Changes in expression of appetite-regulating hormones in the cunner (*Tautogolabrus adspersus*) during short-term fasting and torpor

by

Nicole Babichuk

A thesis submitted to the

School of Graduate Studies

in partial fulfilment of the

requirements for the degree of

Masters of Science

Biology Department, Faculty of Science
Memorial University of Newfoundland

October, 2012

St. John's Newfoundland

Table of contents

Abstract

Feeding in vertebrates is controlled by a number of appetite stimulating (orexigenic) and appetite suppressing (anorexigenic) hormones, whose levels respectively increase and decrease following fasting. Cunners (*Tautogolabrus adspersus*) survive the winter in shallow coastal waters by entering a torpor-like state. In order to better understand the mechanisms regulating appetite/fasting in these fish, quantitative real-time PCR was used to measure transcript expression levels of four appetite-regulating hormones: cholecystokinin (CCK) in the gut, and neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and orexin in the forebrain (hypothalamus and telencephalon) of fed, short-term fasted, induced summer torpor and natural torpor cunners. All hormone mRNA levels decreased during fasting and during natural torpor, but during induced summer torpor brain NPY and orexin mRNA expression levels increased, and CART brain mRNA and CCK gut mRNA expression levels decreased. These results clearly indicate that the cunner has different physiological responses to fasting and torpor.

Acknowledgments

I would like to start by thanking my supervisor, Dr. Helene Volkoff, for her guidance and patience during my time with her, and my lab members Sarah Tuziak and Leah Hoskins for teaching me how to use all the equipment without breaking it. I would also like to thank my supervisory committee, Bob Hooper and Dawn Marshall for all their excellent feedback. Thanks to Ross McGowan for allowing me to use his qPCR machine, and to Allison Jenkins for all her help during my summer at Bonne Bay Marine Station. Finally, thanks to my parents and to James Lee Wright for all their support during the good times and the bad times.

List of Tables

List of Figures

Introduction

The control of food intake and appetite regulation is a complex process in vertebrates. Though individual hormones, pathways and responses may vary from species to species, all vertebrate appetite regulation follows the same general model. This system functions like many other endocrine systems in the body, mainly by interacting with the nervous system to create a feedback system that will inhibit or stimulate itself. One purpose of the feedback system is to help an organism equilibrate with its environment and maintain homeostasis. Environmental conditions are often unpredictable and can be harmful to organisms if they do not have the ability to adapt to those changes. Most environments undergo seasonal variations in temperature and food abundance that have predictable annual cycles of abundance and scarcity. However, both temperature and food abundance can change in a relatively short period of time outside of their predicted range. Peripheral organs and tissues sense these changes and send signals through the nervous system to the brain, which in turn delegates physiological changes within the tissues to adapt to the changing conditions.

A decrease in temperature can have a large impact on heterothermic vertebrates, which depend on the environment to regulate their body temperature. A common mechanism for coping with a decrease in external temperature is to enter a state of dormancy or torpor until temperatures increase or return to normal. Torpor was originally defined in mammals as a state in which body temperature is as low as 10°C and dormancy is shorter than 24 hours (Hudson, 1973). Torpor must

be defined differently for heterotherms than for homeotherms, as heterotherms normally have body temperatures close to or as low as their external environment. In homeotherms torpor is defined by a variety of physiological changes including reduced proteins synthesis, feeding and activity (Campbell et al., 2008). In heterotherms, torpor differs from hibernation, as torpor does not involve advanced physiological preparation; it is temperature-dependent and can be relieved by increasing external temperatures (Geiser et al., 1996, Brett and Groves, 1979). The physiological mechanisms controlling the entry into, and exit from torpor are unknown, but torpor has the ability to override certain endocrine processes such as appetite regulation. Decreases in metabolic rate and protein synthesis reduce an organism's energy consumption, allowing them to survive on little to no food. But how is feeding regulated normally and how does this process change during torpor?

Peripheral and central control of appetite regulation

Although hormones are not the only features influencing appetite regulation, they play the largest role in controlling hunger and feeding. Peripheral factors that influence appetite regulation includes the presence or absence of food in the stomach as well as environmental conditions such as photoperiod and tank color, both of which are hormone mediated (Volkoff et al., 2009). The hormones can be orexigenic hormones, which stimulate appetite or anorexigenic hormones, which suppress appetite. Orexigenic hormones include neuropeptide Y (NPY) and orexin, and anorexigenic hormones include cocaine- and amphetamine-regulated transcript

(CART) and cholecystokinin (CCK). Orexigenic and anorexigenic hormones tend to be widely conserved among different species of vertebrates (Volkoff et al., 2009) and exert their actions mostly on the brain, which acts as the command center responsible for interpreting orexigenic and anorexigenic hormones as feelings of hunger or satiety, respectfully.

Some hormones have been found to act together to increase their effects as orexigenic or anorexigenic agents, while other can have an inhibitory effect on each others activities. For example, NPY and orexin are two orexigenic hormones that appear to be linked as injections of orexin cause an increase in NPY brain mRNA in goldfish (Volkoff and Peter, 2001b) and CART is a potent inhibitor of orexigenic hormones orexin and NPY, as was demonstrated by Volkoff and Peter (2000).

Appetite-regulating hormones are produced in a variety of tissues (peripheral and brain) and travel through the blood to receptors in the brain (crossing the blood-brain barrier) or attach to receptors on projections of the central nervous system such as the vagus nerve (Brightman and Broadwell, 1976). Appetite-regulating hormones can be stimulated directly by the presence/absence of food in the gastrointestinal (GI) tract, or by upstream regulators. One important upstream regulator of appetite is the hormone leptin, which is secreted by adipocytes and inhibits feeding behavior (Volkoff, 2006). Injections of leptin in birds and mammals causes a decrease in food consumption (Neary et al., 2004), and injections of leptin into goldfish appear to inhibit orexigenic hormones NPY and orexin, and stimulate anorexigenic hormones CART and CCK (Volkoff and Peter,

2000; Volkoff et al., 2003). Upstream regulators have many roles, including enhancing the effects of appetite-regulating hormones.

Neural control of appetite regulation

As stated previously, appetite regulation is an intricate system controlled by both the endocrine and nervous systems. Appetite-regulating hormones are released or withheld depending on the nutritional state or feeding status of the organism. The release of these hormones is dependent on the central nervous system, in particular the hypothalamus and adjoining regions of the brain, although certain peripheral tissues can also cause their release. The role of the hypothalamus and other brain regions in feeding was first determined through electrical stimulation studies. In fish and sharks, electrical stimulation of the hypothalamus, telencephalon and optic tectum has been shown to cause increases in feeding behavior (Demski and Knigge, 1971; Demski, 1973; Roberts and Savage, 1978). Electrical stimulation studies were soon followed by brain lesion studies to assess the importance of the hypothalamus in feeding. In mammals, lesions of both the ventromedial hypothalamus (VMH) and the lateral hypothalamus (LH) were performed in numerous studies, with the common result of hyperphagia, or over-eating. In one of the first lesion studies conducted, Anand and Brobeck (1951) showed that rats have specific areas of the hypothalamus that, when removed, inhibit them from eating (Powley and Keesey, 1970; Powley et al., 1980; van den Pol, 1982; Aravich and Scalfani, 1983; Weingarten et al., 1985; Tokunaga et al., 1986;

Bernadis and Berlunger, 1987). Different areas within the hypothalamus have been identified as playing major roles in feeding, and immunohistochemical staining for appetite regulating hormone receptors confirms their roles.

Neuropeptide Y

Neuropeptide Y (NPY) is one of the most powerful orexigenic hormone in fish. NPY was first sequenced from porcine brain (Tatemoto, 1982) and has since been discovered and sequenced in a number of vertebrates such as humans (Minth et al., 1984) and fish, including goldfish (Blomqvist et al., 1992), Atlantic cod (Kehoe and Volkoff, 2007), winter flounder (MacDonald and Volkoff, 2009) and most recently tiger puffer (Kamijo et al., 2011).

The peptide itself is 36 amino acids long. It belongs to the same family of hormones as peptide Y (PY), peptide YY (PYY), and pancreatic polypeptide (PP), together known as the NPY family of peptides (Cerda-Reverter and Larhammar, 2000a). All are thought to have evolved from a common ancestral gene which underwent two distinct gene duplication events, the first event creating NPY and the precursor of PYY/PP, and the second event splitting PYY and PP into two separate genes (Cerda-Reverter et al., 2000a). Most fish can manufacture only NPY and PYY (found within the gut and brain) though some have been found to produce PY (in the brain) as well (Cerda-Reverter et al., 2000a; Volkoff et al., 2009). PYY causes an increase in food consumption in mammals when injected intracerebroventricularly (icv; Stanley, 1993) but decreases food consumption when injected in goldfish

(Gonzalez and Unniappan, 2010). Therefore, role of PYY in appetite regulations needs to be investigated further.

NPY binds to G-protein coupled receptors, which are distributed throughout the brain and peripheral tissues of vertebrates (Fredriksson et al., 2004; Larsson et al., 2005; Larhammar, 1996). Currently, five receptor subtypes have been identified in mammals (Y1, Y2, Y4, Y5 and Y6) and seven receptor subtypes have been identified in fish (Salaneck et al., 2008). In mammals, receptors Y1 and Y5 signal increases in food consumption (Larsson et al., 2005). Not all species of fish possess the same number of receptors (Fallmar et al., 2011). Both species of pufferfish (*Tetraodon nigroviridis* and *Takifugu rubripes*) and a zebrafish (*Danio rerio*) have had their full complement of NPY receptors sequenced (Larsson et al. 2008). Pufferfish appear to have lost Y1,Y5 and Y6 receptor subtypes, while zebrafish have only lost Y5 and Y6 receptors (Larsson et al. 2008). Therefore there may be receptor subtypes other than Y1 and Y5 mediating the eating response to NPY in fish (Larsson et al., 2005).

NPY transcript and receptor mRNA has been found through central and peripheral tissues of all vertebrates. In rats, NPY has been detected in the heart, spleen, liver, ovary, thymus and adrenal gland as well as in pons/medulla, hippocampus, cerebral cortex, olfactory bulb and thalamus, among other regions of the brain (Larhammar et al., 1987). Within the brain, NPY mRNA has been found in areas of the mammalian hypothalamus associated with feeding including the paraventricular nucleus (PVN) and the arcuate nucleus (ARC; Halford et al., 2004).

Expression of NPY has been observed in the telencephalon-preoptic, optic tectum-thalamus and the hypothalamus of several species of fish including goldfish (Narnaware et al., 2000; Narnaware and Peter, 2001; Peng et al., 1994). Some fish peripheral tissues also express NPY transcript and receptor mRNA including the GI tract and the kidney (Cerda-Reverter and Larhammar, 2000b; Fredriksson et al., 2004; Larsson et al., 2005).

The main location of the appetite-regulating effects of NPY is presumed to be the brain. The brain it is a potential site for crosstalk between pathways. For example, NPY has been co-localized with CART, an anorexigenic hormone in the catfish brain, where NPY and CART reactive tissues are located together in the medial olfactory tract, dorsal telencephalon, ventral telencephalon and some regions of the pituitary (Singru et al., 2008).

NPY plays a major role in appetite regulation. The regions of the brain in which both NPY transcript and receptor mRNA have been found include the hypothalamus, telencephalon and optic tectum, all areas that have been implicated as feeding centers. Injection studies in mammals and birds presented the first evidence of NPY as an orexigenic hormone. Intracerebroventricular (icv) injections of NPY in rats resulted in a dramatic (up to 20-fold) increase in feeding behavior following injections (Clark et al., 1987). Icv administration of NPY in goldfish also caused increased feeding (Lopez-Patino et al., 1999). Food deprivation studies followed, and reinforced the theory that NPY is a regulator of appetite. Food deprivation of chinook (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus*

kisutch) for several weeks caused an increase in hypothalamic NPY mRNA (Silverstein et al., 1998). Studies done with goldfish (Lopez-Patino, 1999) salmon and catfish (Silverstein and Plisetskaya, 2000) resulted in similar findings.

Other species of fish have been tested for the effects of food deprivation and there is a large difference in fasting periods required to increase NPY mRNA. Goldfish display an increase in NPY mRNA after only 3 days of fasting, however Atlantic salmon NPY mRNA did not increase after 6 days of fasting (Murashita *et al.* 2009), nor did that of Atlantic cod after 7 days of fasting (Kehoe and Volkoff 2007). It thus appears that some species of fish require longer fasting periods than others to elicit the appetite stimulating effects of NPY. This could be related to the environmental conditions in which the species live. Winter flounder, a species whose range extends along the eastern coast of North America up to and around Newfoundland (Scott et al., 1988) exhibits an increase in brain mRNA following two weeks of fasting (MacDonald and Volkoff 2009). Species that undergo seasonal periods of fasting may regulate the expression of the NPY differently than those in more stable environments. In the winter flounder there was a significant increase in hypothalamic NPY mRNA in the summer but no increase in NPY mRNA in the winter, which is normally a period of fasting for the winter flounder (MacDonald and Volkoff 2009).

Orexin

Orexins are centrally acting appetite-stimulating (orexigenic) hormones. Orexins were discovered by two separate research groups around the same time. Both groups were in search of the corresponding ligands to what we now know are orexin receptors (Sakurai, 2002). Sakurai et al. (1998) identified and described orexin from rat brain tissue, while de Lecea et al. (1998) isolated orexin from the hypothalamus and named it hypocretin.

Orexins originate from a precursor molecule known as prepro-orexin, the gene for which contains two exons, the larger of which forms two mature peptides, orexin A and orexin B (Wong et al., 2011). The mammalian form of orexin A is 53 amino acids longs, while orexin B is the shorter peptide at only 28 amino acids in length (Wong et al., 2011). In teleost fish orexin B is most highly conserved, while in tetrapods orexin A is more conserved (Wong et al., 2011). In mammals, orexin is primarily produced within the lateral hypothalamus but can be also be found within some peripheral tissues including the gut (Kirchgessner and Liu, 1999). Orexin A and B both cause similar effects, however orexin A is a more powerful peptide than orexin B in stimulating feeding (Clapham et al., 2001). This may be due to different receptor binding affinities or to different blood circulation/degradation patterns between the two orexins. Both orexin A and B can travel in the blood, but only orexin A can pass the blood-brain barrier into the brain via simple diffusion and remain intact (Kastin and Akerstrom, 1999). Orexin B is rapidly degraded in the

blood, and no intact peptide is able to make it past the blood-brain barrier (Kastin and Akerstrom, 1999).

There are two orexin receptors in mammals both of which are G-protein-coupled receptors (Sakurai et al., 1998). Orexin-1 receptor (OX1R) has a very high affinity for orexin A, but a 2-3 times lower magnitude of affinity for orexin B (Sakurai et al., 1998). Orexin-2 receptor (OX2R) binds with equal affinity to orexin A and B (Sakurai et al., 1998). Immunohistochemical studies in rats have localized the OX1R and OX2R receptors to several areas within the hypothalamus, as well as the thalamus, midbrain and reticular formation (Hervieu et al., 2001; Cluderay et al., 2002).

Similar to mammals, fish species have two forms of orexin, derived from prepro-orexin (Volkoff, 2006). Orexin remains highly conserved from mammals to fish (Sakurai et al., 1998; Alvarez and Sutcliffe, 2002; Ohkubo et al., 2002). Early in fish orexin research orexin B was established as more highly conserved then orexin A, at least in zebrafish and pufferfish genetic studies (Alvarez and Sutcliffe, 2002; Kaslin et al., 2004). Fish orexin A contains a spacer region where mammalian orexin A does not, making it less conserved among vertebrates (Alvarez and Sutcliffe, 2002; Kaslin et al., 2004, Wong et al., 2011). Though the peptide sequences of fish and mammals are not identical, their three-dimensional structures are very similar (Volkoff 2006). Pufferfish have a highly conserved region of amino acids that forms the secondary structure of an alpha helix, allowing them to preserve their secondary structure without the rest of the sequence being highly conserved when compared

to mammals (Alvarez and Sutcliffe, 2002). Orexins have been localized to two main regions of the fish brain based on studies in goldfish and zebrafish: the hypothalamus and the telencephalon (Kaslin et al., 2004; Huesa et al., 2005). Fish have only one orexin receptor, about which very little is known. The receptor has been identified in zebrafish, pufferfish (Yokogawa et al., 2007), ornate wrasse (Facciolo et al., 2009) and goldfish (Abbott and Volkoff, 2011) and resembles mammalian OX2R (Buckley et al., 2010).

Orexins are implicated in vertebrate appetite regulation and sleep/wakefulness. Rats that have undergone icv injection of orexin have increased food intake (Sakurai et al., 1998), as well as increased movement patterns (Nakamura et al., 2000). Rats also exhibit an increase in prepro-orexin mRNA during fasting, further implicating orexin as an important regulator of feeding (Sakurai et al., 1998). Central injections of orexin in goldfish cause an increase in food intake, with orexin A causing a stronger effect then orexin B (Volkoff et al., 1999). Orexin injections in goldfish also increased locomotion (Volkoff et al., 1999). Further studies in zebrafish and winter flounder, where food deprivation caused an increase in prepro-orexin mRNA, followed the same pattern that was seen in fasted rats (Novak et al., 2005; Buckley et al., 2010). In zebrafish, fibers in the brain containing orexin interact with cholinergic and aminergic neurons, which implicates orexin as a neuropeptide involved in wakefulness (Kaslin et al., 2004). Orexin also appears to be involved in the regulation of seasonal feeding in fish, as in fish such as winter flounder, which have low food consumption in the winter, hypothalamic

prepro-orexin mRNA is higher in the winter than in the summer (Buckley et al., 2010).

Orexin may work in conjunction with NPY to control appetite regulation. Both orexin and NPY separately cause a hyperphagic response when administered by icv injection. When orexin and NPY are injected together they cause a greater increase in food consumption then if NPY was injected alone (Volkoff and Peter, 2001b), suggesting a synergistic action. Using high doses of orexin A to desensitize the orexin pathway also causes a desensitization of the NPY response pathway (Volkoff and Peter, 2001b). This evidence points to some sort of interaction between NPY and orexin, though the exact nature of this interaction still requires further research.

Cocaine- and amphetamine-regulated transcript

Cocaine- and amphetamine-regulated transcript (CART) is a unique peptide that was first discovered as a transcript produced in rats after the administration of pyschostimulant drugs such as cocaine and amphetamine (Douglass et al., 1995). It has since been further characterized as an appetite-regulating hormone in vertebrates and also plays roles in body weight regulation, stress response and endocrine function (Rogge et al., 2008).

The mammalian CART gene can be transcribed into two alternately spliced forms of CART mRNA (Kuhar et al., 2002). The longer prepro-CART sequence produces a peptide that is 102 amino acids while the shorter prepro-CART mRNA translates into a peptide 89 amino acids long (Douglass et al., 1995). Not all

mammals have both splice variants; humans have only short form of the pro-peptide while rodents have both (Thim et al., 1998). Both long and short form pro-CART peptides have cleavage sites that are post-translationally modified by prohormone convertases to form alternately spliced CART peptides (Rogge et al., 2008). Many splice variants of the CART peptide exist, and six have been isolated (Dey et al., 2003). Post-translational modification appears to be tissue-specific in mammals (Thim et al., 1999).

No CART receptors have been positively identified. However recent lab studies point to G-protein-coupled receptor (GPCR) as a possible candidate (Rogge et al., 2008): CART peptide applied to differentiated and non-differentiated PC12 cells exhibit binding properties like a receptor, and the binding is reduced in the presence of a GTP analog, which supports the theory of GPCR binding (Jones and Kuhar, 2008). Mammalian CART is predominantly found in areas of the hypothalamus associated with food regulation including the arcuate and paraventricular nuclei and the lateral hypothalamic area (Douglass et al, 1995; Kristensen et al., 1998). CART is also found in other mammalian tissues including the gastrointestinal (GI) tract (Murphy et al. 2000). CART can circulate in the blood, and in monkeys CART levels have been shown to exhibit a diurnal rhythm (Larsen et al., 2003; Vicentic et al., 2006)

Fish have varying numbers of pre-pro-CART transcripts depending on the species. Goldfish have two CART mRNA sequences, which share 63% sequence identity (Volkoff and Peter, 2001a). In medaka, six different pre-pro-CART

transcripts have been identified, each expressed in a variety of tissues (Murashita and Kurokawa, 2011). Many other fish species including cod (Kehoe and Volkoff, 2007), winter flounder (MacDonald and Volkoff, 2009) and catfish (Kobayashi et al., 2008) have only one CART transcript reported. Gene duplication events may be the cause of multiple CART genes in some fish species (Murashita and Kurokawa, 2011). Across both fish and mammals the structure of the CART gene remains constant, and is composed of three exons and two introns (Murashita and Kurokawa, 2011). Fish and mammalian CART peptides have a preserved tertiary structure maintained by disulfide bonds, indicated by the conserved position of cysteine residues (Volkoff and Peter, 2001). This conserved structure may be the reason early injections of mammalian CART into fish were effective at eliciting an anorexigenic response (Volkoff and Peter, 2001).

The role of CART as an anorexigenic appetite-regulating hormone is evident from injection studies done in mammals and fish. In one of the first injection studies in rats by Lambert et al. (1998), icv administration of CART fragments produced a significant decrease in food intake. The appetite-suppressing effects of CART could be inhibited by administration of a CART antibody, which caused increased feeding in the rats (Lambert et al., 1998). Co-injections of CART with NPY attenuated the appetite-stimulating effects of NPY, indicating the two peptides may have opposing effects on each other (Lambert et al., 1998). NPY is not the only hormone CART affects; co-injections of CART and CCK in mice produced prolonged anorexigenic effects, suggesting a synergistic effect to decrease food intake (Maletinska et al.,

2008). Even when acting alone, CART has strong anorexigenic effects on food consumption. Injections of CART into rat brains inhibited feeding both in mice that were regularly fed and fasted for 24 hours (Kristensen et al., 1998). On a cellular level, injections of CART peptide in rats caused c-Fos expression in areas of the hypothalamus associated with feeding in mammals, indicating CART may exert its anorexigenic actions in the hypothalamus (Vrang et al., 1999).

Injections of CART into rodents and fish support the role of CART as an anorexigenic hormone. Many early studies of the effects of CART on food consumption injected different sized fragments of CART, though there appears to be one form, CART (55-102), that is most effective in eliciting appetite-inhibiting effects in fish (Volkoff and Peter, 2001). In fish, injections of human CART caused decreased food intake in the goldfish, illustrating the highly conserved nature of the CART peptide (Volkoff and Peter, 2000). CART can also attenuate the orexigenic effects of NPY and orexin when co-injected in goldfish (Volkoff and Peter, 2000). CART influences food consumption as an appetite-regulating hormone, but food restriction can also affect the expression of CART. Transcript expression of CART decreased in the telencephalon and hypothalamus of goldfish following 96 hours of fasting in the goldfish (Volkoff and Peter, 2001a). Decreases in CART transcript expression during fasting have also been documented in cod and rat (Kehoe and Volkoff, 2007; Savontaus et al., 2002). In common carp (*Cyprinus carpio*), both CART1 and CART2 gene expression in brain decrease after fasting and increase after refeeding comparing with normal fed controls (Wan et al., 2012).

CART has been established as a regulator of food intake in vertebrates, but CART may also play a role in maintaining body weight. Knockout CART mice, which lack a functional CART gene, have been shown to have higher body weight than wild type mice (Wierup et al., 2005). CART also appears to play a role in insulin secretion in mammals. In the presence of glucose, CART enhanced insulin secretion in rat islet cells (Wierup et al, 2006). Leptin, an anorexigenic hormone, is responsible for inducing the expression of CART (Jequier, 2002). CART appears to play many roles in appetite regulation and may also act as part of an anorexigenic signaling cascade.

Cholecystokinin

Cholecystokinin (CCK) is a peptide found within the GI tract and brain of vertebrates, though its primary site of secretion is the GI tract (Moran and Kinzig, 2004). This makes CCK a peripherally-acting hormone, one of many within the gut that influence food intake by signaling satiety (Volkoff 2006). These peripherally-acting hormones work directly on peripheral tissues, or signal to the central nervous system by binding to receptors on nerves that extend from the peripheral tissue to the brain.

CCK has long been established as a hormone involved in appetite regulation. Ivy and Oldberg (1928) first discovered the peptide while observing the digestive tracts of dogs. They found an unknown peptide causing the gallbladder to contract and release its contents (Ivy and Oldberg, 1928). The hormone was named cholecystokinin and was eventually identified as a factor that also stimulated

enzyme secretion from the pancreas (Jorpes and Mutt, 1966). Although CCK is still most widely known for its ability to stimulate the release of enzymes that cause digestion of fats and proteins, its role as an appetite suppressant is now coming into focus.

Though CCK is a primarily found within the gut, many regions of the brain also contain CCK including the hypothalamus (Moran and Kinzig, 2004). Aside from appetite regulation, there are many possible actions of CCK within the brain and the peptide also appears to act as a neurotransmitter (Crawley and Corwin, 1994). Within the mammalian GI tract, CCK is found in the upper portion of the small intestine, in particular in cells of the duodenum and the jejunum (Larsson and Rehfeld, 1978). These enteroendocrine cells known as l-cells are found in the epithelium of the lumen in the GI tract. They are triangular in shape, with the narrow end of the cell pointed into the lumen to act as a sensor to detect the presence of food in the gut (Buchan et al., 1978). The broad end of the cell projects towards the blood vessels and contains granules of CCK which can be released into the surrounding tissues or blood stream upon stimulation from the apical portion of the cell (Buchan et al., 1978).

The peptide that forms CCK has several biologically active forms that are all derived from the same precursor molecule (Reeve et al., 1994). Prepro-CCK (the precursor peptide) is modified via a post-translation mechanism to form active CCK. CCK is highly conserved among vertebrates, with the carboxyl terminus of the peptide being the most conserved for its biological activity (Moran and Kinzig,

2004). There are three prevalent forms of CCK present in mammals (CCK-58, CCK-33 and CCK-8) however fish contain an unknown number of derivatives (Reeve et al., 1994). Fish have a prepro-CCK precursor peptide that is post-translationally modified into several different forms including the mammalian CCK-8, though it is still unclear how many different forms there are and how they differ from their mammalian counterparts (Jensen, 2001).

CCK exerts its actions by binding to specific G-protein-coupled receptors found within the brain and gut (Moran and Kinzig, 2004). CCK has two different receptor subtypes (CCK_A and CCK_B), which tend to be localized to either the peripheral or central tissues. CCK_A is found within peripheral tissues mainly the GI tract with smaller amounts in localized regions of the brain, while CCK_B is located primarily within the brain and CNS with small amounts found in specific peripheral tissues (Moran and McHugh, 1982). The receptors are also sensitive to post-translational modifications including sulfated tyrosine (Moran and Kinzig, 2004). CCK_A is much more sensitive to sulfated tyrosine and requires a sulfated CCK molecule to bind, while CCK_B binds non-sulfated CCK (Moran et al., 1986). Both receptors are not equally important in mediating the appetite-suppressing response of CCK. CCK_A receptor agonists cause an increase in the appetite-suppressing behavior in mammals, while agonists for CCK_B receptor have no effect on appetite (Asin et al., 1992). Therefore the CCK_A receptor is probably more important in mediating the inhibitory effects of CCK on food intake then the CCK_B receptor. In mammals, the CCK_B receptor is nearly identical to another receptor, the gastrin

receptor (Kopin et al., 1992). Gastrin is a peptide involved in digestion, which stimulates the release of hydrochloric acid from parietal cells into the lumen of the gut (Kopin et al., 1992). This facilitates the digestion of food particles. It has been suggested that gastrin and CCK evolved from the same precursor peptide, as they are produced within the same cells in amphibians and fish (Larsson and Rehfeld, 1977).

CCK is an appetite-suppressing hormone whose actions are initiated by the presence of food in the gut. Certain food macromolecules such as fats and proteins interact with the apical portion of the l-cells that extend into the lumen, stimulating the release of CCK from granules in the base of the cell into the blood stream (Liddle et al, 1985). CCK levels in plasma increase after feeding, and remain elevated after feeding has finished (Liddle et al., 1985). CCK then binds to receptors on the pancreas and gall bladder, causing the release of enzymes and bile to aid in the digestion of dietary fats and proteins (Liddle et al., 1985; Moran and Kinzig, 2004). CCK also acts on muscles of the gut to inhibit gastric emptying, keeping the organism feeling full longer (Moran and Kinzig, 2004).

Many studies have been done with both mammals and fish to determine the direct and appetite-suppressing effects of CCK on vertebrates. In one of the first studies on exogenous CCK administration, Gibbs et al. (1973) injected adult male rats with purified and synthetic CCK and monitored their eating and drinking habits following feeding. The results showed that both the synthetic and purified CCK injections cause a dose-dependent decrease in meal size as well as the duration of

time spent eating (Gibbs et al., 1973). A similar study was then performed with rhesus monkeys, with intravenous infusions of CCK (Gibbs et al., 1976). The monkeys displayed the same pattern as the rats; there was a dose-dependent decrease in food intake (Gibbs et al., 1976). Fish seem to exhibit the same response to CCK administration as mammals. Injections of CCK-8 in goldfish have been shown to cause appetite suppression (Himick and Peter, 1994, Volkoff et al., 2003). Decreasing the rate of gastric emptying is the main mechanism for inhibiting food intake, as was seen when salmonids were injected with CCK (Olsson et al., 1999). Endogenous CCK injections in fish also cause gall bladder contractions and the subsequent release of bile, as was seen in Atlantic salmon (Einarsson et al., 1997). Besides the presence of food in the gut, other physiological conditions such as fasting can also affect CCK levels. Fasted winter flounder exhibited a significant decrease in CCK mRNA expression in the gut when compared with fed fish (MacDonald and Volkoff, 2009). CCK is sensitive to the presence or absence of food in the gut and is thus a good determinant of an organisms feeding state.

The cunner

The cunner (*Tautogolabrus adspersus*) is a member of the labridae family found along the eastern coast of North America. Their range extends from Chesapeake Bay to the island of Newfoundland (Bradbury et al., 1997). Cunners typically inhabit shallow, costal waters, rarely going beyond depths of 10 m (Green and Farwell, 1971). The fish can be found inhabiting a variety of substrates from

sandy bottoms to rocks and weeds during summer months, but they retreat to the safety of large rocks and crevices in the winter to hide (Green and Farwell, 1971). The Cunner is a plentiful local species along the coast and these fish are best known for their extended period of torpor during the winter. This trait was first noted in an observational study of cunners by Johansen (1925), in which he reported that cunners were scarce in the winter and were often seen hiding along the bottom of the ocean. Haugaard and Irving (1943) attributed this scarceness to a decrease in physical activity caused by metabolic depression. The cunners were simply not able to maintain their level of activity when water temperatures dropped in the winter.

Entering into torpor allows cunners to survive winter in the North Atlantic Ocean (Bradbury et al., 1997). Both males and females undergo metabolic depression in late fall when water temperature falls below 5°C, during which they remain inactive and forgo feeding until water temperatures increase in spring (Green and Farwell, 1971). Cunners and their close relative the goldsinny wrasse (*Ctenolabrus rupestris)*, have been shown to decrease their heart rate and oxygen consumption during torpor (Sayer and Davenport, 1996). During the winter, cunners secrete a thick layer of mucous over their skin, which may be used to protect them from contact from ice crystals (Valerio et al., 1989). Their entry into torpor does not appear to be voluntary, as experimental re-warming of cunners in torpor during the winter caused the fish to become active and start feeding (Green and Farwell, 1971). Lewis and Driedzic (2007) measured cunner protein synthesis in a variety of different body tissues including brain, liver, heart and gill in 0°C water

and found a significant decrease in RNA content compared with tissues of fish samples acclimated to spring water temperatures. Assessing changes in levels of appetite-regulating hormones in cunner tissues during the winter would contribute to the understanding of how these hormones might regulate or be regulated by torpor conditions.

Project objectives

The broad goal of this project was to determine the effects of season and fasting on the production of NPY, CART, CCK and orexin in cunner. Because the cuner undergoes a period of winter dormancy during which it ceases to eat and remains inactive, it is a model species to study the effects of long-term and short-term fasting as well as the effects of season on appetite-regulating hormones. The first objective of this study was to determine the genetic sequences of NPY, CART, CCK and orexin in the cunner. Since these sequences had not yet be determined in the cunner but had already been established in other closely-related species of fish I used regions of high homology in these other sequences to design degenerate primers for the cunner hormone genes. Once the genes had been isolated using molecular cloning they were sequenced. Tissue distribution of the genes in the cunner was then determined in central and peripheral tissues samples.

The second objective was to characterize the expression of NPY, CART, CCK and orexin. To determine the effects of season on expression of the genes, tissue samples were taken from fish during their winter hibernation and during their

active summer period and the expression levels of peptides compared using quantitative real-time PCR (qPCR). qPCR allows DNA to be amplified and quantified therefore the relative amount of DNA in a tissue sample can be determined. The effects of feeding on gene expression were determined using tissue samples from fed and fasted summer acclimated fish. Cunners were fasted for three different time periods to give insight into how NPY, CART, CCK and orexin levels change during fasting. The expression levels of the genes in the tissue samples were quantified using qPCR. The final objective was to compare and contrast summer vs. winter gene expression levels with fed vs. fasted gene expression levels and to formulate a general model of expression of the hormones during fasting and torpor.

Though cunners are not a commercially important species, people along the coast fish them recreationally. Cunners are non-migratory, and therefore could serve as indicators of site-specific stress in costal regions, particularly where industries are discharging wastes into the ocean (Auster, 1989).

The significance of the cunner in this study is that it is an easily accessible and manipulated fish species, with a unique survival strategy. By studying the effects of fasting and long periods of torpor on appetite-regulating hormones in the cunner I hope to develop a better understanding of the endocrine control of feeding during metabolic depression. Quantifying the change in appetite-regulating hormones during different seasons and for different feeding regimes in the cunner could provide insight into the profiles of hormones in hibernating species of

vertebrates. It will also contribute towards a general model of appetite-regulation in vertebrates, which is still a poorly understood process.

A greater understanding of appetite-regulating hormones could lead to changes in aquaculture practices for multiple species of fish. With a stronger comprehension of the hormones governing feeding regulation in fish it may be possible to use this knowledge to optimize aquaculture feeding practices resulting in more sustainable aquaculture with a higher yield of fish. Since aquaculture fish are used both as food and for species conservation this knowledge could impact humans and struggling species populations.

Materials and Methods

Study animals

a. Gene characterization and tissue distribution study animals

Summer-acclimated cunner (*Tautogolabrus adspersus*) used for gene characterization and tissue distribution studies were obtained from the Ocean Sciences Center at Memorial University (St. John's, Newfoundland, Canada) in Sept 2010. The fish were a mixture of males and females, and were kept for several weeks in natural photoperiod and water temperatures in 2x2m flow through tanks until sampling.

b. Food deprivation study animals

Cunners (male and female) weighing 7.7-290g were collected off the coast of Norris Point (Norris Point, Newfoundland, Canada) in May 2011 and were acclimated for 2 weeks in 1x1m circular flow-though tanks under natural light and water temperature conditions at Bonne Bay Marine Station (Norris Point, Newfoundland, Canada). The fish were fed frozen chopped herring daily to satiety. At the start of the experiment, the fish were randomly divided into two fed (control) and two fasted tanks. The control group was fed daily to satiety throughout the duration of the experiment whereas fish in the fasted group were completely food deprived for the duration of the experiment. Fasted fish were sacrificed in groups of nine after three days, one week, two weeks and three weeks of fasting. Five control fish were sacrificed at the same time as the fasted fish during each sampling period.

c. Seasonal effects and acute torpor study animals

Cunners weighing 20-164g were collected off the coast of Norris Point in May 2011 and were kept in 1x1m flow though tanks under natural light conditions. Water temperature was kept below 4°C via seawater pumped from the bottom of Bonne Bay (Norris Point, NL, Canada) in order to induce torpor in the fish. Fish were fed frozen chopped herring once a week. Ten cunners were sampled from the "cold" (below 4°C) water temperature after four weeks of torpor. Ten additional cunners were sampled in March 2012 after four months of torpor. These "natural" torpor fish were fed frozen squid once every two weeks and seawater in the tank was <1°C at the time of sampling. All fish were determined to be in torpor based on observations of physiological traits such as reduced feeding and activity levels.

Tissue collection

All cunners sacrificed for tissue collection were anesthetized using 0.05% tricaine methanesulfonate (Syndel Laboratories, Vancouver, British Colombia, Canada) and killed by spinal section. Fish were measured, weighed and samples were collected from central (brain) and peripheral (liver, kidneys, gill, skin, muscle, gut, gonads, heart and spleen) tissues. Tissues were preserved in RNA*later* (Qiagen Inc, Mississauga, Ontario, Canada) and stored at -20°C. All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines.

RNA extraction

In the tissue distribution study brain tissue was dissected to isolate the optic tectum, telencephalon, cerebellum, medulla, pituitary and hypothalamus based on the anatomy of the cod brain (*Gadus morhua*) described in Delfini and Diagne (1985). For gene characterization whole brain RNA was isolated as well as RNA from liver, kidneys, gill, skin, muscle, gut, gonads, heart and spleen. For fasting, seasonal effects and periprandial studies RNA was isolated from the hypothalamus, telencephalon and gut of the fish. RNA extractions were done using a trizol-chloroform and Tri-reagent extraction (BioShop, Burlington, Ontario, Canada). RNA concentrations were quantified at a wavelength of 260-nm using a Nanodrop spectrophotometer (ThermoScientific, Wilmington, North Carolina, USA). All samples used had sample absorbance ratios between 1.8-2.1 at wavelengths of 260 and 280 nm.

cDNA synthesis and cloning

A total of 1 μg of RNA was transcribed to cDNA from each tissue using a QuantiTect Reverse Transcription Kit following the manufacturers protocol (Qiagen Inc). cDNA was then used in a polymerase chain reaction with GoTaq master mix 2X (Promega, Madison, Wisconsin, USA) for a total reaction volume of 25 μl. Primer sequences for gene characterization (Table 1) were a combination of primers from other studies (designed to match winter flounder and orange grouper) and degenerate primers based on sequences from multiple species. Prior to use, all

primer-annealing temperatures were optimized. A negative control consisting of an RT-PCR reaction with no reverse transcription enzyme was included to ensure the absence of contamination.

PCR products were separated by size by gel electrophoresis on a 1.5% agarose gel in TAE buffer (Tris-acetate-EDTA). Gels were imaged using an Epichemi Darkroom Bioimaging System (UVP, Upland, California, USA) equipped with a 12-bit cooled camera. All image management and analysis was done using LabWorks 4.0 software (UVP, Upland, California, USA). Bands containing genes of interest were isolated and removed from the gel, and purified using a GeneJET™ Gel Extraction Kit (Fermentas, Burlington, Ontario, Canada). Purified DNA was ligated into a pGEM easy vector (Promega, Madison, Wisconsin, USA) and minipreps were prepared using a GeneJET™ Plasmid Miniprep Kit (Fermentas). Final gene products were sent to The Center for Applied Genomics (TCAG Sick Kids Hospital; Toronto, Ontario, Canada) for sequencing.

Table 1: Primers used in the cunner for cDNA sequencing and cloning, tissue distributions and qPCR analysis

CART	
Non-specific primers	
CART_F	5' GAGAGTTCCGAGGAGCTGAG 3'
CART_R	5' TTTCGACTGAAGCTTCTCCA 3'
3'RACE primers	
CART_F1_3RACE	5' AGAGTTCCGAGGAGCTGAG 3'
CART_F2_3RACE	5' AGCTGAGCCGCAGAGCGCTG 3'
Gene-specific primers RT-PCR	
CART_F_TisDis	5' AAAGGACCGAACCTGACCTC 3'
CART_R_TisDis	5' GGGACTTGGCCAAACTTTTT 3'
Gene-specific primers qPCR	
CART_F_qPCR	5' AAAGGACCGAACCTGACCTC 3'
CART_R_qPCR	5' GGGACTTGGCCAAACTTTTT 3'
CCK	
Non-specific primers	
CCK_F	5' TTCCTGTGGCTGAGGAGAAT 3'

CCK_R	5’ GCACAGAACCTTTCCTGGAG 3’

3’RACE primers

CCK_F1_3RACE	5’ AGACTCATCTCCTCCAGG 3’
CCK_F2_3RACE	5’ CCTCCAGGAAAGGTTCTGTGC 3’

Gene-specific primers RT-PCR

CCK_F_TisDis	5’ CTCCAGGAAAGGTTCTGTGC 3’
CCK_R_TisDis	5’ CCATCCATCCCAAGTAGTCC 3’

Gene-specific primers qPCR

CCK_F_qPCR	5’ CTCCAGGAAAGGTTCTGTGC 3’
CCK_R_qPCR	5’ CCATCCATCCCAAGTAGTCC 3’

NPY
Non-specific primers

NPY_F	5’ ATGCATCCTAACTTGGTGAG 3’
NPY_R	5’ CCACAATGATGGGTCATATC 3’

Gene-specific primers RT-PCR

NPY_F_TisDis	5’ AGACGGATACCCTGTGAAGC 3’
NPY_R_TisDis	5’ AGCGTGTCTGTGCTTTCCTT 3’

Gene-specific primers qPCR

NPY_F_qPCR	5' AGACGGATACCCTGTGAAGC3'
NPY_R_qPCR	5' TCTGTCTTGTGATGAGGTTGATG 3'

Orexin

Non-specific primers

Ox_F	5' GAGAGTTCCGAGGAGCTGAG 3'
Ox_R	5' TTTCGACTGAAGCTTCTCCA 3'

3'RACE primers

Ox_F1_3RACE	5' TGATGCTCACAGGATGTCTG 3'
Ox_F2_3RACE	5' GGATGTCTGAGTGCTGCAGAC 3'

5'RACE primers

Ox_F1_5RACE	5'AAAGTGAGGATTCCAGCAGC 3'
Ox_F2_5RACE	5'CCAGCAGCAGCATCACCTGTTAG 3'
Ox_R_5RACE	5'ATCGTTAGAATCCCAGCTGC 3'

Gene-specific primers RT-PCR

Ox_F_TisDis	5' GTCGCTCTGGCAGTAAGACC 3'
Ox_R_TisDis	5' CCTCTTGCCCATCGTTAGAA 3'

Gene-specific primers qPCR

Ox_F_qPCR	5' GTCGCTCTGGCAGTAAGACC 3'
Ox_R_qPCR	5' TAAGCGGTCCACGTCTTTTT 3'

Elongation factor

Non-specific primers

EF_F	5' CCTGGACACAGGGACTTCAT 3'
EF_R	5' CGGTGTTGTCCATCTTGTTG 3'

Gene-specific primers RT-PCR

EF_F_TisDis	5' GGTACCTCTCAGGGTGCTGATC 3'
EF_R_TisDis	5' AGCTGCTTCACGCCGAGGGTG 3'

Gene-specific primers qPCR

EF_F_qPCR	5' GGTACATCTCAGGCTGACTGCG 3'
EF_R_qPCR	5' TCACACCGAGGGTGAAGG 3'

18S

Gene-specific primers qPCR

18S_F_qPCR	5' GTGGAGCGATTTGTCTGGTT 3'
18S_R_qPCR	5' GAACGCCACTTGTCCCTCTA 3'

Adaptor primers

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

3' and 5' Rapid Amplification of cDNA Ends

Genes for which only small fragments were obtained using non-specific primers were amplified using 3' and 5' Rapid Amplification of cDNA Ends (3' and 5' RACE). Gene-specific primers for 3' and 5' RACE (Table 1) were designed using the sequenced DNA. For 3'RACE, RNA was reverse transcribed to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen Inc) following the manufacturers protocol. cDNA was subjected to a nested PCR using GoTaq master mix 2X, and dT-AP and AP primers (Table 1) were used with gene-specific primers in first and second round PCR respectively. 5'RACE required reverse transcription of RNA with a gene-specific reverse primer (Table 1). cDNA was purified using a Montage PCR Millipore kit (Bedford, Maine, USA), then tailed with a polyA tail using a Terminal Deoxynucleotidyl Transferase (Invitrogen, Burlington, Ontario, Canada). The final cDNA product was amplified in a nested PCR using gene-specific (Table 1) and dT-AP and AP primers as described above. 3' and 5' RACE PCR products were separated by gel electrophoresis on a 1.5% agarose gel in TAE buffer, cloned and sequenced.

Sequence analysis

Gene sequences were compared with all other known sequences using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Alignments of sequences from multiple species were performed using the ClustalW2 website (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Tissue distribution (RT-PCR)

Samples from active, summer-acclimated cunner obtained from the Ocean Science Center were used for the cunner tissue distribution. RNA was extracted from central (optic tectum, telencephalon, cerebellum, medulla, pituitary and hypothalamus) and peripheral (liver, kidneys, gill, skin, muscle, gut, gonads, heart and spleen) cunner tissues using the methods described above. RNA was reverse transcribed to cDNA using a Quantitect Reverse Transcription kit (Qiagen Inc). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/) and were gene-specific for cunner NPY, CART, CCK and orexin. Genes were amplified by PCR using a GoTaq master mix 2X (Promega). Each PCR reaction was 30 cycles long, with a 94°C melting temperature for 30 seconds, a primer-specific annealing temperature for 30 seconds and a elongation temperature of 72°C for 30 seconds. PCR products were separate by gel electrophoresis on a 1.5% agarose gel and imaged using an Epichemi Darkroom Bioimaging System (UVP, Upland, California, USA). Elongation factor -1 alpha (EF1α) was used as a control gene, with specific primers designed based on winter flounder EF1α (GenBank accession number AW013637).

Quantitative real-time PCR analysis of gene expression

RNA was isolated from hypothalamus, telencephalon and gut using a Tri-reagent extraction (BioShop Inc.). RNA concentration was quantified using a Nanodrop at 260-nm (ThermoScientific). Isolated RNA was reverse transcribed to cDNA with random primers using a Quantitect Reverse Transcription kit (Qiagen

Inc). Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Gene-specific primers were designed for NPY, CART, CCK and orexin to produce 80-125 base pair amplicons of the genes. All primers either spanned across an exon-exon boundary or the two primer locations were separated by an intron of 100 base pairs or more. Forward and reverse primers were designed to have approximately the same melting temperature. RT-PCR products were diluted 1:3 in HPLC-treated water. A mix consisting of 0.2 μl of each primer, 6.2 μl water, 2 μl cDNA and 5 μl SYBR FAST qPCR Master Mix (Kapa Biosystems, Boston, Massachusetts, USA) totaling 10 μl was pipetted onto a 96-well plate (Eppendorf, Mississauga, Ontario, Canada) by an epMotion® 5070 automated robot (Eppendorf). Real-time quantitative PCR was performed using a Mastercycler® ep realplex 2S system (Eppendorf). Optimal primer annealing temperature was determined for the gene-specific primers ($0.98>R^2>1.02$). The basic cycling conditions of the PCR were a denaturing stage at 94°C for 30 seconds, gene-specific annealing temperature for 45 seconds and hybridization stage at 72°C for 60 seconds. A no-template control of water substituted for cDNA was included to ensure the validity of the results. At the end of each qPCR a melting curve was performed to verify that only one PCR product was amplified. EF1∝ was used as a control gene in the fasting and induced torpor studies, and 18S rRNA was used as a control gene in the natural torpor study to ensure results of gene expression levels were solely from experimental conditions.

a) qPCR quantification of NPY, orexin, CART and CCK gene expression

Dissociation curves and melting curves were analyzed with qPCR-specific primers for each gene to ensure specificity. All genes of interest were run in duplicate with a control substituting water for cDNA. Each plate included a gene of interest and a reference gene to ensure the experimental conditions (fasting or torpor) were not affecting gene expression of gene other than those involved in appetite regulation.

qPCR data analysis

All data obtained from qPCR was analyzed using a GraphPad Instat program (GraphPad Software Inc., San Diego, California, USA). Gene expression levels were measured and quantified using a Realplex 1.5 software (Eppendorf). Realplex compared all expression levels using relative quantification ($\Delta\Delta$Ct) to determine relative gene expression levels across multiple samples. Experimental gene expression levels in the fasting and induced torpor studies were normalized to the reference gene EF1$\propto$. Experimental gene expression levels in the natural torpor study were normalized to the reference gene 18S rRNA. Housekeeping gene Ct values during natural torpor and non-torpor were tested using a student's t-test (GraphPad Instat, GraphPad Software Inc). Relative percent expression of the genes of interest was determined by comparing the expression levels of the experimental genes with the reference gene (set at 100% expression). Results were graphed using Excel (Microsoft, Redmond, Washington, USA).

Results

Molecular cloning and sequence analysis

NPY

The cloned cunner NPY mRNA consists of the whole open reading frame for NPY (Genbank accession number JX126916). The open reading frame is 297 base pairs (bp), which codes for a mature peptide 99 amino acids long (Figure 1). When cunner NPY protein sequence was aligned with NPY protein sequences from other vertebrates, the amino acid similarity ranged from 56 to 95% (Figure 2). The highest similarity was with orange grouper (95%), followed closely by winter flounder (94%) and Japanese flounder (93%). The lowest similarity was with winter skate (56%).

Orexin

The cunner orexin mRNA from this experiment consists of a partial sequence (Genbank accession number JX126915). The open reading frame is 285 base pairs long, which codes for a partial mature peptide 95 amino acids long (Figure 3). The translated mature peptides are 43 amino acids (orexin A) and 28 amino acids (orexin B) long (Figure 3). The comparison of cunner orexin protein sequence to that of other species of vertebrates revealed amino acid similarities ranging from 45 to 75% (Figure 4). The highest amino acid similarity was with winter flounder (75%), followed by orange grouper (72%) and Nile tilapia (71%; Figure 4). The lowest similarity was with winter skate (45%) followed by mouse (49%).

CART

The cunner CART mRNA from this experiment is a complete sequence (Genbank accession number JX023541). The open reading frame is 372 base pairs long, which codes for a mature peptide 103 amino acids long. The transcript has a 28 base pair 5'UTR and a 238 base pair 3'UTR (Figure 5). When cunner CART protein sequence was aligned with CART protein sequences of other vertebrates, the amino acid similarity ranged from 34 to 79% (Figure 6). The highest similarity in amino acids was with winter flounder (79%), followed by Atlantic cod (64%) and zebrafish (62%). The lowest similarity was with mouse (34%) followed by winter skate (43%).

CCK

The cunner CCK mRNA from this experiment is a partial sequence (Genbank accession number JX126917). The cloned fragment contains a 209 base pairs long open reading frame, which codes for a partial mature peptide 69 amino acids long as well as a 32 base pair 3'UTR (Figure 7). Based on sequences from other species the whole mature peptide should be approximately 140 amino acids long. When cunner CCK protein sequence was aligned with CCK protein sequences from other vertebrates, the amino acid similarity ranged from 46 to 85% (Figure 8). The highest similarity was with winter flounder and Japanese flounder (both 85%). The lowest similarity was with spiny dogfish (46%) followed by mouse (50%).

```
ATGCATCCTAACTTGGTGAGCTGGCTCGGGACTCTGGGCTTCCTGCTGTGGGCGCTTCTC  60
M  H  P  N  L  V  S  W  L  G  T  L  G  F  L  L  W  A  L  L    20

TGCCTGGGCGCCCTGACAGACGGATACCCTGTGAAGCCGGAGAACCCCGGCGAGGATGCC 120
C  L  G  A  L  T  D  G  Y  P  V  K  P  E  N  P  G  E  D  A    40

CCGGCAGAAGAGCTGGCCAAGTACTACTCAGCCCTGAGACATTACATCAACCTCATCACA 180
P  A  E  E  L  A  K  Y  Y  S  A  L  R  H  Y  I  N  L  I  T    60

AGACAGAGGTATGGAAAGAGGTCCAGTCCTGAGATTCTGGACACGCTGATCTCAGAGCTG 240
R  Q  R  Y  G  K  R  S  S  P  E  I  L  D  T  L  I  S  E  L    80

CTGTTGAAGGAAAGCACAGACACGCTTCCACAGTCAAGATATGACCCATCATTGTGG     297
L  L  K  E  S  T  D  T  L  P  Q  S  R  Y  D  P  S  L  W       99
```

Figure 1. Cloned nucleotide and predicted amino acid sequence of cunner NPY. Grey section indicates translated mature peptide. Bolded letters indicate amino acid sequence. The complete coding sequence minus the stop codon was sequenced

a)

```
Mouse                MLGNKRMGLCGLTLALSLLVCLGILAEGYPSKPDNPGEDAPAEDMARYYSALRHYINLIT 60
Goldfish             MHPNMKMWTGWAACAFLLFVCLGTLTEGYPTKPDNPGEGAPAEELAKYYSALRHYINLIT 60
Japanese_Flounder    MHPNLVSWLGTLGLLLWALLCLSALTEGYPVKPENPGDDAPAEELAKYYSALRHYINLIT 60
Winter_Flounder      MHPNLVSWLGTLGLLLWALLCLSALTEGYPMKPENPGEDAPAEDLAKYYSALRHYINLIT 60
Orange_Grouper       MHPNLVSWLGTLGFLLWALLCLGALTEGYPVKPENPGDDAPAEDLAKYYSALRHYINLIT 60
Cunner               MHPNLVSWLGTLGFLLWALLCLGALTDGYPVKPENPGEDAPAEELAKYYSALRHYINLIT 60
Atlantic_Cod         MHSNLATWLGALGFLLCALICLGTLTEGYPIKPENPGEDAPADELAKYYSALRHYINLIT 60
Winter_Skate         MQNNMKSWLGVFTFIFSMLVCIGTFADAYPSKPDNPGDGASAEQGAKYYTALRHYINLIT 60
                     *  *           :  ::*:. :::.** **:***:.*.*:: *:**:**********

Mouse                RQRYGKRSSP---ETLISDLLMKESTENAPRTRLEDPSMW 97
Goldfish             RQRYGKRSSA---DTLISDLLIGE-TESHPQTRYEDQLVW 96
Japanese_Flounder    RQRYGKRSSPEILDTLVSELLLKESTDTLPQSRY-DPSLW 99
Winter_Flounder      RQRYGKRSSPEILDTLVSELLLKESTDTLPQSRY-DPSLW 99
Orange_Grouper       RQRYGKRSSPEILDTLVSELLLKESTDTLPQSRY-DPSLW 99
Cunner               RQRYGKRSSPEILDTLISELLLKESTDTLPQSRY-DPSLW 99
Atlantic_Cod         RQRYGKRSSPEILDTLVSELVLKESANTLPQSRY-DPSLW 99
Winter_Skate         RQRLGKRSNPE--ALMMTELMLRDNSENFPKFRYDEPSMW 98
                     *** ****..     ::::*:: : ::. *: *  :  :*
```

b)

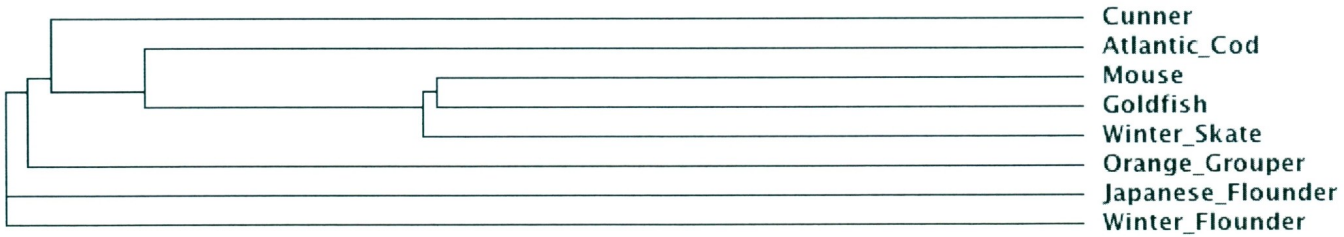


Figure 2. a) Cunner NPY protein alignment from ClustalW with GenBank sequences from Japanese flounder (BAB62409.1), Winter flounder (ACH42755.1), Orange grouper (AAT48713.1), Atlantic cod (ABB79923.1), Goldfish (AAA49186.1), Winter skate (ACH42754.1), and mouse (EDK98613.1) b) ClustalW NPY phylogenetic tree. Amino acids that are identical across species are noted by (*). Amino acids that are somewhat similar across species are noted by (:). Gaps in sequences are noted by (-).

```
CTGCTGCTGGCTCACCTGACTTGTGATGCTCACAGGATGTCTGAGTGCTGCAGACAGCCC   60
L  L  L  A  H  L  T  C  D  A  H  R  M  S  E  C  C  R  Q  P     20

TCCCCCTCCTGTCGCCTCTATGTGCTGCTGTGTCGCTCTGGCAGTAAGACCATCGGGGGA  120
S  P  S  C  R  L  Y  V  L  L  C  R  S  G  S  K  T  I  G  G     40

GCGCTAACAGGTGATGCTGCTGCTGGAATCCTCACTTTGGGTAAACGGAAAAAAGACGTG  180
A  L  T  G  D  A  A  A  G  I  L  T  L  G  K  R  K  K  D  V     60

GACCGCTTACAAAGCCGACTGGCCAACCTCCTCCATGTGTCCAGGAACCCAGCAGCTGGG  240
D  R  L  Q  S  R  L  A  N  L  L  H  V  S  R  N  P  A  A  G     80

ATTCTAACGATGGGCAAGAGGGTGGAGGAGAGGTCTGCAGATTAC                 285
I  L  T  M  G  K  R  V  E  E  R  S  A  D  Y                    95
```

Figure 3. Partially cloned nucleotide and predicted amino acid sequence for cunner orexin. Grey section indicates translated mature peptide (orexin A and orexin B). Bolded letters indicate amino acid sequence.

a)

```
Cunner              LLLAHLTCDAHRMS-ECC-RQPSPSCRLYVLLCR----SGSKTIGGA------LTGDAAA 48
Winter_Flounder     LLLSQLTCDAHSMS-DCC-RQPSRSCRLYALLCR----TGSKTMGGT------LSGDAAA 48
Orange_Grouper      LLLSQLACDAHSVS-ECC-RQPPRNCRLHVLLCR----SGSKNLGGT------LTGDAAA 48
Nile_Tilapia        LLLSQLACDAHSVS-ECC-REPSRPCRLYVLLCR----SGNKGPGGV------LTDDAAA 48
Atlantic_Cod        LLASHTLCDAHSVSASCCSREPPRACRLYVLLLCGPVGGAGRALGGMH-----LGEDASA 55
Goldfish            ALLAHLARDAEGVA-TCC-SSASRSCKLYEILCR----AGRRNDTSIARHIGRFNNDAAV 54
Mouse               ALLSLGVDAQP-LPDCCR--QKTCSCRLYELLHG--------------------AGNHAA 37
Winter_Skate        ALLCSLVSASPRVPKCCC--QQTCSCKVIDLLRG--------------------TGNHAA 38
                     * .        :.  *   . .  *::  :*                       : :.

Cunner              GILTLGKRKKDVDRLQSRLANLLHV-SRNPAAGILTMGKRVE-----------ERSAD-Y 95
Winter_Flounder     GILTLGKRKEEEHRLHSRLHHLLHV-SRNQAAGILTMGKRTE-----------ERAGEPY 96
Orange_Grouper      GILTLGKRREDD-RLQSRLHQLLQG-SRNQAAGILTMGKRTE-----------EMGREQY 95
Nile_Tilapia        GILTLGKRKEDEYRFQSRLQQLLQG-SRNQAAGILTMGKRTR-----------ERAG-QY 95
Atlantic_Cod        GILTLGKREAEEQHFHSRLHQLLRGGARNQAAGILTMGKRSE-----------EEEAVGL 104
Goldfish            GILTLGKRKVGERRVQDRLQQLLHG-SRNQAAGILTVGKRLEDPLQDLMPRPPEDSLDAY 113
Mouse               GILTLGKRRPGPPGLQGRLQRLLQA-NGNHAAGILTMGRRAG----------AELEPHP- 85
Winter_Skate        GILTLGKRKTNAQPLQNRLHHLLHG-LENQATGILTMGKREEPQQVSL----AEDSPGTN 93
                    ********.     .:.** .**:    * *:****:*:*             *
```

b)

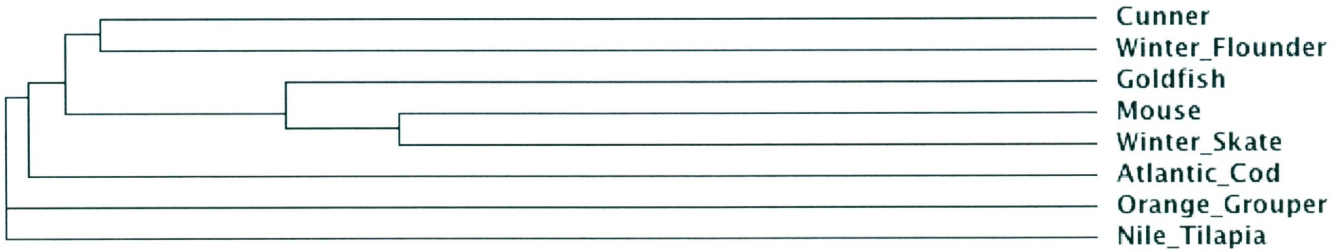


Figure 4. a) Cunner orexin protein alignment from ClustalW with GenBank sequences from Winter flounder (ADJ67994.1), Orange grouper (ADM26763.1), Nile tilapia (ACT65742.1), Atlantic cod (ABF29871.1), Goldfish (ABK58728.1), Winter skate (ADL41188.1), and mouse (NP_034540.1) b) ClustalW orexin phylogenetic tree. Amino acids that are identical across species are noted by (*). Amino acids that are somewhat similar across species are noted by (:). Gaps in sequences are noted by (-).

```
                                                    ttgggacacgatgaggagcacagggacg
28

ATGAGGAGCACAGGGAGCACGAGGAGCTGCAGGCTGCTGCTCTGCGTGCTGCTGCTCTCA    88
M  R  S  T  G  S  T  R  S  C  R  L  L  L  C  V  L  L  L  S      20

GGATCCACCGGAGCAGACCTGACGGAGAACAACTCCCTGACCAGTGAGGACGAGCTGAGC   148
G  S  T  G  A  D  L  T  E  N  N  S  L  T  S  E  D  E  L  S      40

CCCAGAGTGCTGCGCCACTTCTACTCCAAAGGACCGAACCTGACCTCAGAGAAACAGCTG   208
P  R  V  L  R  H  F  Y  S  K  G  P  N  L  T  S  E  K  Q  L      60

TCCGGAGCTCTGCAGGAAGTTCTGGAGAAACTTCAGGCGAAGAAAACGTCTTCATGGGAG   268
S  G  A  L  Q  E  V  L  E  K  L  Q  A  K  K  T  S  S  W  E      80

AAAAAGTTTGGCCAAGTCCCCAGGTGTGACGTCGGGGAGCAGTGTGCAGTGAGAAAAGGC   328
K  K  F  G  Q  V  P  R  C  D  V  G  E  Q  C  A  V  R  K  G      100

TCTCGGATCGGCAGGATGTGTGACTGTCCTCGTAGAGCGTTCTGTAACTTCTGCCTGCTG   388
S  R  I  G  R  M  C  D  C  P  R  R  A  F  C  N  F  C  L  L      120

AAGTGTTTATGAgaaacacacacacaagtcttttattaaaggtcacatttaatgagaa     448
K  C  L  *                                                       123

ccagctgaggagaatgctcagtgaccgtctgcaacacacgaagaagaactacatggtgtt   508

taacagagtctgtgtgaggattgttgtgatgagatggtgagttcaaagttgtgatatcat   568

gtcaacaaacaaatatttgagtctaatgtcggagtctgtctgtcattataataattcata   628

ttcataataaagtgcggatcatgttttaaaaaaaaaaaaaaaaaa                  674
```

Figure 5. Cloned nucleotide and predicted amino acid sequence for cunner CART. Grey section indicates translated mature peptide. Bolded letters indicate amino acid sequence, lower case letters indicate untranslated nucleotides. Stop codon is indicated by an asterix (*). Potential sites for polyadenylation are underlined. Whole coding DNA sequence is shown.

a)

```
Winter_Flounder      --------------------------------MESSEELSRRALRDFYPKGPNLTSEKQL 28
Cunner               MRSTGSTRSCRLLLCVLLLSGSTGADLTENNSLTSEDELSPRVLRHFYSKGPNLTSEKQL 60
Goldfish             MES--SKLWTTAMACAVLVSCIQGAEMDF----DNESDLETRALREFYPKDPNLTNEKQL 54
Salmon               MES--SRLWTRAVVCAVLLSIVLSAEIDYS---DSELDLDTRSVRDFYPKDPNLTNEKQL 55
Atlantic_Cod         MES--SRVWTRALVCAVLLSVVHGADLYNS---ESEEDLSTRALRDFYPKGPNLTNERQL 55
Zebrafish            MES--SSLRMRMAVCALLVCLLTGARANESEP-EIEVELDTRAIRDFYPKDPNLNSEKQL 57
Winter_Skate         MVS--DRLLLAVYFC-VLFSMAVG---------AENSDLEPRALRDFYSKNYYPGSEKEL 48
Mouse                MESSRLRLLPLLGAALLLLLPLLGARA------QEDAELQPRAL-DIYSAVDDASHEKEL 53
                                                     . :*. * : .:*.       *::*

Winter_Flounder      LG-------------ALQEVLEKLQSKRLPLWEKKFGQVPTCDVGEQCAVRKGARIGKMC 75
Cunner               SG-------------ALQEVLEKLQAKKTSSWEKKFGQVPRCDVGEQCAVRKGSRIGRMC 107
Goldfish             LG-------------ALHDVLEKLQSKRISLWEKKFGRVPTCDVGEQCAIRKGSRIGKMC 101
Salmon               LG-------------ALHDVLKKLQTKRLPFWEKKFGQVPTCDVGEQCAVRKGARIGKMC 102
Atlantic_Cod         LG-------------ALHEVLEKLQTRKMPLWEKKFGQVPTCDIGEQCAIRKGARIGKMC 102
Zebrafish            LG-------------ALQEVLEKLQTKRIPPWEKKFGQVPMCDLGEQCAIRKGSRIGKMC 104
Winter_Skate         LG-------------ALHEVLKKLQTKRLPTWEKKYGQGPQCNIGDMCAVRKGPRIWRTC 95
Mouse                PRRQLRAPGAMLQIEALQEVLKKLKSKRIPIYEKKYGQVPMCDAGEQCAVRKGARIGKLC 113
                                  **::**:**:::: . :***:*: * *: *: **:***.** : *

Winter_Flounder      DCPRGAFCNFFLLKCL 91
Cunner               DCPRRAFCNFCLLKCL 123
Goldfish             DCPRGAFCNYFLLKCL 117
Salmon               DCPRGAFCNSYLLKCL 118
Atlantic_Cod         DCPRGSFCNFFLLKCL 118
Zebrafish            DCPRGALCNFFLLKCL 120
Winter_Skate         NCLS-SKCNYFLFKCV 110
Mouse                DCPRGTSCNSFLLKCL 129
                     :*   : **  *:**:
```

b)

Figure 6. a) Cunner CART protein alignment from ClustalW with GenBank sequences from vWinter flounder (ACO34688.1), Goldfish (AAL40907.1), Salmon (NP_001140152.1), Atlantic cod (AAZ94721.1), Zebrafish (NP_001017570.1), Winter skate (ACO06235.1) and mouse (NP_038760.3) b) ClustalW CART phylogenetic tree. Amino acids that are identical across species are noted by (*). Amino acids that are somewhat similar across species are noted by (:). Gaps in sequences are noted by (-).

```
TCCTGTGGCTGAGGAGAATGCAGACTCTCGAGCCAATCTCAGTGAGCTGCTGGCMAGACT  60
P  V  A  E  E  N  A  D  S  R  A  N  L  S  E  L  L  X  R  L    20

CATCTCCTCCAGGAAAGGTTCTGTGCGCAGAAACTCAGTGGCAAACAGAGGCAGCGCACT  120
I  S  S  R  K  G  S  V  R  R  N  S  V  A  N  R  G  S  A  L    40

CAGCGCCAACCACCGGATAGCAGACAGGGACTACTTGGGATGGATGGGTTTCGGCCGCCG  180
S  A  N  H  R  I  A  D  R  D  Y  L  G  W  M  G  F  G  R  R    60

CAGTGCAGAGGAGTACGAGTACTCCTCGTaaagggtgatcacccttacgtacgggcacat  240
S  A  E  E  Y  E  Y  S  S  *                                   69

g                                                              241
```

Figure 7. Partial cloned nucleotide and predicted amino acid sequence for cunner CCK. Grey section indicates translated mature peptide. Bolded letters indicate amino acid sequence, lower case letters indicate untranslated nucleotides. Stop codon is indicated by an asterix (*).

a)

```
Salmon                PYTAEEDGDS--------------------------------RANLSELLAR-LISSRK 26
Rainbow_Trout         PYTAEEDGDS--------------------------------RANLSELLAR-LISSRK 26
Cunner                P-VAEENADS--------------------------------RANLSELLXR-LISSRK 25
Winter_Flounder       P-VAEENGDT--------------------------------RANLSELLAR-LISSRK 25
Japanese_Flounder     P-VAEEDGDS--------------------------------RANLSELLAR-LISSRK 25
Goldfish              PAVSEDGGQSDLGIVMEHTRHTRAAPSSGQLSLLSKAEDDEEPRSSLTELLAR-IIST-K 58
Mouse                 PRRQLRAVLRT---------------------------DGEPRARLGALLARYIQQVRK 32
Spiny_Dogfish         RQRQIRETQSID----------------------LKPLQDSEQRANLGALLTRYLQQVRK 38
                                                          *: *  ** * : .  *

Salmon                GSIRKNSTVNSRASGLSANHRIKDRDYNGWMDFGRRSAEEYEYEYSL 73
Rainbow_Trout         GSLRKNSTVNSRASGLSANHRIKDRDYNGWMDFGRRSAEEYEYSL-- 71
Cunner                GSVRRNSVAN-RGSALSANHRIADRDYLGWMGFGRRSAEEYEYSS-- 69
Winter_Flounder       GSVRRNSTAY--SKGLSPNHRIADRDYLGWMDFGRRSAEEYEYSS-- 68
Japanese_Flounder     GSVRRNSTAY--SKGLSPNHRIADRDYLGWMDFGRRSAEEYEYSS-- 68
Goldfish              GTYRRSPSPK--SKSMGNNHRIKDRDYLGWMDFGRRSAEEYEYSS-- 101
Mouse                 APSGR-MSVLKNLQSLDPSHRISDRDYMGWMDFGRRSAEDYEYPS-- 76
Spiny_Dogfish         GPLGRGTLVGTKLQNMDPSHRIADRDYMGWMDFGRRSAEEYEYAS-- 83
                      ..  :        . :. .*** **** ***.*******:***
```

b)

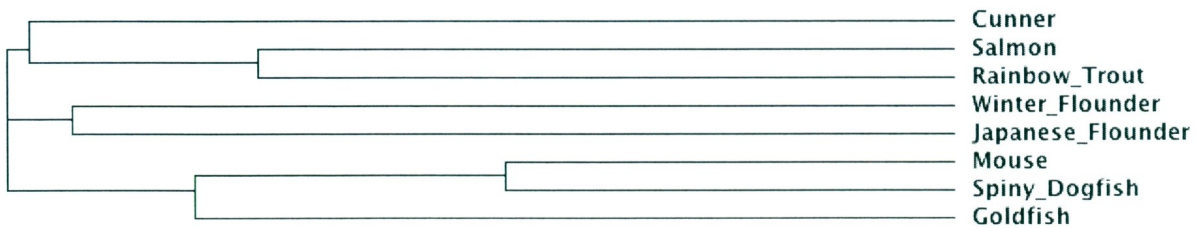


Figure 8. a) Cunner CCK protein alignment from ClustalW with GenBank sequences from Winter flounder (ACH42757.1), Japanese flounder (BAA23734.1), Rainbow trout (NP_001118083.1), Salmon (ACM0982.1), Goldfish (O93464.1), Dogfish (CAB94727.1) and mouse (AAH28487.1) b) ClustalW CCK phylogenetic tree. Amino acids that are identical across species are noted by (*). Amino acids that are somewhat similar across species are noted by (:). Gaps in sequences are noted by (-).

Tissue distribution

Transcript fragments were amplified in each tissue sample using RT-PCR along with a no-template control to verify that any visible amplification was due to the presence of the desired transcript and not contamination. Each tissue sample was also tested with the housekeeping genes (HKG) EF1-α and 18S rRNA to ensure that cDNA was present in all the samples. All tissue expression levels are qualitative and based on comparisons with the control genes.

NPY

For tissue distributions of NPY, a 183 bp region was amplified. In the central tissue distribution, NPY was present in all brain regions except for the pituitary (Figure 9). The brain regions in which NPY was present appeared to have similar strength bands, indicating similar expression levels. NPY was detected in all the peripheral tissues tested, though there was apparent stronger expression in the spleen and kidney than any of the other tissues (Figure 10).

Orexin

The tissue distribution of orexin amplified a 170 bp region of the transcript. Orexin was present in all regions of the brain examined. Based on the strength of bands, there was higher orexin expression in the hypothalamus, optic tectum and telencephalon than other brain regions (Figure 9). In the peripheral tissue

distribution, orexin was present in all the tissues but had apparent higher expression in the ovary, heart, and kidney than other tissues (Figure 10).

CART

For the CART tissue distributions, a 153 bp region was amplified. In the central tissue distribution, CART was present in all regions of the brain except the pituitary (Figure 9). All regions with the transcript present and had high expression in all brain regions with the exception of the cerebellum (Figure 9). In the peripheral tissue distribution, CART was only present in the gill, skin, kidney, gut and ovary (Figure10). Relatively few peripheral tissues express CART.

CCK

For the CCK tissue distribution, a 101 bp region was amplified. In the central tissue distribution, CCK was present in all the brain tissues (Figure 9). The highest expression, based on strength of bands, was in the hypothalamus, optic tectum and telencephalon (Figure 9). In the peripheral tissue distribution, CCK was present in all the tissues, and the highest expression was in the ovary, gill, skin, spleen and kidney (Figure 10).

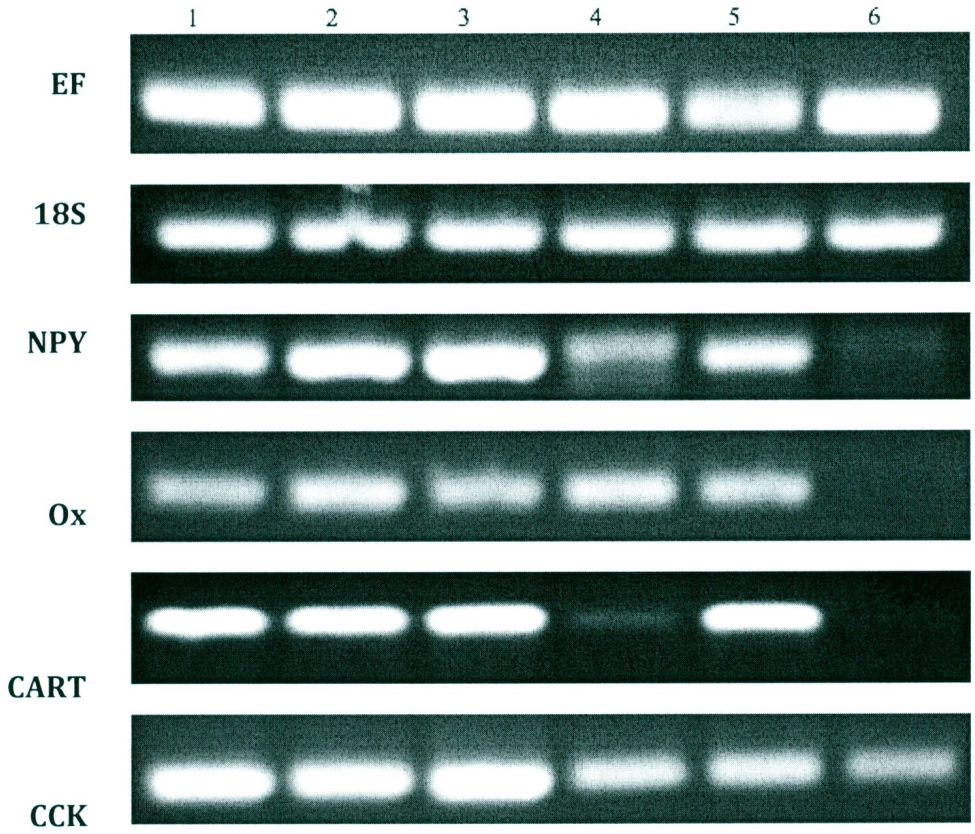


Figure 9. Central tissue distribution of EF, 18S NPY, Ox, CART and CCK in the cunner. Transcript fragments were amplified using RT-PCR and visualized on a 1% agarose gel with ethidium bromide. Samples (from left to right): 1- hypothalamus, 2- optic tectum, 3- telencephalon, 4- cerebellum, 5- medulla, 6- pituitary

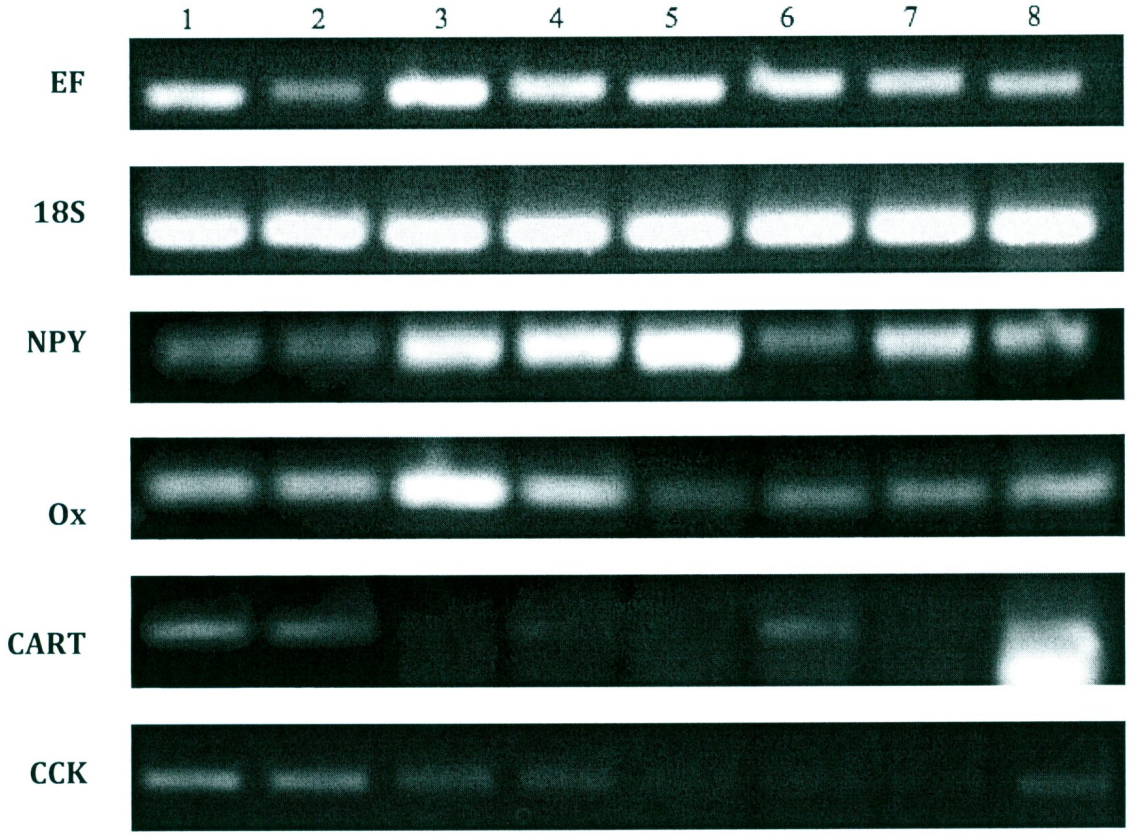


Figure 10. Peripheral tissue distributions of EF, 18S, NPY, orexin, CART and CCK in the cunner. Transcript fragments were amplified using RT-PCR and visualized on a 1% agarose gel with ethidium bromide. Samples (from left to right): 1- gill, 2- skin, 3- spleen, 4- kidney, 5- liver, 6- gut, 7- heart, 8- ovary.

Effects of experimental conditions on gene expression

Effects of fasting on transcript expression in the brain and gut

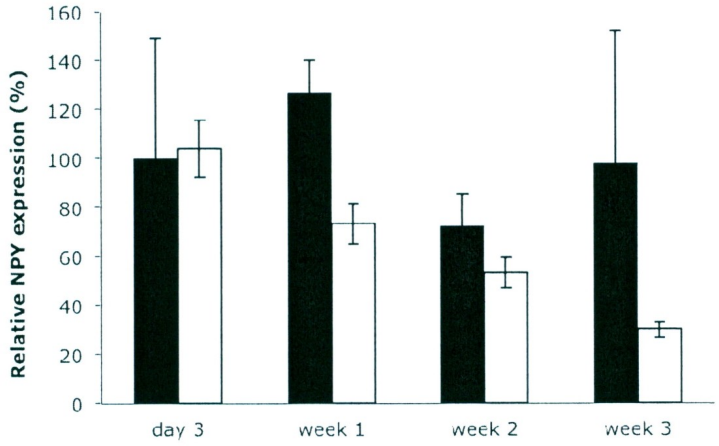
There were different effects of fasting on transcript expression in the two areas of the brain studied in this experiment. In the hypothalamus, NPY showed no significant changes in expression levels over three weeks of fasting (Figure 11a). However fasting did affect NPY expression in the telencephalon, where transcript levels decreased at two and three weeks of fasting compared to fed fish sampled at the same time (Figure 12a).

Orexin expression in the hypothalamus was constant until three weeks of fasting when it significantly decreased and was lower than orexin expression in all the other groups (Figure 11b). There was no effect of fasting on orexin expression in the telencephalon (Figure 12b).

CART transcript expression in the hypothalamus was not significantly effected by fasting (Figure 11c). In the telencephalon, CART expression was significantly lower at weeks one, two and three (Figure 12c) than at day 3.

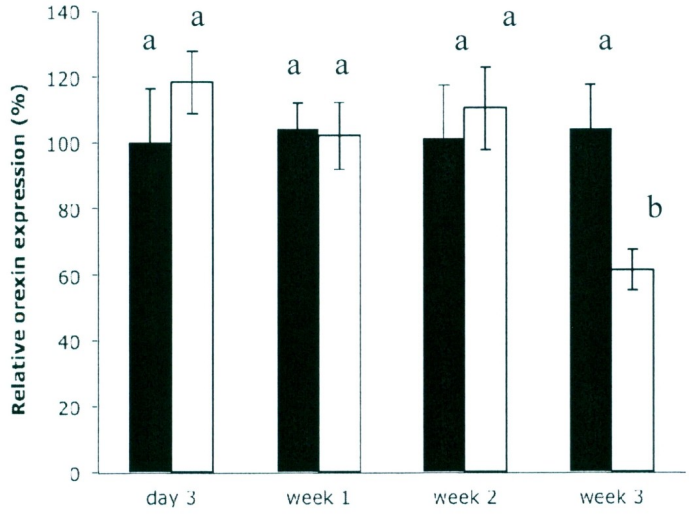
In the gut, only the week 1 samples were used, as errors occurred in sampling or during RNA extraction in all the other groups. There was a significant decrease in CCK expression in the gut after one week of fasting (Figure 13).

a)



NPY

b)



Orexin

c)

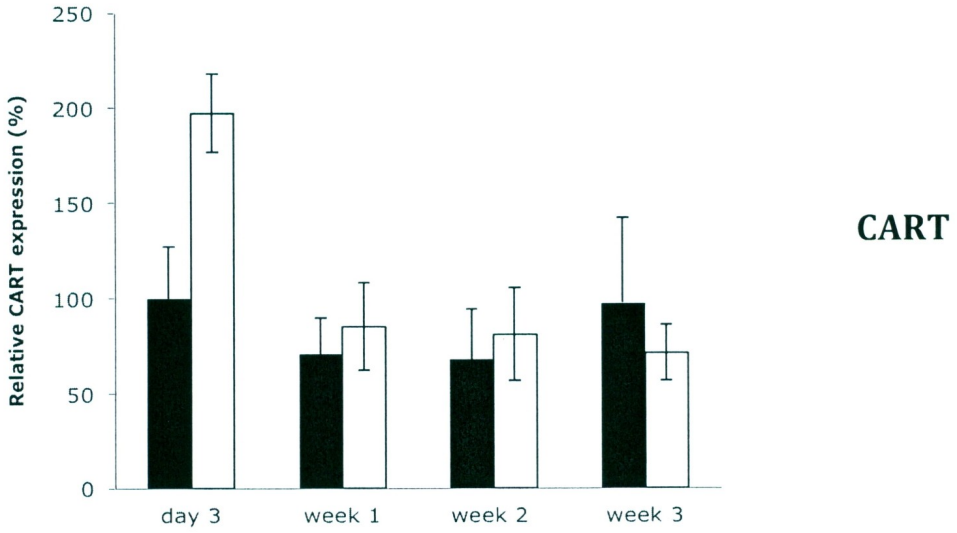
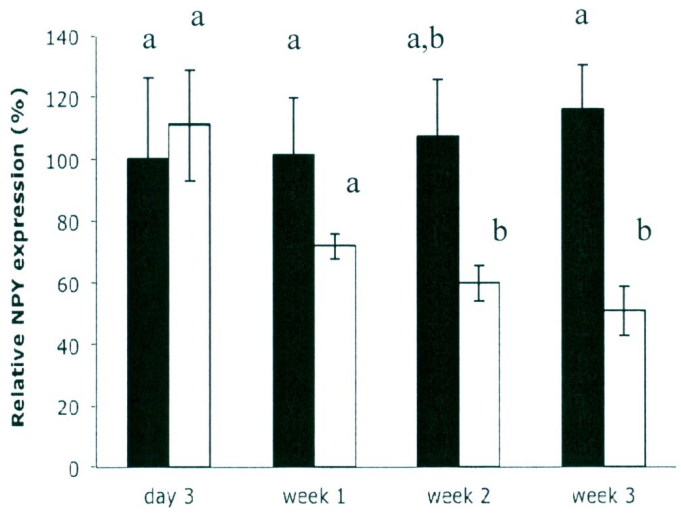


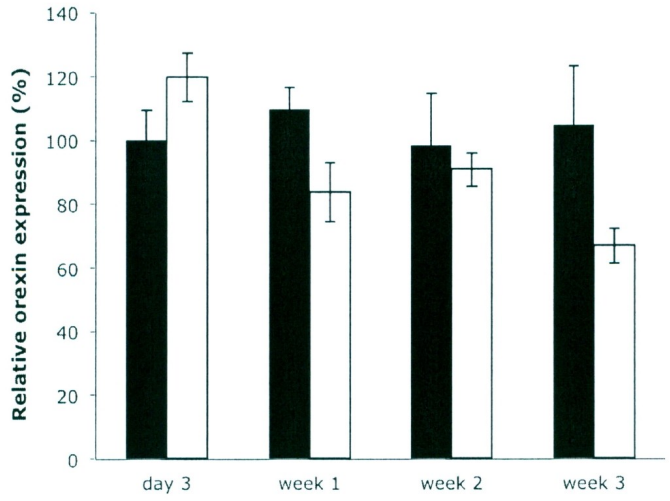
Figure 11. Relative transcript expression of a) NPY, b) orexin and c) CART in the hypothalamus of cunner during summer in fed (*n*=5 fish per group) and fasted (*n*=8 fish per group) fish. Black bars are fed treatments and white bars are fasted treatments. Transcript expression is normalized to day 3 fed treatment. Letters indicate treatments that were significantly different from each other by ANOVA ($p<0.05$). Data are presented as mean ± SEM.

a)



NPY

b)



Orexin

c)

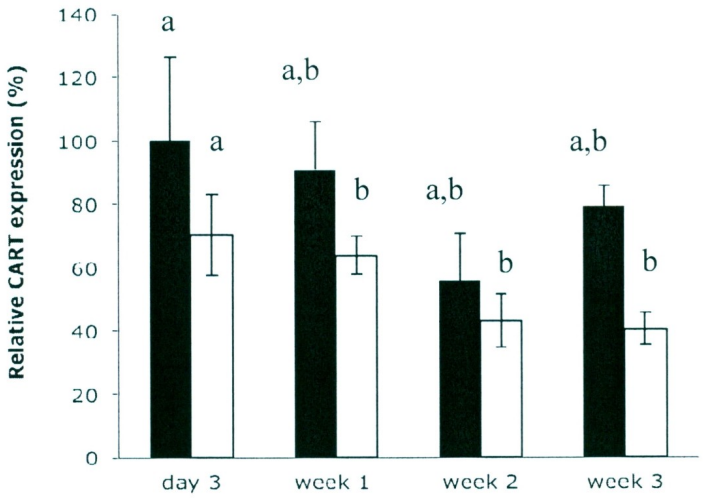


Figure 12. Relative transcript expression of a) NPY, b) orexin and c) CART in the telencephalon of cunner during summer in fed (*n*=5 fish per group) and fasted (*n*=8 fish per group) fish. Black bars are fed treatments and white bars are fasted treatments. Transcript expression is normalized to day 3 fed treatment. Letters indicate treatments that were significantly different from each other by ANOVA ($p<0.05$). Data are presented as mean ± SEM.

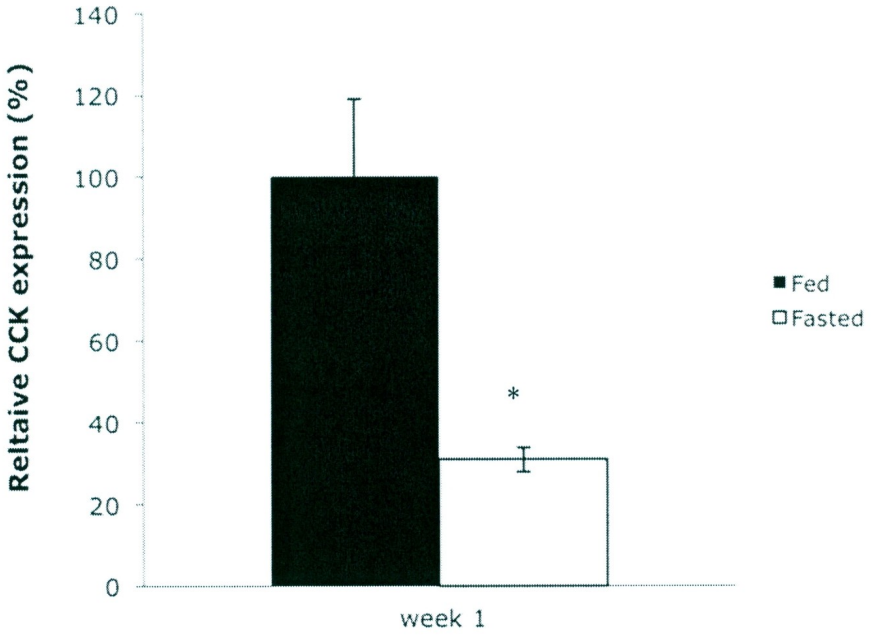


Figure 13. Relative CCK expression in the gut of cunner during summer fed (n=5) and fasted (n=8) fish. Transcript expression is normalized fed fish at week 1. Asterix (*) indicates treatments that are statistically different using t-test ($P<0.05$). Data are presented as mean ± SEM.

Effects of induced torpor on transcript expression in the brain and gut

Induced torpor was achieved by placing fish in 4°C water for 4 weeks, and then sampling their tissues. They were fed once a week, but regular observation showed little to no movement within the tanks and very little feeding activity.

After 4 weeks of induced torpor, NPY expression did not change significantly in the hypothalamus whereas it increased significantly in the telencephalon (Figure 14a).

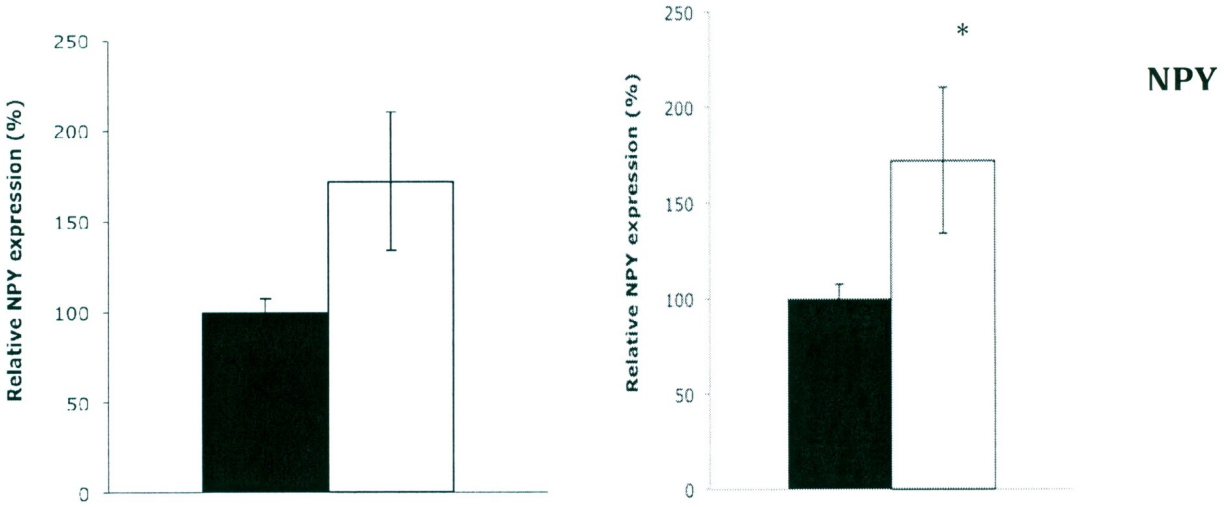
Orexin expression was not affected by induced torpor in either the hypothalamus or the telencephalon (Figure 14b).

CART expression in the hypothalamus was significantly lower in the induced torpor fish compared to non-torpor fish (Figure 14c). There was on significant effect of induced torpor on CART expression in the telencephalon (Figure 14c).

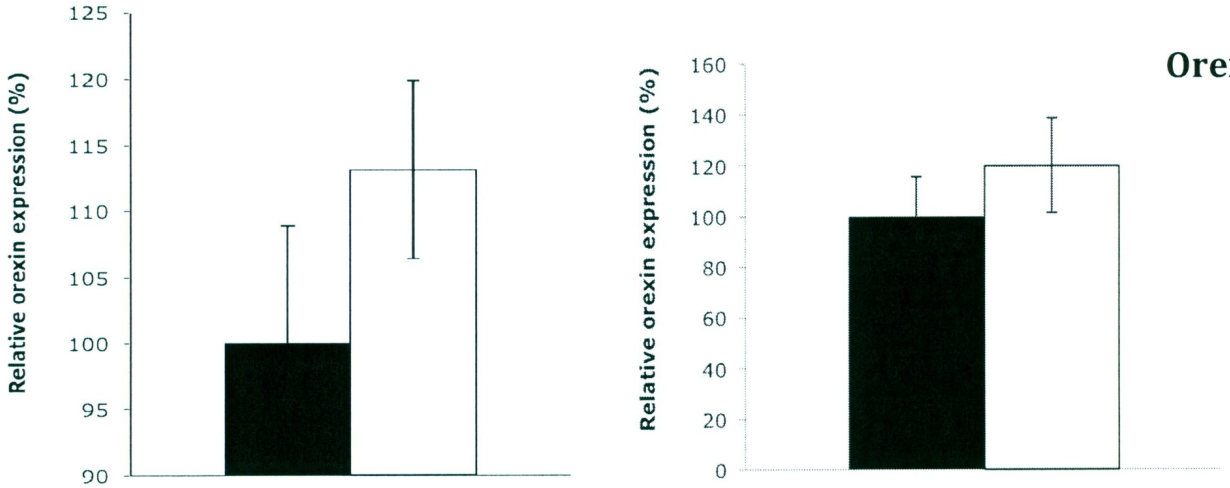
CCK expression in the gut was significantly lower in the induced torpor samples compared to the non-torpor samples (Figure 15).

H **T**

a)


Relative NPY expression (%)
250
200
150
100
50
0
*
NPY

b)


Relative orexin expression (%)
125
120
115
110
105
100
95
90
160
140
120
100
80
60
40
20
0
Orex

c)

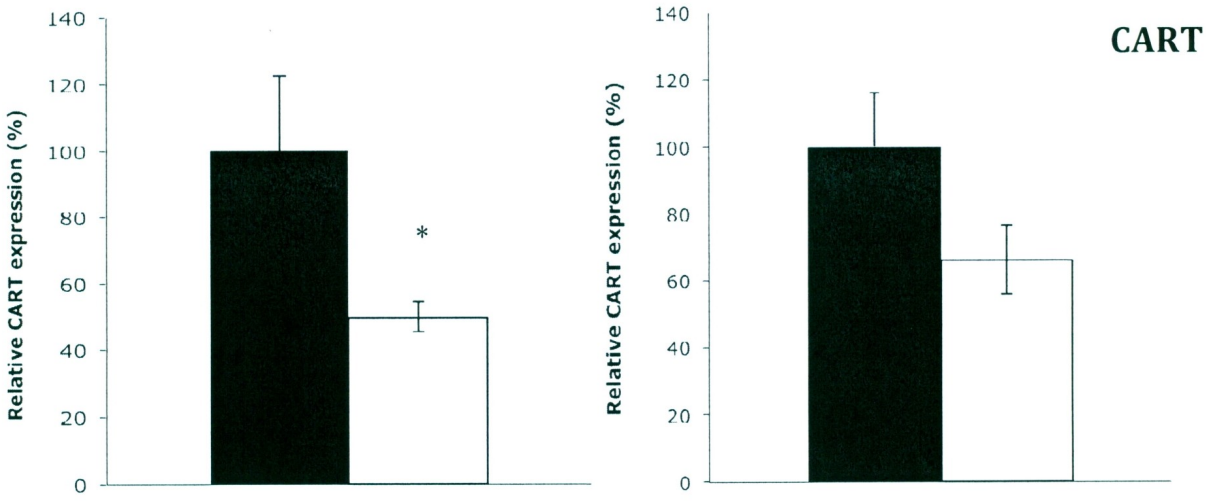


Figure 14. Relative transcript expression of a) NPY, b) orexin and c) CART in the hypothalamus (H) and telencephalon (T) of the cunner during non-torpor (*n*=6) and induced torpor (*n*=8) experiment. Black bars represent non-torpor fish and white bars represent torpor treatments. Transcript expression is normalized to non-torpor fish. Asterix (*) indicates treatments that are statistically different using t-test ($P<0.05$). Data are presented as mean ± SEM.

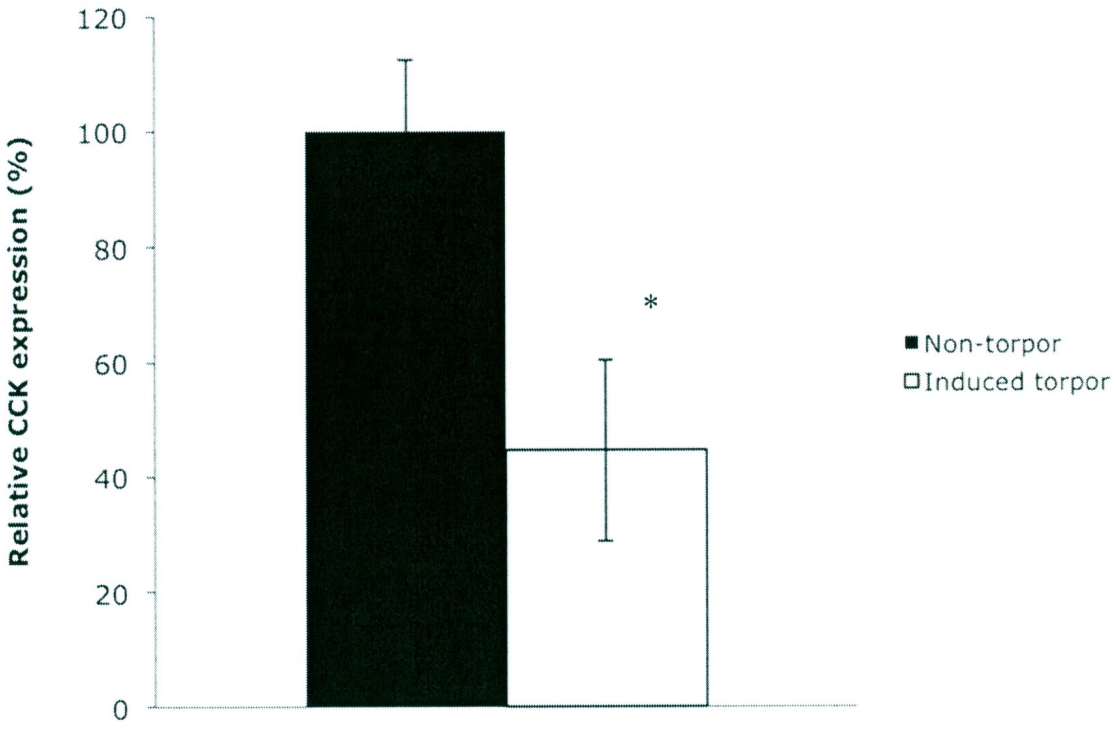


Figure 15. Relative CCK expression in the gut during induced torpor (n=8) and non-torpor (n=6). Transcript expression is normalized to non-torpor fish. Asterix (*) indicates treatments that are statistically different using t-test ($P<0.05$). Data are presented as mean ± SEM.

Effects of natural torpor on transcript expression in the brain and gut

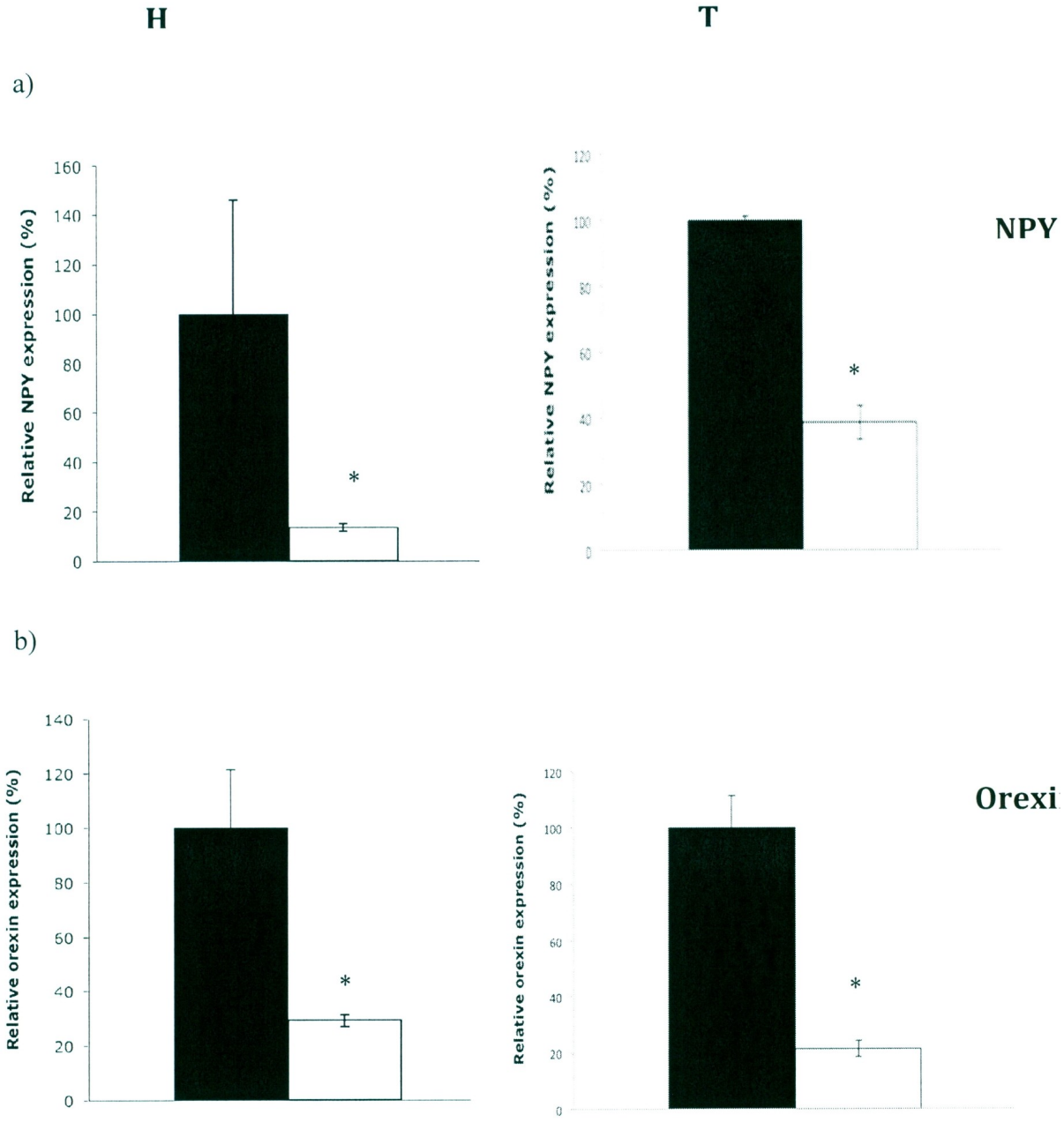
The natural torpor fish were kept at natural water temperatures and photoperiod and fed once every two weeks. They were sampled in March after approximately 5 months of torpor.

Although the HKG used for both induced torpor and fasting studies was EF1-α as its expression was constant between treatment groups, a significant decrease in EF1-α expression was seen in both brain and gut of torpor fish compared to non-torpor fish (Table 2). Several other HKG were thus tested. Significantly decreased transcript expression levels during torpor were seen for beta actin-α, RPL7 and tubulin beta (Table 2). 18S rRNA was the only HKG tested with similar expression levels in torpor and non-torpor brain samples (Table 2), and was therefore used as the HKG for natural torpor transcript expression studies.

There was a significant decrease in NPY, orexin and CART expression levels in both the hypothalamus and telencephalon of natural torpor fish compared to non-torpor fish (Figure 16 a,b and c). It was not possible to measure relative CCK expression levels in the gut, as the housekeeping gene used in the gut (18S rRNA) had significantly increased expression during natural torpor (Figure 17).

Table 2. Average Ct values for housekeeping genes (HKG) tested in non-torpor and torpor brain samples (hypothalamus and telencephalon) of the cunner. Data are presented as mean ± SEM.

Gene	Non-torpor	Torpor
18S	13.13 ± 0.26	12.70 ± 0.84
Tubulin beta	19.44 ± 0.21	20.98 ± 0.31
RPL7	16.75 ± 0.41	19.48 ± 0.52
Beta-α	17.19 ± 0.1	19.83 ± 0.26
EF1-α	20.07 ± 0.16	22.37 ± 0.41


H
T
a)
Relative NPY expression (%)
160
140
120
100
80
60
40
20
0
*
Relative NPY expression (%)
120
100
80
60
40
20
0
*
NPY
b)
Relative orexin expression (%)
140
120
100
80
60
40
20
0
*
Relative orexin expression (%)
120
100
80
60
40
20
0
*
Orexi

c)

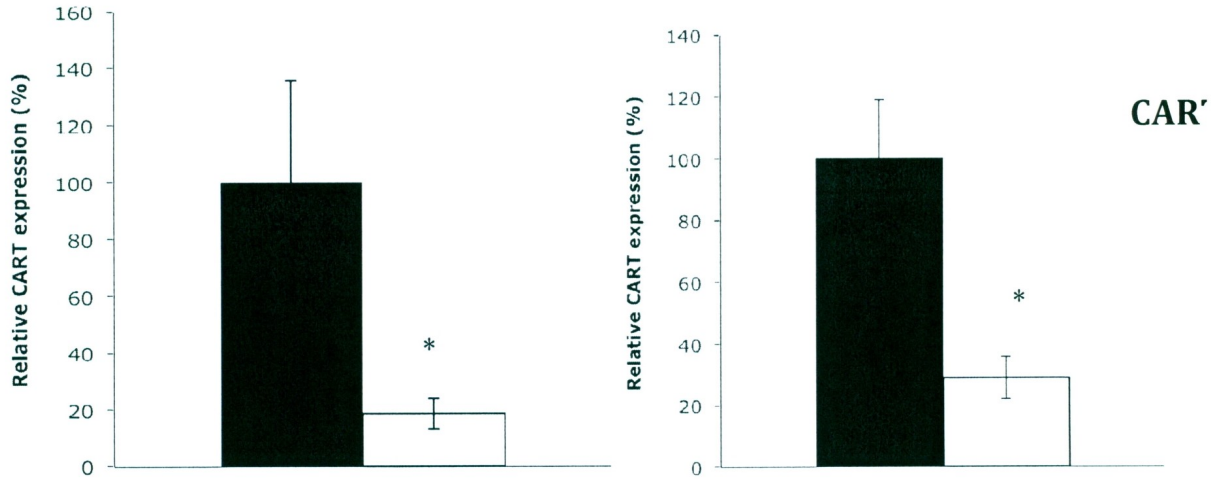


Figure 16. Relative transcript expression a) NPY, b) orexin and c) CART in the hypothalamus of the cunner during non-torpor (n=6) and natural torpor (n=7). Transcript expression is normalized to day 3 fed treatment. Asterix (*) indicates treatments that are statistically different using t-test ($P<0.05$). Data are presented as mean ± SEM.

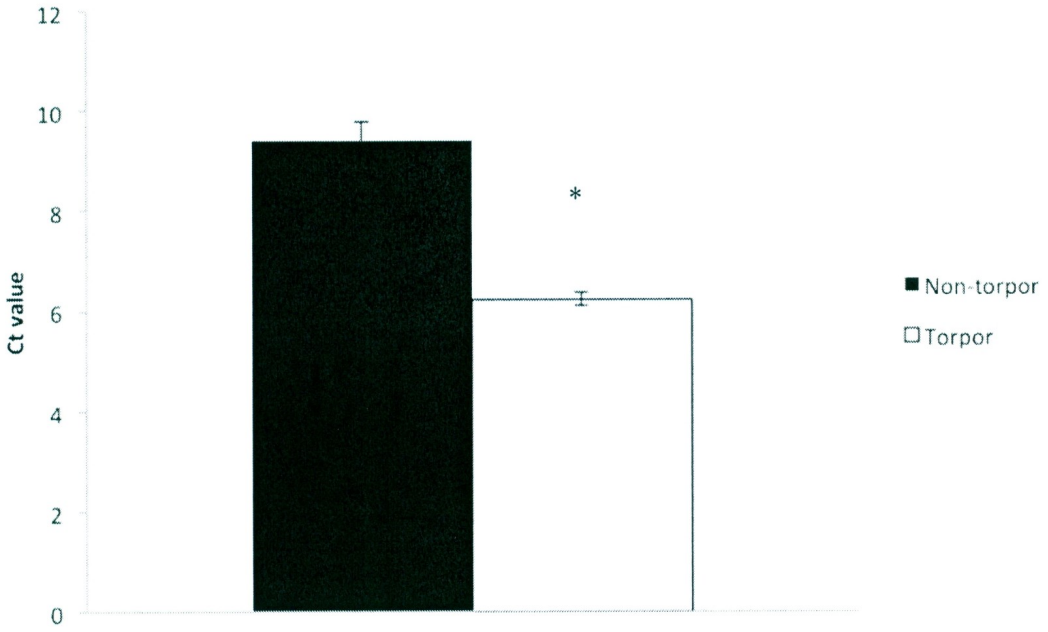


Figure 17. 18S Ct values in the gut during natural torpor (n=5) and non-torpor (n=5). Asterix (*) indicates treatments that are statistically different using t-test ($P<0.05$). Data are presented as mean ± SEM.

Discussion

Cloning and sequence analysis

In this study NPY, orexin, CART and CCK were cloned and sequenced for the first time in the cunner. These sequences will contribute towards the understanding of the evolution of appetite-regulating hormones in vertebrates. Genes that play important roles in everyday functions are often highly conserved, especially in areas coding for the mature peptide. This is why injecting proteins obtained from one species into another species often induce biological reactions similar to those that would be observed if using a species-specific peptide.

NPY was the most highly conserved of the four hormones examined in this study. It exhibited the highest degree of homology with other species of fish, which may correlate to its important functions in appetite regulation and stress response. When compared with translated DNA sequences from other species of fish, the highest degree of homology was with orange grouper NPY sequence (95%), followed closely by winter flounder (94%) and Japanese flounder (93%). Orange grouper is a member of the Perciformes order, as is the cunner, so it is no surprise they share highly similar amino acid sequences. Winter flounder and Japanese flounder are members of the Pleuronectiformes order, which is very closely related to the Perciformes therefore they also should share a high degree of homology with the cunner protein sequences. NPY also had low homology with mouse and skate sequences (64% and 56% respectively). Skates are in the class Chondrichthyes, which separated from the class Actinopterygii (to which cunners also belong) sometime between 400-500 million years ago (Martin, 2001). Mice are members of the class Mammalia, which is also highly divergent from

Actinopterygii, which explains the lower degree of homology of skate and mouse with cunner protein sequences. However, the translated mature peptide portion of the transcript is highly conserved: of the 36 amino acids in the mature peptide 25 are completely identical among all the species.

Orexin was also very conserved. The cunner orexin transcript had the highest degree of homology with winter flounder (75%), closely followed by orange grouper (72%) and Nile tilapia (71%). Nile tilapia is also a member of the Perciformes order, which explains the high homology with cunner orexin sequences. Orexin had the lowest homology with protein sequences from winter skate (45%) and mouse (49%). The orexin A translated mature peptide was only similar among all the species in the alignment for 8 of the 43 amino acids, and the orexin B translated mature peptide was only similar for 10 of the 28 amino acids aligned with other species.

The cunner CART transcript exhibited the highest degree of homology with winter flounder (79%). The CART transcripts from Atlantic cod (64%), and zebrafish (62%) were also very similar to cunner CART peptide sequence. Atlantic cod is a member of the Gadiformes order and zebrafish is a member of the Cypriniformes order and are therefore not as closely related to the cunner as the winter flounder. The lowest transcript homology was seen with mouse (35%) and winter skate (43%). Of the 103 amino acids in the translated mature peptide, only 32 were conserved across all the species in the alignment. This low degree of protein conservation may be due to multiple CART copies in a species, such as goldfish, which has two forms of CART transcripts (Volkoff and Peter, 2001).

The cunner CCK had the highest degree of homology with CCK sequences from winter flounder and Japanese flounder (both 85%). The lowest degree of homology was with spiny dogfish (46%) and mouse (50%). Spiny dogfish is a member of the class Chondrichthyes, as is the winter skate, and share approximately similar degrees of homology as skate with cunner sequences. The translated mature peptide is very highly conserved, with 6 of the 8 amino acids identical amongst all the species in the alignment. This high degree of conservation of the mature peptide may indicate the importance of the role CCK plays in appetite regulation and other physiological processes in vertebrates.

Tissue distribution

NPY, orexin, CART and CCK were amplified using RT-PCR from various regions of the brain and peripheral tissues to determine their sites of expression and their relative expression levels based on the strength of the bands.

NPY was expressed in the forebrain (hypothalamus, telencephalon, optic tectum), as well as the medulla and cerebellum of the cunner, and all the bands had the same intensities. The presence of NPY transcripts in the forebrain of the cunner was expected, as NPY has been found in the same regions in other species of fish such as salmon (Silverstein et al., 1998), Atlantic cod (Kehoe and Volkoff, 2007), winter flounder (MacDonald and Volkoff, 2009) and goldfish (Peng et al., 1994), among others.

NPY was expressed in all the peripheral tissues tested. There was apparent higher expression in the spleen and the kidney. NPY expression in the porcine spleen has been linked to vasoconstriction and inhibiting cAMP formation (Lundberg et al., 1988). In the mammalian kidney, NPY is also linked to vasoconstrictor activity via the release of

angiotensin (Pernow et al., 1987). This vasoconstrictor activity is important for maintaining cardiovascular homeostasis, but has not been linked to appetite regulation (Pedrazzini et al., 1998). In addition to appetite regulation and cardiovascular homeostasis, NPY has also been implicated in sex reversal of the bluehead wrasse (Kramer and Imbriano, 1997), and in increased heart rate in the dogfish (Xiang et al, 1994).

NPY is widely expressed throughout different tissues and causes many different physiological responses. For the purpose of this study, the appetite-regulating effects of NPY have been studied in the brain, where it has been shown NPY mRNA levels fluctuate with feeding and fasting (MacDonald and Volkoff, 2009).

Orexin was expressed in all the central tissues of the cunner brain with the exception of the pituitary. The strongest orexin expression was observed in the regions of the forebrain including the hypothalamus, telencephalon and optic tectum. This same pattern of expression has been documented in other species such as goldfish (Huesa et al., 2005), zebrafish (Kaslin et al., 2004), cod (Xu and Volkoff, 2007), winter flounder (Buckley et al., 2010) and orange grouper (Yan et al., 2011).

Orexin was expressed in all the peripheral cunner tissues, but was most strongly expressed in liver, kidney, heart and ovary. Orexin mRNA has not been detected in any mammalian liver, and has been found only in small quantities in the liver of fish such as orange grouper (Yan et al., 2011). Orexin is found in the kidneys of both mammals (Heinonen et al., 2008) and fish (Yan et al., 2011) but its exact function there is unknown. Orexin is found within the mammalian heart, and injections of orexin A into the heart ventricles of rats cause a rise in blood pressure and heart rate (Zhang et al., 2005).

Zebrafish express orexin in the nerve fibers extending to aminergic nuclei in the heart, suggesting that orexin may play a role in the release of aminergic factors (Kaslin et al., 2004). The presence of orexin expression in the gonads of fish is not well understood, but there is some evidence that orexin may be able to inhibit reproductive behavior by inhibiting the release of gonadotropin-releasing hormone (Hoskins et al., 2008).

CART is expressed in the forebrain (hypothalamus, telencephalon and optic tectum) as well as in the medulla and cerebellum of the cunner. It is most highly expressed in the hypothalamus, telencephalon, optic tectum and the medulla. CART has been detected in the medulla of rats, and is thought to be involved in stress, and may help regulate cardiovascular responses (Hwang et al., 2004). CART transcript in the forebrain has also been documented in goldfish (Volkoff and Peter, 2001a), cod (Kehoe and Volkoff, 2007), salmon (Murashita et al., 2009) and winter flounder (MacDonald and Volkoff, 2009).

In the peripheral tissues, CART is found in the gill, skin, kidney, gut and ovary. The presence of CART in the gill and skin has no known purpose, neither does CART found within the kidney. CART may play a role in follicular atresia in mammals, but there is no link to the same function in the fish ovary (Kobayaski et al., 2004). In mammals, CART has been found in some of the muscles surrounding the stomach, though its exact actions there are unknown (Couceyro et al., 1998).

Overall the main site of synthesis of CART appears to be within the forebrain, where CART transcript levels have been shown to rise and fall with feeding and fasting (MacDonald and Volkoff, 2009).

Within the cunner brain, CCK is expressed in all the areas, but is most strongly expressed in the forebrain (hypothalamus, telencephalon and optic tectum). CCK has also been found in the forebrains of goldfish (Peyon et al., 1999), rainbow trout (Jensen et al., 2001), and flounder (Kurokawa et al., 2003). Both rainbow trout and flounder have multiple CCK transcripts, Japanese flounder having two (Kurokawa et al., 2003) and rainbow trout having three (Jensen et al., 2001).

CCK was expressed in all the peripheral tissues, with the highest expression in the skin, gill, kidney, spleen and ovary. In the pufferfish, nerves extending to mucous glands under the skin contain CCK (Funakoshi et al., 1998). There is no known function of CCK in the kidney and spleen, but its presence in these tissues has been documented in other species of fish (MacDonald and Volkoff, 2009).

One of the main sites of synthesis of CCK is within the gut. CCK transcript expression did not appear very strong in the peripheral tissue distribution of the gut, but the presence of CCK has been well documented in the gut of fish. In goldfish, CCK immunoreactivity has been seen in the endocrine cells and in some nerve fibers (Himick and Peter, 1994) and in rainbow trout CCK immunoreactivity was detected in the small intestine and the pyloric caeca (Vigna et al., 1985).

Fasting experiment

In the fasting experiment, cunners were deprived of food for three weeks, and sampled at three days, one week, two weeks, and three weeks of fasting. Changes in expression were examined in the gut, hypothalamus and telencephalon. Samples from

fasted fish were compared with those of fed fish sampled at the same time, to determine the effects of fasting on transcript expression of NPY, orexin, CART and CCK.

There was no significant effect of fasting on NPY transcript expression in the hypothalamus. This is similar to the fasting response seen in cod, which showed no changes in NPY expression in the forebrain after one week of fasting (Kehoe and Volkoff, 2007). However, long-term fasting has been shown to induce increases in hypothalamic NPY expression in other fish species. Salmon that were food-deprived for 3 weeks exhibited increased NPY transcript expression in the hypothalamus (Silverstein et al. 1998). Similar results were seen in winter flounder, which exhibited an increase in hypothalamic NPY expression after both 2 weeks and 4 weeks of fasting (MacDonald and Volkoff, 2009). In the telencephalon of the cunner, NPY expression significantly decreased at three weeks of fasting. This result seems to be unique to the cunner, as other species exhibit increases in telencephalic NPY expression during fasting. Goldfish fasted for 72 hours display an increase in telencephalon NPY expression (Narnaware and Peter, 2001). Brazilian flounder fasted for 2 weeks showed whole brain increases in NPY expression (Campos et al., 2010). One of the few species that did not show an increase in NPY expression in the telencephalon following fasting was the tiger puffer fish, which exhibited no change in NPY expression following one week of fasting (Kamijo et al., 2011). It is noteworthy that in our study, there were also no significant changes in telencephalic NPY expression after either one or two weeks of fasting. The variation in the duration of fasting times used in fish fasting experiments make it difficult to make good comparisons between studies. Cunners appear to maintain their NPY transcript expression levels for up to three weeks during fasting, after which there is a decrease in

NPY expression levels in some tissues. If the study were to be extended to four or five weeks, it is possible that even more of the tissues would have decreased NPY transcript levels.

Orexin expression in the hypothalamus was significantly decreased at three weeks of fasting. This pattern of orexin transcript expression seems to be unique to cunners, and has not been documented in other species of fish. Zebrafish that have been calorie-restricted for two weeks show an increase in whole brain orexin expression (Novak et al., 2005). Goldfish fasted for 3 or 10 days shown an increase in hypothalamic orexin expression (Nakamachi et al., 2006; Abbott and Volkoff, 2011). Hypothalamic orexin levels are also significantly higher in fasted winter flounder (Buckley et al., 2010). As for NPY, the decrease in expression might indicate a metabolic shutdown. Orexin expression in the cunner telencephalon was not significantly different during three weeks of fasting. This result is similar to winter flounder orexin expression in the telencephalon, which also did not change after 4 weeks of fasting (Buckley et al., 2010).

CART transcript expression in the hypothalamus was not significantly different after three weeks of fasting. These results are similar to findings in winter flounder, which did not exhibit any significant different in hypothalamic CART expression after 4 weeks of fasting (MacDonald and Volkoff, 2009). However not all fish show the same pattern of expression, as goldfish showed decreased hypothalamic CART expression after 10 days of fasting (Abbott and Volkoff, 2011). In fish, whole brain or forebrain CART expression seems to follow a consistent trend of down regulation during fasting, as seen in the common carp (Wan et al., 2012), cod (Kehoe and Volkoff, 2007), catfish (Kobayashi et al., 2008) and salmon (Murashita et al., 2009). Cunner CART expression

in the telencephalon was significantly lower at one, two and three weeks but not from fed fish sampled at the same time, only from the first set of fed fish (sampled at day 3). The fed fish CART expression was not significantly different over the three weeks of the experiment.

CCK transcript expression in the cunner gut was significantly lower after one week of fasting. This is a similar trend to the one seen in winter flounder, where CCK expression was significantly lower in the gut after two weeks of fasting (MacDonald and Volkoff, 2009) and in yellowtail, where a decrease in CCK gut expression is seen after 72 hours of fasting (Murashita et al., 2006).

The general trend of NPY, orexin, CART and CCK transcript expression during fasting is a decrease in expression. It appears as though NPY, orexin and CART each have a specific area of the brain in which it is affected by fasting. NPY and CART transcript expressions decrease in the telencephalon but not in the hypothalamus during fasting. Orexin transcript expression decreases in the hypothalamus but not in the telencephalon during fasting. It seems most likely that when cunners are food deprived for extended periods of time they enter a survival mode and down-regulate appetite regulating hormones. While it is not surprising that anorexigenic hormones CART are CCK are down regulated during fasting, other fish exhibit usually exhibit increases in expression of the orexigenic hormones NPY and orexin. One factor that could be affecting the fish might be their proximity to their natural spawning period. During the starvation study, the cunners all had very large, well-developed gonads. The cunner's proximity to spawning time in this study could have affected how they responded to food deprivation. Some fish species, such as smelt, abstain from eating during spawning (Foltz

and Nordem, 1977). If cunners naturally fast during spawning, then perhaps they entered their natural period of fasting in preparation for spawning, when there was no more food available to them. This may explain why both orexigenic and anorexigenic transcript levels were decreased during fasting, as opposed to the increased orexigenic hormone expression seen in other species of food-deprived fish. Another possibility is that cunners are very resistant to changes in transcript expression during the initial stages of fasting, but then as fasting continues they start to shut down transcript expression. This would certainly explain why there was very little change in any of the brain transcript expression levels before three weeks, and that if the study had continued and food had been continued to be withheld then other hormones that remained unchanged in some areas of the brain may have started to show decreased expression as well. The pattern of transcript expression seen in the starvation study may be caused by multiple factors including proximity to spawning. The cunner appears to have a longer response time during fasting and maintain their hormone levels for a period of one to three weeks before decreasing their expression levels in an attempt to conserve energy.

Acute torpor experiment

To submit the cunners to acute torpor, the fish were placed into cold seawater intake tanks (~2°C) tanks for four weeks and fed once a week. After four weeks the hypothalamus, telencephalon and gut were sampled and tested for transcript expression of NPY, orexin, CART and CCK. These expression levels were then compared with fed fish sampled at the same time. During the experiment cunners were observed eating very little (when compared to fed fish) and made almost no movement, staying hidden beneath

rocks for the majority of the time. No other studies have ever examined the effects of fish put acutely into torpor on gene expression. The cunners response to this fed, short-term torpor is reflective of both fasting experiments and seasonal difference experiments done in other fish.

During the acute torpor experiment, NPY transcript expression was not significantly changed in the hypothalamus. In the telencephalon, there was an increase in NPY expression compared with fed, non-torpor fish.

Orexin expression did not change in either the hypothalamus or telencephalon during induced torpor. The fish placed into acute torpor were relatively immobile, spending most of their time hidden under rocks. Orexin also plays a role in sleep/wakefulness, and injections of orexin in goldfish have been shown to increase locomotor and foraging activity (Volkoff et al., 1999). Hypothalamic orexin expression in goldfish is higher during the day, when the fish are active, than during the night when they rest (Hoskins and Volkoff, 2011). In the induced torpor fish, there may be a trade-off between the actions of orexin, because while not eating much, which makes them hungry (which would increase orexin expression), they also need to conserve energy and might decrease the expression of arousal hormones. This balance might explain the lack of visible change in orexin expression between summer and induced torpor fish.

CART expression was significantly decreased in the hypothalamus, but was unaffected by induced torpor in the telencephalon. These results are similar to the response seen in fish when they are fasted. Both cod (Kehoe and Volkoff, 2007) and goldfish (Volkoff and Peter, 2001) show decreased hypothalamic CART expressions

when fasted. The decrease in CART expression is consistent with the "fasted state" of the induced torpor cunners who had access to food but ate very little.

CCK expression was significantly decreased in the gut during induced torpor. This seems to follow the general trend of anorexigenic hormones decreasing during the induced torpor. During winter and during fasting, winter flounder significantly decrease their CCK transcript expression in the gut (MacDonald and Volkoff, 2009).

For induced torpor, the fish appeared very much as if they were in a natural torpor; there was very little movement within the tank and the fish ate very little food compared to non-torpor fish. Their appetite-regulating hormone expression during the induced torpor was similar to patterns of expression seen during starvation in other species of fish. The mRNA expressions of orexigenic hormones increased, and that of anorexigenic hormones decreased. There is obviously some mechanism at work that overrides the fish desire to eat, in order to conserve energy. The starvation response in the cunner appears to be to decrease both orexigenic and anorexigenic hormone expression, while the induced torpor response seems to mimic starvation responses seen in other species of fish.

The hormone mRNA expression profiles seen during induced torpor could also be attributed partly to a stress response; the fish were taken from normal temperature seawater and acutely put into cold water, while the photoperiod was maintained the same. Fish subjected to rapid changes in temperature often shown signs of stress. Decreases in temperature have been shown to causes increases in oxygen consumption and opercular beat frequency in goldfish (Freeman, 1950), which is associated with thermal stress (Heath et al. 1973). Trout subjected to an acute 13°C change in water temperature

showed increased plasma cortisol levels (Strange et al., 1977). Chronically high levels of cortisol, a hormone associated with stress response in vertebrates, have been shown to increase mortality due to disease and infection in fish (Pickering and Pottinger, 1989). In future studies it would be interesting to test plasma cortisol levels of cunners in induced torpor, to determine if they are in a stressed state. Stress caused by acute changes in temperature could affect transcript expression in the cunner. Stress coupled with torpor could be the explanation for the hormone expression seen in this study. The induced torpor experiment was not an accurate replication of natural torpor, but did produce interesting results that should further be investigated.

Seasonal torpor experiment

Natural torpor fish were collected in the wild after four months of natural torpor, and transcript expression levels of NPY, orexin, CART and CCK were compared to that of fish collected during the summer.

Both NPY and orexin expressions were significantly lower in both the hypothalamus and the telencephalon in natural torpor fish than in summer fish. Cunners eat little to nothing during their time in torpor, and also move as little as possible to conserve energy (Bradbury et al., 1997). Previous studies have show that cunners exhibit a decrease in protein synthesis and RNA content in their tissues during torpor (Lewis and Dreidzic, 2007); therefore it is not surprising that appetite-regulating hormones are down regulated during the winter months. However other seasonal fish such as winter flounder exhibit increases in hypothalamic NPY (MacDonald and Volkoff, 2009) and orexin (Buckley et al., 2010) during the winter. This difference in transcript expression between

the two species may be explained by different survival strategies. Although winter flounder have been recorded eating and moving less during the winter (He, 2003; MacDonald and Volkoff, 2009), they remain in a more active state than the dormant cunner, which enters a state of winter metabolic depression (Lewis and Dreidzic, 2007).

The fall in orexin expression levels may also be linked to the role orexins play in sleep/wakefulness. The fall in orexin expression levels in the natural torpor fish may be contributing to the reduction in physical activity.

CART expression was significantly lower in the hypothalamus and telencephalon of natural torpor fish. This result is different from that seen in winter flounder, where there was no effect of seasonal variation on CART transcript expression (MacDonald and Volkoff, 2009). CART could be down regulated as cunners are eating very little during the winter and therefore do not need high expression of anorexigenic hormones, and could also be down regulated due to the general decrease in protein synthesis in cunner tissues during torpor.

In the gut, 18S expression was not consistent with non-torpor values. 18S Ct values were significantly lower in the torpor gut samples, indicating that there was higher 18S expression during torpor. This is unusual, as the other HKGs tested in this study in the brain showed decreased expression during torpor. 18S translated into a ribosome, which is responsible for catalyzing the formation of proteins. It has already been documented that protein synthesis of various tissues decreases during torpor (Lewis and Driedzic, 2007), however protein synthesis in the gut has never been measured. It is possible that some other process is occurring in the gut during torpor that requires the gut to increase its 18S production. Further study into the effects of torpor on other HKG such

as those tested in the brain will help determine what sort of processes are occurring in the cunner gut during torpor.

It was not possible to measure CCK expression in the gut as the HKG expression was no consistent between torpor and non-torpor samples, but the Ct values do indicated that CCK expression is reduced in the gut during natural torpor. This is similar to the results seen in the winter flounder gut, where there is a significant decrease in gut CCK transcript expression during the winter (MacDonald et al., 2009). The reduction in CCK expression in the gut during winter could also be caused by atrophy of the gut epithelium as CCK is released from enteric cells in the lining of the intestine. It is possible that during the winter, the gut epithelium degrades in order to conserve energy, which would induce a very low CCK expression. Although we have not performed a histological study of the gut epithelium, this phenomenon of gut shedding has been observed in other fish species. For example, Atlantic salmon show a decrease in mass and length of their GI tract over 40 days of fasting, both of which increase to normal levels upon re-feeding (Krogdahl and Bakke-McKellep, 2005). Cunners could be doing the same thing over the winter months, as the gut can be very energetically expensive to maintain.

Natural torpor hormone expression very nicely supports the theory of metabolic shutdown in the cunner during the winter. Lewis and Driedzic (2007) documented a decrease in RNA content in the cunner tissues during the winter months, though until now it was uncertain if appetite-regulating hormones were included. Our results suggest that the expressions of orexigenic and anorexigenic hormones in the brain and the gut are all significantly reduced during natural torpor.

General conclusions

The cunner serves as a unique model for appetite regulation because of the extended period of torpor it undergoes every winter. The cunner has a unique survival response that is unlike other fish seen so far. Overall a long-term, but not short-term, fasting in the summer results in a general decrease in the expression of all appetite-related peptides whether they are orexigenic or anorexigenic. This suggests that cunner have the ability to maintain their appetite-regulating protein levels during short-term fasting and then might "shutdown" this production and enter a metabolic depressed state (which includes a decrease in protein synthesis) to preserve energy, as food supplies are not restored. A similar "shutdown" is seen in animals undergoing natural torpor in the winter. Our induced torpor experiment seems to suggest that fish stop eating and enter a low activity state when the temperature drops, even if the photoperiod remains that of the summer months and food is available. This state however appears to be different from natural the winter torpor or summer fasted state, as it presents different expression profiles for appetite regulating hormones. It is also possible that some of the differences in hormone expression between induced torpor and natural torpor fish could be due to the different photoperiod during winter and summer. The induced torpor state could also be representative of stressed animals as the latter were submitted to acute changes in temperature. Cunners are a very physiologically interesting species, and their physiological responses to environmental conditions should continue to be investigated. Cunners are a non-migratory species, and therefore may serve as indicator of environmental conditions in an area. Cunners have already been used in this manner to assess the effects of effluents from pulp and paper mills in Newfoundland (Billiard and

Khan, 2003). Hopefully physiological studies of this organism will continue to help us better understand it, and how it responds to changes in its environment.

Literature Cited

Abbott M, Volkoff H. 2011. Thyrotropin releasing hormone (TRH) in goldfish (*Carassius auratus*): Role in the regulation of feeding and locomotor behaviors and interactions with the orexin system and cocaine- and amphetamine regulated transcript (CART). Horm Behav 59(2):236-45.

Alvarez CE, Sutcliffe JG. 2002. Hypocretin is an early member of the incretin gene family. Neurosci Lett 324(3):169-72.

Anand BK, Brobeck JR. 1951. Localization of a "Feeding center" in the hypothalamus of the rat. Proc Soc Exp Biol Med 77:323-5.

Aravich PF, Scalfani A. 1983. Paraventricular hypothalamic lesions and medial hypothalamic knife cuts produce similar hyperphagia syndromes. Behav Neurosci 97(6): 970-983.

Auster PJ. 1989. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (North and Mid-Atlantic): Tautog and Cunner[abstract]. Connecticut Univ Groton Marine Sciences Inst, Biological report vol. 82.

Asin KE, Gore Jr. PA, Bednarz L, Holladay M, Nadzan AM. 1992. Effects of selective CCK receptor agonists on food intake after central or peripheral administration in rats. Brain Res 571(1):169-74.

Bernadis LL, Berlinger LL. 1987. The dorsomedial hypothalamic nucleus revisited. Brain Res 434(3):321–381.

Billiard SM, Khan RA. 2003. Chronic stress in cunner, *Tautogolabrus adspersus*, exposed to municipal and industrial effluents. Ecotoxicol Environ Saf 55(1):9-18.

Blomqvist AG, Söderberg C, Lundell I, Milner RJ, Larhammar D. 1992. Strong evolutionary conservation of neuropeptide Y: Sequences of chicken, goldfish, and torpedo marmorata DNA clones. PNAS 899(6):2350-4.

Brightman, MW, Broadwell, RD. 1976. The morphological approach to the study of normal and abnormal brain permeability. Adv Exp Med Bio 69:41-54.

Bradbury C, Green JM, Bruce-Lockhart M. 1977. Daily And seasonal activity patterns of female cunner, tautogolabrus adspersus (labridae), in newfoundland. FISH BULL 95(4):646-52.

Brett JR, Groves TDD. 1979. Chapter 6 Physiological energetics. In: Fish physiology.

Academic Press. 279 p.

Buchan AM, Polak JM, Solcia E, Capella C, Hudson D, Pearse AG. 1978. Electron immunohistochemical evidence for the human intestinal I cell as the source of CCK. Gut 19(5):403-7.

Buckley C, MacDonald EE, Tuziak SM, Volkoff H. 2010. Molecular cloning and characterization of two putative appetite regulators in winter flounder (pleuronectes americanus): Preprothyrotropin-releasing hormone (TRH) and preproorexin (OX). Peptides 31(9):1737-47.

Campbell HA, Fraser KPP, Bishop CM, Peck LS, Egginton S. 2008. Hibernation in an antarctic fish: On ice for winter. PLoS ONE (3): e1743.

Campos V, Collares T, Deschamps J, Seixas F, Dellagostin O, Lanes C, Sandrini J, Marins L, Okamoto M, Sampaio L, et al. 2010. Identification, tissue distribution and evaluation of brain neuropeptide Y gene expression in the brazilian flounder *Paralichthys orbignyanus*. Journal of Biosciences 35(3):405-13.

Cerda-Reverter JM, Larhammar D. 2000a. Neuropeptide Y family of peptides: Structure, anatomical expression, function, and molecular evolution. Biochemistry and Cell Biology 78(3):371-392.

Cerdá-Reverter JM, Martínez-Rodríguez G, Anglade I, Kah O, Zanuy S. 2000b. Peptide YY (PYY) and fish pancreatic peptide Y (PY) expression in the brain of the sea bass (*Dicentrarchus labrax*) as revealed by in situ hybridization. The Journal of Comparative Neurology 426(2):197-208.

Clapham JC, Arch JRS, Tadayyon M. 2001. Anti-obesity drugs: A critical review of current therapies and future opportunities. Pharmacology and Therapeutics 89(1):81-121.

Clark JT, Sahu A, Kalra PS, Balasubramaniam A, Kalra SP. 1987. Neuropeptide Y (NPY)-induced feeding behavior in female rats: Comparison with human NPY ([Met17]NPY), NPY analog ([norLeu4]NPY) and peptide YY. Regul Pept 17(1):31-9.

Cluderay JE, Harrison DC, Hervieu GJ. 2002. Protein distribution of the orexin-2 receptor in the rat central nervous system. Regul Pept 104(1-3):131-44.

Couceyro P, Paquet M, Koylu E, Kuhar MJ, Smith Y. 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.

Crawley JN, Corwin RL. 1994. Biological actions of cholecystokinin. Peptides 15(4):731-55.

Cutler DJ, Morris R, Sheridhar V, Wattam TAK, Holmes S, Patel S, Arch JRS, Wilson S, Buckingham RE, Evans ML, et al. 1999. Differential distribution of orexin-A and orexin-B immunoreactivity in the rat brain and spinal cord. Peptides 20(12):1455-70.

De Lecea L, Kilduff TS, Peyron C, Gao X-, Foye PE, Danielson PE, Fukuhara C, Battenberg ELF, Gautvik VT, Bartlett II FS, et al. 1998. The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci U S A 95(1):322-7.

Delfini C, Diagne M. 1985. Brain of the cod (Gadus morhua morhua, Linné 1758) (Pisces, Paracanthopterygii). Qualitative and quantitative analysis of major subdivisions. J Hirnforsch 26(4):439-49.

Demski LS. 1973. Feeding and aggressive behavior evoked by hypothalamic stimulation in a cichlid fish. Comparative Biochemistry and Physiology Part A: Physiology 44(3):685-92.

Demski LS, Knigge KM. 1971. The telencephalon and hypothalamus of the bluegill (*Lepomis macrochirus*): Evoked feeding, aggressive and reproductive behavior with representative frontal sections. The Journal of Comparative Neurology 143(1):1-16.

Dey A, Xhu X, Carroll R, Turck CW, Stein J, Steiner DF. 2003. Biological processing of the cocaine and amphetamine-regulated transcript precursors by prohormone convertases, PC2 and PC1/3. Journal of Biological Chemistry 278(17):15007-14.

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

Einarsson S, Davies SP, Talbot C. 1997. Effect of exogenous cholecystokinin on the discharge of the gallbladder and the secretion of trypsin and chymotrypsin from the pancreas of the atlantic salmon, *Salmo salar L.* Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 117(1):63-7.

Facciolo RM, Crudo M, Giusi G, Alò R, Canonaco M. 2009. Light- and dark-dependent orexinergic neuronal signals promote neurodegenerative phenomena accounting for distinct behavioral responses in the teleost *Thalassoma pavo.* Journal of Neuroscience Research 87(3):748-57.

Fällmar H, Sundström G, Lundell I, Mohell N, Larhammar D. 2011. Neuropeptide Y/peptide YY receptor Y2 duplicate in zebrafish with unique introns displays distinct peptide binding properties. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 160(4):166-73.

Foltz JW, Norden CR. 1977. Seasonal changes in food consumption and energy content of smelt (*Osmerus mordax*) in Lake Michigan. Transactions of the American Fisheries Society 106(3):230-234.

Fredriksson R, Larson ET, Yan Y-, Postlethwait J-, Larhammar D. 2004. Novel neuropeptide Y Y2-like receptor subtype in zebrafish and frogs supports early vertebrate chromosome duplications. J Mol Evol 58(1):106-14.

Freeman JA. 1950. Oxygen consumption, brain metabolism and respiratory movements of goldfish during temperature acclimatization, with special reference to lowered temperatures. Biol Bull 99(3):416-424.

Funakoshi K, Kadota T, Atobe Y, Nakano M, Goris RC, Kishida R. 1998. Gastrin/CCK-ergic innervation of cutaneous mucous gland by the supramedullary cells of the puffer fish *Takifugu niphobles*. Neurosci Lett 258(3):171-4.

Geiser F, Song X, Körtner G. 1996. The effect of He–O2 exposure on metabolic rate, thermoregulation and thermal conductance during normothermia and daily torpor. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 166(3):190-6.

Gibbs J, Falasco JD, McHugh PR. 1976. Cholecystokinin-decreased food intake in Rhesus monkeys. Am J Physiol 230(1):15-8.

Gibbs J, Young RC, Smith GP. 1973. Cholecystokinin decreases food intake in rats. Journal of Comparative and Physiological Psychology 84(3):488-95.

Gonzalez R, Unniappan S. 2010. Molecular characterization, appetite regulatory effects and feeding related changes of peptide YY in goldfish. Gen Comp Endocrinol 166(2):273-9.

Green JM, Farwell M. 1971. Winter habits of the cunner, *Tautogolabrus adspersus* (walbaum 1792), in Newfoundland. Can J Zool 49(12):1497-9.

Halford JCG, Cooper GD, Dovey TM. 2004. The pharmacology of human appetite expression. Current Drug Targets 5(3):221-40.

Haugaard N, Irving L. 1943. The influence of temperature upon the oxygen consumption of the cunner (*Tautogolabrus adspersus Walbaum*) in summer and in winter. J Cell Comp Physiol 21(1):19-26

He P. 2003. Swimming behaviour of winter flounder (*Pleuronectes americanus*) on natural fishing grounds as observed by an underwater video camera. Fisheries Research 60(2–3):507-14.

Heath AG. 1973. Ventilatory responses of teleost fish to exercise and thermal stress. Amer Zool 13(2):491-503.

Heinonen MV, Purhonen AK, Mäkelä KA, Herzig KH. Functions of orexins in peripheral tissues. Acta Physiologica 192(4):471-485.

Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA. 2001. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 103(3):777-97.

Himick BA, Peter RE. 1994. Cck/gastrin-like immunoreactivity in brain and gut, and cck suppression of feeding in goldfish. Am J Physiol 267(3):841-851.

Hoskins LJ, Xu M, Volkoff H. 2008. Interactions between gonadotropin-releasing hormone (GnRH) and orexin in the regulation of feeding and reproduction in goldfish (*Carassius auratus*). Horm Behav 54(3):379-85.

Hoskins LJ, Volkoff H. 2012. The comparative endocrinology of feeding in fish: Insights and challenges. Gen Comp Endocrinol 176(3):327-35.

Hudson JW. 1973. Torpidity in mammals (torpor and hibernation physiology in mammals covering evolution, hypothermia, energy conservation, cell and organ adaptations, nervous and cardiovascular system changes, etc) Special Aspects of Thermoregulation pp. 97-165.

Huesa G, Van den Pol AN, Finger TE. 2005. Differential distribution of hypocretin (orexin) and melanin-concentrating hormone in the goldfish brain. J Comp Neurol 488(4):476-91.

Hwang LL, Chen CT, Li TL, Chiu CZ, Chi SF. 2004. Central pressor effects of CART peptides in anesthetized rats. Neuropeptides 38(2–3):69-76.

Ivy AC, Oldberg E. 1928. A hormone mechanism for gall-bladder contraction and evacuation. Am J Physiol 86(3):599-613.

Jensen H, Rourke IJ, Møller M, Jønson L, Johnsen AH. 2001. Identification and distribution of CCK-related peptides and mRNAs in the Rainbow trout, *Oncorhynchus mykiss*. Biochimica Et Biophysica Acta (BBA) - Gene Structure and Expression 1517(2):190-201.

Jensen J. 2001. Regulatory peptides and control of food intake in non-mammalian

vertebrates. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 128(3):469-77.

Jequier E. 2002. Leptin signaling, adiposity, and energy balance. Annals of the New York Academy of Sciences 967(1):379-388.

Johansen F. 1925. Natural history of the cunner (*Tautogolabrus adspersus Walbaum*). Can Biol Fish 2a(2):423-467.

Jones DC, Kuhar MJ. 2008. CART receptor binding in primary cell cultures of the rat nucleus accumbens. Synapse 62(2):122-127.

Jorpes E, Mutt V. 1966. Cholecystokinin and pancreozymin, one single hormone? Acta Physiol Scand 66(1):196-202.

Kalra SP, Dube MG, Sahu A, Phelps CP, Kalra PS. 1991. Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. Proceedings of the National Academy of Sciences 88(23):10931-5.

Kamijo M, Kojima K, Maruyama K, Konno N, Motohashi E, Ikegami T, Uchiyama M, Shioda S, Ando H, Matsuda K. 2011. Neuropeptide Y in Tiger puffer (*Takifugu rubripes*): Distribution, cloning, characterization, and mRNA expression responses to prandial condition. Zool Sci 28(12):882-90.

Kaslin J, Nystedt JM, Ostergard M, Peitsaro N, Panula P. 2004. The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. J Neurosci 24(11):2678-2689.

Kastin AJ, Akerstrom V. 1999. Orexin A but not orexin B rapidly enters brain from blood by simple diffusion. J Pharmacol Exp Ther 289(1):219-223.

Kehoe AS, Volkoff H. 2007. Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in atlantic cod (gadus morhua). Comp Biochem Physiol A-Mol Integr Physiol 146(3):451-61.

Kirchgessner AL, Liu M. 1999. Orexin synthesis and response in the gut. Neuron 24(4):941-51.

Kobayashi Y, Peterson BC, Waldbieser GC. 2008. Association of cocaine- and amphetamine-regulated transcript (CART) messenger RNA level, food intake, and growth in Channel catfish. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 151(2):219-25.

Kobayashi Y, Jimenez-Krassel F, Li Q, Yao J, Huang R, Ireland JJ, Coussens PM, Smith GW. 2004. Evidence that cocaine- and amphetamine-regulated transcript is a

novel intraovarian regulator of follicular atresia. Endocrinology 145(11):5373-83.

Konturek SJ, Pepera J, Zabielski K, Konturek PC, Pawlik T, Szlachcic A, Hahn EG. Brain-gut axis in pancreatic secretion and appetite control. J Physiol Pharmacol (3):293-317.

Kopin AS, Lee YM, McBride EW, Miller LJ, Lu M, Lin HY, Kolakowski LF, Beinborn M. 1992. Expression cloning and characterization of the canine parietal cell gastrin receptor. Proceedings of the National Academy of Sciences 89(8):3605-9.

Kramer CR, Imbriano MA. Neuropeptide Y (NPY) induces gonad reversal in the protogynous bluehead wrasse, thalassoma bifasciatum (teleostei: Labridae). Journal of Experimental Zoology 279(2):133-144.

Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, et al. 1998. Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393:72-76.

Krogdahl Å, Bakke-McKellep MA. 2005. Fasting and refeeding cause rapid changes in intestinal tissue mass and digestive enzyme capacities of Atlantic salmon (*Salmo salar L.*). Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 141(4):450-60.

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

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

Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar MJ. 1998. CART peptides in the central control of feeding and interactions with neuropeptide Y. Synapse 29(4):293-298.

Larhammar, D. 1996. Structural diversity of receptors for Neuropeptide Y, peptide YY and pancreatic polypeptide. Regul Peptides 65(1):165-174.

Larhammar D, Ericsson A, Persson H. 1987. Structure and expression of the rat neuropeptide Y gene. PNAS 84(7):2068-972.

Larsen PJ, Seier V, Fink-Jensen A, Holst JJ, Warberg J, Vrang N. 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):219-226.

Larsson LI, Rehfeld JF. 1978. Distribution of gastrin and CCK cells in the rat gastrointestinal tract. Histochemistry and Cell Biology 58(1):23-31.

Larsson LI, Rehfeld JF. 1977. Evidence for a common evolutionary origin of gastrin and cholecystokinin. Nature 269:335-8.

Larsson T, Olsson F, Sundstrom G, Lundin LG, Brenner S, Venkatesh B, Larhammar D. 2008. Early vertebrate chromosome duplications and the evolution of the neuropeptide Y receptor gene regions. BMC Evolutionary Biology 8(1):184.

Larsson TA, Olsson F, Sundstrom G, Brenner S, Venkatesh B, Larhammar D. 2005. Pufferfish and zebrafish have five distinct NPY receptor subtypes, but have lost appetite receptors Y1 and Y5. Ann N Y Acad Sci 1040:375-7.

Lewis JM, Driedzic WR. 2007. Tissue-specific changes in protein synthesis associated with seasonal metabolic depression and recovery in the north temperate labrid, *Tautogolabrus adspersus*. Am J Physiol Reg Integr Comp Physiol 293(1):474-81.

Liddle RA, Goldfine RD, Rosen MS, Taplitz RA, Williams JA. 1985. Cholecystokinin bioactivity in human plasma. molecular forms, responses to feeding, and relationship to gallbladder contraction. J Clin Invest 75(4):1144-52.

López-Patiño MA, Guijarro AI, Isorna E, Delgado J, Alonso-Bedate M, de Pedro N. 1999. Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (carassius auratus). Eur J Pharmacol 377(2–3):147-53.

Lundberg JM, Hemsén A, Larsson O, Rudehill A, Saria A, Fredholm BB. 1988. Neuropeptide Y receptor in pig spleen: Binding characteristics, reduction of cyclic AMP formation and calcium antagonist inhibition of vasoconstriction. Eur J Pharmacol 145(1):21-9.

MacDonald E, Volkoff H. 2009. Neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) in Winter skate (*Raja ocellata*): CDNA cloning, tissue distribution and mRNA expression responses to fasting. Gen Comp Endocrinol 161(2):252-61.

Maletinska L, Maixnerova J, Matyskova R, Haugvicova R, Pirnik Z, Kiss A, Zelezna B. 2008. Synergistic effect of CART (cocaine- and amphetamine-regulated transcript) peptide and cholecystokinin on food intake regulation in lean mice. BMC Neuroscience 9(1):101.

Martin A. 2001. The phylogenetic placement of chondrichthyes: Inferences from analysis of multiple genes and implications for comparative studies. Genetica 111(1):349-57.

Minth CD, Bloom SR, Polak JM, Dixon JE. 1984. Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine. PNAS 81(14):4577-81.

Moran TH, Kinzig KP. 2004. Gastrointestinal satiety signals - II. cholecystokinin. Am J Physiol -Gastroint Liver Physiol 286(2):G183-8.

Moran TH, McHugh PR. 1982. Cholecystokinin suppresses food intake by inhibiting gastric emptying. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 242(5):R491-7.

Moran TH, Robinson PH, Goldrich MS, McHugh MR. 1986. Two brain cholecystokinin receptors: implications for behavioral actions. Brain Res 362(1):175-179.

Murashita K and Kurokawa T. 2011. Multiple cocaine- and amphetamine-regulated transcript (CART) genes in Medaka, *Oryzias latipes*: Cloning, tissue distribution and effect of starvation. Gen Comp Endocrinol 170(3):494-500.

Murashita K, Kurokawa T, Ebbesson LOE, Stefansson SO, Rønnestad I. 2009. Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (*Salmo salar*). Gen Comp Endocrinol 162(2):160-71.

Murphy K, Abbott C, Mahmoudi M, Hunter R, Gardiner J, Rossi M, Stanley S, Ghatei M, Kuhar M, Bloom S. 2000. Quantification and synthesis of cocaine- and amphetamine-regulated transcript peptide (79-102)-like immunoreactivity and mRNA in rat tissues. Journal of Endocrinology 166(3):659-68.

Nakamachi T, Matsuda K, Maruyama K, Miura T, Uchiyama M, Funahashi H, Sakurai T, Shioda S. 2006. Regulation by orexin of feeding behaviour and locomotor activity in the goldfish. Journal of Neuroendocrinology 18(4):290-297.

Nakamura T, Uramura K, Nambu T, Yada T, Goto K, Yanagisawa M, Sakurai T. 2000. Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. Brain Res 873(1):181-7.

Narnaware YK, Peter RE. 2001. Effects of food deprivation and refeeding on neuropeptide Y (NPY) mRNA levels in goldfish. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 129(2-3):633-7.

Narnaware YK, Peyon PP, Lin XW, Peter RE. 2000. Regulation of food intake by neuropeptide Y in goldfish. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 279(3):R1025-34.

Näslund E, Hellström PM. 2007. Appetite signaling: From gut peptides and enteric nerves to brain. Physiol Behav 92(1-2):256-62.

Neary NM, Goldstone AP, Bloom SR. 2004. Appetite regulation: From the gut to the hypothalamus. Clinical Endocrinology 60:153-60.

Novak CM, Jiang XL, Wang CF, Teske JA, Kotz CM, Levine JA. 2005. Caloric restriction and physical activity in Zebrafish (*Danio rerio*). Neurosci Lett 383(1-2):99-104.

Ohkubo T, Boswell T, Lumineau S. 2002. Molecular cloning of chicken prepro-orexin cDNA and preferential expression in the chicken hypothalamus. Biochimica Et Biophysica Acta (BBA) - Gene Structure and Expression 1577(3):476-80.

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

Pedrazzini T, Seydoux J, Künstner P, Aubert JF, Grouzmann E, Beermann F, Brunner HR. 1998. Cardiovascular response, feeding behavior and locomotor activity in mice lacking the NPY Y1 receptor. Nature Medicine 4(6):722-726.

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

Pernow J, Lundberg JM, Kaijser L. 1987. Vasoconstrictor effects in vivo and plasma disappearance rate of neuropeptide Y in man. Life Sci 40(1):47-54.

Peyon P, Saied H, Lin X, Peter RE. 1999. Postprandial, seasonal and sexual variations in cholecystokinin gene expression in goldfish brain. Mol Brain Res 74(1–2):190-6.

Pickering AD, Pottinger TG. 1989. Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. Fish Physiol Biochem 7(1-6):253-258.

Powley TL, Keesey RE. 1970. Relationship of body weight to the lateral hypothalamic feeding syndrome. J Compar Physiol Psyc 70(1):25-36.

Powley TL, Opsahl CH, Cox JE, Weingarten HP. 1980. The role of the hypothalamus in energy homeostasis. in Handbook of the hypothalamus. Part A: behavioral studies of the hypothalamus, eds Morgane PJ, Panskepp J (Dekker, New York), pp 211–298.

Reeve JR, Eysselein VE, Ho FJ, Chew P, Vigna SR, Liddle RA, Evans C. 1994. Natural

and synthetic CCK-58. Annals of the New York Academy of Sciences 713(1):11-21.

Roberts MG, Savage GE. 1978. Effects of hypothalamic lesions on the food intake of the Goldfish *(Carassius auratus)*. Brain, Behavior and Evolution 15(2):150-64.

Rogge G, Jones D, Hubert GW, Lin Y, Kuhar MJ. 2008. CART peptides: Regulators of body weight, reward and other functions. Nature Reviews Neuroscience 9(10):747-758.

Sahu A, Sninsky CA, Phelps CP, Dube MG, Kalra PS, Kalra SP. 1992. Neuropeptide Y release from the paraventricular nucleus increases in association with hyperphagia in streptozotocin-induced diabetic rats. Endocrinology 131(6):2979-85.

Sakurai T. 2002. Roles of orexins in the regulation of feeding and arousal. Sleep Med 3, Supplement 2(0):S3-9.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, et al. 1998. Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92(4):573-85.

Salaneck E, Larsson TA, Larson ET, Larhammar D. 2008. Birth and death of neuropeptide Y receptor genes in relation to the teleost fish tetraploidization. Gene 409(1-2):61-71.

Savontaus E, Conwell IM, Wardlaw SL. 2002. Effects of adrenalectomy on AGRP, POMC, NPY and CART gene expression in the basal hypothalamus of fed and fasted rats. Brain Res 958(1):130-8.

Sayer MDJ, Davenport J. 1996. Hypometabolism in torpid goldsinny wrasse subjected to rapid reductions in seawater temperature. J Fish Biol 49(1):64-75.

Scott GK, Davies PL, Kao MH, Fletcher GL. 1988. Differential amplification of antifreeze protein genes in the pleuronectinae. J Mol Evol 27(1):29-35.

Silverstein JT, Plisetskaya EM. 2000. The effects of NPY and insulin on food intake regulation in fish. Amer Zool 40(2):296-308.

Silverstein JT, Breininger J, Baskin DG, Plisetskaya EM. 1998. Neuropeptide Y-like gene expression in the salmon brain increases with fasting. Gen Comp Endocrinol 110(2):157-65.

Singru PS, Mazumdar M, Barsagade V, Lechan RM, Thim L, Clausen JT, Subhedar N.

2008. Association of cocaine- and amphetamine-regulated transcript and neuropeptide Y in the forebrain and pituitary of the Catfish, *Carias batrachus*: A double immunofluorescent labeling study. J Chem Neuroanat 36(3-4):239-50.

Stanley BG. 1993. Neuropeptide Y in multiple hypothalamic sites controls eating behaviour, endocrine and autonomic systems for body energy balance. In: The biology of neuropeptide Y and related peptides Colmers WF and Wahlestedt C, editors. Totowa NJ: Humana Press. 457 p.

Strader AD, Woods SC. 2005. Gastrointestinal hormones and food intake. Gastroenterology 128(1):175-91.

Strange RJ, Shreck CB, Golden JT. 1977. Corticoid stress responses to handling and temperature in salmonids. T Am Fish Soc 106(3):213-218.

Tatemoto K. 1982. Neuropeptide Y: Complete amino acid sequence of the brain peptide. PNAS 79(18):5485-9.

Thim L, Kristensen P, Larsen PJ, Wulff BS. 1998. CART, a new anorectic peptide. Int J Biochem Cell Biol 30(12):1281-4.

Thim L, Kristensen P, Nielsen PF, Wulff BS, Clausen JT. 1999. Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. Proceedings of the National Academy of Sciences 96(6):2722-7.

Tokunaga K, Fukushima M, Kemnitz JW, Bray GA. 1986. Comparison of ventromedial and paraventricular lesions in rats that become obese. Am J Physiol 251(6):1221–1227.

Valen R, Jordal A-O, Murashita K, Rønnestad I. 2011. Postprandial effects on appetite-related neuropeptide expression in the brain of Atlantic salmon, *Salmo salar*. Gen Comp Endocrinol 171(3):359-66.

Valerio PF, Kao MH, Fletcher GL. Thermal hysteresis activity in the skin of the cunner, *Tautogolabrus adspersus*. Can J Zool 68(5):1065-7.

Van den Pol AN. 1982. Lateral hypothalamic damage and body weight regulation: role of gender, diet, and lesion placement. Am J Physiol 242(3):265–274.

Vicentic A, Lakatos A, Jones D. 2006. The CART receptors: Background and recent advances. Peptides 27(8):1934-7.

Vigna SR, Fischer BL, Morgan JLM, Rosenquist GL. 1985. Distribution and molecular heterogeneity of cholecystokinin-like immunoreactive peptides in the brain

and gut of the Rainbow trout, *Salmo gairdneri*. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 82(1):143-6.

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 & Integrative Physiology 144(3):325-31.

Volkoff H, Eykelbosh AJ, Peter RE. 2003. Role of leptin in the control of feeding of goldfish Carassius auratus: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. Brain Res 972(1-2):90-109.

Volkoff H, Peter RE. 2001a. Characterization of two forms of cocaine- and amphetamine-regulated transcript (CART) peptide precursors in Goldfish: Molecular cloning and distribution, modulation of expression by nutritional status, and interactions with leptin. Endocrinology 142(12):5076-88.

Volkoff H, Peter RE. 2001b. Interactions between orexin A, NPY and galanin in the control of food intake of the Goldfish, *Carassius auratus*. Regul Pept 101(1-3):59-72.

Volkoff H, Peter RE. 2000. Effects of CART peptides on food consumption, feeding and associated behaviors in the Goldfish, *Carassius auratus*: Actions on neuropeptide Y- and orexin A induced feeding. Brain Res 887(1):125-33.

Volkoff H, Suraj U, Scott PK. 2009. Chapter 9 the endocrine regulation of food intake. In: Fish physiology. Academic Press. 421 p.

Volkoff H, Bjorklund JM, Peter RE. 1999. Stimulation of feeding behavior and food consumption in the Goldfish, *Carassius auratus*, by orexin-A and orexin-B. Brain Res 846(2):204-9.

Vrang N, Tang-Christensen M, Larsen PJ, Kristensen P. 1999. Recombinant CART peptide induces c-fos expression in central areas involved in control of feeding behaviour. Brain Res 818(2):499-509.

Wan Y, Zhang Y, Ji P, Li Y, Xu P, Sun X. 2012. Molecular characterization of CART, AgRP, and MC4R genes and their expression with fasting and re-feeding in Common carp (*Cyprinus carpio*). Molecular Biology Reports 39(3):2215-23.

Weingarten HP, Chang P, McDonald TJ. 1985. Comparisons of the metabolic and behavioral disturbances following paraventricular and ventromedial hypothalamic lesions. Brain Res Bull 14:1551–1559.

Wierup N, Björkqvist M, Kuhar MJ, Mulder H, Sundler F. 2006. CART regulates islet

hormone secretion and is expressed in the β-cells of type 2 diabetic rats. Diabetes 55(2):305-11.

Wierup N, Richards WG, Bannon AW, Kuhar MJ, Ahrén B, Sundler F. 2005. CART knock out mice have impaired insulin secretion and glucose intolerance, altered beta cell morphology and increased body weight. Regul Pept 129(1–3):203-11.

Wong KKY, Ng SYL, Lee LTO, Ng HKH, Chow BKC. 2011. Orexins and their receptors from fish to mammals: A comparative approach. Gen Comp Endocr 171(2):124-130.

Xiang H, Taylor EW, Whitely N, Randall DJ. 1994. Modulation of noradrenergic action by neuropeptide Y in Dogfish (*Squalus acanthias*) hearts. Physiological Zoology 67(1):204-215.

Xu M, Volkoff H. 2007. Molecular characterization of prepro-orexin in Atlantic cod (*Gadus morhua*): Cloning, localization, developmental profile and role in food intake regulation. Mol Cell Endocrinol 271(1–2):28-37.

Yan A, Zhang L, Tang Z, Zhang Y, Qin C, Li B, Li W, Lin H. 2011. Orange-spotted grouper (*Epinephelus coioides*) orexin: Molecular cloning, tissue expression, ontogeny, daily rhythm and regulation of NPY gene expression. Peptides 32(7):1363-70.

Yokogawa T, Marin W, Faraco J, Pézeron G, Appelbaum L, Zhang J, Rosa F, Mourrain P, Mignot E. 2007. Characterization of sleep in zebrafish and insomnia in hypocretin receptor mutants. PLoS Biol 5(10):2379-97.

Zhang W, Fukuda Y, Kuwaki T. 2005. Respiratory and cardiovascular actions of orexin-A in mice. Neurosci Lett 385(2):131-6.