPGEGAPAEELAKYYSALRHYINLIT 60
Halibut         MHPNLVSWLGTLGLLLWALLCLSALTEGYPVKPENPGDDAPAEELAKYYSALRHYINLIT 60
Ray             MQTNMKFWLGVFTFAFCMLICIGTFADAYPSKPDNPGEGAPAEDLAKYYSALRHYINLIT 60
Mouse           MLGNKRMGLCGLTLALSLLVCLGILAEGYPSKPDNPGEDAPAEDMARYYSALRHYINLIT 60
Trout           MHPNLGTWLGAVTLLVWTFICIGTLAEGYPVKPENPGEDAPTEELAKYYSALRHYINLIT 60
Zebrafish       MNPNMKMWMSWAACAFLLFVCLGTLTEGYPTKPDNPGEDAPAEELAKYYSALRHYINLIT 60
Skate           MQNNMKSWLGVFTFIFSMLVCIGTFADAYPSKPDNPGDGASAEQGAKYYTALRHYINLIT 60
                *              .  ::*:. :.:.** **:***:.*.:: *:**:**********

Flounder        RQRYGKRSSPEILDTLVSELLLKESTDTLPQSRYD-PSLW 99
Cod             RQRYGKRSSPEILDTLVSELVLKESANTLPQSRYD-PSLW 99
Eel             RQRYGKRSSPE--G-VMSELLFGDNSEHNQRSRYDDSYMW 97
Grouper         RQRYGKRSSPEILDTLVSELLLKESTDTLPQSRYD-PSLW 99
Goldfish        RQRYGKRSSA---DTLISDLLIGE-TESHPQTRYEDQLVW 96
Halibut         RQRYGKRSSPEILDTLVSELLLKESTDTLPQSRYD-PSLW 99
Ray             RQRYGKRSSPE--ALMMTDLMLRENAESFPKYRYDEPFMW 98
Mouse           RQRYGKRSSP---ETLISDLLMKESTENAPRTRLEDPSMW 97
Trout           RQRYGKRSSPDTLDTLISELLLKESTDTLPQSRYDEPSLW 100
Zebrafish       RQRYGKRSSA---DTLISDLLIGE-TESRPQTRYEDHLAW 96
Skate           RQRLGKRSNPE--ALMMTELMLRDNSENFPKFRYDEPSMW 98
                *** ****..     ::::*:: : ::   : * :    *
```

B



A

```
Flounder        MESS----------------------------EELSRRALRDFYPKGPNLTSEKQLLG- 30
Cod             MESSRVWTRALVCAVLLSVVHGADLYNS--ESEEDLSTRALRDFYPKGPNLTNERQLLG- 57
Goldfish        MESSRLKTRMAVCALLICLLTGAKANESEPEIEVELDARAIRDFYPKDPNLTSEKQLLG- 59
Mouse           MESSRLRLLPLLGAALLLLLPLLGARAQ---EDAELQPRAL-DIYSAVDDASHEKELPRR 56
Trout           MESSRLWIRAVVCAVLLSIVLSADIDYS--DSELDLDTRSVRDFYPKDPNLTNEKQLLG- 57
Zebrafish       MESSKIWSTAMVCAVLLSCIQAAEMDF---DNESDLETRALREFYPKDPNLTNEKQLLG- 56
Skate           ------AVTQHCPAR------------------SLEPRALRDFYSKNYYPGSEKELLG- 40
                ****                            .*. *:: ::*.       *::*

Flounder        ------------ALQEVLEKLQSKRLPLWEKKFGQVPTCDVGEQCAVRKGARIGKMCDCP 78
Cod             ------------ALHEVLEKLQTRKMPLWEKKFGQVPTCDIGEQCAIRKGARIGKMCDCP 105
Goldfish        ------------ALQEVLEKLQTKRIPPWEKKFGQVPMCDLGEQCAIRKGSRIGKMCDCP 107
Mouse           QLRAPGAMLQIEALQEVLKKLKSKRIPIYEKKYGQVPMCDAGEQCAVRKGARIGKLCDCP 116
Trout           ------------ALHDVLKKLQTKRLPFWEKKFGQVPTCDVGEQCAVRKGARIGKLCDC- 104
Zebrafish       ------------ALHDVLEKLQSKRISLWEKKFGRVPTCDVGEQCAIRKGSRIGKMCDCP 104
Skate           ------------ALHEVLKKLQTKRLPTWEKKYGQGPQCNIGDMCAVRKGPRIWRTCNCL 88
                            **::**:**::::::. :***:*: * *: *: **:***.** : *:*

Flounder        RGAFCNFFLLKCL 91
Cod             RGSFCNFFLLKCL 118
Goldfish        RGAFCNFFLLKCL 120
Mouse           RGTSCNSFLLKCL 129
Trout           -------------
Zebrafish       RGAFCNYFLLKCL 117
Skate           S-SKCNYFLFKCV 100
                    ** ** **
```

B

Figure 8. A. CART amino acid sequence alignment acquired with Clustal W of winter flounder and winter skate with Cod, *Gadus morhua* (GenBank accession number DQ167210); Goldfish CART II, *Carrasius auratus* (GenBank accession number AF288811); Mouse, *Mus musculus* (GenBank accession number BC056431); Lake trout, *Salvelinus namaycush* (GenBank accession number DQ836925); Zebrafish, *Danio rerio* (GenBank accession number XM_680337). B. The phylogenetic tree of CART. (*) indicates identical amino acids; (:) indicates similar amino acids; (-) indicates gaps introduced by the Clustal W algorithm when aligning the sequences.

Figure 9. A. CCK amino acid sequence alignment acquired with Clustal W of winter flounder (GenBank accession number EU684055) and winter skate (GenBank accession number EUEU684054) with spiny dogfish, *Squalus acanthias* (GenBank Accession number Z97375); Japanese eel, *Anguilla japonica* (GenBank Accession number AB109556); Bastard halibut, *Paralichthys olivaceus* (GenBank Accession number AB009281); Mouse, *Mus musculus* (GenBank Accession number NM_031161); Rainbow trout, *Oncorhynchus mykiss* (GenBank Accession number NM_001124345); Zebrafish, *Danio rerio* (GenBank Accession number XM_001346104). B. The phylogenetic tree of CCK. (*) indicates identical amino acids; (:) indicates similar amino acids; (-) indicates gaps introduced by the Clustal W algorithm when aligning the sequences.

A

```
Flounder        MTTGLCVYVLLAVLCTSC-LGHPLSSQHLEEGQRSVSAASEALLEADTHSLGEPHLRQRR 59
Dogfish         MNSGICVCVFLAVLSSGC-MGRLATGSDDGGPTGSELKQSVAMRQ--------RQIRETQ 51
Eel             MNGGICVCVLLAALSTSC-LGRP-SSNTQDE-SRAAQSQVDTVLS--------EHMREAR 49
Halibut         MTAGLCVCVLLAVLCTSC-LGHPISSQHLDEGQRSISTPSEALLEADTHSLGEPHLRQSR 59
Mouse           MKSGVCLCVVMAVLAAGA-LAQPVVPAEATDPV--EQRAQEAPR---------RQLRAVL 48
Trout           MNAGICVCVLLAAFSGSS-LGRP---SHSQDEDKPEPPQLDSVMSP-------QHTRHTR 49
Zebrafish       MNAGLCVCALLAALSTSSCLSLP---VHSEDGVQSN---VGSATG---------HTRHTR 45
Skate           MNSGICVCVLLAVLSSGG-LARPDGATERDGERPHGPLHQRPLRE--------APYYGLL 51
                *. *:*: ..:*.:. .  :.                    .

Flounder        SAP----QLN-ALPVAEENGDTRANLSELLARLISS-RKGSVRRNSTAYS--KGLSPNHR 111
Dogfish         SIDL----------KPLQDSEQRANLGALLTRYLQQVRKGPLGRGTLVGTKLQNMDPSHR 101
Eel             STP----LSDQQKPKAEEGVDSRASLTELLARLIS--RKGNTRRNSTINSRASGLSANHR 103
Halibut         SAP----QLK-SLPVAEEDGDSRANLSELLARLISS-RKGSVRRNSTAYS--KGLSPNHR 111
Mouse           RT---------------DGEPPARLGALLARYIQQVRKAPSGR-MSVLKNLQSLDPSHR 91
Trout           SAPSSGQLIPFSKPAEDEAEDPRTSLRELLARLIS--RKGSLQRSSSLSSEASGPGPSHK 107
Zebrafish       AAPPAGQINLLTKPEDDEE--PRSSLTELLARIIS--TKGSYRRSPAANSRTMG--ASHR 99
Skate           KPR--------------LNSEQGPGLVALLATYLHKDNTGSRAG------TVRSVDASHR 91
                                 . *  **:  :    ..            .  ..*:

Flounder        IADRDYLGWMDFGRRSAEEY-EYSS 135
Dogfish         IADRDYMGWMDFGRRSAEEY-EYAS 125
Eel             IKDRDYLGWMDFGRRSAEEY-EYSS 127
Halibut         IADRDYLGWMDFGRRSAEEY-EYSS 135
Mouse           ISDRDYMGWMDFGRRSAEDY-EYPS 115
Trout           IKDRDYLGWMDFGRRSAEEYEEYSS 132
Zebrafish       IKDRDYLGWMDFGRRSAEEY-EYSS 123
Skate           ITNRDYMGWMDFGRRGAEDY-DYPS 115
                * :***:********.**:* :*.*
```

B

Tissue Distribution

Winter flounder NPY, CART and CCK

Reverse transcription PCR (RT-PCR) was used to amplify NPY, CART and CCK in different brain regions as well as in several peripheral tissues of winter flounder (Figures 10 and 11). A 300 bp fragment was amplified for NPY, a 123 bp fragment for CART, and an 87 bp fragment for CCK. Each primer pair was used to amplified with no cDNA as the negative control. No expression was detected in any control amplification, thus verifying the absence of contamination. To ensure that cDNA was present in all tissues examined, samples were all amplified with a control gene, EF-1_. All samples amplified with EF-1_ produced a band of expected size (201 bp) and upon visual inspection, of similar intensity. The PCRs are end-point reactions and it is assumed that there is an equal amount of RNA in each reaction in order to compare the fluorescence between samples.

Within the brain, NPY, CART and CCK expression was detected in all brain regions examined (Figure 10). Expression levels may be lower in the cerebellum compared to the other brain regions on the basis of visual intensity (Figure 10). NPY, CART and CCK expression was detected in all peripheral tissues examined (Figure 11). CART expression may be higher in the gill, gut, liver, kidney and gonad compared to the other tissues. No apparent differences in expression between tissues could be detected for either CCK or NPY.

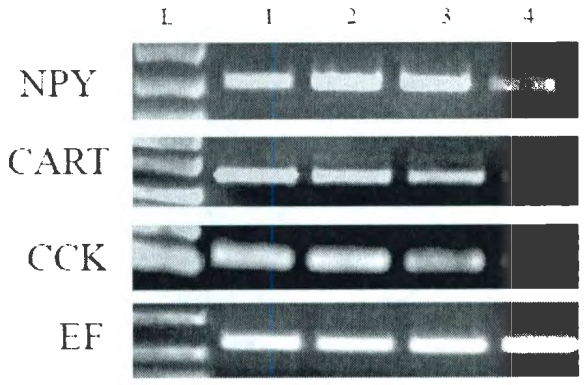


Figure 10. RT-PCR distribution of NPY (300 bp), CART (123 bp), CCK (87 bp) and EF (201 bp) in different brain regions of the winter flounder. Samples were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. L, New England Biolabs PCR maker; 1, hypothalamus; 2, telencephalon; 3, optic tectum; 4, cerebellum.

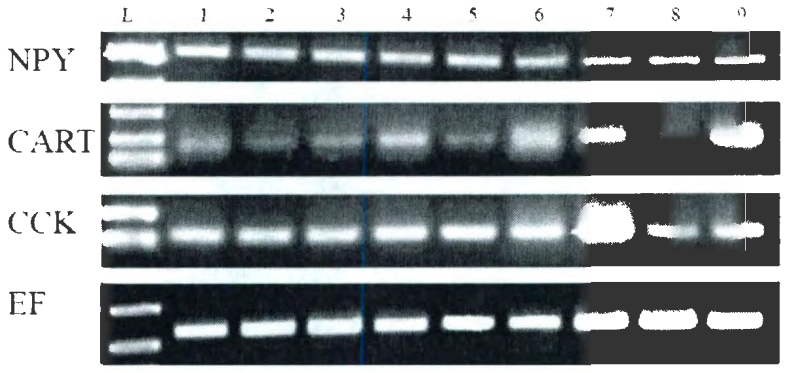


Figure 11. RT-PCR distribution of NPY (300 bp), CART (123 bp), CCK (87 bp) and EF (201 bp) in different peripheral tissues of the winter flounder. Samples were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. L, New England Biolabs PCR maker; 1, gill; 2, heart; 3, stomach; 4, gut; 5, spleen; 6, liver; 7, kidney; 8, muscle; 9, gonad.

Winter skate NPY, CART and CCK

The reverse transcription PCR (RT-PCR) was used to amplify NPY, CART and CCK in different brain regions as well as several peripheral tissues of winter skate (Figures 12 and 13). A 285 bp fragment was isolated for NPY, a 92 bp fragment for CART, and a 120 bp fragment for CCK. No expression was detected in any negative control samples. A 247 bp fragment of similar intensity was amplified in each sample using the control gene EF-1_.

Within the brain, NPY, CART and CCK expressions were detected in all regions examined (Figure 12). Whereas NPY and CCK expression was similar in all brain regions, CART expression may be higher in the hypothalamus and the telencephalon compared to the optic tectum and the cerebellum on the basis of visual inspection.

CART, NPY and CCK expression were detected in all peripheral tissues examined (Figure 13). NPY expression may be higher in the heart, gut, liver, muscle and gonad. Both CART and CCK displayed relatively constant expression levels, with potentially slightly higher expression levels in the gut, liver and kidney.

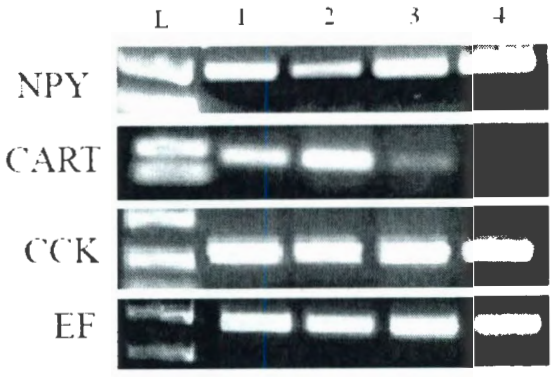


Figure 12. RT-PCR distribution of NPY (285 bp), CART (92 bp), CCK (120 bp) and EF (247 bp) in different brain regions of the winter skate. Samples were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. L, New England Biolabs PCR maker; 1, hypothalamus; 2, telencephalon; 3, optic tectum; 4, cerebellum.

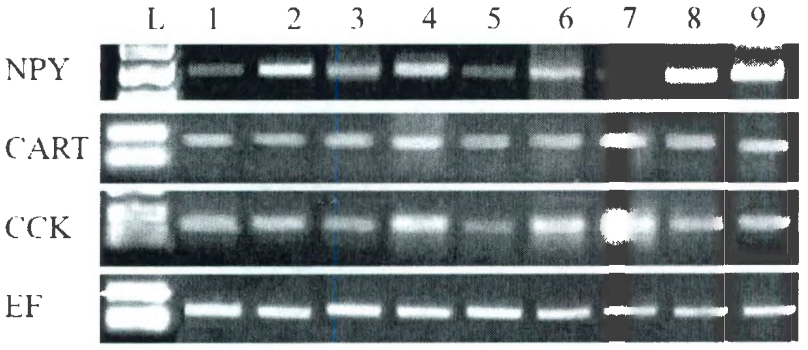


Figure 13. RT-PCR distribution of of NPY (285 bp), CART (92 bp), CCK (120 bp) and EF (247 bp) in different peripheral tissues of the winter skate. Samples were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. L, New England Biolabs PCR maker; 1, gill; 2, heart; 3, stomach; 4, gut; 5, spleen; 6, liver; 7, kidney; 8, muscle; 9, gonad.

Food intake

Flounder consumed an average of 2.12 ± 0.2 g of food per fish per feeding during the winter (0°C) and an average of 12.7 ± 0.89 g of food per fish per feeding during the summer (11°C), which was significantly higher compared to the winter experiment (Figure 14).

Skates held in the summer (11°C) consumed an average of 59.19 g ± 6.1 of food per fish per feeding. No experiment was conducted in the winter months for skates.

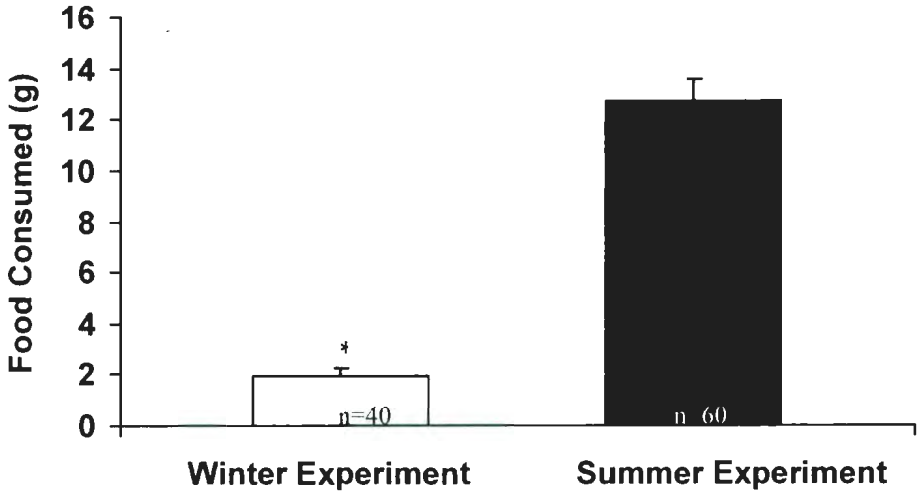


Figure 14. Average food consumed by flounder at each feeding was higher in the summer experiment compared to the winter experiment. Flounder consumed a statistically higher amount of food in the summer compared to the winter ($p<0.05$). Data presented as mean ± SEM. * indicates significant difference.

Gene Expression

Effects of starvation on gene expression: Winter flounder winter experiment

Based on the results from the tissue distribution, the highest expression of NPY, and CART was most frequently found in the hypothalamus and in the gut for the CCK which is why gene expression was done on these tissues. There were no significant differences in NPY expression in the hypothalamus of flounder between fed fish and starved at either two, four or six weeks of starvation (Figure 15). The NPY expression levels were similar at two, four and six weeks for both fed and starved groups (Figure 15)

CART expression levels in the fed group were significantly higher in fed two weeks compared to starved four weeks, fed six weeks and starved six weeks ($p<0.05$) (Figure 16).

There were no significant differences in CCK expression levels in the gut of flounder both between fed and starved fish, or between collection dates (Fig. 17). Expression levels of CCK were extremely low in all samples and this made quantification difficult.

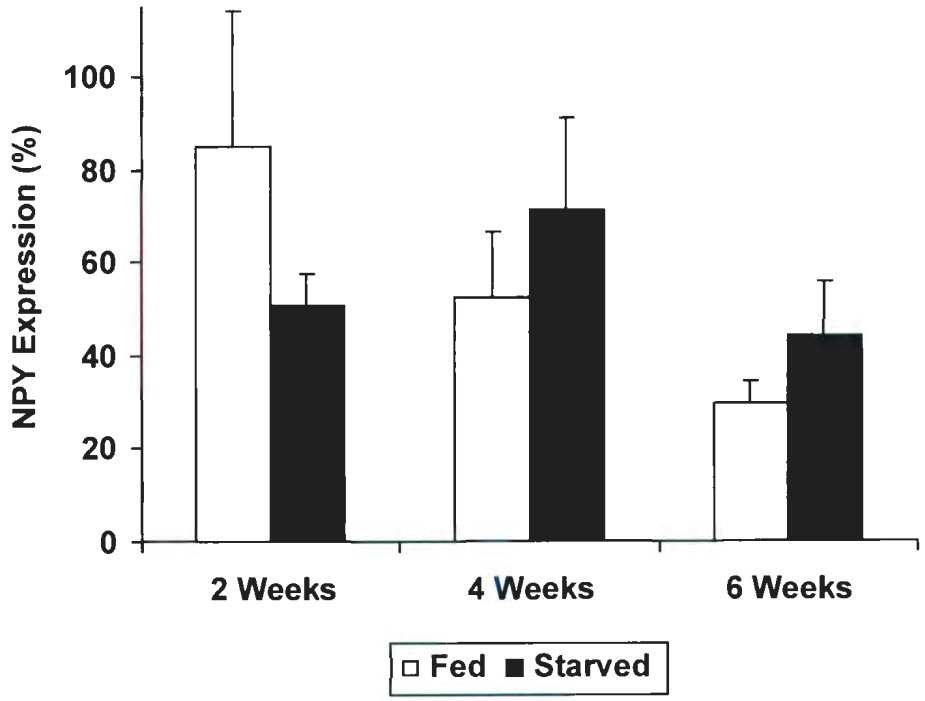


Figure 15. NPY expression in the hypothalamus of winter flounder had no change over the winter experiment. Ten fish were sampled from both fed and starved groups at each collection. NPY expression is expressed as a percentage normalized to the control group. Data is presented as mean ± SEM.

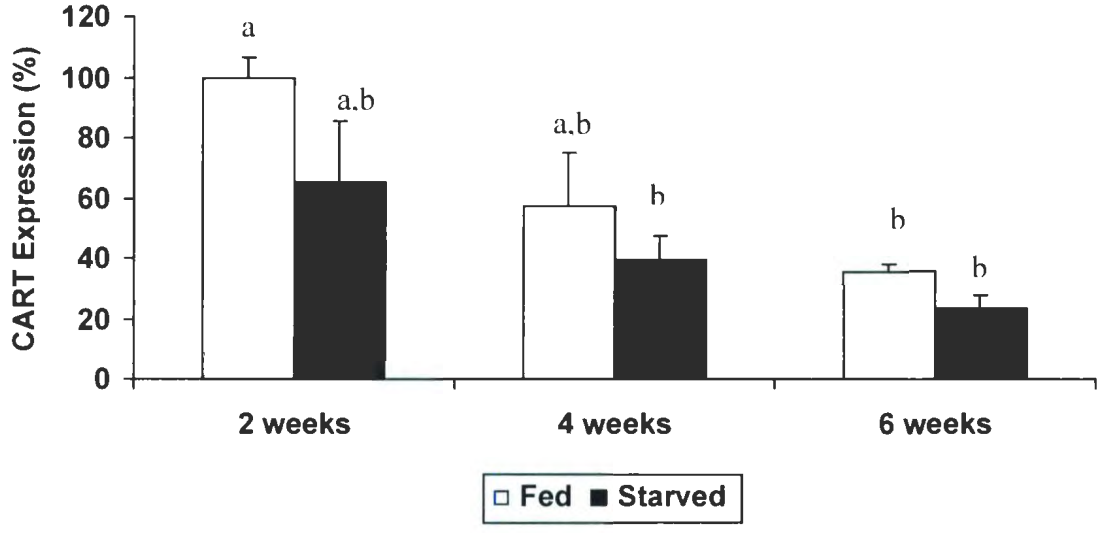


Figure 16. CART expression in the hypothalamus of winter flounder during the winter experiment decreased between sampling dates. Ten fish were sampled from both fed and starved groups at each collection. CART expression expressed as a percentage normalized to the control group. The flounder that were fed for two weeks expressed CART at a significantly higher level compared to the starved fed from six weeks and those fed for six weeks ($p<0.05$). Data is presented as mean ± SEM. Letters a, ab, and b are used to indicated which columns are similar and which are different.

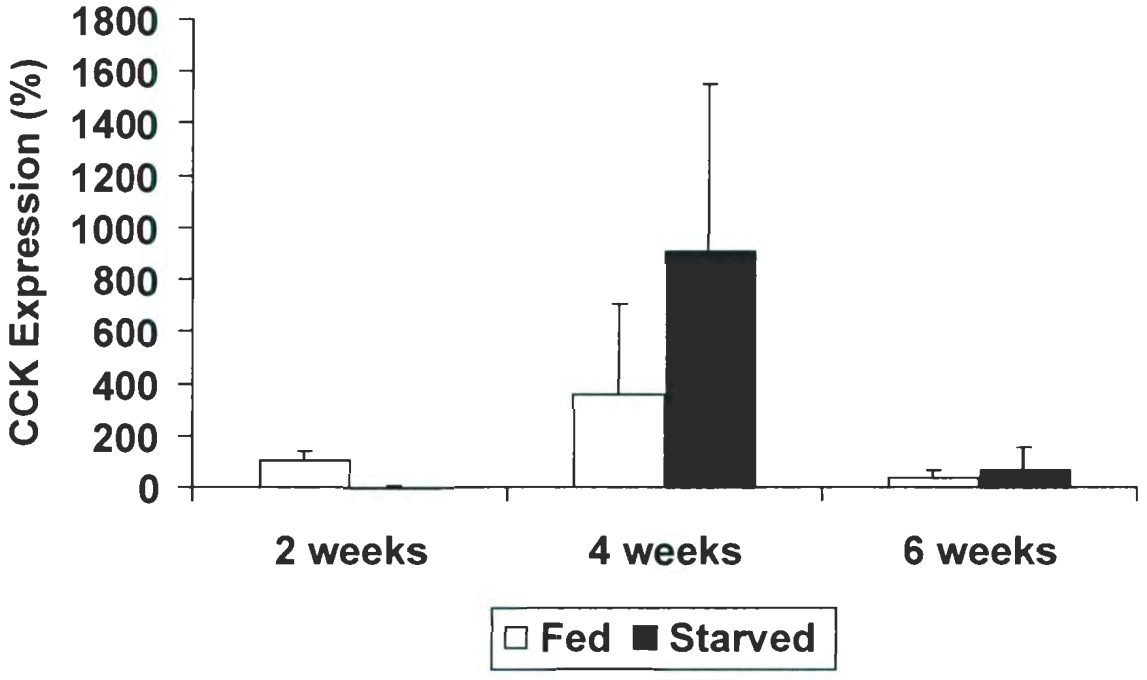


Figure 17. CCK expression in the gut of winter flounder had no change during the winter experiment. Ten fish were sampled from both fed and starved groups at each collection. CCK expression is expressed as a percentage normalized to the control group. Data is presented as mean ± SEM.

Effects of starvation on gene expression: Winter flounder summer experiment

At both two and four weeks of starvation, NPY expression in the hypothalamus of flounder was significantly higher in fasted fish compared to fed fish (Figure 18). While in fed fish, NPY expression levels were similar at two and four weeks. In fasted fish, there was a significant increase in NPY expression at four weeks compared to fish at two weeks.

There were no significant changes in CART mRNA expression in the hypothalamus of the winter flounder between the fed and starved groups at either two or four weeks of starvation or between both collections (Figure 19).

CCK mRNA levels were similar in fed fish at two and four weeks. There was a significant decrease in CCK expression in the gut of starved fish at four weeks compared to starved fish at two weeks. There were no significant differences in CCK expression between fed and fasted fish at either two or four weeks (Figure 20).

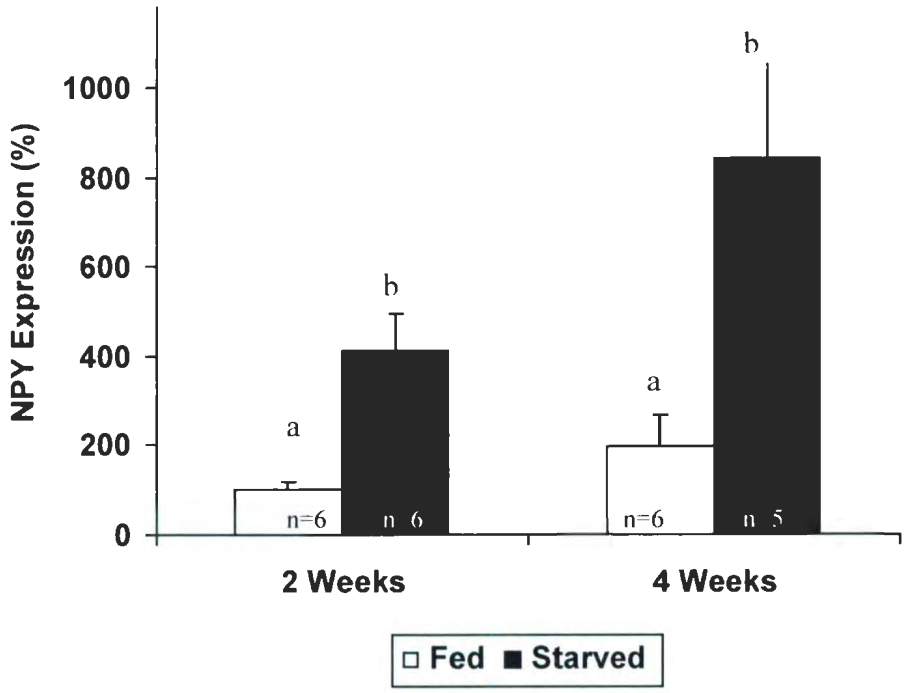


Figure 18. NPY expression in the hypothalamus of winter flounder during the summer experiment was higher in starved fish compared to fed fish. NPY is expression expressed as a percentage normalized to the control group. At both two weeks and four weeks there was a significantly higher NPY expresssion in starved fish compared to fed fish ($p<0.05$). There was a statistically significant increase in NPY expression in the starved group at four weeks starvation compared to two weeks starvation ($p<0.05$). Data is presented as mean ± SEM. Letters, a and b, are used to indicated which columns are the same, and which are different.

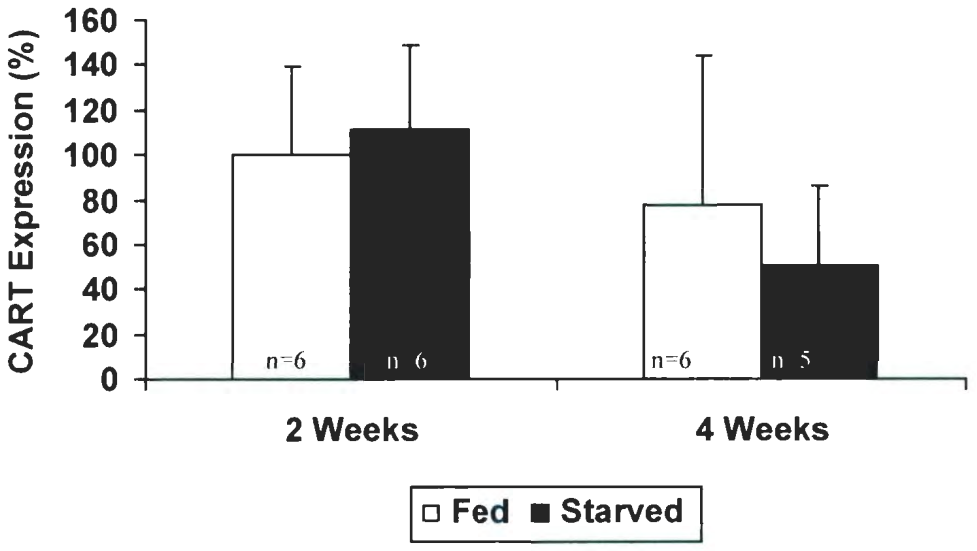


Figure 19. CART expression in the hypothalamus of winter flounder did not change during the summer experiment. CART expression is expressed as a percentage normalized to the control group. Data is presented as mean ± SEM.

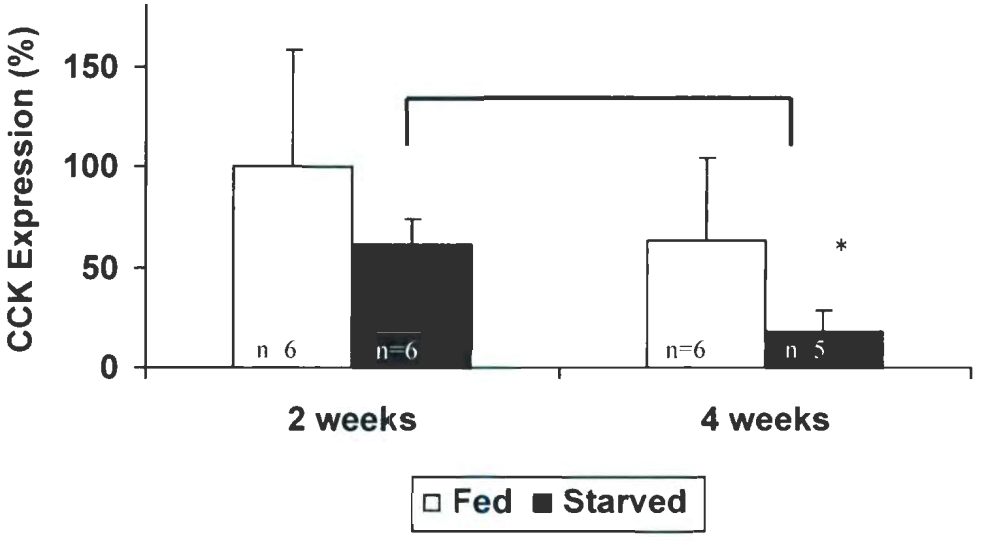


Figure 20. CCK expression in the gut of winter flounder decreased in starved fish during the summer experiment. CCK expression is expressed as a percentage normalized to the control group. CCK expression levels in the gut were significantly lower after four weeks of starvation compared to two weeks ($p<0.05$). Data is presented as mean ± SEM. * indicates a significant difference.

Effects of season on gene expression

When examining the effect different seasons have on hormone expression we found a significant decrease in NPY expression in the winter flounder hypothalamus in the summer experiment compared to the winter experiment (Figure 21). In contrast there were no differences in CART expression between summer and winter in the flounder hypothalamus (Figure 22). When hormone expression was looked at in the periphery we found a significant increase in CCK expression in the winter flounder gut in the summer experiment compared to the winter experiment (Figure 23). There are changes occurring between seasons in the hormone profile of the winter flounder.

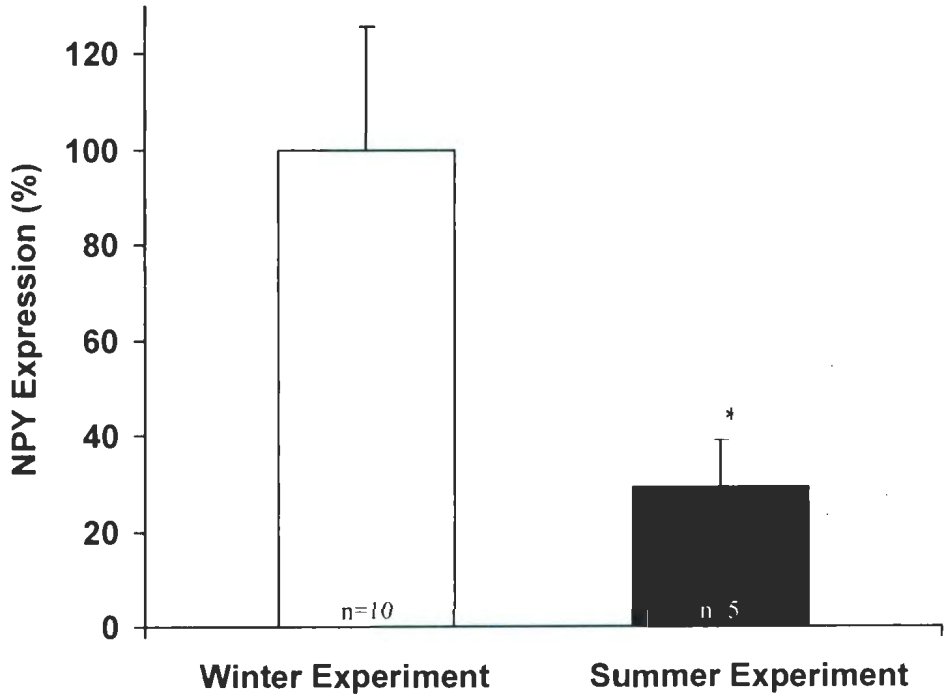


Figure 21. NPY expression in the hypothalamus of fed winter flounder collected at four weeks decreased in the summer experiment. NPY expression is expressed as a percentage normalized to the control group. NPY levels in the summer were significantly lower than in the winter ($p<0.05$). Data is presented as mean ± SEM. * indicates a significant difference.

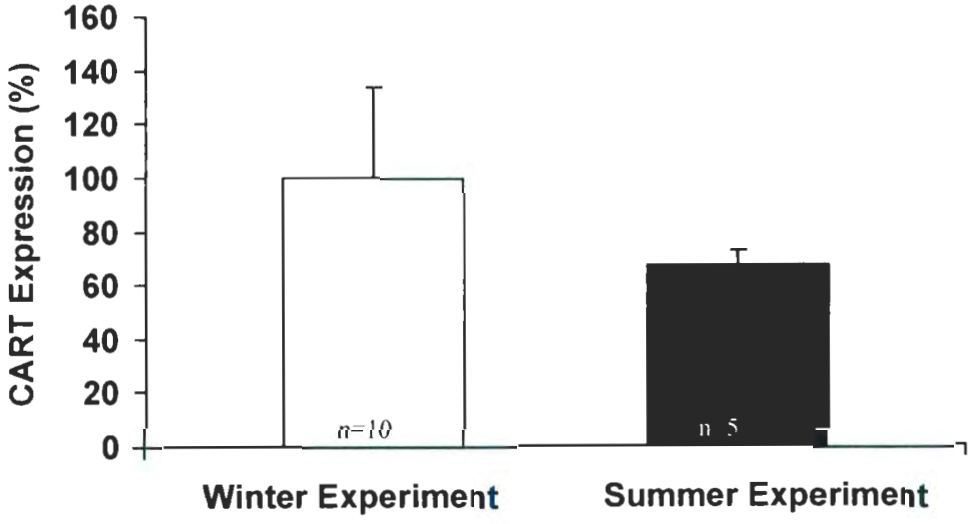


Figure 22. CART expression in the hypothalamus of fed winter flounder collected at four weeks did not change over both experiments. CART expression expressed as a percentage normalized to the control group. Data is presented as mean ± SEM.

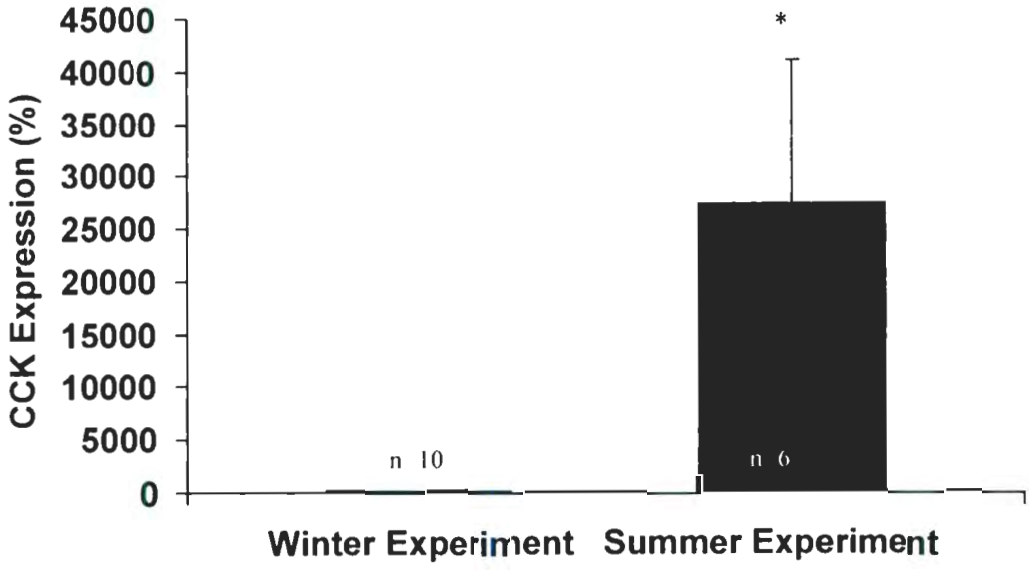


Figure 23. CCK expression in the hypothalamus of fed winter flounder collected at four weeks was higher in the summer experiment. CCK expression is expressed as a percentage normalized to the control group. There was a statistically significant increase in CCK expression in the summer compared to the summer ($p<0.05$). Data is presented as mean ± SEM. * indicates a significant difference.

Effects of starvation on gene expression in winter skate

There were no significant changes in NPY expression in the hypothalamus of the winter skate brain between fed and starved animals (Figure 24). CART expression was not analyzed due to time constraints.

There was a significant increase in CCK expression in the skate gut of starved animals compared to fed animals (Figure 25). In contrast to the winter flounder experiment that was conducted around the same time, NPY and CCK expression levels do not change in response to starvation.

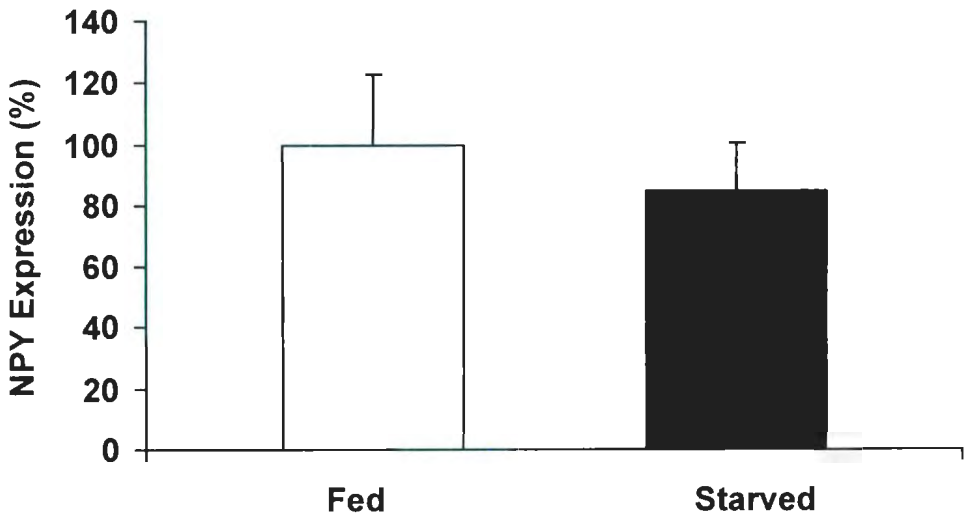


Figure 24. NPY expression in the hypothalamus of winter skate was not different between fed and starved animals. Data is presented as mean ± SEM. Eight skates were sampled for each group. NPY expression is expressed as a percentage normalized to the control group.

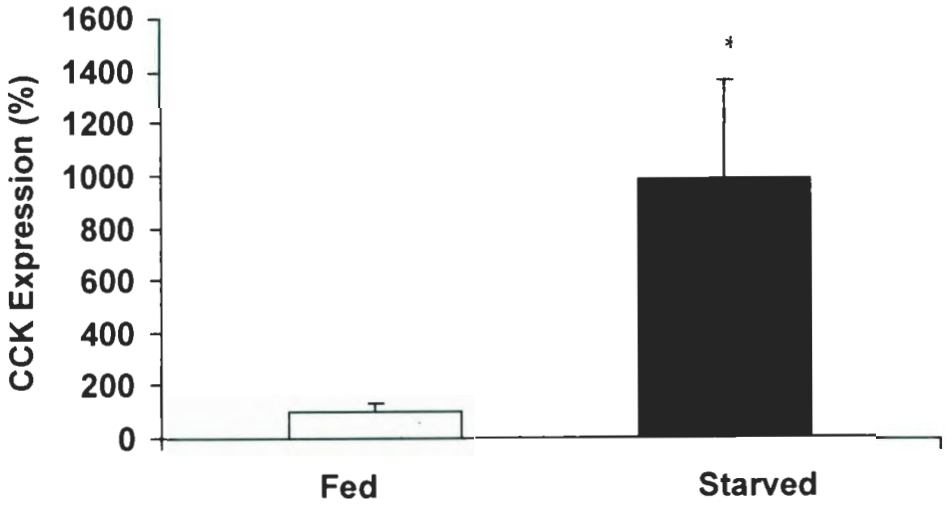


Figure 25. CCK expression in the hypothalamus of winter skate was higher in starved fish. Eight skates were sampled for each group. CCK expression is expressed as a percentage normalized to the control group. There was a statistically significant increase in CCK expression in the starved fish compared to the fed fish ($p<0.05$). Data is presented as mean ± SEM. * indicates a significant difference.

Discussion

Cloning

Winter flounder NPY, CART and CCK

The cDNA sequences for winter flounder NPY, CART and CCK were successfully cloned and characterized for the first time.

As in other fish species, the winter flounder NPY gene appears to have four exons and three possible polyadenylation sites. Winter flounder preproNPY shows a relatively high degree of homology with NPY of other fish. The highest sequence similarity is found with another flat fish, the bastard halibut (96%) and with orange spotted grouper (96%), while winter flounder and trout NPY sequences have 80% similarity. Further examination shows that the majority of the mature peptide is extremely well conserved, with a stretch of 20 amino acids possessing 100% identity with other teleosts (Figure 7). The high sequence similarity of winter flounder preproNPY and mature NPY with other fish NPY sequences may indicate that NPY in winter flounder has conserved physiological roles as in those related fish species.

Winter flounder preproCART cDNA has 84% amino acid similarity with cod preproCART, 83% similarity to goldfish CART II and 79% similarity to zebrafish sequences (Figure 8). The winter flounder CART cDNA starts with the characteristic MESS amino acid sequence found in all vertebrate CARTs but appears to lack the subsequent 28 amino acid residues. This is the first report of a "short" proCART sequence. Prior to now, CART has been cloned in a small number of mammalian and

fish species. Further studies will be needed to determine the physiological significance, if any, of this shorter sequence.

There is good conservation of the arrangement of cysteine residues within CART proteins between species. The location of the six cysteine residues is identical in all species (Figure 8). Cysteine residues are essential for the three dimensional structural conformation of a protein because of their thiol group. The two sulfurs located in the thiol groups can form a disulfide bond which is required to make the folded biologically active form of the protein. When rats are injected with unfolded CART peptides there is no decrease in feeding behaviour which demonstrates that these disulfide bonds are essential for the biological activity of CART (Couceyro *et al.* 2003). The complete conservation of the cysteine residues in different animals suggests the structured characteristics of CART may be conserved between species.

Winter flounder CCK gene appears to have two introns and one exon, determined by comparing winter flounder CCK with other fish CCK sequences. The winter flounder preproCCK sequence shows 91% sequence similarity with its relative the bastard halibut. When comparing the mature peptide sequence, CCK-8, there is 100% sequence similarity with other teleosts, and only one amino acid difference with other species (Fig. 9). Some teleosts, such as the rainbow trout have more than one form of CCK, but the current experimental results suggest there is only one form of CCK in winter flounder (Jensen *et al.* 2001). The extremely well conserved mature peptide and nucleotide sequence conservation with related species suggests the physiological function of the peptide may be well conserved.

Winter skate NPY, CART and CCK

NPY, CART and CCK were cloned for winter skate using RT-PCR with degenerate primers and RACE. Winter skate NPY has three exons divided by two introns, and three possible polyadenylation sites. As the winter skate is an elasmobranch and evolutionarily distant from teleost fish, it was expected that the hormone sequences would be more similar to other elasmobranchs than other teleosts. Winter skate preproNPY displays a high similarity (81%) with electric ray NPY, yet much lower degrees of similarity with NPYs from teleost fish (at most 55% similarity to rainbow trout NPY). Mature NPY peptide is much more highly conserved among species, including a stretch of 13 identical amino acids at the c-terminus. This high degree of conservation suggests that NPY might have similar physiological action among fish, including teleosts and elasmobranchs.

The partially characterized winter skate CART gene has three exons but the 5' region has not yet been isolated. It was expected that there would be differences between the skate cDNA sequence and CART sequences from teleost fish. The sequence that was cloned suggests that there may be an additional intron in winter skate CART compared to other species. There are no known CART sequences cloned from other elasmobranchs so the closest homology is with winter flounder (57%) followed by goldfish (53%). Further examination of the cloned sequence shows that there is conservation among the cysteine residues which are vital to the final conformation of the CART peptide. The six cysteine residues are identical in all the species listed (Figure 8). The good sequence homology with winter flounder and goldfish and the excellent conservation of cysteine residues

among all species, is evidence that the cloned fragment is winter skate CART and suggests similar physiological functions across the taxa.

The cloned winter skate CCK sequence contains two exons and one intron. Winter skate preproCCK shows a very low degree of similarity with CCKs from other fish; the highest sequence similarity is 46% similarity to spiny dogfish CCK, another elasmobranch. On the other hand, there is a very high degree of identity between the mature CCK-8 regions among all species, with only one variable amino acid. These facts indicate that the cloned sequence is indeed winter skate CCK and that CCK must have conserved biological functions among fish species.

Tissue Distribution

Winter flounder

NPY

Using RT-PCR NPY expression was examined in four brain regions and in eight peripheral tissues. High levels of NPY mRNA expression were found in the winter flounder forebrain including the hypothalamus, telencephalon and optic tectum with lower levels in the cerebellum. High NPY expression in the forebrain has previously been shown in other fish species including salmon, sea bass, catfish, sole and goldfish (Peng *et al.* 1994; Silverstein *et al.* 1998; Cerda-Reverter *et al.* 2000; Marchetti *et al.* 2000; Leonard *et al.* 2001; Rodriguez-Gomez *et al.* 2001; Narnaware *et al.* 2002). High forebrain NPY expression is consistent with its role in the regulation of feeding (Narnaware *et al.* 2002), in processing olfactory inputs (Pirone *et al.* 2003) and in the

control of pituitary secretions (Cerda-Reverter *et al.* 2000; Rodriguez-Gomez *et al.* 2001).

NPY mRNA expression was detected in the gill, heart, stomach, gut, spleen, liver, kidney, muscle and gonad. This widespread distribution of NPY has been shown in several fish species and is consistent with the putative role of NPY in a number of physiological functions. NPY has been shown to induce coronary artery contraction (Bjenning *et al.* 1993), cause contractions in gut arteries (Preston *et al.* 1998), increase heart rate (Xiang 1994), and act on cells that secrete somatostatin for the regulation of puberty in sea bass (Peyon *et al.* 2003). The expression of NPY in the gonad has been previously shown and implicated in the regulation of reproduction (Peng *et al.* 1994; Leonard *et al.* 2001; Gaikwad *et al.* 2005).

However, one of the major functions of NPY is related to the regulation of feeding. This important role is reflected by high NPY mRNA expression levels in both brain and gastrointestinal tract (Kehoe *et al.* 2007). In addition, peripheral administration of NPY causes an increase in food intake (Kiris GA (Kiris 2007).

CART

Similar to NPY expression, CART mRNA expression was also more pronounced in the forebrain regions, the hypothalamus, telencephalon and optic tectum compared to the cerebellum. CART immunoreactivity (Singru *et al.* 2007) and CART mRNA expression (Volkoff *et al.* 2001; Kehoe *et al.* 2007) have both been shown in brain regions including the hypothalamus, telencephalon and optic tectum of other fish species.

CART mRNA expression was found in the cerebellum of the winter flounder, which contrasts with cod where no CART mRNA expression was found in this area (Kehoe *et al.* 2007). The diverse expression of CART in the winter flounder brain suggests that it has many physiological roles, including a role in the control of feeding. In goldfish and cod, the expression of CART mRNA in the hypothalamus and telencephalon decreases when fish are fasted (Volkoff *et al.* 2001; Kehoe *et al.* 2007) consistent with the anorexigenic properties of CART.

Winter flounder CART mRNA expression was high in the gut, liver, kidney and gonad compared to a lower expression in gill, heart, stomach, spleen and muscle. CART mRNA expression has been found in the bovine ovary, where it inhibits estradiol production (Kobayashi *et al.* 2004). CART mRNA expression has been found throughout the rat central nervous system (Couceyro *et al.* 1997). In goldfish CART mRNA expression has been shown in the gill, kidney and gonad but not in the liver, gut or the muscle (Volkoff *et al.* 2001). In cod, CART mRNA expression is only found in the brain and the ovary (Kehoe *et al.* 2007). It thus appears that CART mRNA expression in winter flounder has a more widespread distribution among peripheral tissues than that found in any other species examined to date. In particular, previous studies have detected CART peptides in the gut (Couceyro *et al.* 1998; Kuhar *et al.* 1999), yet until now CART mRNA has not been found in the gastrointestinal tract. This discovery of CART mRNA in the gut of flounder suggests that CART might act as a brain-gut peptide.

CCK

Winter flounder CCK mRNA expression levels appeared to be highest in the hypothalamus, telencephalon and optic tectum and lower in the cerebellum. The mRNA expression of CCK in the brain of fish has been widely documented (Peyon *et al.* 1998; Kurokawa *et al.* 2003; Murashita *et al.* 2006). In trout, three different forms of CCK mRNA are found in the brain CCK-L, CCK-N and CCK-T (Jensen *et al.* 2001) whereas mammals only express one form of CCK. CCK mRNA levels in the brain have also been shown to change in response to feeding. In goldfish and yellowtail, CCK mRNA increases after a meal (Peyon *et al.* 1999; Murashita *et al.* 2007).

Winter flounder CCK mRNA was expressed in all the peripheral tissues examined, for example gill, heart, stomach, gut, spleen, liver, kidney, muscle and gonad. Slightly higher expression was found in the liver and relatively lower expression was found in the muscle. CCK mRNA in yellowtail is found in stomach, pyloric caeca (a sac used to increase gut surface area), anterior intestine, posterior intestine, and rectum (Murashita *et al.* 2006). In trout the three different forms of CCK mRNA are expressed throughout the fish including the stomach, intestine, liver and muscle (Jensen *et al.* 2001). The mRNA expressions of CCK in the gill, heart and spleen that were found in winter flounder have not been previously noted in other fish species. In response to feeding, CCK mRNA levels increase in the yellowtail digestive tract, pyloric caeca, following a meal (Murashita *et al.* 2007). The presence of CCK mRNA in this area of the digestive tract suggests a role as an appetite hormone.

Winter skate

NPY

Within the winter skate brain, NPY mRNA expression was detected in hypothalamus, telencephalon, optic tectum and cerebellum. NPY mRNA expression has been shown in the forebrain of a number of teleost fish species (Silverstein *et al.* 1998; Cerda-Reverter *et al.* 2000; Leonard *et al.* 2001; Kehoe *et al.* 2007) including the winter flounder (this study). NPY immunoreactive cells have also been found in the central nervous system of elasmobranchs (Chiba 2000; Sueiro *et al.* 2007). The expression of NPY mRNA in the cerebellum has not been well documented in other species. Changes in forebrain NPY mRNA expression levels in goldfish, catfish and cod around feeding time seem to indicate that NPY is a possible regulator of food intake (Silverstein *et al.* 1998; Narnaware *et al.* 2000; Kehoe *et al.* 2007).

In the periphery, NPY mRNA expression was found in every tissue examined, with apparent high expression levels in the heart, stomach, gut, liver, muscle and gonad. NPY mRNA has not been found in the gill, spleen or liver in other fish species with the exception of the winter flounder as repeated here. In elasmobranchs, NPY inhibits both gastric emptying and stomach contractions (Dumont *et al.* 1992; Bjenning *et al.* 1993; Bjenning *et al.* 1993) providing further support of the role of NPY as a brain gut peptide.

CART

CART mRNA expression was high in the hypothalamus and telencephalon of the winter skate brain and lower in the optic tectum and the cerebellum. These expression

patterns were similar to those found in previous studies in cod and goldfish (Volkoff *et al.* 2001; Kehoe *et al.* 2007). CART mRNA expression in the brain decreases with fasting, so its presence in the brain supports its role as a brain-gut peptide (Volkoff *et al.* 2001).

CART mRNA expression showed relatively constant expression in all of the peripheral tissues examined. Similar to winter flounder, CART mRNA has not been previously detected in the gut of mammals and fish, only CART peptides (Couceyro *et al.* 1998; Kuhar *et al.* 1999).

CCK

CCK mRNA expression was found in the winter skate hypothalamus, telencephalon, optic tectum and cerebellum. Not including winter flounder, CCK mRNA expression has to date not been identified in the fish cerebellum. On the other hand, its high expression in the hypothalamus and telencephalon has been well documented (Peyon *et al.* 1998; Kurokawa *et al.* 2003; Murashita *et al.* 2006). In addition, CCK binding sites have been found in the brain of elasmobranchs (Oliver *et al.* 1996), and CCK mRNA levels in the brain change in response to feeding (Peyon *et al.* 1999; Murashita *et al.* 2007) suggesting CCK peptides have a role in the brain gut regulation of feeding and digestion.

CCK mRNA expression in the winter skate was found in all the peripheral tissues tested, with higher expression in the gut, liver and kidney. CCK can cause gallbladder contraction in the killifish (Honkanen *et al.* 1988), inhibit gastric secretions in cod

(Holstein 1982), increase gut motility in dogfish (Aldman *et al.* 1989) and cod (Forgan *et al.* 2007) and slow gastric emptying in trout (Olsson *et al.* 1999), suggesting its role in appetite regulation. The presence of CCK mRNA in the gastrointestinal tract combined with evidence of a role of CCK in the regulation of digestive functions supports the role of CCK in appetite regulation.

Summary of tissue distribution and cloning

The sequences and tissue distribution of NPY, CART and CCK mRNA in winter flounder and winter skate have been successfully determined. Sequence comparison and examination of the literature suggests a role in appetite regulation for each of these hormones in these fish. With this information, experiments were designed to examine the effect of starvation on the expression of these three appetite hormones in both fish species. In the future, a screening of neuropeptide expression with relation to season may give more insight into their roles in appetite regulation.

Effects of Starvation on Gene Expression

Winter flounder winter experiment

Over the course of the winter experiment, samples were taken every two weeks. mRNA from the hypothalamus and gut was isolated and used to examine the effects of starvation on the expression of NPY, CART and CCK.

NPY mRNA expression in the hypothalamus displayed no change between either collections, or between starved and fed fish. These results differ from previous studies in

salmon and goldfish that show that NPY expression in the brain increases in starved fish compared to fed fish (Silverstein *et al.* 1998; Narnaware *et al.* 2000; Narnaware *et al.* 2001). In goldfish, mRNA expression in the hypothalamus and telencephalon was high before feeding and decreased after feeding (Narnaware *et al.* 2000) and was shown to be regulated by macronutrient content (Narnaware *et al.* 2002). In the chinook and coho salmon mRNA expression in the forebrain increased in fasted fish compared to fed fish (Silverstein *et al.* 1998). Similar to our results, in cod, however, NPY mRNA expression in the brain does not change in response to starvation (Kehoe *et al.* 2007). Similar to cod, winter flounder is capable of withstanding long periods of fasting in the wild. The lack of changes in NPY expression could be indicative of this feeding adaptation. A longer period of fasting might be necessary to induce changes in NPY expression.

It is also important to note that during the winter experiment, winter flounder ate very little food (~2 g per feeding), which may be an indicator of other physiological processes. Winter flounder have been previously observed to undergo a dormancy-like phase in the colder winter months where their movement and food consumption decrease (Martell *et al.* 1994; Stoner *et al.* 1999; Meise *et al.* 2003). Winter flounder in the winter showed very little swimming activity in the tanks compared to animals held in the summer (personal observation). The fact that there was no change in mRNA expression of NPY in starved fish compared to fed fish may be related to the inactivity of winter flounder in the winter months and possibly a "shutdown" of the NPY system.

During the winter, mRNA expression of CART in the hypothalamus was not affected by starvation at 2, 4 or 6 weeks. These results contrast with previous studies in

goldfish showing that CART brain mRNA levels decrease in response to starvation (Volkoff *et al.* 2001). Similar to NPY expression, the absence of variation in CART expression might be due to the dormant-like state of the animals and a shutdown of normal regulatory functions that are controlled by the brain. Interestingly, CART mRNA levels in fed fish decreased from week 2 to week 6. The reasons for this decline are unclear as one would expect the mRNA expression of CART to remain constant in the fed group. CART mRNA presence in the ovary has been previously implicated in reproduction (Volkoff *et al.* 2001; Kobayashi *et al.* 2004; Kehoe *et al.* 2007) and winter flounder are known to spawn in the winter (Scott *et al.* 1988).

CCK mRNA expression was examined in the gut of winter flounder for the winter experiment. There were no significant changes in CCK mRNA expression. In fact, the expression of CCK in the gut was so low that it was very difficult to quantify. CCK has been well documented as a digestion regulating peptide in fish (Honkanen *et al.* 1988; Aldman *et al.* 1989; Olsson *et al.* 1999; Olsson *et al.* 1999; Forgan *et al.* 2007) as well as acting as a satiety factor (Himick *et al.* 1994; Volkoff *et al.* 2003). In yellowtail, there was an 80 fold higher expression of CCK mRNA levels in the brain compared to the pyloric caeca (Murashita *et al.* 2006), which might have been a better area to examine CCK mRNA expression. The low expression levels of CCK in winter flounder suggest that other regulatory processes must be occurring in the winter months to induce low activity and low feeding rates. In the winter months carp have been shown to slow not only their heart contractions in response to the cold water but also to produce different types of myosin in the heart compared to the summer (Vornanen 1994; Tiitu V 2001).

Other fish species such as *Rutilus rutilus*, also undergo fasting during the winter months (Mendez G 1993) accompanied by metabolic depression (Holker 2003) and a reliance on energy reserves in white muscle (van Dijk PLM 2002). This evidence that adaptations to cold water occur in other fish species suggests that winter flounder could be undergoing similar physiological changes. All energy is focused on surviving the winter and not eating, due to lack of prey and also the harshness of winter. The physiological functions involved in surviving the cold may override the appetite regulatory functions, especially since food is not available.

Winter flounder summer experiment

In the summer experiment, mRNA expression of NPY in the hypothalamus was higher in the starved animals compared to the fed animals at both two and four weeks of stravation. The higher expression of NPY mRNA in the brain of starved fish is similar to expression patterns found in other fish (Silverstein *et al.* 1998; Narnaware *et al.* 2001). The higher expression of NPY mRNA in starved fish supports a role for NPY as an appetite regulating peptide.

In the summer, CART mRNA expression in the hypothalamus did not change significantly over the course of the experiment. CART mRNA tended to be lower in the starved group compared to the fed group after four weeks of starvation, but this decrease was not significant. In both goldfish and cod (Volkoff *et al.* 2001; Kehoe *et al.* 2007), brain CART mRNA expression levels decrease following starvation, so it was expected that CART mRNA in the hypothalamus would also decrease in starved flounder

compared to fed flounder. CART mRNA expression in the hypothalamus was relatively low, and lower than NPY. It is possible that differences in expression levels were too small to be detected by real-time quantitative PCR.

There were no significant differences in gut CCK expression levels between fed and starved animals. These results contrast with previous studies showing decreases in CCK gut levels following fasting in several fish species, including yellowtail, dogfish and rainbow trout (Aldman *et al.* 1989; Olsson *et al.* 1999; Murashita *et al.* 2006). However, CCK expression levels were lower in fasted animals after four weeks of starvation compared to two weeks of starvation, suggesting that CCK might be involved in the regulation of digestive processes and feeding in winter flounder. If CCK is an anorexigenic peptide then one would expect that the longer the starvation period the lower the amount of CCK production.

Effect of season on gene expression in winter flounder

In order to examine the effects of season on gene expression, I compared the expression of NPY, CART and CCK in fed animals in the winter and the summer.

NPY mRNA expression was significantly lower in the fed fish in the summer compared to the winter. As food consumption is higher in the summer compared to the winter and NPY has been shown to be an orexigenic peptide in fish, one would have expected an increase in NPY expression in summer animals. High NPY expression levels in the winter might be indicative of a stimulation of appetite-related NPY pathways in the brain by an empty gut and a down regulation on NPY receptors within the brain. If

food is normally not accessible in the winter there is no point wasting valuable energy reserves telling the body that it needs food. As well no NPY expression differences between fed and starved fish in the winter were found and yet the differences were clear in the summer. There must be other regulatory factors occurring during the winter in winter flounder which are overriding the appetite regulatory functions of NPY.

There were no significant differences in CART mRNA expression in the hypothalamus of winter flounder in the winter experiment compared to the summer experiment. The lack of difference in CART mRNA expression could be due to small sample size and higher variability of CART expression compared to NPY or to lower expression levels of CART. CART mRNA expression is higher in the telencephalon compared to other brain regions in cod, catfish and goldfish (Narnaware *et al.* 2000; Leonard *et al.* 2001; Kehoe *et al.* 2007). CART mRNA expression might also be higher in the winter flounder telencephalon and this could be where the effect of starvation is more evident. Another factor to consider is the cloned CART sequence itself. Goldfish has two forms of CART, I and II, and CART I is more sensitive to feeding studies compared to the other (Volkoff *et al.* 2001). Winter flounder may also have two versions of CART and we only cloned one of them. Finally, it is also possible that CART may not have a strong role in feeding regulation in winter flounder because not only was there no difference in CART mRNA expression between seasons, but there was no difference between starved and fed winter flounder in the summer experiment.

CCK gut mRNA expression was higher in fed winter flounder in the summer compared to the winter. Given that fish had higher food consumption and were fed to

satiety in the summer and that CCK acts as an anorexigenic peptide in fish, higher levels of CCK in the fed fish in the summer were expected. The expression levels of CCK in the winter were so low they were almost undetectable. This low expression in the winter again emphasizes the fact that there must be other regulatory functions occurring during the winter months, or no regulation at all.

The low expression of CCK in addition to relatively constant expression of NPY and CART in the winter is indicative that the fish are not responding to starvation in the winter as they do in the summer. A number of fish, including winter flounder, have been shown to display both decreased growth rates and food consumption in colder water compared to warmer water (Martell *et al.* 1994; Stoner *et al.* 1999; Meise *et al.* 2003; Kehoe *et al.* 2007). It is noteworthy that during the winter, winter flounder produce antifreeze proteins to protect themselves from freezing (Gauthier *et al.* 2005). Metabolites have been shown to affect feeding as glucose administration and hyperglycemia causes slower feeding and a decrease in food intake (Banos *et al.* 1998; Kuz'mina 2005). It is possible that high antifreeze protein levels affect appetite related hormonal systems. This may be another factor to consider when examining food intake with future studies.

Skate experiment

Only the summer experiment was conducted for skate because of lack of availability of winter skates in the winter. This experiment had one sampling because only a small number of animals were available.

There were no significant differences in NPY expression in the hypothalamus of fed winter skates compared to starved winter skates. This could be largely due to the length of starvation. In goldfish NPY, mRNA levels increased after 72 hours of food deprivation (Narnaware *et al.* 2001) whereas in salmon, NPY mRNA levels changed after two to three weeks starvation (Silverstein *et al.* 1998). Winter skates live mostly on the ocean floor unlike goldfish and salmon which spend most of the time in the water column. Thus a different starvation regime may be required to examine mRNA expression in winter skate, like examining the effect of a longer starvation period.

Gut CCK mRNA expression was significantly higher in the starved fish compared to the fed fish. As for NPY this unexpected result could be due to the short duration of the study, or the physiology and life history of the winter skate. The majority of the fish used in feeding experiments are teleosts, and the elasmobranchs may have slightly different appetite regulatory systems because the teleosts are a more recent evolutionary lineage and display different life histories.

General conclusions

My results suggest that there are many factors other than appetite regulation contributing to the differences in winter flounder NPY, CART and CCK expression between the winter months and the summer months. Future studies are needed to examine what exactly causes the lack of feeding and activity during the winter, if it is temperature related alone or if more factors come into play, such as maintaining energy

for reproduction. The mechanisms regulating the dormant like state also need further examination.

One definite conclusion is that as more fish species are examined it is evident that fish are proving to be much more diverse in their feeding habits than was originally predicted when appetite studies were solely conducted on mammals. Fish exhibit diverse phylogeny, morphology, ecology, behaviour, migration and reproductive strategies which each must be considered when studying species-specific appetite regulation mechanisms.

Literature Cited

Adams, L. D., W. Gong, S. D. Vechia, R. G. Hunter and M. J. Kuhar (1999). "CART: from gene to function." Brain Research **848**(1-2): 137-40.

Akabayashi, A., C. Wahlestedt, J. T. Alexander and S. F. Leibowitz (1994). "Specific inhibition of endogenous neuropeptide Y synthesis in arcuate nucleus by antisense oligonucleotides suppresses feeding behavior and insulin secretion." Brain Research **21**(1-2): 55-61.

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

Aldman, G., A. C. Jonsson, J. Jensen and S. Holmgren (1989). "Gastrin/CCK-like peptides in the spiny dogfish, *Squalus acanthias*; concentrations and actions in the gut." Comparative Biochememistry and Physiology C., Comparative Pharmacology and Toxicology **92**(1): 103-8.

Aldman, G., A. C. Jonsson, J. Jensen and S. Holmgren (1989). "Gastrin/CCK-like peptides in the spiny dogfish, *Squalus acanthias*; concentrations and actions in the gut." Comparative and Biochemical Physiology C **92**(1): 103-8.

Arvidsson, A. K., A. Wraith, A. C. Jonsson-Rylander and D. Larhammar (1998). "Cloning of a neuropeptide Y/peptide YY receptor from the Atlantic cod: the Yb receptor." Regululatory Peptides **75-76**: 39-43.

Asnicar, M. A., D. P. Smith, D. D. Yang, M. L. Heiman, N. Fox, Y. F. Chen, H. M. Hsiung and A. Koster (2001). "Absence of cocaine- and amphetamine-regulated transcript results in obesity in mice fed a high caloric diet." Endocrinology **142**(10): 4394-400.

Banos, N., J. Baro, C. Castejon, I. Navarro and J. Gutierrez (1998). "Influence of high-carbohydrate enriched diets on plasma insulin levels and insulin and IGF-I receptors in trout." Regulatory Peptides **77**(1-3): 55-62.

Barrenechea, M. A., J. Lopez and A. Martinez (1994). "Regulatory Peptides in Gastric Endocrine-Cells of the Rainbow-Trout *Oncorhynchus mykiss* - General Distribution and Colocalizations." Tissue & Cell **26**(3): 309-321.

Beck, B., M. Jhanwar-Uniyal, A. Burlet, M. Chapleur-Chateau, S. F. Leibowitz and C. Burlet (1990). "Rapid and localized alterations of neuropeptide Y in discrete hypothalamic nuclei with feeding status." Brain Research **528**(2): 245-9.

Beinfeld, M. C. (2003). "Biosynthesis and processing of pro CCK: recent progress and future challenges." Life Sciences **72**(7): 747-57.

Ben Khemis, I., C. Audet, R. Fournier and J. de la Noue (2003). "Early weaning of winter flounder (*Pseudopleuronectes americanus* Walbaum) larvae on a commercial microencapsulated diet." Aquaculture Research **34**(6): 445-452.

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

Bjenning, C., N. Hazon, A. Balasubramaniam, S. Holmgren and J. M. Conlon (1993). "Distribution and activity of dogfish NPY and peptide YY in the cardiovascular system of the common dogfish." American Journal of Physiology **264**(6 Pt 2): R1119-24.

Bjenning, C., S. Holmgren and A. P. Farrell (1993). "Neuropeptide Y potentiates contractile response to norepinephrine in skate coronary artery." American Journal of Physiology **265**(2 Pt 2): H661-5.

Blomqvist, A. G., C. Soderberg, I. Lundell, R. J. Milner and D. Larhammar (1992). "Strong evolutionary conservation of neuropeptide Y: sequences of chicken, goldfish, and *Torpedo marmorata* DNA clones." Proceedings of the National Academy of Sciences of the United States of America **89**(6): 2350-4.

Bosi, G., A. Di Giancamillo, S. Arrighi and C. Domeneghini (2004). "An immunohistochemical study on the neuroendocrine system in the alimentary canal of the brown trout, *Salmo trutta*, L., 1758." General and Comparative Endocrinology **138**(2): 166-181.

Broberger, C., K. Holmberg, M. J. Kuhar and T. Hokfelt (1999). "Cocaine- and amphetamine-regulated transcript in the rat vagus nerve: A putative mediator of cholecystokinin-induced satiety." Proceedings of the National Academy of Sciences of the United States of America **96**(23): 13506-11.

Cerda-Reverter, J. M., I. Anglade, G. Martinez-Rodriguez, D. Mazurais, J. A. Munoz-Cueto, M. Carrillo, O. Kah and S. Zanuy (2000). "Characterization of neuropeptide Y expression in the brain of a perciform fish, the sea bass (*Dicentrarchus labrax*)." Journal of Chemical Neuroanatomy **19**(4): 197-210.

Cerda-Reverter, J. M. and D. Larhammar (2000). "Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution." Biochemistry and Cell Biology **78**(3): 371-92.

Chiba, A. (2000). "Immunohistochemical cell types in the terminal nerve ganglion of the cloudy dogfish, *Scyliorhinus torazame*, with special regard to neuropeptide Y/FMRFamide-immunoreactive cells." Neuroscience Letters **286**(3): 195-8.

Cho, S. H. (2005). "Compensatory growth of juvenile flounder *Paralichthys olivaceus* L. and changes in biochemical composition and body condition indices during starvation and after refeeding in winter season." Journal Of The World Aquaculture Society **36**(4): 508-514.

Conlon, J. M., C. Bjenning and N. Hazon (1992). "Structural characterization of neuropeptide Y from the brain of the dogfish, *Scyliorhinus canicula*." Peptides **13**(3): 493-7.

Couceyro, P., M. Paquet, E. Koylu, M. J. Kuhar and Y. Smith (1998). "Cocaine- and amphetamine-regulated transcript (CART) peptide immunoreactivity in myenteric plexus neurons of the rat ileum and co-localization with choline acetyltransferase." Synapse **30**(1): 1-8.

Couceyro, P. R. and T. Fritz (2003). "Production of recombinant CART peptides in Escherichia coli with agonist and antagonist effects on food intake in rats." Protein Expression and Purification **32**(2): 185-93.

Couceyro, P. R., E. O. Koylu and M. J. Kuhar (1997). "Further studies on the anatomical distribution of CART by in situ hybridization." Journal of Chemical Neuroanatomy **12**(4): 229-41.

Davies, L. and J. L. Marks (1994). "Role of hypothalamic neuropeptide Y gene expression in body weight regulation." American Journal of Physiology **266**(5 Pt 2): R1687-91.

de Montgolfier, B., C. Audet and Y. Lambert (2005). "Growth of early juvenile winter flounder (*Pseudopleuronectes americanus* Walbaum)." Aquaculture Research **36**(16): 1595-1601.

Demski, L. S. (1973). "Feeding and Aggressive Behavior Evoked by Hypothalamic Stimulation in a Cichlid Fish." Comparative Biochemistry and Physiology **44**(Na3): 685-692.

Demski, L. S. and K. M. Knigge (1971). "Telencephalon and Hypothalamus of Bluegill (*Lepomis Macrochirus*) - Evoked Feeding, Aggressive and Reproductive Behavior with Representative Frontal Sections." Journal of Comparative Neurology **143**(1): 1-&.

Douglass, J. and S. Daoud (1996). "Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript." Gene **169**(2): 241-5.

Douglass, J., A. A. McKinzie and P. Couceyro (1995). "PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine." Journal of Neuroscience **15**(3 Pt 2): 2471-81.

Doyon, C., K. M. Gilmour, V. L. Trudeau and T. W. Moon (2003). "Corticotropin-releasing factor and neuropeptide Y mRNA levels are elevated in the preoptic area of socially subordinate rainbow trout." General and Comparative Endocrinology **133**(2): 260-271.

Dube, M. G., T. L. Horvath, P. S. Kalra and S. P. Kalra (2000). "Evidence of NPY Y5 receptor involvement in food intake elicited by orexin A in sated rats." Peptides **21**(10): 1557-60.

Dumont, Y., J. C. Martel, A. Fournier, S. St-Pierre and R. Quirion (1992). "Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues." Progress in Neurobiology **38**(2): 125-67.

Ekblad, E., M. Kuhar, N. Wierup and F. Sundler (2003). "Cocaine- and amphetamine-regulated transcript: distribution and function in rat gastrointestinal tract." Neurogastroenterology and Motility **15**(5): 545-57.

Elias, C. F., C. Aschkenasi, C. Lee, J. Kelly, R. S. Ahima, C. Bjorbaek, J. S. Flier, C. B. Saper and J. K. Elmquist (1999). "Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area." Neuron **23**(4): 775-86.

Fetissov, S. O., J. Kopp and T. Hokfelt (2004). "Distribution of NPY receptors in the hypothalamus." Neuropeptides **38**(4): 175-88.

Forgan, L. G. and M. E. Forster (2007). "Effects of potential mediators of an intestinal brake mechanism on gut motility in Chinook salmon (*Oncorhynchus tshawytscha*)." Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **146**(3): 343-347.

Fredette, M., J. Batt and J. Castell (2000). "Feeding stimulant for juvenile winter flounders." North American Journal Of Aquaculture **62**(2): 157-160.

Frisk, M. G. and T. J. Miller (2006). "Age, growth, and latitudinal patterns of two Rajidae species in the northwestern Atlantic: little skate (*Leucoraja erinacea*) and winter skate (*Leucoraja ocellata*)." Canadian Journal Of Fisheries And Aquatic Sciences **63**(5): 1078-1091.

Gaikwad, A., K. C. Biju, P. L. Muthal, S. Saha and N. Subhedar (2005). "Role of neuropeptide Y in the regulation of gonadotropin releasing hormone system in the forebrain of *Clarias batrachus* (Linn.): immunocytochemistry and high performance liquid chromatography-electrospray ionization-mass spectrometric analysis." Neuroscience **133**(1): 267-79.

Gardiner, J. V., W. M. Kong, H. Ward, K. G. Murphy, W. S. Dhillo and S. R. Bloom (2005). "AAV mediated expression of anti-sense neuropeptide Y cRNA in the arcuate nucleus of rats results in decreased weight gain and food intake." Biochemical and Biophysical Research Communications **327**(4): 1088-93.

Gauthier, S. Y., C. B. Marshall, G. L. Fletcher and P. L. Davies (2005). "Hyperactive antifreeze protein in flounder species. The sole freeze protectant in American plaice." The Febs Journal **272**(17): 4439-49.

Gehlert, D. R. (1999). "Role of hypothalamic neuropeptide Y in feeding and obesity." Neuropeptides **33**(5): 329-38.

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

Hanson, J. M. and S. C. Courtenay (1996). "Seasonal use of estuaries by winter flounder in the southern Gulf of St Lawrence." Transactions Of The American Fisheries Society **125**(5): 705-718.

He, P. G. (2003). "Swimming behaviour of winter flounder (*Pleuronectes americanus*) on natural fishing grounds as observed by an underwater video camera." Fisheries Research **60**(2-3): 507-514.

Hebb, C. D., J. D. Castell, D. M. Anderson and J. Batt (2003). "Growth and feed conversion of juvenile winter flounder (*Pleuronectes americanus*) in relation to different protein-to-lipid levels in isocaloric diets." Aquaculture **221**(1-4): 439-449.

Himick, B. A. and R. E. Peter (1994). "Cck/Gastrin-Like Immunoreactivity in Brain and Gut, and Cck Suppression of Feeding in Goldfish." American Journal of Physiology **267**(3): R841-R851.

Himick, B. A., S. R. Vigna and R. E. Peter (1996). "Characterization of cholecystokinin binding sites in goldfish brain and pituitary." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **40**(1): R137-R143.

Holker, F. (2003). "The metabolic rate of roach in relation to body size and temperature." Journal of Fish Biology **62**(3): 565-579.

Holstein, B. (1982). "Inhibition of gastric acid secretion inthe Atlantic cod, *Gadus morhua*, by sulphated and desulphated gastrin, caerulein, and CCK-octapeptide." Acta Physiologica Scandinavia **114**(3): 453-9.

Honkanen, R. E., J. W. Crim and J. S. Patton (1988). "Effects of cholecystokinin peptides on digestive enzymes in killifish in vivo." Comparative Biochememistry and Physiology A **89**(4): 655-60.

Horvath, T. L., S. Diano and A. N. van den Pol (1999). "Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations." Journal of Neuroscience **19**(3): 1072-87.

Hwa, J. J., M. B. Witten, P. Williams, L. Ghibaudi, J. Gao, B. G. Salisbury, D. Mullins, F. Hamud, C. D. Strader and E. M. Parker (1999). "Activation of the NPY Y5 receptor regulates both feeding and energy expenditure." American Journal of Physiology **277**(5 Pt 2): R1428-34.

Imsland, A. K. and T. M. Jonassen (2001). "Regulation of growth in turbot (*Scophthalmus maximus* Rafinesque) and Atlantic halibut (*Hippoglossus hippoglossus* L.): aspects of environment x genotype interactions." Reviews in Fish Biology and Fisheries **11**(1): 71-90.

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

Johnsen, A. H., L. Jonson, I. J. Rourke and J. F. Rehfeld (1997). "Elasmobranchs express separate cholecystokinin and gastrin genes." Proceedings of the National Academy of Sciences of the United States of America **94**(19): 10221-10226.

Jonsson, A. C. (1991). "Regulatory Peptides in the Pancreas of 2 Species of Elasmobranchs and in the Brockmann Bodies of 4 Teleost Species." Cell and Tissue Research **266**(1): 163-172.

Jonsson, A. C., S. Holmgren and B. Holstein (1987). "Gastrin Cck-Like Immunoreactivity in Endocrine-Cells and Nerves in the Gastrointestinal-Tract of the Cod, *Gadus morhua*, and the Effect of Peptides of the Gastrin Cck Family on Cod Gastrointestinal Smooth Muscle." General and Comparative Endocrinology **66**(2): 190-202.

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

Kamisaka, Y., G. K. Totland, M. Tagawa, T. Kurokawa, T. Suzuki, M. Tanaka and I. Ronnestad (2001). "Ontogeny of cholecystokinin-immunoreactive cells in the digestive tract of Atlantic halibut, *Hippoglossus hippoglossus*, larvae." General and Comparative Endocrinology **123**(1): 31-37.

Kawano, K., T. Hirashima, S. Mori, Y. Saitoh, M. Kurosumi and T. Natori (1992). "Spontaneous Long-Term Hyperglycemic Rat with Diabetic Complications - Otsuka Long-Evans Tokushima Fatty (Oletf) Strain." Diabetes **41**(11): 1422-1428.

Kehoe, A. S. and H. Volkoff (2007). "Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod

(Gadus morhua)." Comparative Biochemistry and Physiology A Molecular Integrative Physiology **146**(3): 451-61.

Kiris GA (Kiris, G. A., Kumlu M (Kumlu, M.), Dikel S (Dikel, S.) (2007). "Stimulatory effects of neuropeptide Y on food intake and growth of *Oreochromis niloticus*." Aqaculture **264**(1-4): 383-389.

Kobayashi, Y., F. Jimenez-Krassel, Q. Li, J. Yao, R. Huang, J. J. Ireland, P. M. Coussens and G. W. Smith (2004). "Evidence that cocaine- and amphetamine-regulated transcript is a novel intraovarian regulator of follicular atresia." Endocrinology **145**(11): 5373-83.

Korner, J., E. Savontaus, S. C. Chua, Jr., R. L. Leibel and S. L. Wardlaw (2001). "Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus." Journal of Neuroendocrinology **13**(11): 959-66.

Kristensen, P., M. E. Judge, L. Thim, U. Ribel, K. N. Christjansen, B. S. Wulff, J. T. Clausen, P. B. Jensen, O. D. Madsen, N. Vrang, P. J. Larsen and S. Hastrup (1998). "Hypothalamic CART is a new anorectic peptide regulated by leptin." Nature **393**(6680): 72-6.

Kuhar, M. J., S. Adams, G. Dominguez, J. Jaworski and B. Balkan (2002). "CART peptides." Neuropeptides **36**(1): 1-8.

Kuhar, M. J. and L. L. Yoho (1999). "CART peptide analysis by Western blotting." Synapse **33**(3): 163-71.

Kurokawa, T., T. Suzuki and H. Hashimoto (2003). "Identification of gastrin and multiple cholecystokinin genes in teleost." Peptides **24**(2): 227-35.

Kushi, A., H. Sasai, H. Koizumi, N. Takeda, M. Yokoyama and M. Nakamura (1998). "Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice." Proceedings of the National Academy of Sceinces of the United States of America **95**(26): 15659-64.

Kuz'mina, V. V. (2005). "Regulation of feeding behavior in fishes: humoral regulation." Zhurnal Evoliutsionno_Biokhimii I Fiziologii **41**(3): 224-35.

Lambert, P. D., P. R. Couceyro, K. M. McGirr, S. E. Dall Vechia, Y. Smith and M. J. Kuhar (1998). "CART peptides in the central control of feeding and interactions with neuropeptide Y." Synapse **29**(4): 293-8.

Larsen, P. J., V. Seier, A. Fink-Jensen, J. J. Holst, J. Warberg and N. Vrang (2003). "Cocaine- and amphetamine-regulated transcript is present in hypothalamic neuroendocrine neurones and is released to the hypothalamic-pituitary portal circuit." Journal of Neuroendocrinology **15**(3): 219-26.

Larsson, T. A., E. T. Larson, R. Fredriksson, J. M. Conlon and D. Larhammar (2006). "Characterization of NPY receptor subtypes Y2 and Y7 in rainbow trout *Oncorhynchus mykiss*." Peptides **27**(6): 1320-7.

Larsson, T. A., E. T. Larson and D. Larhammar (2007). "Cloning and sequence analysis of the neuropeptide Y receptors Y5 and Y6 in the coelacanth *Latimeria chalumnae*." General Comparative Endocrinology **150**(2): 337-42.

Lazar, G., M. Calle, E. W. Roubos and T. Kozicz (2004). "Immunohistochemical localization of cocaine- and amphetamine-regulated transcript peptide in the

central nervous system of the frog *Rana esculenta*." Journal of Comparative Neurology **477**(3): 324-39.

Leonard, J. B., G. C. Waldbieser and J. T. Silverstein (2001). "Neuropeptide Y sequence and messenger RNA distribution in channel catfish (*Ictalurus punctatus*)." Marine Biotechnology (NY) **3**(2): 111-8.

Leonard, J. B., G. C. Waldbieser and J. T. Silverstein (2001). "Neuropeptide Y sequence and messenger RNA distribution in channel catfish (*Ictalurus punctatus*)." Marine Biotechnology (NY) **3**(2): 111-8.

Liang, X. F., G. Z. Li, W. Yao, L. W. Cheong and W. Q. Liao (2007). "Molecular characterization of neuropeptide Y gene in Chinese perch, an acanthornorph fish." Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology **148**(1): 55-64.

Litvak, M. K. (1999). "The development of winter flounder (*Pleuronectes americanus*) for aquaculture in Atlantic Canada: current status and future prospects." Aquaculture **176**(1-2): 55-64.

Lopez-Patino, M. A., A. I. Guijarro, E. Isorna, M. J. Delgado, M. Alonso-Bedate and N. de Pedro (1999). "Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (*Carassius auratus*)." European Journal on Pharmacology **377**(2-3): 147-53.

Magalhaes, G. S., I. L. Junqueira-de-Azevedo, M. Lopes-Ferreira, D. M. Lorenzini, P. L. Ho and A. M. Moura-da-Silva (2006). "Transcriptome analysis of expressed sequence tags from the venom glands of the fish *Thalassophryne nattereri*." Biochimie **88**(6): 693-9.

Marchetti, G., B. Cozzi, M. Tavanti, V. Russo, S. Pellegrini and O. Fabiani (2000). "The distribution of neuropeptide Y-immunoreactive neurons and nerve fibers in the forebrain of the carp *Cyprinus carpio* L." Journal of Chemical Neuroanatomy **20**(2): 129-39.

Martell, D. J. and G. McClelland (1994). "Diets Of Sympatric Flatfishes, *Hippoglossoides platessoides*, *Pleuronectes ferrugineus*, *Pleuronectes americanus*, From Sable Island Bank, Canada." Journal Of Fish Biology **44**(5): 821-848.

Meise, C. J., D. L. Johnson, L. L. Stehlik, J. Manderson and P. Shaheen (2003). "Growth rates of juvenile winter flounder under varying environmental conditions." Transactions Of The American Fisheries Society **132**(2): 335-345.

Mendez G, W. W. (1993). "Meatbolic Responses to Fodd-Deprivation and Refeeding in Juveniles of *Rutilus-rutilus* (Telesotei, Cyprinidae)." Environmental Biology of Fishes **36**(1): 73-81.

Mercier, L., C. Audet, J. de la Noue, B. Parent, C. C. Parrish and N. W. Ross (2004). "First feeding of winter flounder (*Pseudopleuronectes americanus*) larvae: use of Brachionus plicatilis acclimated at low temperature as live prey." Aquaculture **229**(1-4): 361-376.

Montpetit, C. J., V. Chatalov, J. Yuk, I. Rasaratnam and J. H. Youson (2005). "Expression of neuropeptide Y family peptides in the brain and gut during stages of the life cycle of a parasitic lamprey (*Petromyzon marinus*) and a nonparasitic

lamprey (Ichthyomyzon gagei)." Annuals of the New York Academy of Science **1040**: 140-9.

Moons, L., T. F. C. Batten and F. Vandesande (1992). "Comparative Distribution of Substance-P (Sp) and Cholecystokinin (Cck) Binding-Sites and Immunoreactivity in the Brain of the Sea Bass (*Dicentrarchus labrax*)." Peptides **13**(1): 37-46.

Moran, T. H., L. F. Katz, C. R. Plata-Salaman and G. J. Schwartz (1998). "Disordered food intake and obesity in rats lacking cholecystokinin A receptors." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **43**(3): R618-R625.

Moran, T. H. and K. P. Kinzig (2004). "Gastrointestinal satiety signals II. Cholecystokinin." American Journal of Physiology and Gastrointestinal Liver Physiology **286**(2): G183-8.

Moran, T. H. and K. P. Kinzig (2004). "Gastrointestinal satiety signals II. Cholecystokinin." American Journal of Physiology and Gastrointestinal Liver Physiology **286**(2): G183-8.

Murashita, K., H. Fukada, H. Hosokawa and T. Masumoto (2006). "Cholecystokinin and peptide Y in yellowtail (*Seriola quinqueradiata*): molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting." General Comparative Endocrinology **145**(3): 287-97.

Murashita, K., H. Fukada, H. Hosokawa and T. Masumoto (2007). "Changes in cholecystokinin and peptide Y gene expression with feeding in yellowtail (*Seriola quinqueradiata*): Relation to pancreatic exocrine regulation." Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology **146**(3): 318-325.

Murray, H. M., C. L. Hew, K. R. Kao and G. L. Fletcher (2002). "Localization of cells from the winter flounder gill expressing a skin type antifreeze protein gene." Canadian Journal Of Zoology-Revue Canadienne De Zoologie **80**(1): 110-119.

Narnaware, Y. K. and R. E. Peter (2001). "Neuropeptide Y stimulates food consumption through multiple receptors in goldfish." Physiology & Behaviour **74**(1-2): 185-90.

Narnaware, Y. K. and R. E. Peter (2002). "Influence of diet composition on food intake and neuropeptide Y (NPY) gene expression in goldfish brain." Regululatory Peptides **103**(2-3): 75-83.

Narnaware, Y. K., P. P. Peyon, X. Lin and R. E. Peter (2000). "Regulation of food intake by neuropeptide Y in goldfish." American Journal of Physiology. Regulatory, Integrative and Comparative Physiology **279**(3): R1025-34.

Okumura, T., H. Yamada, W. Motomura and Y. Kohgo (2000). "Cocaine-amphetamine-regulated transcript (CART) acts in the central nervous system to inhibit gastric acid secretion via brain corticotropin-releasing factor system." Endocrinology **141**(8): 2854-60.

Oliver, A. S. and S. R. Vigna (1996). "CCK-X receptors in the endothermic mako shark (*Isurus oxyrinchus*)." General and Comparative Endocrinology **102**(1): 61-73.

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

Olsson, C., G. Aldman, A. Larsson and S. Holmgren (1999). "Cholecystokinin affects gastric emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss*." Journal of Experimental Biology **202**(Pt 2): 161-70.

Peng, C., W. Gallin, R. E. Peter, A. G. Blomqvist and D. Larhammar (1994). "Neuropeptide-Y gene expression in the goldfish brain: distribution and regulation by ovarian steroids." Endocrinology **134**(3): 1095-103.

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

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

Peyon, P., S. Vega-Rubin de Celis, P. Gomez-Requeni, S. Zanuy, J. Perez-Sanchez and M. Carrillo (2003). "In vitro effect of leptin on somatolactin release in the European sea bass (*Dicentrarchus labrax*): dependence on the reproductive status and interaction with NPY and GnRH." General Comparative Endocrinology **132**(2): 284-92.

Pirone, A., L. Betti, G. Mascia, G. Giannaccini, A. Lucacchini and O. Fabiani (2003). "Autoradiographic distribution of neuropeptide Y binding sites in the brain of the carp *Cyprinus carpio* L. (Cyprinidae, Teleostei)." Comparative Biochemistry and Physiology Part A. Molecular & Integrative Physiology **134**(4): 757-62.

Plante, S., C. Audet, Y. Lambert and J. de la Noue (2003). "Comparison of stress responses in wild and captive winter flounder (*Pseudopleuronectes americanus* Walbaum) broodstock." Aquaculture Research **34**(10): 803-812.

Preston, E., A. C. Jonsson, C. D. McManus, J. M. Conlon and G. P. Courtice (1998). "Comparative vascular responses in elasmobranchs to different structures of neuropeptide Y and peptide YY." Regulatory Peptides **78**(1-3): 57-67.

Rajjo, I. M., S. R. Vigna and J. W. Crim (1988). "Cholecystokinin Immunoreactivity in the Digestive-Tract of Bowfin (*Amia calva*), Bluegill (*Lepomis macrochirus*), and Bullfrog (*Rana catesbeiana*)." General and Comparative Endocrinology **70**(1): 133-144.

Roberts, M. G. and G. E. Savage (1978). "Effects of Hypothalamic-Lesions on Food-Intake of Goldfish (*Carassius auratus*)." Brain Behavior and Evolution **15**(2): 150-164.

Rodriguez-Gomez, F. J., C. Rendon-Unceta, C. Sarasquete and J. A. Munoz-Cueto (2001). "Distribution of neuropeptide Y-like immunoreactivity in the brain of the Senegalese sole (*Solea senegalensis*)." Anatomical Record **262**(3): 227-37.

Rubio, V. C., F. J. Sanchez-Vazquez and J. A. Madrid (2008). "Role of cholecystokinin and its antagonist proglumide on macronutrient selection in European sea bass *Dicentrarchus labrax*, L." Physiology & Behavior **93**(4-5): 862-869.

Sahu, A., M. G. Dube, C. P. Phelps, C. A. Sninsky, P. S. Kalra and S. P. Kalra (1995). "Insulin and insulin-like growth factor II suppress neuropeptide Y release from the nerve terminals in the paraventricular nucleus: a putative hypothalamic site for energy homeostasis." Endocrinology **136**(12): 5718-24.

Salaneck, E., D. H. Ardell, E. T. Larson and D. Larhammar (2003). "Three neuropeptide Y receptor genes in the spiny dogfish, *Squalus acanthias*, support en bloc duplications in early vertebrate evolution." Molecular Biology and Evolution **20**(8): 1271-80.

Salaneck, E., R. Fredriksson, E. T. Larson, J. M. Conlon and D. Larhammar (2001). "A neuropeptide Y receptor Y1-subfamily gene from an agnathan, the European river lamprey. A potential ancestral gene." European Journal of Biochemistry **268**(23): 6146-54.

Schaffhauser, A. O., A. Stricker-Krongrad, L. Brunner, F. Cumin, C. Gerald, S. Whitebread, L. Criscione and K. G. Hofbauer (1997). "Inhibition of food intake by neuropeptide Y Y5 receptor antisense oligodeoxynucleotides." Diabetes **46**(11): 1792-8.

Scott, G. K., P. L. Davies, M. H. Kao and G. L. Fletcher (1988). "Differential amplification of antifreeze protein genes in the pleuronectinae." Journal of Molecualr Evolution **27**(1): 29-35.

Silverstein, J. T., V. M. Bondareva, J. B. Leonard and E. M. Plisetskaya (2001). "Neuropeptide regulation of feeding in catfish, *Ictalurus punctatus*: a role for glucagon-like peptide-1 (GLP-1)?" Comparative Biochemisty and Biology. Part B, Biochemistry and Molecular Biology **129**(2-3): 623-31.

Silverstein, J. T., J. Breininger, D. G. Baskin and E. M. Plisetskaya (1998). "Neuropeptide Y-like gene expression in the salmon brain increases with fasting." General and Comparative Endocrinology **110**(2): 157-65.

Singru, P. S., M. Mazumdar, A. J. Sakharkar, R. M. Lechan, L. Thim, J. T. Clausen and N. K. Subhedar (2007). "Immunohistochemical localization of cocaine- and amphetamine-regulated transcript peptide in the brain of the catfish, *Clarias batrachus* (Linn.)." Journal of Comparative Neurology **502**(2): 215-35.

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

Stoner, A. W., A. J. Bejda, J. P. Manderson, B. A. Phelan, L. L. Stehlik and J. P. Pessutti (1999). "Behavior of winter flounder, *Pseudopleuronectes americanus*, during the reproductive season: laboratory and field observations on spawning, feeding, and locomotion." Fishery Bulletin **97**(4): 999-1016.

Sueiro, C., I. Carrera, S. Ferreiro, P. Molist, F. Adrio, R. Anadon and I. Rodriguez-Moldes (2007). "New insights on *Saccus vasculosus* evolution: a developmental and immunohistochemical study in elasmobranchs." Brain, Behavior and Evolution **70**(3): 187-204.

Sulikowski, J. A., P. C. W. Tsang and W. H. Howell (2004). "An annual cycle of steroid hormone concentrations and gonad development in the winter skate, *Leucoraja ocellata*, from the western Gulf of Maine." Marine Biology **144**(5): 845-853.

Tatemoto, K. (1982). "Neuropeptide Y: complete amino acid sequence of the brain peptide." Proceedings for the National Academy of Science of the United States of America **79**(18): 5485-9.

Thim, L., P. Kristensen, P. F. Nielsen, B. S. Wulff and J. T. Clausen (1999). "Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat." Proceedings for the National Academy of Science for the United States of America **96**(6): 2722-7.

Tiitu V, V. M. (2001). "Cold adaptation suppresses the contractility of both atrial and ventricular muscle of the crucian carp heart." Jourlan of Fish Biology **59**(1): 141-156.

Tokunaga, K., M. Fukushima, J. W. Kemnitz and G. A. Bray (1986). "Comparison of ventromedial and paraventricular lesions in rats that become obese." American Journal of Physiology **251**(6 Pt 2): R1221-7.

Treberg, J. R. and W. R. Driedzic (2006). "Maintenance and accumulation of trimethylamine oxide by winter skate (*Leucoraja ocellata*): reliance on low whole animal losses rather than synthesis." American Journal Of Physiology-Regulatory Integrative And Comparative Physiology **291**(6): R1790-R1798.

van Dijk PLM, S. G., Hardewig I (2002). "The effect of fasting and refeeding on temperature preference, activity and growth of roach, *Rutilus rutilus*." Oecologia **130**(4): 496-504.

Vicentic, A., G. Dominguez, R. G. Hunter, K. Philpot, M. Wilson and M. J. Kuhar (2004). "Cocaine- and amphetamine-regulated transcript peptide levels in blood exhibit a diurnal rhythm: regulation by glucocorticoids." Endocrinology **145**(9): 4119-24.

Vishnuvardhan, D. and M. C. Beinfeld (2000). "Role of tyrosine sulfation and serine phosphorylation in the processing of procholecystokinin to amidated cholecystokinin and its secretion in transfected AtT-20 cells." Biochemistry **39**(45): 13825-30.

Vishnuvardhan, D. and M. C. Beinfeld (2002). "Biosynthesis and post-translational processing of site-directed endoproteolytic cleavage mutants of Pro-CCK in AtT-20 cells." Biochemistry **41**(2): 570-8.

Volkoff, H. (2006). "The role of neuropeptide Y, orexins, cocaine and amphetamine-related transcript, cholecystokinin, amylin and leptin in the regulation of feeding in fish." Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology **144**(3): 325-31.

Volkoff, H., A. J. Eykelbosh and R. E. Peter (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 Research **972**(1-2): 90-109.

Volkoff, H. and R. E. Peter (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 Research **887**(1): 125-33.

Volkoff, H. and R. E. Peter (2001). "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**(12): 5076-88.

Volkoff, H. and R. E. Peter (2001). "Interactions between orexin A, NPY and galanin in the control of food intake of the goldfish, *Carassius auratus*." Regulatory Peptides **101**(1-3): 59-72.

Volkoff, H. and R. E. Peter (2006). "Feeding Behavior of Fish and Its Control." Zebrafish **3**(2): 131-140.

Vornanen, M. (1994). "Seasonal and temperature-induced changes in myosin heavy chain composition of crucian carp hearts." American Journal of Physiology **267**(6 Pt 2): R1567-73.

Vrang, N., M. Tang-Christensen, P. J. Larsen and P. Kristensen (1999). "Recombinant CART peptide induces c-Fos expression in central areas involved in control of feeding behaviour." Brain Research **818**(2): 499-509.

Wang, C., C. J. Billington, A. S. Levine and C. M. Kotz (2000). "Effect of CART in the hypothalamic paraventricular nucleus on feeding and uncoupling protein gene expression." Neuroreport **11**(14): 3251-5.

Williams, G., C. Bing, X. J. Cai, J. A. Harrold, P. J. King and X. H. Liu (2001). "The hypothalamus and the control of energy homeostasis: different circuits, different purposes." Physiology and Behaviour **74**(4-5): 683-701.

Xiang, H. (1994). "Comparative aspects of the role of neuropeptide Y in the regulation of the vertebrate heart." Cardioscience **5**(4): 209-13.

Yokosuka, M., M. G. Dube, P. S. Kalra and S. P. Kalra (2001). "The mPVN mediates blockade of NPY-induced feeding by a Y5 receptor antagonist: a c-FOS analysis." Peptides **22**(3): 507-14.

Zheng, H., C. Patterson and H. R. Berthoud (2001). "Fourth ventricular injection of CART peptide inhibits short-term sucrose intake in rats." Brain Research **896**(1-2): 153-6.