CHARACTERIZATION OF APPETITE-RELATED NEUROPEPTIDES IN GOLDFISH (CARASSIUS AURATUS) AND ATLANTIC COD (GADUS MORHUA): DAILY RHYTHMS IN GENE EXPRESSION AND EFFECTS OF LONG-TERM ADMINISTRATION

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PAS : PER-ARNT-SIM; PER = Period, ARNT = aryl hydrocarbon receptor nuclear translocator, SIM = single-minded

PCR : Polymerase chain reaction

Per : Period

qPCR : quantitative real-time RT-PCR

RNA : Ribonucleic acid

RT-PCR : Reverse transcription-polymerase chain reaction

SCN : Suprachiasmatic nucleus of the hypothalamus

inhibits further expression of the Bmal1 gene (Gekakis *et al.*, 1998). Next, Per and Cry proteins form heterodimers which inhibit further Clock-Bmal1 mediated transcription of Per and Cry genes, while Per protein drives the transcription of the Bmal1 gene, thereby initiating a new cycle (Sangoram *et al.*, 1998; Shearman *et al.*, 2000). In this manner, Per and Cry transcripts are expressed in antiphase of Bmal1 transcripts, while the Clock gene is constitutively expressed (Oishi *et al.*, 1998; Leloup and Goldbeter, 2003). This molecular mechanism generates 24 hour-cycling transcripts which are the foundation of circadian rhythm generation.

The cyclical expression of circadian genes regulates the cyclical expression of other downstream target genes, either directly by the core genes or indirectly via a cascade of other circadian transcription factors (Panda *et al.*, 2002; Oishi *et al.*, 2003). Rhythmic gene expression may promote or entrain other physiological rhythms, such as rhythms in growth, reproduction and appetite.

Circadian rhythms in fish

Although a region analogous to the mammalian SCN has not been identified in fish, both the pineal gland and the eyes have been proposed to be important regions for regulating circadian rhythms, as both are sites of rhythmic synthesis of the nighttime-secreted hormone melatonin (Falcon, 1999; Hirayama *et al.*, 2005). However, whereas some behavioural and physiological rhythms, such as locomotor activity, feeding and plasma cortisol rhythms, persist after pinealectomy in Asian stinging catfish (*Heteropneustes fossilis*; Garg and Sundararaj, 1986) and rainbow trout (*Oncorhynchus mykiss*; Sanchez-

Vazquez *et al.*, 2000), an intact hypothalamus is essential for sustaining daily locomotor activity rhythms in the primitive hagfish and lamprey (Ookasouda and Kabasawa, 1988; Weigle *et al.*, 1996) and it appears that multiple oscillators are present throughout the brain and various peripheral tissues in zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*; Cermakian *et al.*, 2000; Cahill, 2002; Velarde *et al.*, 2009). Together, this evidence suggests that the circadian system of fish differs from that of mammals.

In fish, circadian rhythms are generated by an endogenous molecular feedback loop similar to that of mammals (Ishikawa *et al.*, 2002). Taxonomic differences lie in how the expressions of circadian genes are regulated (*e.g.* in response to photic input) and in the number of copies of these genes (Cermakian *et al.*, 2000; 2002). Due to a whole genome duplication event that occurred early during teleost evolution, circadian regulatory genes are often present in several forms (Wang, 2008a; 2008b), some of which may have functionally diverged over time or been lost altogether in certain species. Circadian regulatory genes and how their expressions are regulated are only beginning to be understood in fish models.

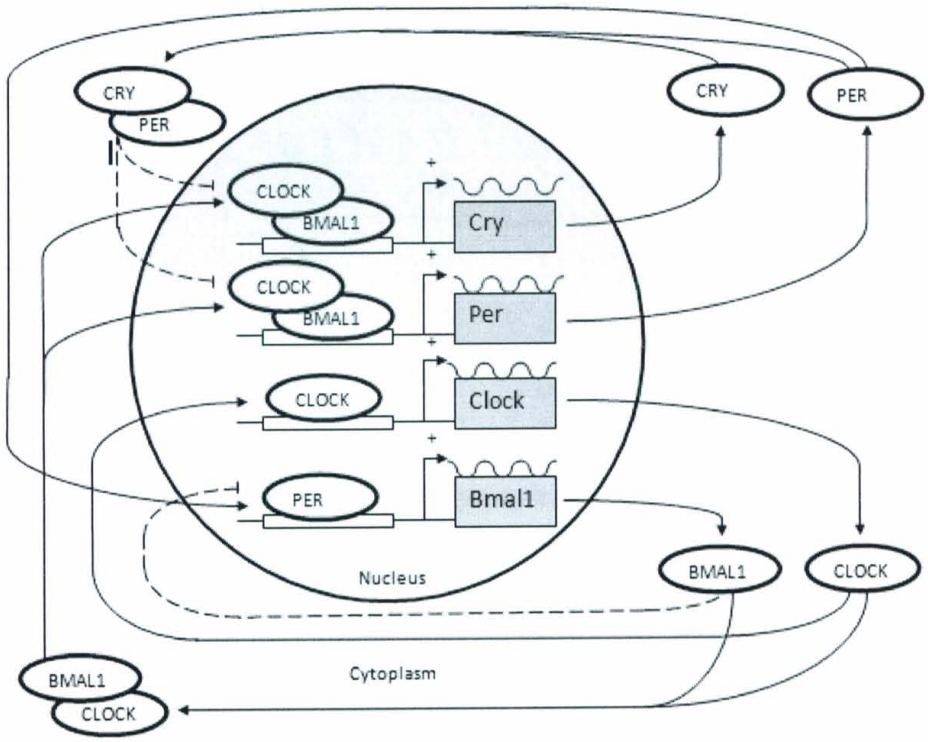


Figure 1.1 Simplified molecular feedback mechanism for circadian regulation in mammals. Dashed lines indicate an inhibitory response. The Clock gene is constitutively expressed.

1.2 Circadian regulatory proteins

1.2.1 Clock

The Clock gene, first discovered and characterized in mice, was initially found to regulate circadian rhythms of activity in mammals (Vitaterna *et al.*, 1994). The protein encoded by this gene is a member of the bHLH (basic helix-loop-helix) PAS (PER-ARNT-SIM; a protein module first identified in *Drosophila* Period (PER) protein, the human aryl hydrocarbon receptor nuclear translocator (ARNT) protein and the *Drosophila* single-minded (SIM) protein) superfamily of transcription factors which bind DNA and mediate transcription following dimerization (King *et al.*, 1997). Although it is constitutively expressed in mammals (Oishi *et al.*, 1998; Maywood *et al.*, 2003), Clock protein forms periodic associations with Bmal1 protein to activate transcription of target genes, including the circadian regulatory genes Cry and Per (Gekakis *et al.*, 1998), and that of downstream target genes involved in a variety of physiological processes.

Microarray analyses of mice have identified over 100 cycling transcripts whose expressions are reduced in Clock gene-mutant mice compared to wild-type (Oishi *et al.*, 2003). These transcripts are involved in a variety of physiological processes such as lipid metabolism, proteolysis and immune function. In addition, Clock gene-mutant mice exhibit altered sleep homeostasis, hyperphagia and obesity, with abnormal hypothalamic levels of appetite-regulating peptides, including orexin (Naylor *et al.*, 2000; Turek *et al.*, 2005). This evidence implicates Clock in the regulation of a number of physiological processes, including energy balance and sleep-wake activity.

Clock in fish

Several forms of the Clock gene have recently been identified in fish (Wang, 2008b) but functional differences between these forms and whether they are under differential regulation has not yet been determined. As opposed to mammals, for which Clock is constitutively expressed (Oishi *et al.*, 1998), in zebrafish, the Clock gene is transcribed cyclically (Whitmore *et al.*, 1998) such that Clock and Bmal1 mRNA levels oscillate in antiphase of Per and Cry mRNA levels (Cahill, 2002). Whether this is a common mode of circadian regulation amongst all teleosts has not been established.

Other than its role in circadian regulation, Clock has been implicated in other aspects of physiology in fish, in particular the timing of seasonal events in salmonids. In rainbow trout, expression of the Clock gene is strongly associated with spawning date (Leder *et al.*, 2006) and in Chinook salmon (*Oncorhynchus tshawytscha*), variations in Clock alleles are associated with variations in timing of migration and spawning (O'Malley *et al.*, 2007). This suggests that in fish, the Clock protein may regulate the periodic transcription of genes involved in other physiological processes, such as reproduction and feeding.

1.2.2 Period

The Per gene was first discovered and isolated in *Drosophila* when mutations at that particular locus were found to trigger altered behavioural rhythms (Konopka and Benzer, 1971; Reddy *et al.*, 1984). In later years, three forms of the Per gene, designated Per1,

Per2 and Per3, were identified in mammals; these produce proteins that are members of the bHLH-PAS superfamily of transcription factors (Albrecht *et al.*, 1997; Sun *et al.*, 1997; Tei *et al.*, 1997; Zylka *et al.*, 1998). Each of the Per genes is transcribed cyclically but in different phases from one another within the mammalian SCN (Albrecht, 2002), which suggests that the three forms are under differential transcriptional regulation. Although the exact functions of the three forms are not fully understood, they are not redundant as each has its own targets and produce different output responses (Bae *et al.*, 2001; Zheng *et al.*, 2001). For instance, in rodents, both Per1 and Per2 transcription can be induced by an acute nocturnal light pulse (Takumi *et al.*, 1998), while Per3 transcription is unaffected by this treatment (Zylka *et al.*, 1998).

Aside from its role in circadian regulation, Per has been implicated in physiological processes such as energy homeostasis. For example, fasting in mice causes increased Per1 transcript levels in peripheral tissues (Kawamoto *et al.*, 2006), food restriction in rats alters Per1 and Per2 transcription patterns in the hypothalamus and peripheral tissues (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Minana-Solis *et al.*, 2009) and administration of glucose to rat fibroblast cells downregulates Per1 and Per2 transcription (Hirota *et al.*, 2002). This data suggests that Per transcription is affected by feeding status, although the mechanisms regulating that interaction are not clear.

In addition, Per has been linked to sleep homeostasis in mammals. For example, in sleep-deprived mice, Per1 and Per2 transcript levels in the forebrain increase linearly with respect to time kept awake, but return to normal following a few hours of recovery sleep (Franken and Dijk, 2009). This association between circadian genes and sleep is not

surprising given that circadian genes regulate daily rhythms and that sleep is a typical daily cyclical process.

Period in fish

As with Clock, several copies of Per genes are present in teleosts (Wang, 2008a). The majority of studies involving Per in fish have been conducted in zebrafish embryos and cultured cell lines, and the role that different forms play in circadian regulation has not been fully established. In embryo-derived cell lines, Per1, Per2 and Per3 are rhythmically transcribed in different phases (Pando *et al.*, 2001). When held under constant darkness, Per1 and Per3 transcript levels continue to oscillate with rhythms degenerating over several days, while the rhythmic expression of Per2 transcripts is abolished. Additionally, Per2 transcript levels are elevated during the light phase in zebrafish cultured cells as well as *in vivo* in embryo brain and pineal gland (Cahill, 2002; Ziv and Gothilf, 2006). This evidence suggests that Per2 transcription may be more responsive to light than that of Per1 and Per3, and may be involved in entrainment to light-dark cycles.

Few studies have examined Per expression in fish species other than zebrafish (Park *et al.*, 2007; Sugama *et al.*, 2008; Davie *et al.*, 2009; Velarde *et al.*, 2009). In Atlantic salmon (*Salmo salar*), Per2 is rhythmically transcribed in the whole brain and various peripheral tissues under a short-day photoperiod (Davie *et al.*, 2009) and in goldfish, each of Per1, Per2 and Per3 is rhythmically transcribed in different phases in the retina (Velarde *et al.*, 2009). In addition, Per2 and Per3 transcripts are rhythmically expressed in different phases in the goldfish gut, while only Per3 transcripts are expressed cyclically in the liver (Velarde *et al.*, 2009). In the golden rabbitfish (*Siganus guttatus*), Per1 is

rhythmically transcribed in the whole brain, retina, liver and pineal gland (Park *et al.*, 2007), while Per2 transcription fluctuates over the day in whole brain, pineal gland and other peripheral tissues (Sugama *et al.*, 2008). As in zebrafish, expression of Per2 in rabbitfish is light-sensitive as its transcription in the pineal is induced by a nocturnal light pulse.

1.3 Appetite-related peptides

Energy homeostasis in fish, as in other vertebrates, is a complex process in which energy output is stringently balanced with energy input (for a see review on appetite regulation see Volkoff *et al.*, 2005). Energy input, or food intake, is regulated by chemical signals secreted by the brain and peripheral tissues which communicate with each other in order to control appetite. These chemical signals include hormones, some of which stimulate hunger and initiate feeding behaviour [orexigenic; *e.g.* Neuropeptide Y (NPY), orexin (OX)], while others induce satiety and thereby stop food intake [anorexigenic; *e.g.* cholecystokinin (CCK), Cocaine- and amphetamine regulated transcript (CART), amylin). How endocrine factors interact to regulate appetite is only partially understood.

1.3.1 Orexin

Orexins are neuropeptides that were originally isolated from the mammalian brain as the ligands of two orphan G-protein coupled receptors, now known as orexin receptor 1 (OXR1) and orexin receptor 2 (OXR2; Sakurai *et al.*, 1998). Orexins are produced from

the cleavage of a precursor molecule, preproorexin, into two mature peptides, orexin-A (OX-A) or hypocretin 1 and orexin-B (OX-B) or hypocretin 2 (De Lecea *et al.*, 1998). From here on, they shall be referred to as orexins, emphasizing their orexigenic, or appetite-stimulating, effects.

The vast majority of literature on the physiological functions of OX to date has come from mammalian studies. The widespread distribution of OX neurons (Date *et al.*, 1999; van den Pol, 1999; Voisin *et al.*, 2003) and receptors (Trivedi *et al.*, 1998; Voisin *et al.*, 2003) in regions of the brain and body that are associated with sleep-wake regulation, appetite homeostasis, circadian rhythms and neuroendocrine regulation implicates OX in all of these processes.

In mammals, both direct and indirect connections between OX neurons and the SCN (Date *et al.*, 1999; Aston-Jones *et al.*, 2001) suggest a possible role for OX in circadian rhythm modulation. Daily patterns of endogenous OX levels have been observed in rodents, including fluctuations of hypothalamic expression, OX neuronal activity and OX peptide levels of hypothalamic extracellular fluid and cerebrospinal fluid, with highest levels generally observed during the animals' active period and lowest levels during rest/sleep (Taheri *et al.*, 2000; Yoshida *et al.*, 2001; Martinez *et al.*, 2002; Deboer *et al.*, 2004; Zhang *et al.*, 2004; Lee *et al.*, 2005; Mileykovskiy *et al.*, 2005). In rats, an abolishment of OX fluctuations in the cerebrospinal fluid is seen following surgical lesion of the SCN (Deboer *et al.*, 2004; Zhang *et al.*, 2004), suggesting that the master circadian pacemaker might modulate the OX system and *vice versa*.

The high expression of OX observed during an animals' active period is consistent with the role of OX in stimulating wakefulness, which has been observed following brain injections in rodents (Nakamura *et al.*, 2000; Piper *et al.*, 2000). This OX-induced increase in arousal may contribute to increased foraging behaviour and may explain the orexigenic role of OX (for a review see Rodgers *et al.*, 2002).

The role of OX in non-mammalian vertebrates has been less thoroughly studied than in mammals (Shibahara *et al.*, 1999; Alvarez and Sutcliffe, 2002; Ohkubo *et al.*, 2002). Among teleost fish, preproOX sequences have been characterized in several species, including goldfish, three-spine stickleback (*Gasterosteus aculeatus*), Japanese medaka (*Oryzias latipes*), zebrafish and Atlantic cod (*Gadus morhua*; Kaslin *et al.*, 2004; Faraco *et al.*, 2006; Xu and Volkoff, 2007; Hoskins *et al.*, 2008). Similarities exist between fish and mammals in terms of both the sequence and the distribution of OX neurons throughout the brain (Kaslin *et al.*, 2004; Huesa *et al.*, 2005; Amiya *et al.*, 2007), with OX neurons projecting to brain regions known for their roles in appetite regulation and wakefulness.

Evidence suggests that OX increases arousal in fish, as both central injections of OX in goldfish (Volkoff *et al.*, 1999; Volkoff and Peter, 2000) and peripheral injections in ornate wrasse (*Thalassoma pavo*; Facciolo *et al.*, 2009) promote increased locomotor activity. Furthermore, in a zebrafish bioluminescence study, in which a fluorescent protein was linked to OX neurons, bioluminescent signals increased during the daily active period (Naumann *et al.*, 2010).

Aside from its role in alertness, the appetite regulatory effects of OX have been examined in several fish species. Both central injections of OX in goldfish (Volkoff *et al.*, 1999) and peripheral injections in ornate wrasse (Facciolo *et al.*, 2009) stimulate food intake, whereas central injections of anti-OX serum in goldfish decrease food intake (Nakamachi *et al.*, 2006). Furthermore, OX transcription increases around mealtime in Atlantic cod forebrain (Xu and Volkoff, 2007) and in response to fasting in both zebrafish (Novak *et al.*, 2005) and goldfish (Nakamachi *et al.*, 2006) brain. Together, this evidence indicates a feeding stimulatory role for OX in fish.

1.3.2 Neuropeptide Y

NPY is a 36 amino acid peptide that was originally isolated from the pig hypothalamus and is one of the most highly conserved peptides known, showing little sequence variation between fish and other vertebrates, including humans (Tatemoto *et al.*, 1982; Cerda-Reverter and Larhammar, 2000). NPY was named due to the presence of an amidated tyrosine (Y) residue at the C-terminal end of the peptide. In mammals, several G-protein-coupled NPY receptor subtypes have been identified and are classified as Y1, Y2, Y4, Y5 and Y6 (Michel *et al.*, 1998), each displaying a unique expression pattern throughout the brain and peripheral tissues (Dumont *et al.*, 1992; Fetissov *et al.*, 2004). These receptors have functional differences allowing NPY to exert a variety of different physiological effects, some of which include cardiovascular control (Walker *et al.*, 1991),

anxiety reactions (Erickson *et al.*, 1996), circadian rhythm modulation (Glass *et al.*, 2010) and endocrine and appetite regulation (Horvath *et al.*, 1999; Silva *et al.*, 2002).

The major role of NPY, and that which has been most extensively studied, appears to be the regulation of feeding processes (for a review see Beck, 2006). NPY is a potent appetite stimulator as seen in rodents, where central administration of NPY strongly increases food intake (Clark *et al.*, 1984; Stanley *et al.*, 1985; Kalra *et al.*, 1988). In addition, NPY secreted from neurons in the mammalian gastrointestinal (GI) tract has a variety of effects on digestive processes (Sundler *et al.*, 1983; Sheikh, 1991; Dumont *et al.*, 1992), which further supports its role in regulating the physiology of feeding.

NPY has been implicated in the modulation of circadian rhythms in mammals (Morin, 1994; Golombek *et al.*, 1996; Huhman *et al.*, 1996; Kim and Harrington, 2008; Glass *et al.*, 2010). A neuronal tract connecting the eyes to the SCN utilizes NPY to convey environmental information, such as lighting, to the master pacemaker. In this manner, NPY acts as a non-photic cue to reset rhythms generated by the SCN, possibly by eliciting a change in SCN neuronal firing rhythms. Whereas the appetite-stimulating effects of NPY are mediated, at least in part, by Y1 and Y5 receptors (Wyss *et al.*, 1998; Polidori *et al.*, 2000), the phase shifting effect of NPY on SCN-generated rhythms is likely exerted via Y2 receptors (Golombek *et al.*, 1996; Huhman *et al.*, 1996). Together, this evidence indicates that NPY plays a role in modulating circadian rhythmicity, at least in mammals.

Daily patterns of NPY levels have been exclusively studied in rodents. In rats, peaks of NPY peptide levels in the SCN coincide with the timing of lights on and lights off (Calza *et al.*, 1990; Shinohara *et al.*, 1993), suggesting that NPY conveys light information to the SCN. However, when rats are housed in constant darkness, NPY levels peak early in the subjective night (segment corresponding to the dark phase of an entrained light-dark cycle), which coincides with their usual feeding time. When rats are food restricted and offered a single meal during the day, hypothalamic NPY secretion increases in anticipation of the oncoming meal, decreases over the course of the meal and continues if food is withheld (Kalra *et al.*, 1991), likely because these rats are still hungry. Collectively, this evidence suggests that daily NPY patterns in rodents are affected by both lighting conditions and the timing of food availability. No information is available to determine if this is also true in other vertebrates, such as fish.

In fish, NPY sequences have been identified and characterized in a number of species, including goldfish (Peng *et al.*, 1994), Atlantic cod (Kehoe and Volkoff, 2007), Atlantic salmon (Murashita *et al.*, 2009) and winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009a), with peptide mRNA widely expressed throughout the brain, especially the forebrain region (Peng *et al.*, 1994; Leonard *et al.*, 2001; Kehoe and Volkoff, 2007), and in various peripheral tissues (Kah *et al.*, 1989; Kehoe and Volkoff, 2007). Fish NPY receptors, some of which are structurally similar to those in mammals and some which are distinct fish subtypes, are distributed in the brain and peripheral tissues in a species-specific manner (Ringvall *et al.*, 1997; Pirone *et al.*, 2003; Larsson *et*

al., 2008), which suggests that the functions of NPY may differ between species of fish and between fish and mammals.

As in mammals, NPY appears to be an important stimulator of appetite in fish, such that food intake increases following central injections of NPY in several species, including goldfish (Lopez-Patino *et al.*, 1999; Narnaware *et al.*, 2000; Volkoff and Peter, 2000), channel catfish (*Ictalurus punctatus*) (Silverstein and Plisetskaya, 2000) and rainbow trout (Aldegunde and Mancebo, 2006). In addition, forebrain NPY expression increases around a daily scheduled meal in both goldfish (Narnaware *et al.*, 2000) and Atlantic cod (Kehoe and Volkoff, 2007) and fasting increases hypothalamic NPY mRNA expression in goldfish (Narnaware *et al.*, 2000), Chinook and coho salmon (*Oncorhynchus kisutch*; Silverstein *et al.*, 1998), suggesting a role for endogenous NPY in stimulating appetite.

1.3.3 Cholecystokinin

CCK was first described in the 1920s as a substance released from the small intestine of dogs and cats that induces contraction of the gallbladder (Johnsen, 1998) and was later characterized as an anorexigenic peptide hormone produced in the mammalian digestive tract (Jorpes and Mutt, 1959; Gibbs *et al.*, 1973). CCK exists endogenously in several forms of varying lengths, arising from different cleavage sites on the preprohormone (Beinfeld, 2003). The C-terminal octapeptide, named CCK-8, is the physiologically active portion of the propeptide and has been highly conserved over evolutionary time (Johnsen, 1998), suggesting the possibility of similar effects of CCK among the taxa.

In mammals, CCK peptides and CCK receptors, designated as CCK-A or CCK-1 receptor and CCK-B or CCK-2 receptor, have been localized to neurons and endocrine cells of the gut and digestive organs, including the gall bladder and pancreas, as well as throughout the brain, including regions implicated in appetite regulation, such as the hypothalamus and brainstem (Polak *et al.*, 1975; Beinfeld *et al.*, 1981; Moran *et al.*, 1986; Moran and Kinzig, 2004). The wide distribution of CCK receptors suggests that CCK may influence satiation both centrally and peripherally.

The secretion and effects of CCK on the digestive system are similar between mammals and fish (Jonsson *et al.*, 1987; Rajjo *et al.*, 1988; Plantikow *et al.*, 1992; Himick and Peter, 1994; Koven *et al.*, 2002; Forgan and Forster, 2007; Dockray, 2009). CCK is secreted from endocrine cells bordering the gut lumen in response to the presence of food in the small intestine, which induces the secretion of pancreatic enzymes and contraction of the gallbladder, and inhibits both gastric motility and emptying. In this manner, digestive enzymes and bile enter the gut and the entry of food into the small intestine is slowed, thereby regulating the amount of food entering the gut.

In mammals, both peripheral (Gibbs *et al.*, 1973; Gutzwiller *et al.*, 2000) and central (Tsai *et al.*, 1984; Willis *et al.*, 1984) injections of CCK cause a transient decrease in food intake, which suggests that CCK may act on both the gut and brain. In addition, endogenous CCK levels in the rat hypothalamus increase immediately following a meal (McLaughlin *et al.*, 1985). CCK appears to regulate appetite at the level of the brain via the vagus nerve, which provides a direct connection from the gut to the brain (Moran *et al.*, 1990; Wang *et al.*, 1998; Berthoud *et al.*, 2004; Halford *et al.*, 2004; Whited *et al.*,

2006). Nerve terminals of the vagus lie in close apposition to CCK secretory cells of the small intestine, allowing the meal-terminating signal to be conveyed to the brainstem. From here, signals are relayed to other areas of the brain, including known appetite-regulating centers, such as the hypothalamus (Moran *et al.*, 1986). CCK receptors are also present in the brain, where CCK likely acts directly as well, to reduce food intake.

In fish, as in mammals, food intake is transiently reduced following either peripheral or central injections of CCK in both goldfish (Himick and Peter, 1994) and channel catfish (Silverstein and Plisetskaya, 2000). In goldfish, the peripheral, but not central, effects of CCK are blocked by capsaicin (Kang *et al.*, 2010), which destroys the vagus, suggesting that the same relay mechanism exists in fish, as in mammals. In addition, CCK mRNA expression increases in the goldfish brain (Peyon *et al.*, 1999) and yellowtail gut (*Seriola quinqueradiata*; Murashita *et al.*, 2007) a few hours following a meal. CCK transcripts and peptides that have been identified in a number of species, including rainbow trout (Jensen *et al.*, 2001), yellowtail (Murashita *et al.*, 2006), winter flounder (MacDonald and Volkoff, 2009a) and goldfish (Himick and Peter, 1994; Peyon *et al.*, 1998) are predominantly localized in the brain and GI tract and although only one CCK receptor has been identified in fish (Volkoff *et al.*, 2005), CCK binding sites are widely distributed (Moons *et al.*, 1992; Himick *et al.*, 1996). In summary, this evidence suggests that CCK may act as a short-term inhibitor of appetite in fish by acting on both the gut and brain.

1.3.4 Amylin

Amylin or islet amyloid polypeptide (IAPP) is a peptide that is co-secreted with insulin from β cells of the vertebrate pancreas in response to food intake (Westermark *et al.*, 2002; Woods *et al.*, 2006). In mammals, this peptide is known to inhibit gastric emptying, gastric acid secretion, gluconeogenesis, insulin secretion and enzyme secretion from the pancreas and stomach, which together, act to terminate a meal (Lutz, 2005; Woods *et al.*, 2006). Peripheral or central injections of amylin reduce food intake in rats and goldfish (Chance *et al.*, 1991; Rushing *et al.*, 2000; Lutz *et al.*, 2001; Mollet *et al.*, 2004; Thavanathan and Volkoff, 2006), while chronic central and peripheral infusion of amylin in rats also reduces food intake, as well as body weight and fat stores (Rushing *et al.*, 2000; Lutz *et al.*, 2001), suggesting that amylin is involved in regulating short-term and long-term food intake in both mammals and fish.

In mammals, amylin secreted from the pancreas acts centrally to reduce food intake. Circulating amylin reaches the brain, where it crosses the blood-brain-barrier and appears to act on the brainstem (which may relay the signal to higher forebrain structures) and on the hypothalamus, as amylin binding sites have been identified in both regions (van Rossum *et al.*, 1994; Banks *et al.*, 1995; Rowland *et al.*, 1997; Becskei *et al.*, 2007). Although amylin is predominantly synthesized in the mammalian pancreas, it has been detected in other tissues, including the brain, where highest levels are observed in the hypothalamus (Chance *et al.*, 1991; D'Este *et al.*, 2001; Dobolyi, 2009). In other vertebrates, including chickens and teleosts, although the pancreas is still a site of amylin

production, amylin mRNA expression tends to be higher in other tissues, in particular the brain (Fan *et al.*, 1994; Westermark *et al.*, 2002; Martinez-Alvarez *et al.*, 2008).

1.4 Interactions between appetite-related peptides

1.4.1 NPY and OX

In vertebrates, both NPY and OX are important appetite-stimulating neuropeptides synthesized mainly in the hypothalamus. Direct interactions between the NPY and OX systems have been observed in neuronal distribution studies which show that OX neurons form dense, direct reciprocal connections with NPY neurons in the mammalian hypothalamus (Broberger *et al.*, 1998; Elias *et al.*, 1998; Horvath *et al.*, 1999) and that OX receptors may be present in over 90% of NPY neurons (Backberg *et al.*, 2002). Similarly, OX nerve terminals in the goldfish hypothalamus lie in close apposition to NPY neurons, and *vice versa* (Matsuda *et al.*, 2009), which suggests that this neuronal distribution is a conserved feature among vertebrates.

The interaction between OX and NPY is further demonstrated by injection studies which show that central administration of OX stimulates NPY neuronal activity in the rat hypothalamus, and *vice versa* (Yamanaka *et al.*, 2000; Niimi *et al.*, 2001) and that central injections of OX increase hypothalamic NPY mRNA expression in both rats and goldfish (Volkoff and Peter, 2001b; Lopez *et al.*, 2002). In addition, blocking NPY receptors by pre-treatment with NPY receptor antagonists reduces the orexigenic effect of centrally administered OX in both rats and goldfish (Dube *et al.*, 2000; Jain *et al.*, 2000; Volkoff

and Peter, 2001b). Likewise, NPY-elicited food intake is abolished when OX receptors are blocked (Niimi *et al.*, 2001; Volkoff and Peter, 2001b; Matsuda *et al.*, 2009). Furthermore, simultaneous central injections of OX and NPY in goldfish and rats increase food intake to a greater extent than that elicited by either of the peptides alone (Volkoff and Peter, 2001b; Sahu, 2002), which indicates a synergistic effect on appetite stimulation.

1.4.2 NPY and CART

Cocaine- and amphetamine-regulated transcript (CART) is a neuropeptide whose transcription was originally observed to be regulated by administration of cocaine and amphetamine (Douglass *et al.*, 1995). It is thought to be anorexigenic, as is demonstrated by decreased food intake following central administration of CART in goldfish (Volkoff and Peter, 2000) and rats (Kristensen *et al.*, 1998).

As opposing factors in the regulation of appetite, CART and NPY may interact, at least in part, to modulate food intake. In both rats and goldfish (Kristensen *et al.*, 1998; Volkoff and Peter, 2000), central injections of CART inhibit the orexigenic effect of NPY, which suggests that CART has an inhibitory effect on the NPY system. However, one study shows that administration of CART to rat hypothalamic explants induces the secretion of NPY and *vice versa* (Dhillo *et al.*, 2002), suggesting there may be two populations of CART neurons in the hypothalamus that either stimulate or inhibit feeding (Vrang *et al.*,

1999a) and where CART is co-stored with either orexigenic (*e.g.* melanin-concentrating hormone) or other anorexigenic (*e.g.* pro-opiomelanocortin) peptides.

Aside from appetite modulation, CART and NPY may interact to influence other physiological processes, such as seizure control, as central co-injection of the two peptides in goldfish substantially reduce CART-induced tremors, likely because of the anticonvulsant role of NPY (Vezzani *et al.*, 1999; Volkoff and Peter, 2000). Interactions between CART and NPY may be facilitated by co-localization in hypothalamic neurons, such as occurs in the human hypothalamus (Menyhert *et al.*, 2007), or the close proximity of neurons containing these peptides, as is seen in the rat hypothalamus (Broberger, 1999; Vrang *et al.*, 1999a) and regions of the walking catfish (*Clarias batrachus*) brain (Singru *et al.*, 2008). In addition, close apposition of CART and NPY neurons in the olfactory bulbs of walking catfish may be indicative of an interaction in sensory information processing.

1.4.3 NPY and Amylin

No study has ever examined the interactions between the NPY and amylin systems in fish, but these peptides appear to be linked in the regulation of short- and long-term energy balance in mammals. In the rodent hypothalamus, amylin acutely inhibits the orexigenic effects of NPY such that pre-treatment with central injections of amylin or central co-injections of NPY and amylin inhibit NPY-elicited feeding (Balasubramaniam *et al.*, 1991; Morris and Nguyen, 2001). Chronic peripheral infusions of amylin reduce food intake and increase hypothalamic NPY transcript and peptide levels (Arnelo *et al.*,

2000; Roth *et al.*, 2006), whereas these levels appear to be unaffected by single peripheral or daily central amylin injections (Morris and Nguyen, 2001; Barth *et al.*, 2003). This evidence suggests that amylin may inhibit NPY-induced food intake in a single treatment or feeding episode, but NPY expression may increase to compensate for the anorexigenic effects of persistently high levels of amylin.

1.4.4 CCK and OX

In mammals, CCK and OX have been shown to interact to regulate energy homeostasis such that central injections of OX attenuate the anorexigenic effects of peripherally administered CCK (Asakawa *et al.*, 2002) and pre-treatment with peripheral injections of OX attenuate CCK-induced vagal nerve activity (Burdyga *et al.*, 2003). This suggests that OX might reduce the sensitivity of the brain or vagal neurons to the satiating effects of CCK. However, other studies suggest that CCK may directly up-regulate the OX system, as peripheral injections of CCK increase OX brainstem levels (Gallmann *et al.*, 2006) and application of CCK to slices of mouse hypothalamus induce OX neuronal activity (Tsujino *et al.*, 2005), which indicates that OX up-regulation could act to reduce the satiating effects of CCK. Similar studies examining direct interactions between the CCK and OX systems have not been performed in fish models.

In mammals, CCK, OX and their receptors are present in endocrine cells and neurons of the gut where interactions between the CCK and OX systems have been observed (Kirchgessner and Liu, 1999; Larsson *et al.*, 2003; Dockray, 2009). For instance,

application of OX to cultured neuroendocrine intestinal cells induces the secretion of CCK from these cells (Larsson *et al.*, 2003). OX also potentiates the CCK-elicited increase in intestinal cell calcium stores, which is part of a process that helps neutralize stomach acid passing into the gut (Flemstroem *et al.*, 2001). In addition, OX-induced inhibition of intestinal glucose absorption appears to be modulated by CCK (Hirsh and Cheeseman, 1998; Ducroc *et al.*, 2007). The mechanism by which CCK and OX interact within the GI tract is uncertain in mammals and has not been examined in fish.

1.4.5 CCK and CART

A number of studies of rodents have shown that CCK interacts synergistically with CART to relay a meal-terminating signal from the vagus nerve to the brain. For instance, peripheral co-administration of CCK and CART increases neuronal activity in both the brainstem and hypothalamus, while prolonging the anorexigenic effect normally elicited by each peptide alone (Maletinska *et al.*, 2008; De Lartigue *et al.*, 2010; Pirnik *et al.*, 2010). Interestingly, the pattern of hypothalamic neuronal activity induced by central injections of CART is similar to that induced by peripheral injections of CCK (Olson *et al.*, 1992; Vrang *et al.*, 1999b). In addition, pre-treatment with peripheral injections of CCK increases the anorexigenc effect of centrally injected CART (Maletinska *et al.*, 2008). This evidence suggests that CCK and CART have common targets and act together to synergistically reduce food intake.

It is likely that CCK modulates the effects of CART, as CART neurons of the brainstem region and approximately half of all vagal neurons, which contain CART, also possess CCK receptors on their surface (Broberger *et al.*, 1999). As well, CART peptide secretion in vagal neurons increases following peripheral injections of CCK or direct application of CCK to these neurons *in vitro* (De Lartigue *et al.*, 2010). Furthermore, CART expression in the brainstem and other brain regions is significantly decreased in mutant rats lacking CCK receptors (Abraham *et al.*, 2009). These observations suggest that CCK may mediate the anorexigenic effects of CART by directly stimulating CART-containing neurons. There have been no studies examining the direct interactions between CCK and CART in any fish model.

1.4.6 CCK and Amylin

CCK and amylin are secreted upon food intake from the gut and pancreas, respectively, and both act to decrease further food intake with similar potency, partly by inhibiting gastric emptying (Reidelberger *et al.*, 2001). In rodents, amylin has been shown to inhibit some of the effects of CCK in the GI tract by inhibiting CCK-induced enzyme secretion from the rat pancreas (Young *et al.*, 2005) and the contractile response of smooth muscle in the guinea pig intestine (Ochiai *et al.*, 2001). However, with respect to both neuronal activity in the brain and food intake, CCK and amylin appear to interact synergistically.

The appetite-inhibiting interaction between the CCK and amylin systems has been demonstrated in several studies using rodents (Bhavsar *et al.*, 1998; Lutz *et al.*, 2000;

Trevaskis *et al.*, 2010) and goldfish (Thavanathan and Volkoff, 2006). In rodents, co-administration of both peptides by either peripheral acute injections or chronic infusions leads to a synergistic decrease in both food intake and neuronal activity in the brainstem region, where the two peptides are thought to interact in mammals (Bhavsar *et al.*, 1998; Becskei *et al.*, 2007; Trevaskis *et al.*, 2010). The anorexigenic effect of CCK can be attenuated by peripheral (Lutz *et al.*, 2000) or central (Thavanathan and Volkoff, 2006) injections of amylin receptor antagonists in rats and goldfish, respectively. Additionally, in goldfish, central injections of CCK receptor antagonist decrease the anorexigenic effect of amylin and co-injections of both peptides decrease food intake with an inhibitory effect that is greater than that caused by either peptide alone. This evidence further suggests that CCK may mediate the effects of amylin, and *vice versa*, to create a synergistic inhibitory effect on appetite.

1.5 Objectives of this study

Currently, the understanding of how appetite is regulated is limited in fish. For example, daily patterns of appetite-related neuropeptides have not been measured and the effects of long-term administration of these neuropeptides have never been assessed. In this study I attempted to expand knowledge in this area by (1) determining daily rhythms in mRNA expression of circadian regulatory peptides and important appetite-related neuropeptides, in two teleost species, goldfish and Atlantic cod and (2) assessing the effects of long-term administration of appetite-related hormones on both feeding and mRNA expression. For my first objective, I determined if fish have circadian rhythms by examining the daily

hypothalamic mRNA expression profiles of the circadian regulatory proteins, Clock and Period. Next, daily profiles of hypothalamic mRNA expression for the appetite-related neuropeptides, OX and NPY, were determined. The influence of the act of feeding on these profiles was explored in both species, and the effects of different environmental lighting regimes on these profiles were determined for goldfish. For my second objective, the effects of continuous long-term peripheral and central administration of NPY and CCK on both food intake and hypothalamic mRNA expression of OX, CART and amylin were examined in goldfish.

2.0 Daily rhythms of appetite-related neuropeptides in goldfish and Atlantic cod

2.1 Introduction

Circadian, or daily, rhythms of behaviour and physiology have been observed in nearly all animals studied (Young, 2000; Dunlap *et al.*, 2007; Dong and Golden, 2008; Harmer, 2009). Most of the literature concentrates on circadian patterns, such as those of sleep-wake/rest-activity, feeding and gene expression, in rodent and fruit fly models, however, a body of evidence is growing which suggests that daily cycles are prevalent in fish, as well.

Behavioural cycles, such as locomotor activity and feeding patterns, have been observed in several teleost species, including Atlantic salmon (*Salmo salar*), goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*; Richardson and McCleave, 1974; Sanchez-Vazquez *et al.*, 1996; Sanchez-Vazquez and Tabata, 1998). In addition, a number of physiological daily rhythms have been identified in fish, including: (1) daily heart rate patterns (Aissaoui *et al.*, 2000); (2) daily production and secretion of the circadian-regulated pineal-derived hormone melatonin (Falcon *et al.*, 1989; Kezuka *et al.*, 1989; Bolliet *et al.*, 1996; Oliveira *et al.*, 2009) and (3) daily variations of plasma levels of hormones such as cortisol, growth hormone and thyroid hormones (Boujard *et al.*, 1993; Holloway *et al.*, 1994), to name a few.

Interestingly, daily mRNA expression profiles have received little or no attention in fish, aside from daily expression patterns of light signalling peptides, such as melanopsin and rhodopsin, in a cichlid, *Astatotilapia burtoni* (Grone *et al.*, 2007) and hormones, such as

prolactin, somatolactin and growth hormone, in golden rabbitfish (*Siganus guttatus*; Ayson and Takemura, 2006). Additionally, daily rhythms in mRNA expression of several proteins involved in generating and regulating circadian rhythms, such as Clock and Period (Per), have been demonstrated for zebrafish (*Danio rerio*; Cahill, 2002; Wang, 2008a), goldfish (Velarde *et al.*, 2009), Atlantic salmon (Davie *et al.*, 2009) and golden rabbitfish (Park *et al.*, 2007).

In mammals, circadian cycles are generated in pacemaker cells located in the suprachiasmatic nucleus of the hypothalamus (SCN), where an autonomous molecular feedback loop results in the cyclic transcription and translation of circadian regulatory proteins, such as Clock and Per (Reppert and Weaver, 2001). Although similar regulatory proteins have been identified in fish (Wang, 2008a; 2008b), it is unknown whether the same molecular mechanism exists to produce daily oscillations in fish. In mammals, molecular oscillations generated in the SCN drive the rhythmic transcription of downstream target genes (Panda *et al.*, 2002; Oishi *et al.*, 2003), which may result in observable rhythms of behaviour and physiology that are either (1) true circadian rhythms, which are sustained under constant environmental conditions, but can be adjusted by external influences, or (2) are dependent upon cycles in the environment (*e.g.* light-dark cycles) to set the rhythm.

Light is an important timing cue that can alter and entrain the expression of core circadian genes (Takumi *et al.*, 1998; Cermakian *et al.*, 2002). In zebrafish, a light-dark cycle seems to be necessary to sustain oscillations of both Clock and Per transcription, as these rhythms degenerate rapidly when fish are subjected to constant darkness (Whitmore *et al.*,

1998; Pando *et al.*, 2001; Ziv and Gothilf, 2006; Dekens and Whitmore, 2008). Other endogenous rhythms can be entrained to light-dark cycles and become synchronized with environmental cues, such as locomotor activity in zebrafish (Lopez-Olmeda and Sanchez-Vazquez, 2009) and feeding patterns in rainbow trout trained to operate a lever to obtain food (Bolliet *et al.*, 2001). In the absence of light-dark cycles (*i.e.* constant light), a number of endogenous behavioural and physiological rhythms may be disrupted, as has been shown for daily heart rate pattern in gilt-head seabream (*Sparus aurata*; Aissaoui *et al.*, 2000), locomotor activity rhythms in Atlantic salmon and sharpsnout seabream (*Diplodus puntazzo*; Richardson and McCleave, 1974; Vera *et al.*, 2006), pituitary mRNA expression patterns of growth hormone and somatolactin in golden rabbitfish (Ayson and Takemura, 2006) and melatonin synthesis and secretion rhythms in a variety of teleost species (Falcon *et al.*, 2010), indicating the importance of light-dark cues in sustaining endogenous rhythms.

Although photoperiod is generally the most powerful entraining cue, feeding time may also synchronize endogenous rhythms. Whereas in some fish species, such as rainbow trout, photoperiod is the most important entraining cue for locomotion (Bolliet *et al.*, 2001), in other species, such as goldfish (Aranda *et al.*, 2001), zebrafish (Sanchez and Sanchez-Vazquez, 2009) and European catfish (*Siluris glanis*; Bolliet *et al.*, 2001), a daily scheduled meal can induce food anticipatory activity (FAA), seen as increased locomotor activity prior to mealtime, regardless of photoperiod. This evidence indicates that daily behavioural rhythms can be synchronized to feeding cycles and/or light-dark cycles, and

that species-specific differences exist with respect to which cycle is the most potent entraining cue.

Feeding cues may also alter the core molecular oscillator, as Per transcription is altered in fasted (Kawamoto *et al.*, 2006) or food restricted (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Minana-Solis *et al.*, 2009) rodents. Food restricted rodents exhibit FAA, in terms of increased activity and body temperature, prior to mealtime (Boulos and Terman, 1980; Bodosi *et al.*, 2004), but FAA is abolished in mice containing a mutation in the Per2 gene (Feillet *et al.*, 2006), which suggests that Per2 may be involved in generating the daily changes associated with meal anticipation.

Two orexigenic neuropeptides, orexin (OX) and Neuropeptide Y (NPY), have been implicated in daily FAA (Kalra *et al.*, 1991; Mistlberger *et al.*, 2003; Akiyama *et al.*, 2004). OX is a potent stimulator of appetite and wakefulness (Piper *et al.*, 2000; Rodgers *et al.*, 2002; Mileykovskiy *et al.*, 2005). In nocturnal rodents, OX neuronal activity is normally highest during the night when animals are active and eating, but when feeding is restricted to a single daily meal, animals exhibit FAA and OX neuronal activity peaks around the scheduled mealtime (Mistlberger *et al.*, 2003; Akiyama *et al.*, 2004). Targeted OX neuron ablation abolishes FAA, in mice but not in rats, suggesting a possible species-specific role of OX in FAA.

NPY, an orexigenic neuropeptide (Beck, 2006), has also been implicated in FAA, as hypothalamic NPY secretion in rats increases in anticipation of an oncoming meal, with secretion decreasing over the course of the meal (Kalra *et al.*, 1991). This evidence

indicates that neuropeptide rhythms may become entrained to daily feeding cycles. Short-term periprandial fluctuations in mRNA expression have been observed for OX in Atlantic cod (*Gadus morhua*; Xu and Volkoff, 2007) and for NPY in both goldfish (Narnaware *et al.*, 2000) and Atlantic cod (Kehoe and Volkoff, 2007). However, daily patterns of neuropeptides, such as OX and NPY, both of which have been implicated in circadian rhythmicity in mammals (OX: Date *et al.*, 1999; Deboer *et al.*, 2004; Zhang *et al.*, 2004, NPY: Kim and Harrington, 2008; Glass *et al.*, 2010), have never been examined in fish.

Objectives of this study

The present study aimed at determining the daily hypothalamic mRNA expression profiles for two core circadian regulatory proteins, Clock and Per, for the first time in goldfish, a temperate freshwater teleost, and Atlantic cod, a marine teleost living in cold waters of the North Atlantic ocean. As molecular oscillations generated in the hypothalamus are thought to drive the rhythmic transcription of other physiologically important genes, I wanted to quantify the daily hypothalamic mRNA expression profiles of OX and NPY, two neuropeptides that are known to regulate activity/wakefulness and food intake, both activities that fluctuate over the day.

As the expression of core circadian genes and appetite-regulators can be modulated by external cues, the effects of feeding (*i.e.* whether the fish are fed or unfed on sampling day) on Clock, Per, OX and NPY mRNA expression profiles were examined for both species. In addition, as photoperiod often plays a key modulatory role in the transcription of circadian genes in mammals and may influence the expression of appetite-regulators,

the effects of photoperiod (*i.e.* 16L:8D photoperiod *vs*. constant light) on Clock, Per, OX and NPY mRNA expression were examined in the goldfish hypothalamus.

2.2 Daily rhythms of hypothalamic mRNA expression in goldfish

2.2.1 Materials and methods

Experimental animals

Goldfish (*Carassius auratus*) were purchased from Ozark Fisheries (Martinsville, IN, USA). A mixture of 88 males and females ranging in size from 12 to 20 g were housed in groups of 8 individuals in each of 11 aquaria (15 gal) in fresh water that was continuously filtered and aerated at 20°C. Fish were fed daily at 12:00 with an approximately 2% wet body weight (BW) ration of commercially prepared trout pellets (Corey Aquafeeds, Fredericton, NB, Canada). Fish were acclimated to this feeding schedule and either a simulated photoperiod of 16 h light:8 h dark (16L:8D) with lights on at 06:00 and lights off at 22:00, or constant light (LL) conditions for 2 weeks prior to the experiment (prior to LL conditions, fish had been held under a photoperiod of 16L:8D). All experiments were conducted according to the principles published in the Canadian Council on Animal Care guidelines on the Care and Use of Fish in Research, Teaching and Testing.

Experimental design

To determine circadian mRNA expression profiles, goldfish were sampled at different time points over the course of one day. Up until the day of sampling, all fish were fed at their regularly scheduled feeding time. On the day of sampling, 56 fish in 7 tanks were fed as scheduled and 32 fish in 4 tanks were not fed. This was necessary to determine if the act of feeding had any effect on mRNA expression. Fish were sampled and whole brains excised at seven sampling times: 02:00, 07:00, 10:00, 12:00, 14:00, 17:00 and 21:00. Sampling began at 02:00. 8 fish were sampled at each of 02:00, 07:00 and 10:00 (n

= 8 for each group). At 12:00 half of the fish were offered food and half were left unfed. Sampling began 5 minutes after offering the food, to allow the fed group to eat. Fish were sampled from both fed and unfed tanks (n = 8 for each group) at 12:00 and upon subsequent sampling intervals at 14:00, 17:00 and 21:00. At each sampling time, individuals were randomly collected from different tanks to eliminate tank bias. Sampling at night was conducted with the aid of red flashlights.

The same experiment was carried out with fish that were maintained at the two different photoperiods.

Sampling, RNA extraction and cDNA synthesis

At each sampling interval, goldfish were anaesthetized in a concentrated solution of tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) and killed by spinal section. Brains were excised and placed in RNA Later solution (Qiagen, Mississauga, ON, Canada) and frozen at -20°C for further processing. Hypothalami were subsequently dissected from the brains and total RNA was extracted using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. 1 μL of RNA grade glycogen (20 mg/mL; Fermentas, Canada) was added after homogenization of tissue to act as an inert carrier of nucleic acids to facilitate the recovery of RNA from small pieces of brain tissue. Quality and quantity of total RNA were assessed using a NanoDrop spectrophotometer (ND-2000; Thermo Scientific, Wilmington, DE, USA). Quality was assessed by determining the ratio of sample absorbance measured at 260 nm and 280 nm with only samples with ratios between 1.8 and 2.0 being used for further processing. Concentration of total RNA was determined by

the absorbance reading measured at 260 nm. cDNA was synthesized from 1 μg total RNA using a QuantiTect Reverse Transcription kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol.

Brain and tissue distribution by Reverse transcription PCR (RT-PCR)

To determine which goldfish tissues transcribe Clock and Per1, brain and tissue distribution studies were performed. Various brain and peripheral tissue samples were obtained from a single goldfish and total RNA was extracted and reverse transcribed to cDNA using the procedures described above. cDNA samples were submitted to a PCR using specific primers for Clock, Per1 and Elongation factor 1α (EF1α; Table 2.1), designed from cloned sequences of goldfish Clock (GenBank accession # HM161712), or goldfish Per1 (GenBank accession #EF690698) and goldfish EF1α (GenBank accession # AB056104) using Vector NTI Advance 10 software (Invitrogen, Carlsbad, CA, USA). EF1α was used as an internal control transcript. Samples were subsequently submitted to electrophoresis on a 1.2% agarose gel stained with ethidium bromide and visualized using an Epichemi Darkroom BioImaging system (UVP, Upland, CA, USA) equipped with a 12-bit cooled camera. Image processing and analysis were performed using LabWorks 4.0 software (UVP, Upland, CA, USA).

Quantitative real-time RT-PCR (qPCR)

The hypothalamus of the brain was used for mRNA quantification studies as this region has been shown to express all four genes of interest [Clock: Fig. 2.1A, Per1: Fig. 2.1A, OX (Huesa *et al.*, 2005) and NPY(Peng *et al.*, 1994)].

Specific primers were designed from cloned sequences of goldfish Clock (GenBank accession # HM161712), goldfish Per1 (GenBank accession # EF690698), goldfish preproorexin (GenBank accession # DQ923590), goldfish NPY (GenBank accession # M87297) and goldfish EF1α (GenBank accession # AB056104) using Vector NTI Advance 10 software and are listed in Table 2.1.

1 μg of each cDNA sample was diluted 1:2 in RNAase-free water and submitted to a SYBR green PCR using specific primers (Table 2.1). All PCRs were prepared using an epMotion 5070 automated pipetting system (Eppendorf, Mississauga, ON, Canada) with a final reaction volume of 10 μL, which included 2 μL of diluted sample cDNA, 5 μL of master mix from a SYBR green QuantiFast PCR kit (Qiagen, Mississauga, ON, Canada), 0.2 μL of each sense and antisense primer (final concentration of 1 μM for each) and 2.6 μL of RNAase-free water. All reactions were performed using a Mastercycler ep realplex 2S system (Eppendorf, Mississauga, ON, Canada). Initial validation PCRs were performed for each primer pair to determine optimal primer annealing temperature and to ensure that PCRs were reproducible (R^2 coefficient > 0.98) and that each gene of interest had equivalent PCR efficiencies (calculated using the formula: $E = 10^{(-1/slope)} - 1$): Clock = 1.04, Per1 = 0.94, OX = 0.99, NPY = 1.01 and EF1α at 58°C = 0.98 or at 60°C = 0.95. After validation, cDNA samples from each sampling time and group (fed or unfed; n = 5 to 7 at each sampling time for both fed and unfed) were submitted to qPCR on 96 well plates to target each of the four genes of interest under the following conditions: 95°C for 4 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds for OX and NPY annealing or 58°C for 15 seconds for Clock and Per1 annealing, followed by 68°C

for 20 seconds. Each cDNA sample was run in duplicate with a no-template control (in which cDNA sample was replaced with water) included on each plate. Experiments were almost always performed at least twice.

mRNA expression analysis was performed using Mastercycler ep realplex 1.5 software (Eppendorf, Mississauga, ON, Canada). The ΔΔCt, or relative cycle threshold (Ct), method was used to compare mRNA expression levels relative to a reference housekeeping gene (EF1α). Briefly, each sample was analyzed using the formula:

$$\Delta\Delta Ct \text{ sample} = (\Delta Ct \text{ transcript of interest} - \Delta Ct \text{ EF1}\alpha) - \Delta Ct \text{ calibrator}$$

where the ΔCt for each sample was calculated by subtracting the average Ct of the reference housekeeping gene for the sample from the average Ct of the transcript of interest (*i.e.*, Clock, Per1, OX or NPY) for the same sample. The ΔΔCt for each sample was then calculated by subtracting the ΔCt of a calibrator sample (chosen to be a 07:00 sample) from the ΔCt of each sample. The resulting number represents the amount of mRNA of the gene of interest relative to the calibrator and normalized by EF1α.

Statistical analysis

Statistical tests were performed using GraphPad Prism 5.02 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as mean ± SEM. One-way Analysis of Variance (ANOVA) with Tukey's multiple comparison post-hoc analysis was used to determine differences in mRNA expression levels over time for 1) fed, 2) unfed, 3) 16L:8D and 4) LL groups. Two-way ANOVA with Bonferroni's post-hoc analysis was used to determine how mRNA expression levels were affected by two factors: 1)

circadian time and photoperiod and 2) circadian time and feeding status. Results were considered significant at $p < 0.05$.

Table 2.1 Sequences of primers used for qualitative and quantitative RT-PCR analysis in goldfish daily mRNA expression profiles study

Primer	Sequence
Clock	
clock-F	5'-*TTCTTAGATCACAGGGCCTCTC*-3'
clock-R	5'-*CTATCATGTGGACGACCTGGAG*-3'
Period 1	
per1-F	5'-*AAACAGAGTAGCTTAGAAGCCCTG*-3'
per1-R	5'-*GTCCAGTCCACCCAGGAAAGAGCT*-3'
Orexin-A	
OX-F	5'-*ACTGCACAGCCAAGAGAGTTC*-3'
OX-R	5'-*ATAATTTGCAGGACCTTGACG*-3'
NPY	
NPY-F	5'-*CATCAACCTCATAACAAGGCA*-3'
NPY-R	5'-*CAGACAAGATATGAGGACCAGTT*-3'
Elongation factor 1α (internal control)	
EF-F	5'-*GAAGAACGTGTCTGTCAAGG*-3'
EF-R	5'-*GTTCAGGATGATGACCTGAG*-3'

2.2.2 Results

2.2.2.1 Clock and Per1 mRNA expression

Brain and tissue distribution by RT-PCR

Clock and Per1 mRNA expression were localized in various brain regions and peripheral tissues of the goldfish using RT-PCR analysis to amplify Clock and Per1 mRNA (Fig. 2.1). The amplified fragments of Clock and Per1 were 95 bp and 97 bp, respectively. In the brain, both Clock and Per1 were found in all regions examined. In the periphery, Clock was found in all tissues examined with higher expression in eye, pituitary, skin, heart and gonads, while Per1 was found to be highly expressed in all tissues examined with lowest expression in muscle. RT-PCR was also used to amplify EF-1α (control gene) in each of the samples and produced fragments of expected size (108 bp). Control reactions (with no DNA template) showed no RT-PCR products, verifying a lack of contamination.

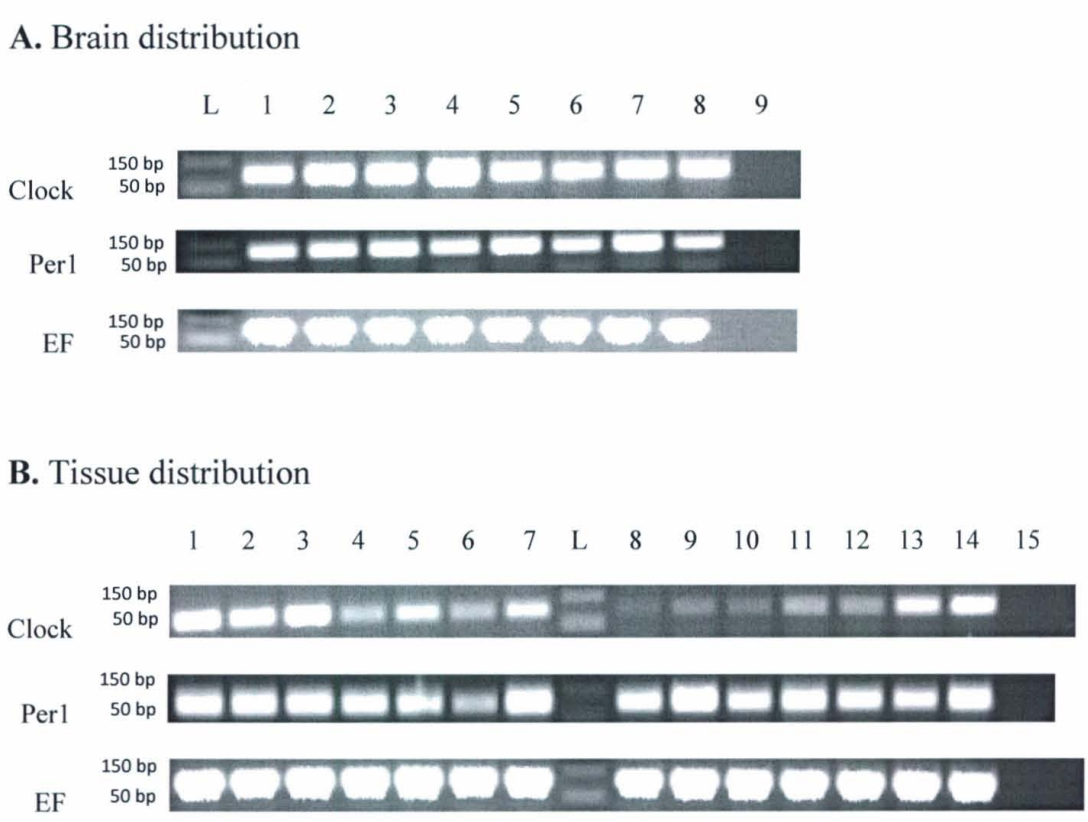


Figure 2.1 RT-PCR distribution of Clock (95 bp), Per1 (97 bp) and EF (108 bp) transcripts in different brain regions of the goldfish (A): L, Ladder (sizes of fragments to left of figures); 1, olfactory bulb; 2, telencephalon; 3, optic tectum; 4, hypothalamus; 5, cerebellum; 6, vagal lobes; 7, thalamus; 8, spinal cord; 9, control; and in different peripheral tissues of the goldfish (B): 1, eye; 2, whole brain; 3, pituitary; 4, gill; 5, skin; 6, muscle; 7, heart; L, Ladder (sizes of fragments to left of figures); 8, spleen; 9, kidney; 10, liver; 11, foregut; 12, midgut; 13, testis; 14, ovary; 15, control. Samples were visualized by electrophoresis on 1.2% agarose gel stained with ethidium bromide.

Quantitative real-time RT-PCR

Clock

Under a 16L:8D photoperiod, goldfish showed robust daily variations in hypothalamic Clock mRNA expression levels over time (Fed fish: $F(6, 25) = 10.52$, $p = 0.000008$, solid line in Fig. 2.2A; Unfed fish: $F(6, 24) = 21.07$, $p < 0.000001$, solid line in Fig. 2.2B) with lowest levels observed in the early morning and a peak in expression observed at 17:00 (see Appendix 1 for post hoc analysis). Under LL, Clock mRNA expression varied over time (Fed fish: $F(6, 32) = 7.923$, $p = 0.000028$, dashed line in Fig. 2.2A; Unfed fish: $F(6, 32) = 7.209$, $p = 0.000063$, dashed line in Fig. 2.2B) with lowest expression observed at 07:00 and 10:00 (see Appendix 1 for post hoc analysis). There was a significant interaction effect between photoperiod and time on Clock mRNA expression (Fed fish: $F(6, 57) = 9.22$, $p < 0.000001$, Fig. 2.2 A; Unfed fish: $F(6, 56) = 13.77$, $p < 0.000001$, Fig. 2.2B) as the expression profile evident under a 16L:8D photoperiod was abolished under LL. No significant differences were observed between fed and unfed fish at either photoperiod (16L:8D fish: $F(3, 29) = 0.61$, $p = 0.61$; LL fish: $F(3, 34) = 0.94$, $p = 0.43$).

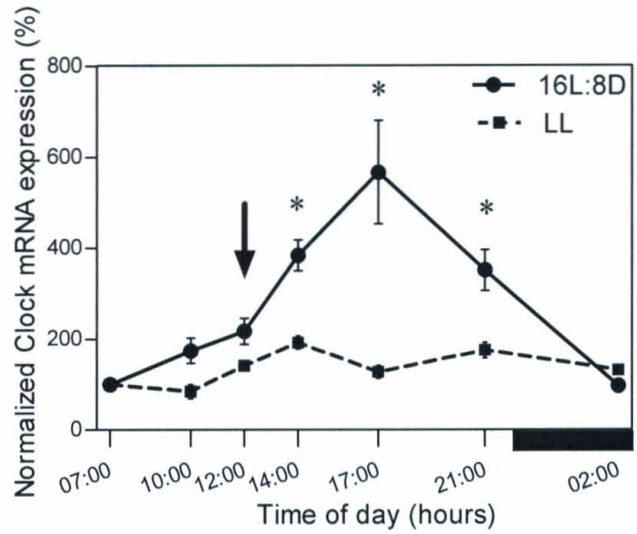
Per1

Goldfish held under a 16L:8D photoperiod showed significant daily variations in hypothalamic Per1 mRNA expression levels (Fed fish: $F(6, 34) = 6.93$, $p = 0.000072$, solid line in Fig. 2.3A; Unfed fish: $F(6, 34) = 4.99$, $p = 0.0009$, solid line in Fig. 2.3B) with highest levels observed in the morning and a trough in expression observed in the evening (see Appendix 2 for post hoc analysis). This mRNA expression profile appears to be in antiphase of the Clock mRNA expression profile. Goldfish held under LL showed

significant variations in Per1 mRNA expression over time only in unfed fish (Fed fish: $F(6, 31) = 2.39$, $p = 0.052$, dashed line in Fig. 2.3A; Unfed fish: $F(6, 34) = 3.497$, $p = 0.0084$, dashed line in Fig. 2.3B; see Appendix 2 for post hoc analysis). There was a significant interaction effect between photoperiod and time on Per1 mRNA expression levels (Fed fish: $F(6, 65) = 3.23$, $p = 0.0078$, Fig. 2.3A; Unfed fish: $F(6, 68) = 2.8$, $p = 0.017$, Fig. 2.3B) with generally higher levels of expression seen in LL. In fish held under 16L:8D, there was a significant interaction effect between feeding status and time where higher Per1 mRNA expression was observed in fed fish at the regularly scheduled feeding time (12:00) than in unfed fish at this time (16L:8D fish: $F(3, 36) = 17.76$, $p < 0.0001$, Fig. 2.3C; LL fish: $F(3,33) = 2.32$, $p = 0.094$).

Figure 2.2 Daily hypothalamic Clock mRNA expression profiles in goldfish. Clock mRNA expression is compared between fish held under a 16L:8D photoperiod and constant light (LL) in fed (A) and unfed (B) fish. mRNA expression levels are expressed as a percentage normalized to the 07:00 group (n = 5 to 6 at each sampling time for fed and unfed groups at both photoperiods). Data are presented as mean ± SEM. Stars (*) indicate significant differences between 16L:8D and LL at a given time point ($p < 0.05$) as analyzed by Two-way ANOVA with Bonferroni post-hoc analysis. The black bar directly below the x axis indicates the dark phase of the 16L:8D photoperiod. The black arrow indicates the regularly scheduled feeding time (12:00).

A. Fed goldfish



B. Unfed goldfish

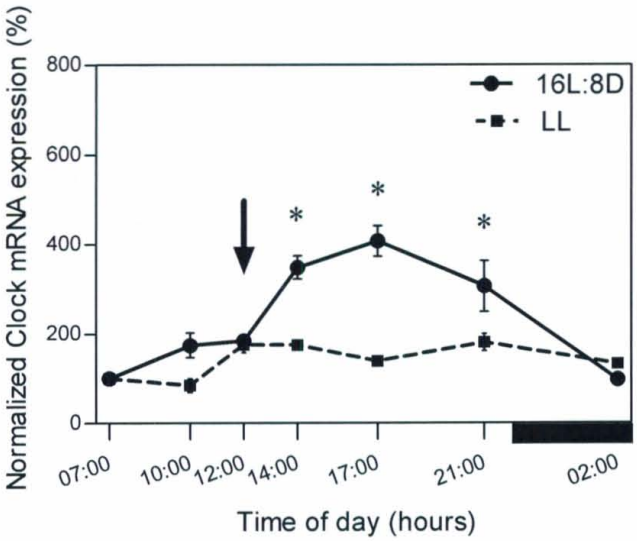
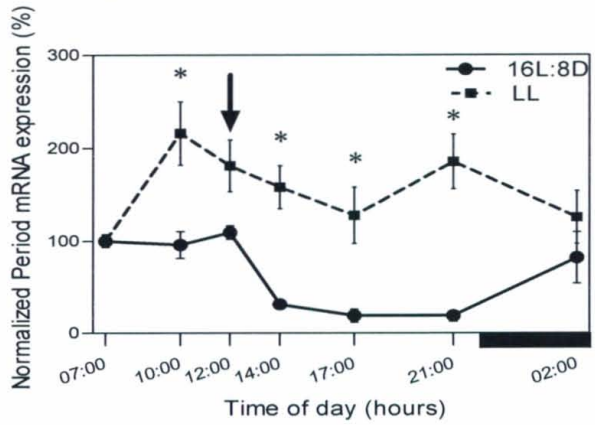
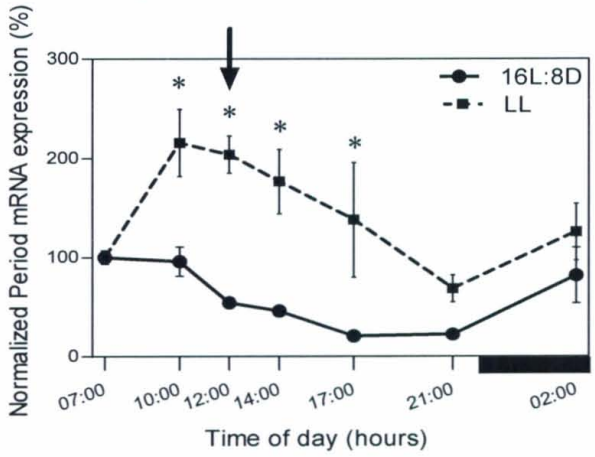


Figure 2.3 Daily hypothalamic Per1 mRNA expression profiles in goldfish. Per1 mRNA expression is compared between fish held under a 16L:8D photoperiod and constant light (LL) in (A) fed and (B) unfed fish and (C) Per1 mRNA expression is compared between fed and unfed fish held under a 16L:8D photoperiod. Per1 mRNA expression levels are expressed as a percentage normalized to the 07:00 group (n = 5 to 7 at each sampling time for fed and unfed groups at both photoperiods). Data are presented as mean ± SEM. Stars (*) indicate significant differences ($p < 0.05$) between (A,B) photoperiods or (C) fed and unfed fish at a given time point as analyzed by Two-way ANOVA with Bonferroni post-hoc tests. The black bar directly below the x axis indicates the dark phase of the 16L:8D photoperiod. The black arrow indicates the regularly scheduled feeding time (12:00).

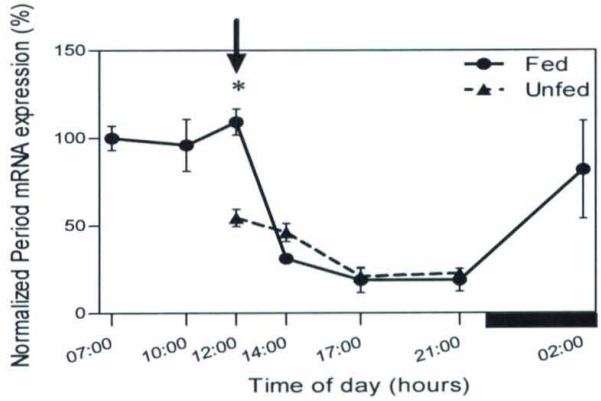
A. Fed goldfish



B. Unfed goldfish



C. 16L:8D goldfish



2.2.2.2 OX and NPY mRNA expression

Quantitative real-time RT-PCR

OX

Under a 16L:8D photoperiod, fed goldfish showed daily variations in hypothalamic OX mRNA expression levels ($F(6, 32) = 2.492$, $p = 0.0431$, solid line in Fig. 2.4A) with peaks observed just prior to feeding time at 10:00 and again, prior to the onset of darkness at 21:00 (see Appendix 3 for post hoc analysis). The pattern of mRNA expression was similar in unfed goldfish held under the same photoperiod, but expression did not decrease as sharply after peaking at 10:00 and daily variations did not reach statistical significance ($F(6, 30) = 2.14$, $p = 0.078$, solid line in Fig. 2.4B). Under LL, neither fed nor unfed goldfish showed daily variations in OX mRNA expression (Fed fish: $F(6, 32) = 1.23$, $p = 0.32$, dashed line in Fig. 2.4A; Unfed fish: $F(6, 33) = 1.052$, $p = 0.41$, dashed line in Fig. 2.4B). A significant interaction effect between photoperiod and time was seen in fed goldfish (Fed fish: $F(6, 64) = 2.61$, $p = 0.025$, Fig. 2.4A; Unfed fish: $F(6, 63) = 1.61$, $p = 0.16$), such that prior to feeding (at 10:00), OX mRNA expression was significantly lower in fish held under LL. No significant differences were observed between fed and unfed fish at any time point under either photoperiod (16L:8D fish: $F(3, 36) = 0.32$, $p = 0.81$; LL fish: $F(3, 35) = 1.09$, $p = 0.36$).

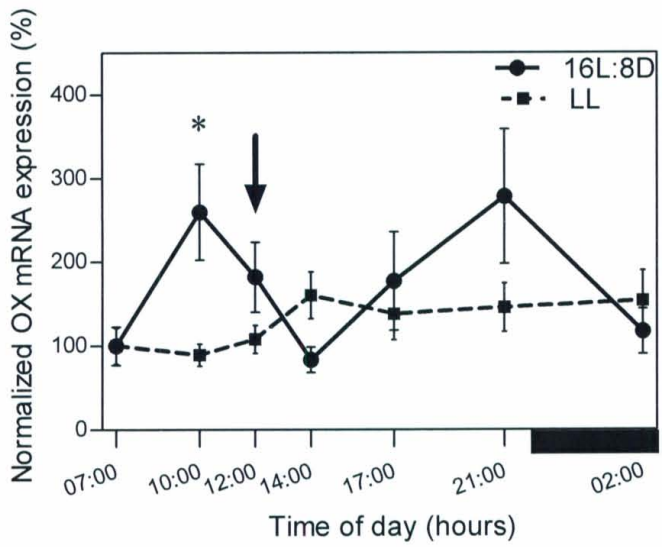
NPY

Neither fed nor unfed goldfish held under 16L:8D showed significant daily variations in hypothalamic NPY mRNA expression (Fed fish: $F(6, 31) = 1.69$, $p = 0.16$, solid line in Fig. 2.5A; Unfed fish: $F(6, 32) = 0.98$, $p = 0.45$, solid line in Fig. 2.5B). Fish held under

LL showed significant daily variations (Fed fish: $F(6, 32) = 2.78$, p 0.027, dashed line in Fig. 2.5A; Unfed fish: $F(6, 33) = 3.29$, $p = 0.012$, dashed line in Fig. 2.5B) with a peak in NPY mRNA expression observed prior to feeding time at 10:00 (see Appendix 4 for post hoc analysis). There was a significant interaction effect between photoperiod and time (Fed fish: $F(6, 60) = 2.67$, $p = 0.023$, Fig. 2.5A; Unfed fish $F(6, 62) = 3.63$, $p = 0.0037$, Fig. 2.5B) with a significantly higher peak in expression at 10:00 in fish held under LL. No significant differences were observed between fed and unfed fish at any time point under either photoperiod (16L:8D fish: $F(3, 35) = 1.35$, $p = 0.27$; LL fish: $F(3, 35) = 0.16$, $p = 0.92$).

Figure 2.4 Daily hypothalamic OX mRNA expression profiles in goldfish. OX mRNA expression is compared between fish held under a 16L:8D photoperiod and LL in fed (A) and unfed (B) fish. mRNA expression levels are expressed as a percentage normalized to the 07:00 group (n = 5 to 6 at each sampling time for fed and unfed groups at both photoperiods). Data are presented as mean ± SEM. Stars (*) indicate significant differences ($p < 0.05$) between 16L:8D and LL at a given time point, as analyzed by Two-way ANOVA with Bonferroni post-hoc tests. The black bar directly below the x-axis indicates the dark phase of the 16L:8D photoperiod. The black arrow indicates the regularly scheduled feeding time (12:00).

A. Fed goldfish



B. Unfed goldfish

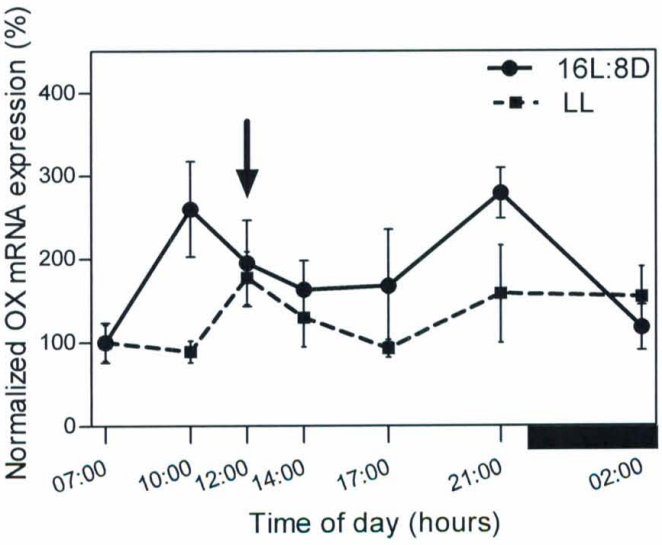
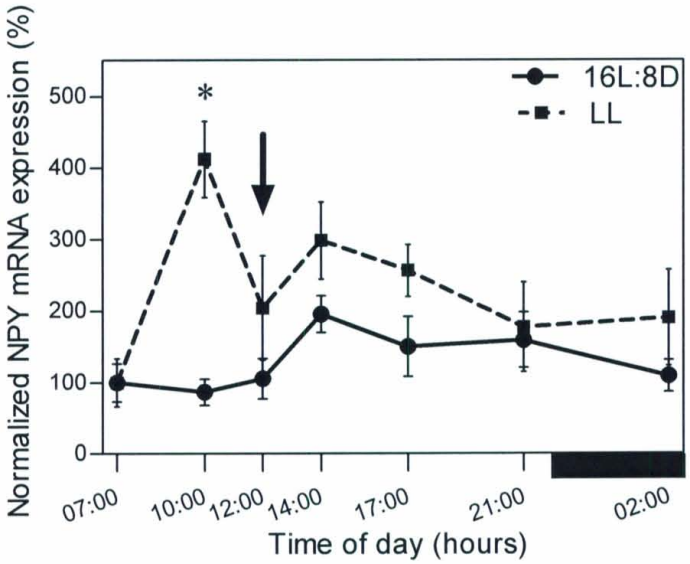
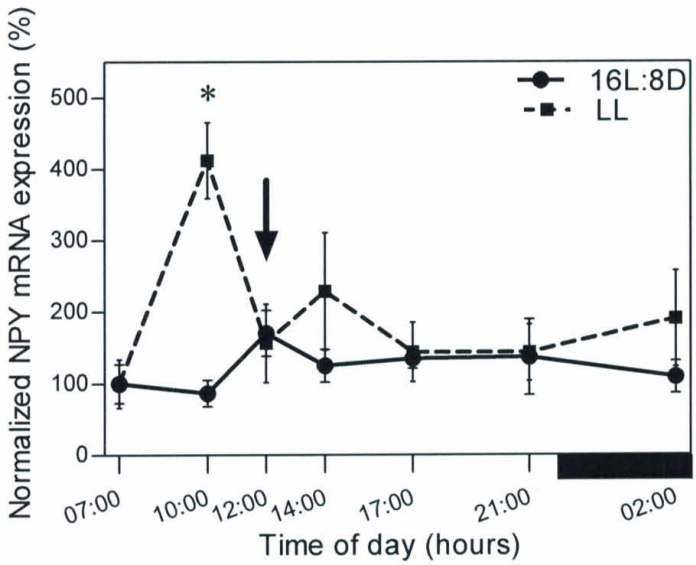


Figure 2.5 Daily hypothalamic NPY mRNA expression profiles in goldfish. NPY mRNA expression is compared between fish held under a 16L:8D photoperiod and LL in fed (A) and unfed (B) fish. NPY mRNA expression levels are expressed as a percentage normalized to the 07:00 group ($n = 5$ to 6 at each sampling time for fed and unfed groups at both photoperiods). Data are presented as mean ± SEM. Stars (*) indicate significant differences ($p < 0.05$) between 16L:8D and LL at a given time point, as analyzed by Two-way ANOVA with Bonferroni post-hoc tests. The black bar directly below the x-axis indicates the dark phase of the 16L:8D photoperiod. The black arrow indicates the regularly scheduled feeding time (12:00).

A. Fed goldfish



B. Unfed goldfish



2.2.3 Discussion

This study examined the daily patterns in mRNA expressions of circadian regulatory peptides and important appetite-related neuropeptides in the goldfish hypothalamus. Transcripts of the circadian regulatory proteins, Clock and Per1, cycled rhythmically in antiphase of each other providing evidence of circadian regulation in this species. Transcript levels of OX and NPY, important neuropeptides involved in feeding and wakefulness/activity, fluctuated over the day and night and often varied under different photoperiods and in response to the act of feeding.

Daily profiles of circadian regulatory proteins

This study shows that both Clock and Per1 mRNAs are expressed throughout the goldfish brain and peripheral tissues in all regions and tissues examined. This is consistent with previous studies in mammals (Yamamoto *et al.*, 2001; Balsalobre, 2002) and other fish species, including zebrafish and Atlantic salmon (Whitmore *et al.*, 1998; Wang, 2008a; Davie *et al.*, 2009) which show that components of the molecular circadian clock are expressed throughout the whole brain and body.

This study also demonstrates, for the first time in goldfish, rhythmic hypothalamic mRNA expression of Clock and Per1 under a light-dark cycle. These rhythms occur in antiphase of one another such that while Clock transcription reaches a peak in the early evening, Per1 is at a trough of mRNA expression. Whereas Clock is constitutively transcribed in mammals (Oishi *et al.*, 1998), birds (Yoshimura *et al.*, 2000) and frogs (Zhu *et al.*, 2000), antiphasic transcription of Clock and Per has been shown to occur in zebrafish

(Cermakian *et al.*, 2000; Cahill, 2002) and *Drosophila* (Bae *et al.*, 1998). This study provides further evidence that antiphasic transcription cycles of Clock and Per may be common to the teleost mode of circadian regulation.

Clock mRNA expression peaked in the early evening with levels decreasing overnight and remaining low throughout the early morning, which is similar to the pattern observed in zebrafish brain (Whitmore *et al.*, 1998), but different from the pattern observed in Atlantic salmon brain, in which Clock mRNA expression is highest during the dark phase (Davie *et al.*, 2009). Species-specific differences or procedural differences between experiments, such as previous history of animals (*e.g.* entrainment to a specific photoperiod), tissue examined (*e.g.* using whole brain *versus* specific brain regions has the possibility of diluting differential fluctuations in distinct regions) and method of expression analysis often encumber any direct comparisons among these types of studies and might explain inconsistencies between the present study and previous studies on fish.

Hypothalamic Per1 expression in this study showed highest levels in the early morning hours with levels declining in the early afternoon before increasing again after lights off. This is consistent with previous observations where Per1 mRNA was rhythmically expressed in goldfish retina, with a peak at midnight (Velarde *et al.*, 2009), and is similar to the rhythmic Per1 mRNA expression profile observed in a zebrafish embryo-derived cell line (Pando *et al.*, 2001). This profile contrasts, however, with that of the golden rabbitfish where Per1 rhythmic mRNA expression peaks near dusk in the whole brain, retina, liver and pineal gland (Park *et al.*, 2007). As for Clock mRNA profiles, Per1

mRNA profiles appear to be species-specific and might differ from one experiment to another due to different methodologies.

The feeding status of fish (whether fish were fed or unfed) had no effect on Clock mRNA expression at any given sampling interval. On the other hand, Per1 mRNA expression was significantly higher at feeding time in fed fish as compared to those who were not offered food, which differs from Per1 mRNA expression in zebrafish brain, which entrains to a light-dark cycle, regardless of fasting or feeding schedule (Sanchez and Sanchez-Vazquez, 2009). In the present study, the actual presence and consumption of food appears to be responsible for the upregulation of Per1 transcription. Similarly, previous studies in mammals indicate that Per expression is affected by feeding status (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Kawamoto *et al.*, 2006; Minana-Solis *et al.*, 2009) perhaps by changing the redox state of cells which affects the binding ability of the Clock-Bmal1 [Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like] heterodimer and its ability to initiate transcription of its target genes, including Per (Li and Li, 2004). This suggests a possible mechanism for food-modulated transcriptional regulation of Per1 in goldfish.

Interestingly, the more pronounced effects on mRNA expression profiles in this study were observed when fish were held under constant light. The presence of light-dark cycles appears to be necessary to sustain oscillating Clock transcription in the goldfish hypothalamus, as this rhythmic pattern was abolished under constant light. It has been suggested that in response to a change in photoperiod, the rhythmic transcription of circadian genes within individual cells becomes desynchronized and must entrain to the

new photoperiod (Abe *et al.*, 2002; Carr and Whitmore, 2005), which may be the case for Clock transcription in the goldfish hypothalamus. It is possible that extended duration of light exposure, and not constant light *per se*, is responsible for arrhythmic Clock mRNA expression observed in the present study, as Clock mRNA expression is rhythmic when Atlantic salmon (Davie *et al.*, 2009) and hamsters (Tournier *et al.*, 2003) are held under a short day photoperiod (8L:16D), but is arrhythmic when animals are held under a long day photoperiod (16L:8D). Further studies using different photoperiods are necessary to determine if this is the case for goldfish.

Exposure to constant light also affected the rhythmic transcription of Per1. Fluctuations similar to that observed under a light-dark cycle - with respect to increments and decrements in expression levels – were seen in unfed fish but not in fed fish. These results are consistent with studies showing that in the whole brain of golden rabbitfish and embryo-derived cell lines and whole brain of zebrafish, Per1 transcription rhythms degenerate and undergo a gradual desynchronization under continuous lighting (Carr and Whitmore, 2005; Park *et al.*, 2007; Sanchez and Sanchez-Vazquez, 2009), which may explain why significant variations in Per1 mRNA expression were observed in some fish but not others.

Interestingly, Per1 mRNA expression was significantly elevated during the subjective day in both fed and unfed goldfish held under constant light, as compared to under a light-dark cycle. This would be explained by an induction of Per1 transcription by light, as suggested in mammals (Takumi *et al.*, 1998; Daan *et al.*, 2001). Another explanation may be associated with rest deprivation which may occur in the absence of regular day-night

cycles, as Per1 mRNA expression has been shown to increase in response to sleep deprivation in mice (Franken and Dijk, 2009). Although fish do not exhibit the characteristic stages of mammalian sleep, many fishes do exhibit distinct stages of activity and rest periods (Zhdanova, 2006), which may be disrupted in constant light, leading to rest deprivation and, in turn, elevated Per1 expression.

Daily profiles of appetite-related neuropeptides

Under a light-dark cycle, hypothalamic OX mRNA expression fluctuated significantly over the day with lowest levels observed during the night. This overall daily expression profile is similar to that seen in mammals, where highest values occur during activity and lowest values during the rest period (Yoshida *et al.*, 2001; Martinez *et al.*, 2002; Lee *et al.*, 2005; Mileykovskiy *et al.*, 2005), and is consistent with the wake-promoting actions of this neuropeptide. A peak in expression was seen two hours prior to feeding and returned to baseline values by two hours after feeding time. As OX stimulates feeding, has been linked to FAA in mice (Yoshida *et al.*, 2001; Akiyama *et al.*, 2004) and shows periprandial fluctuations in mRNA expression in Atlantic cod (Xu and Volkoff, 2007), this preprandial peak might be indicative of an increased appetite that would be expected prior to the daily meal.

OX mRNA expression peaked again one hour before lights off and decreased to baseline values overnight. This second peak in OX mRNA expression is surprising as one might expect a decrease in OX expression in preparation for rest. Recent studies in zebrafish (Yokogawa *et al.*, 2007; Appelbaum *et al.*, 2009) have suggested a dual role model in which OX would promote both wakefulness *and* sleep-consolidation: wakefulness would

be due to the activation of wake-promoting brain regions by OX neurons, while sleep consolidation may be due to an interaction between OX and melatonin, an important sleep-inducing hormone secreted from the pineal gland (Falcon *et al.*, 2010). As OX neurons have been shown to innervate the pineal gland and induce the secretion of melatonin in zebrafish (Appelbaum *et al.*, 2009), the nightime secretion of melatonin could be preceded by an increase in OX levels, as in the present study in which OX mRNA expression peaks just before lights out.

As with hypothalamic Clock mRNA expression, the pattern of OX mRNA expression was abolished when goldfish were exposed to constant light, which differs from previous studies in rats where daily fluctuations of OX levels in the cerebrospinal fluid persist in constant light or constant dark (Deboer *et al.*, 2004; Zhang *et al.*, 2004). It is unlikely that in the absence of a light-dark cycle, altered feeding and locomotor patterns would lead to decreased constitutive levels of OX mRNA expression, as fish were fed at the same time and daily food intake (Lopez-Olmeda *et al.*, 2006) and locomotor activity (Iigo and Tabata, 1996) do not change between goldfish held under light-dark photoperiods and constant light. Similar to the rhythm of Clock mRNA expression, it is probable that exposure to constant light desynchronizes the regular OX mRNA expression pattern such that no peaks are observed.

No differences in OX mRNA expression were observed between fed and unfed fish at any given sampling interval, under either photoperiod, suggesting that the act of feeding does not play a significant role in the short-term regulation of OX transcription. A previous study observed that hypothalamic OX mRNA expression increases in goldfish that have

been fasted for two or six days (Nakamachi *et al.*, 2006), but it is probable that a 24 hour fast, as in the present study, is not sufficient to upregulate OX mRNA expression in this species.

The present study shows that hypothalamic NPY mRNA expression did not vary significantly over the day in a 16L:8D photoperiod and no significant differences were observed between fed and unfed fish at any given sampling time. Both fed and unfed fish displayed an increasing trend in expression in the hours prior to mealtime which is consistent with a previous study showing that hypothalamic NPY mRNA expression in goldfish increases three hours before a scheduled meal (Narnaware *et al.*, 2000). However, the previous study observed a decrease in expression after mealtime unless the meal was withheld, in which case expression continued to increase. This was not observed in fed and unfed fish in the present study for reasons which are unclear, but could reflect differences in photoperiod used or time of feeding.

Hypothalamic NPY mRNA expression did not peak at the transition from light to dark and dark to light, as has been observed in rats (Calza *et al.*, 1990; Shinohara *et al.*, 1993). Exposure to constant light had a dramatic effect on NPY mRNA expression, with a significant peak observed two hours prior to feeding time, which may be indicative of increased appetite in anticipation of the daily meal. This is consistent with a previous study in which goldfish held under constant light exhibited FAA and a preprandial rise in NPY mRNA expression (Vera *et al.*, 2007). However, when fish in the previous study were fed at random, they did not exhibit any preprandial changes in activity or NPY mRNA expression, suggesting that these rhythms were entrained to a regularly scheduled

daily meal. Likewise, in the present study, a scheduled meal appears to entrain hypothalamic NPY mRNA expression in the absence of light-dark cues.

2.3 Daily rhythms of hypothalamic mRNA expression in Atlantic cod

2.3.1 Materials and methods

Experimental animals

Juvenile Atlantic cod (*Gadus morhua*) were reared and maintained in the Dr. Joe Brown Aquatic Research Building at the Ocean Sciences Centre (Memorial University of Newfoundland, Logy Bay, Canada). An approximately equal mixture of males and females (total of 220 individuals) with an average body weight of 170 g were maintained in 4 flow-through 1000 L tanks (55 fish per tank) that were continuously aerated at 13°C under a photoperiod of 16L:8D (lights on at 06:00). Only 130 of these fish were sampled, but because Atlantic cod is a social species, tanks were maintained at higher densities to avoid stress. Fish were fed once daily at 10:00 with EWOS Marine Diet pellets at a ration of approximately 1% body weight, a ration close to satiation (Xu and Volkoff, 2007). Fish were acclimated to this photoperiod and feeding schedule for at least two weeks prior to the start of the experiment. All experiments were conducted according to the principles published in the Canadian Council on Animal Care guidelines on the Care and Use of Fish in Research, Teaching and Testing.

Experimental design

To study daily mRNA expression profiles, cod were sampled at different time points over the course of one day. Fish from two of the tanks were fed their usual ration daily, up to and including sampling day, while fish in the other two tanks were fasted for 3 days prior to and including sampling day, to determine if short-term fasting has any effect on mRNA

expression levels. On sampling day, whole brains were excised from fish in both fed and fasted tanks at 07:00 and 09:00 (n = 8 at each sampling time for both fed and fasted groups). At 10:00, fed tanks were offered their usual daily ration of food. Fish were then sampled from both fed and fasted tanks (n = 8 per group) 10 minutes after feeding, as well as in subsequent sampling intervals at 11:00, 13:00, 17:00, 22:00 and 03:00 (the next day; n = 8 at each sampling time per group). At each sampling time, individuals were randomly collected from duplicate tanks (4 fish per tank) to eliminate tank bias. Sampling at night was conducted with the aid of red flashlights.

Sampling, RNA extraction and cDNA synthesis

At each sampling interval, cod were anaesthetized and brains were excised as previously described (Section 2.2.1). Hypothalami were subsequently dissected from the brains and total RNA was extracted and cDNA synthesized as described previously (Section 2.2.1).

Cloning of Per in Atlantic cod

A small fragment of the unknown Per sequence was isolated by performing PCR amplifications using degenerate primers (CPer-F and CPer-R, Table 2.2) designed in regions of high similarity between zebrafish (Genbank accession # BC163543), Japanese medaka (*Oryzias latipes*; GenBank accession # AB383146), goldfish (GenBank accession # EF690698), rainbow trout (GenBank accession # AF228695) and mouse (GenBank accession # AK148202) Per1 sequences using Vector NTI Advance 10 software (Invitrogen, Carlsbad, CA, USA). PCR reactions were carried out in a volume of 25 µL using GoTaq Green Master Mix (Promega, Madison, WI, USA) with 0.8µM each primer. PCR products were submitted to electrophoresis on a 1.2% agarose gel stained with

ethidium bromide and visualized using an Epichemi Darkroom BioImaging system (UVP, Upland, CA, USA) equipped with a 12-bit cooled camera. Image processing and analysis were performed using LabWorks 4.0 software (UVP, Upland, CA, USA). Bands of predicted size (approximately 620 bp) were isolated and purified with the GeneJET™ Gel Extraction Kit (Fermentas, Burlington, ON, Canada), cloned using the pGEM-T Easy vector system (Promega, Madison, WI, USA) and sequenced by the The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON, Canada).

Brain and tissue distribution by RT-PCR

Brain and tissue distribution studies were performed using semi-quantitative RT-PCR analysis to localize Clock and Per2 expression. Tissues were dissected from an additional fed fish and total RNA was extracted and reverse transcribed to cDNA as described previously (Section 2.2.1). cDNA samples were submitted to PCR amplifications using specific primers for Atlantic cod Clock, Per2 and EF1α transcripts (Table 2.2) designed from Atlantic cod Clock (GenBank accession # HM161713), my cloned partial cDNA sequences of Per2 and Atlantic cod EF1α (GenBank accession # CO541952) using Vector NTI Advance 10 software. EF1α was used as an internal control transcript. Samples were subsequently submitted to electrophoresis as previously described (Section 2.2.1).

Quantitative real-time RT-PCR

The hypothalamus of the brain was used for mRNA quantification studies as this region has been shown to transcribe all four genes of interest [Clock and Per2: Fig. 2.7A; OX: Xu and Volkoff (2007); NPY: Kehoe and Volkoff (2007)].

Specific primers were designed from the cloned sequence of Atlantic cod Per2 and from Atlantic cod Clock (GenBank accession # HM161713), preproorexin (GenBank accession # EU096315), NPY (GenBank accession # DQ256082) and EF1α (GenBank accession # CO541952) using Vector NTI Advance 10 software and are listed in Table 2.2.

1 μg of each cDNA sample was diluted 1:2 in RNAase-free water and submitted to a SYBR green PCR using specific primers. Quantitative real-time PCR was performed on cDNA samples as previously described (Section 2.2.1). Initial validation PCRs ensured that each gene of interest had equivalent PCR efficiencies (Clock = 1.06, Per2 = 0.95, OX = 0.96, NPY = 0.98 and EF1α = 1.09). After validation, cDNA samples from each sampling time and group (fed or fasted; n = 5 to 7 for each sampling time per group) were submitted to qPCR on 96 well plates to target each of the four genes of interest under the following conditions: 95°C for 4 minutes followed by 40 cycles of 95°C for 15 seconds, 58°C for 15 seconds and 68°C for 20 seconds. mRNA expression analysis was performed as previously described (Section 2.2.1), where the 10:00 fed group was chosen to be the calibrator.

Statistical analysis

Statistical tests were performed using GraphPad Prism 5.02 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as mean ± SEM. One-way ANOVA with Tukey's multiple comparison post-hoc analysis was used to determine differences in mRNA expression levels over time for 1) fed and 2) fasted groups. Two-way ANOVA with Bonferroni's post-hoc test was used to determine how mRNA expression levels were

affected by two factors: circadian time and feeding status. Results were considered significant at $p < 0.05$.

Table 2.2 Sequences of primers used for cDNA cloning and qualitative and quantitative RT-PCR analysis in Atlantic cod (*Gadus morhua*) daily mRNA expression profiles study

Primer	Sequence
Clock	
cod_clock-F	5'-*TGTCACAAGCACCTCAATGCAG*-3'
cod_clock-R	5'-*GGCTGCAGACGCACTACTACAT*-3'
Period	
Primers for cloning	
CPer-F	5'-*GTCKTSCTTTATCAAYCCMTGGAGC*-3'
CPer-R	5'-*TACTCNTACCAGCAGATCAACTG*-3'
Specific primers for RT-PCR	
Cod_per2-F	5'-*CAGGAGATCTGCAAGGGGCTTCAC*-3'
Cod_per2-R	5'-*TACTCCTATCAGCAGATCAACTGCC*-3'
Orexin-A	
OX-T1	5'-*TCAGTGCTCAAGAGAATCCAA*-3'
OX-T2	5'-*TTCCCTAGAGTGAGGATGCC*-3'
NPY	
C_NPY-F	5'-*GACAAAGGTACGGGAAGAGG*-3'
C_NPY-R	5'-*CAATGACGGGTCATATCTGC*-3'
Elongation factor 1α (internal control)	
EF-C1	5'-*CAACGCCCAGGTCATCATCC*-3'
EF-C2	5'*ACGCTCTTGGGCAGATCCTC*-3'

2.3.2 Results

2.3.2.1 Clock and Per2 mRNA expression

Cloning and sequencing of Per2 in Atlantic cod

Alignment and phylogenetic analyses show the partial Per amino acid sequence obtained from cloning has more identities with teleost Per2 protein, showing 54% identity with fire clownfish (*Amphiprion melanopus*) Per2, 53% identity with goldfish Per2 and 52% identity with both zebrafish Per2 and Somalian cavefish (*Phreatichthys andruzzi*) Per2. The cloned sequence has fewer identities with Per1 protein from higher vertebrate species, showing 40% identity with golden hamster (*Mesocricetus auratus*) Per1 and 39% identity with common marmoset (*Callithrix jacchus*) Per1 (Fig. 2.6).

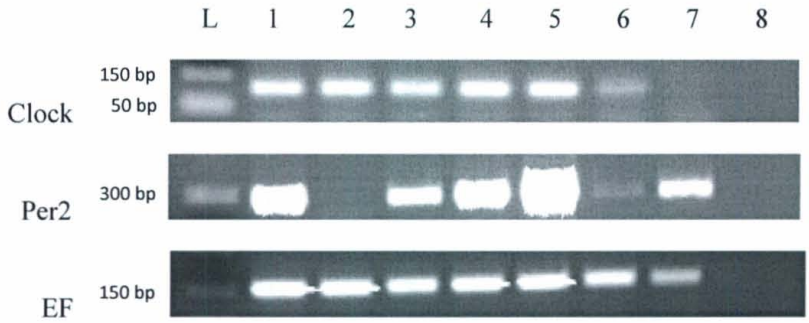
Brain and tissue distribution by RT-PCR

Using semi-quantitative RT-PCR, Clock and Per2 mRNA expression were localized in various brain regions and peripheral tissues of cod (Fig. 2.7). The amplified fragments of Clock and Per2 were 100 bp and 282 bp, respectively. Clock mRNA was present in the olfactory bulbs and all regions of the forebrain and cerebellum, with lower expression in the medulla oblongata and little to none in the spinal cord. Per2 was present in all brain regions examined except for the telencephalon, with highest expression in the cerebellum, olfactory bulbs and hypothalamus and lowest expression in the medulla oblongata. In peripheral tissues, Clock was present in all tissues examined, with highest expression in the pituitary and was also highly expressed in muscle, spleen, ovary and intestine. Per2 was present in all tissues examined except for pyloric cecae, with highest expression seen

in the skin, pituitary and ovary and was also highly expressed in the eye, gill, spleen and liver. Comparatively lower expression was seen in the other peripheral tissues examined. RT-PCR was used to amplify EF-1α (control transcript) in each of the samples and produced fragments of expected size (162 bp). Control reactions (with no DNA template) showed no RT-PCR products, verifying a lack of contamination.

Figure 2.6 Atlantic cod partial Per amino acid sequence alignment with fire clownfish (*Amphiprion melanopus*) Per2 (GenBank accession # ADI59666), Somalian cavefish (*Phreatichthys andruzzi*) Per2 (GenBank accession # ADL62690), zebrafish (*Danio rerio*) Per2 (GenBank accession # AAI63549), goldfish (*Carassius auratus*) Per2 (GenBank accession # ABU93787), golden hamster (*Mesocricetus auratus*) Per1 (GenBank accession # AAQ99158) and common marmoset (*Callithrix jacchus*) Per1 (GenBank accession # XP_002748008).

A. Brain distribution



B. Tissue distribution

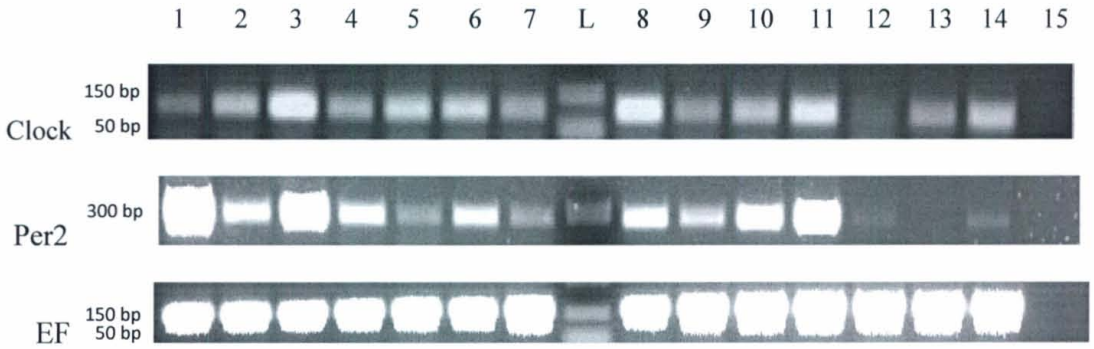


Figure 2.7 RT-PCR distribution of Clock (100 bp), Per2 (282 bp) and EF (162 bp) transcripts in different brain regions of the Atlantic cod (A): L, Ladder (sizes of fragments to left of figures); 1, olfactory bulb; 2, telencephalon; 3, optic tectum; 4, hypothalamus; 5, cerebellum; 6, medulla oblongata; 7, spinal cord; 8, control; and in different peripheral tissues of Atlantic cod (B): 1, skin; 2, eye; 3, pituitary; 4, gill; 5, muscle; 6, spleen; 7, kidney; L, Ladder (sizes of fragments to left of figures); 8, whole brain; 9, heart; 10, liver; 11, ovary; 12, stomach; 13, pyloric cecae; 14, intestine; 15, control. Samples were visualized by electrophoresis on 1.2% agarose gel stained with ethidium bromide.

Quantitative real-time RT-PCR

Clock

Cod showed significant variation in hypothalamic Clock mRNA expression over the day (Fed fish: $F(7, 35) = 2.82$, $p = 0.02$, solid line in Fig. 2.8; Fasted fish: $F(7, 29) = 6.34$, $p = 0.00014$, dashed line in Fig. 2.8) with highest levels in the late afternoon (see Appendix 5 for post hoc analysis). There was a significant interaction effect between feeding status and time, *i.e.*, Clock mRNA expression response to time depends on feeding status, and *vice versa* ($F(7, 64) = 3.16$, $p = 0.0062$, Fig. 2.8). No significant differences were observed between fed and fasted fish at any single time point.

Per2

Hypothalamic Per2 mRNA expression did not vary significantly over the day (Fed fish: $F(7, 35) = 0.35$, $p = 0.93$; Fasted fish: $F(7, 32) = 1.06$, $p = 0.41$, Fig. 2.9). There was no interaction effect between circadian time and feeding status ($F(7, 67) = 0.46$, $p = 0.86$).

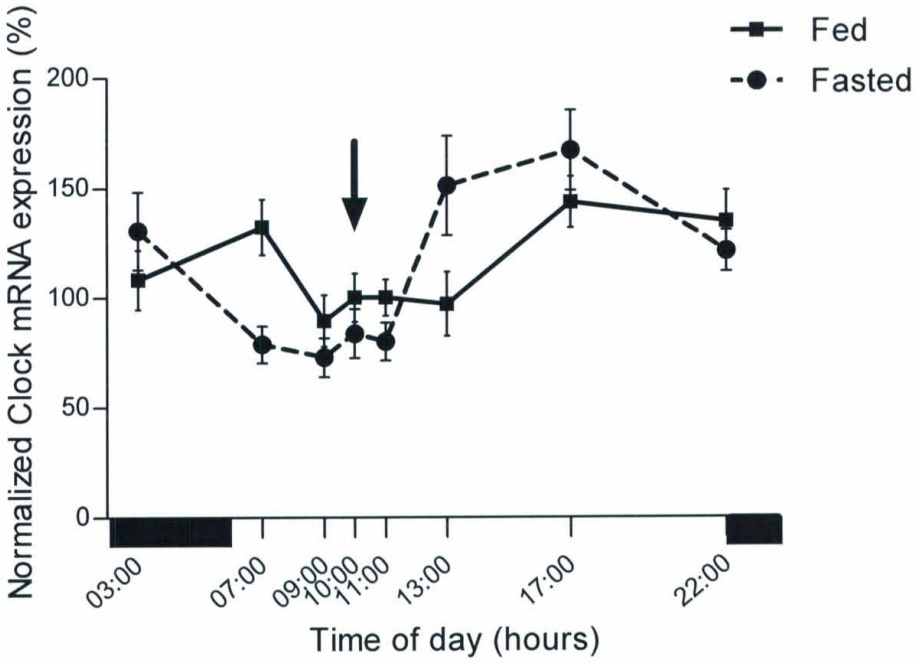


Figure 2.8 Daily hypothalamic Clock mRNA expression profiles in fed and fasted cod. Clock mRNA expression levels are expressed as a percentage normalized to the 10:00 fed group (n = 5 to 6 at each sampling time per group). Data are presented as mean ± SEM. The black bars directly below the x axis indicate the dark phase of the photoperiod. The black arrow indicates the regularly scheduled feeding time (10:00).

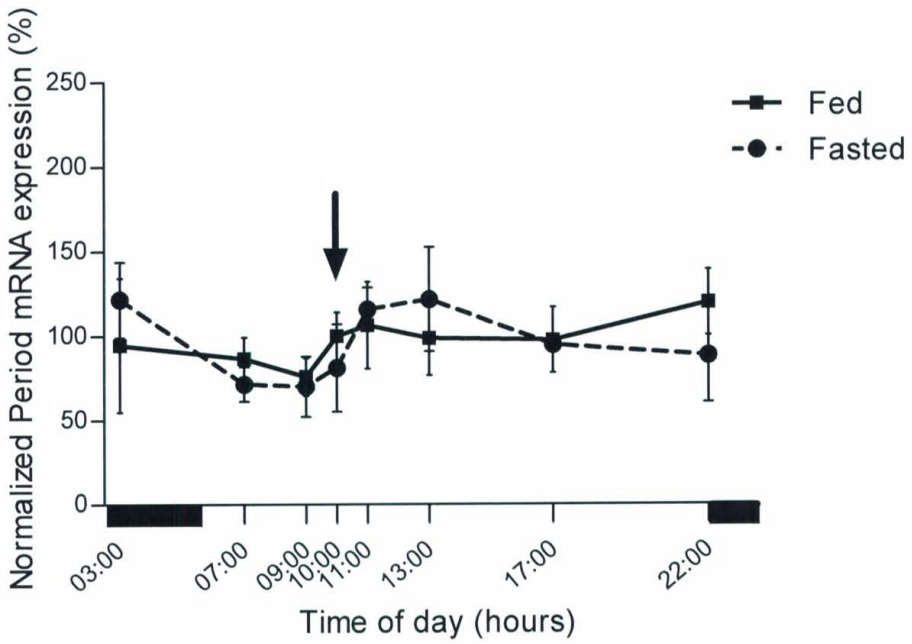


Figure 2.9 Daily hypothalamic Per2 mRNA expression profiles in fed and fasted cod. Per2 mRNA expression levels are expressed as a percentage normalized to the 10:00 fed group (n = 5 to 6 at each sampling time per group). Data are presented as mean ± SEM. The black bars directly below the x axis indicate the dark phase of the photoperiod. The black arrow indicates the regularly scheduled feeding time (10:00).

2.3.2.2 OX and NPY mRNA expression

OX

Cod showed significant variation in OX mRNA expression over the day (Fed fish: $F(7, 36) = 3.32$, $p = 0.0079$, solid line in Fig. 2.10; Fasted fish: $F(7, 40) = 2.59$, $p = 0.027$, dashed line in Fig. 2.10). In fed fish, OX mRNA expression was lowest in the early morning, increased at mealtime and stayed high for the rest of the lights on period (see Appendix 7 for post hoc analysis). In fasted fish, there was a trend for increased OX mRNA expression at 09:00 (prior to mealtime), a decrease at mealtime (10:00) with levels remaining high until lights off (see Appendix 7 for post hoc analysis). There was no significant interaction effect between feeding status and time, *i.e.*, OX mRNA expression response to time did not depend on feeding status, and *vice versa* ($F(7, 76) = 1.55$, $p = 0.16$). Two-way ANOVA analysis shows that the variance in the data comes from separate effects of time ($F(7, 76) = 4.97$, $p = 0.0001$) and feeding status ($F(1, 76) = 11.97$, $p = 0.0009$). No significant differences were observed between fed and fasted groups at any time point.

NPY

Fed cod did not show significant variation in NPY mRNA expression over the day ($F(7, 30) = 2.009$, $p = 0.087$, solid line in Fig. 2.11), but there was a trend for higher expression from lights on to feeding time, with a decrease thereafter. Fasted cod showed significant daily variations in NPY mRNA expression ($F(7, 32) = 2.61$, $p = 0.03$, dashed line in Fig. 2.11) with expression levels decreasing after the usual mealtime (10:00) until 13:00 before rising again. Two-way ANOVA indicated that the interaction effect between feeding status and time was not significant ($F(7, 62) = 2.05$, $p = 0.063$) because the

significant variance in the data comes from separate effects of time ($F(7, 62) = 2.72$, $p = 0.016$) and feeding status ($F(1, 62) = 17.94$, $p = 0.00008$). Post hoc analysis revealed that NPY mRNA expression was significantly higher in fed fish compared to fasted fish at 07:00 (Fig. 2.11).

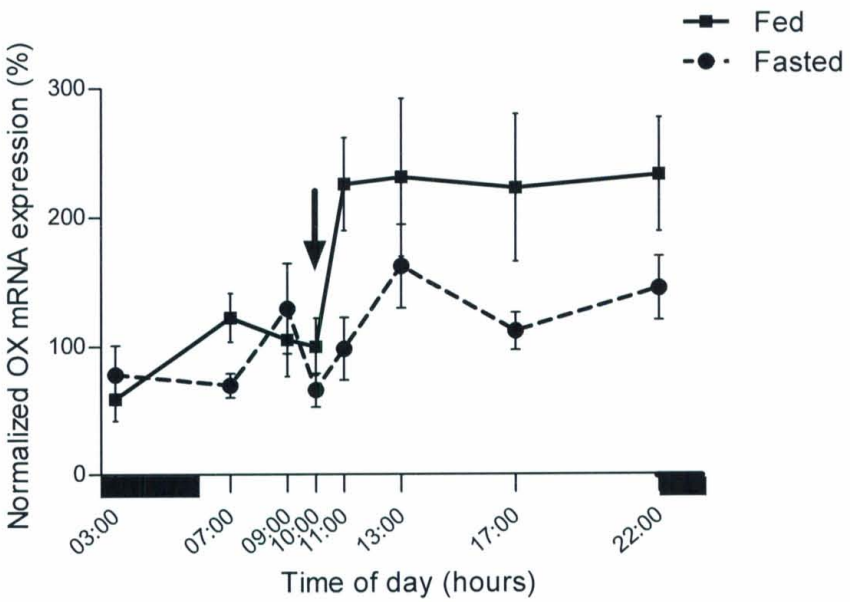


Figure 2.10 Daily hypothalamic OX mRNA expression profiles in fed and fasted cod. OX mRNA expression levels are expressed as a percentage normalized to the 10:00 fed group (n = 5 to 7 at each sampling time per group). Data are presented as mean ± SEM. The black bars directly below the x axis indicate the dark phase of the photoperiod. The black arrow indicates the regularly scheduled feeding time (10:00).

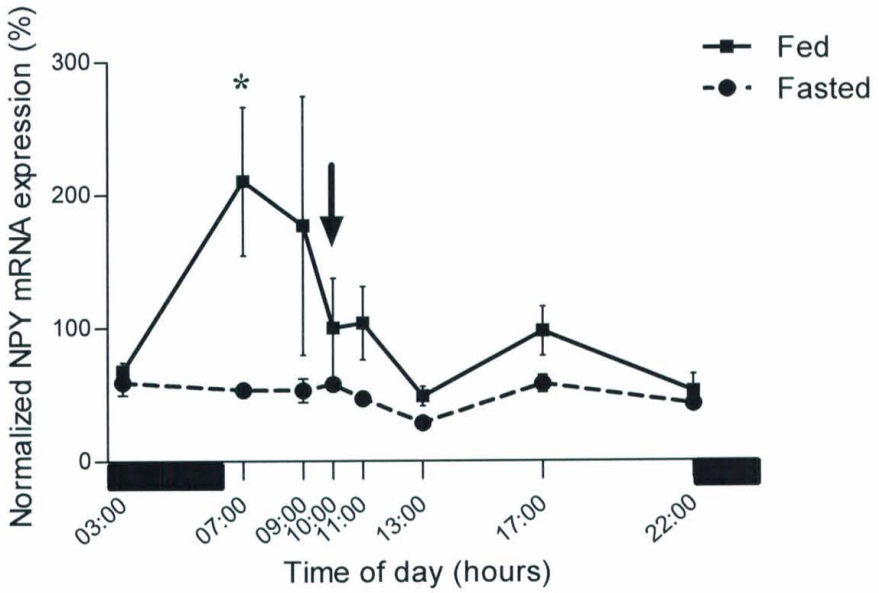


Figure 2.11 Daily hypothalamic NPY mRNA expression profiles in fed and fasted cod. NPY mRNA expression levels are expressed as a percentage normalized to the 10:00 fed group (n = 5 to 6 at each sampling time per group). Data are presented as mean ± SEM. A star (*) indicates a significant difference between fed and fasted groups at a specific time point (07:00). The black bars directly below the x axis indicate the dark phase of the photoperiod. The black arrow indicates the regularly scheduled feeding time (10:00).

2.3.3 Discussion

The present study examined the daily patterns of mRNA expressions of circadian regulatory peptides and important appetite-related neuropeptides in the Atlantic cod hypothalamus. Transcripts of the circadian regulatory protein Clock fluctuated over the day and night, while Per2 levels showed no variations. Transcript levels of OX, an important neuropeptide involved in feeding and wakefulness, were highest during the day, but no significant differences were observed between fed cod and those which had been fasted for several days. Transcript levels of NPY, another important appetite-related neuropeptide, differed between fed and fasted cod several hours before the regularly scheduled mealtime.

Daily profiles of circadian regulatory proteins

The circadian regulatory proteins, Clock and Per2, are transcribed widely throughout the brain and peripheral tissues of Atlantic cod. This is consistent with previous studies showing that both transcripts are expressed widely in the brain and body of mammals (Sakamoto *et al.*, 1998; Balsalobre, 2002) and other fish species, including zebrafish, golden rabbitfish, Atlantic salmon and goldfish (Whitmore *et al.*, 1998; Sugama *et al.*, 2008; Davie *et al.*, 2009; Velarde *et al.*, 2009). Both Clock and Per2 transcripts were highly expressed in the pituitary and gonads, which suggests that these circadian regulatory proteins may be involved in endocrine regulation and reproduction.

This study shows, for the first time in Atlantic cod, daily hypothalamic mRNA expression profiles of Clock and Per2. Significant variation in Clock mRNA expression was

observed over the day in both fed and fasted cod, with highest expression levels observed in the late afternoon, which is similar to the rhythmic pattern observed in zebrafish, where mRNA expression rises in the late afternoon to peak early at night (Whitmore *et al.*, 1998), but different from the rhythmic pattern observed in Atlantic salmon, where mRNA expression is highest during the dark phase (Davie *et al.*, 2009). Differences in expression patterns between these experiments may be due to species differences, entrainment to different photoperiods (*i.e.* 16L:8D in the present study, 14L:10D for zebrafish study and 8L:16D for salmon study) and tissue-specific expression patterns (*i.e.* hypothalamic expression in the present study as compared to whole brain expression in the other studies).

Interestingly, the circadian profile of Clock mRNA expression was more robust in fasted fish than in fed fish, but no differences between fed and fasted fish were observed at any given sampling interval, suggesting that feeding status does not significantly affect the daily transcription of Clock. Previous studies have shown that Clock is constitutively transcribed in mammals (Oishi *et al.*, 1998), birds (Yoshimura *et al.*, 2000), and frogs (Zhu *et al.*, 2000), whereas Clock transcription is cyclical in both zebrafish (Whitmore *et al.*, 1998) and Atlantic salmon (Davie *et al.*, 2009). The present observations in cod suggest that cyclical transcription of Clock may be the common mode of teleost circadian regulation.

In contrast to Clock, hypothalamic Per2 mRNA expression remained constant over the day, and no differences were observed between fed and fasted fish at any given sampling interval. This contrasts with previous studies which show that Per2 transcription

fluctuates from morning to night in the brain, pineal gland and other peripheral tissues of the golden rabbitfish (Sugama *et al.*, 2008) and is rhythmically transcribed in goldfish retina and gut (Velarde *et al.*, 2009) and in a zebrafish embryo-derived cell line and embryonic pineal gland (Pando *et al.*, 2001; Ziv and Gothilf, 2006). As Per2 transcription has been shown to be induced by light in mammals (Takumi *et al.*, 1998) and the pineal gland of zebrafish embryos and a zebrafish embryo-derived cell line (Pando *et al.*, 2001; Ziv and Gothilf, 2006), I expected Per2 transcription in the cod hypothalamus to increase in the hours after light onset. Several reasons may account for this difference. First, different tissues and different developmental stages were examined in previous studies and this study. Previous studies have shown that circadian clocks within peripheral tissues of fish often cycle in different phases from central oscillators and are directly responsive to light stimulation (Cermakian *et al.*, 2000; Cahill, 2002; Velarde *et al.*, 2009). As Per2 mRNA was expressed in various peripheral tissues of the Atlantic cod, with high levels in the skin, cod peripheral tissues may be light-responsive, as is the case in zebrafish.

Also, it is possible that although Per2 transcription did not cycle in Atlantic cod at the level of the whole hypothalamus, it may cycle within the SCN or other extra-hypothalamic brain regions, as studies in rats have shown that Per genes are cyclically transcribed in different phases in different brain regions, while some regions transcribe Per constitutively (Hastings *et al.*, 1999; Abe *et al.*, 2002; Albrecht, 2002). Alternatively, cod might have another form of Per which oscillates to control circadian regulation.

One should also consider that cod is a groundfish species which normally swim at an ocean depth of 100 m or more, where they are exposed to blue light only (Boeuf and Le

Bail, 1999). It has been suggested that in the light-responsive cell line derived from embryonic zebrafish, Per2 may be upregulated via a signal transduction pathway whereby light is received by the circadian regulatory photoreceptors, or cryptochromes, which transcribe the Cryptochrome (Cry) gene and are sensitive to blue light (Cermakian *et al.*, 2002). Blue light received by cryptochromes in cod might regulate daily molecular oscillations. Future studies examining the daily profiles of Cry transcription in cod will determine the importance of this gene in circadian regulation.

Finally, it is noteworthy that the absence of oscillating transcription does not necessarily preclude an oscillation for the Per2 protein levels as these can be subject to post-transcriptional or post-translational regulation. Indeed, a mammalian cell line generated to constitutively transcribe Per2, displays rhythmic secretion of Per2 due to post-transcriptional regulation (Kawamoto *et al.*, 2006). Further studies examining the daily accumulation of Per2 protein in Atlantic cod might help determine if protein levels oscillate in this species. Regardless of the presence of oscillations, Per2 may still be involved in circadian regulation, as the mammalian circadian proteins, Clock and Timeless, are constitutively expressed, but their physical associations with other oscillating circadian proteins are necessary to maintain rhythmicity of the molecular feedback loop (Sangoram *et al.*, 1998).

Another crucial factor in determining the expression profiles of circadian regulatory genes is the photoperiod under which animals are maintained, as mRNA expression of both Clock and Per2 is cyclical in Atlantic salmon brain when fish are held under a short-day photoperiod (8L:16D), but not when fish are held under a long-day photoperiod

(16L:8D; Davie *et al.*, 2009). In the present study, cod were held under a long-day photoperiod, so it is possible that, as in salmon, cyclical Per2 mRNA expression and more robust Clock mRNA expression rhythms may have been observed under a shorter day photoperiod.

Daily expression of appetite-related neuropeptides

Both fed and fasted cod displayed significant daily fluctuations in hypothalamic OX mRNA expression. In fed cod, OX mRNA expression increased at or soon after mealtime when fish were actively eating, and remained high for the rest of the day before decreasing during the nightly period of inactivity, which is consistent with the roles of OX in appetite stimulation and wakefulness. A previous study showed higher forebrain OX mRNA expression at mealtime than two hours before or after mealtime (Xu and Volkoff, 2007). The absence of a decrease in OX expression postprandially in this study could be explained by the fact that meal size affects the periprandial profile of OX mRNA expression in this species (Xu and Volkoff, 2007), and as fish in my study were fed higher rations of food than in the previous study, a longer time may have been spent foraging which might keep OX levels high for a longer duration after meal administration. The previous study also examined OX mRNA expression in the forebrain as a whole in which differential expressions in each of the telencephalon, optic tectum and hypothalamus may have resulted in overall highest OX expression at mealtime when pooled together as forebrain samples. In fasted fish, high OX expression around mealtime indicates that periprandial fluctuations of expression still occur even when the regular

daily meal is withheld, likely because fish had become entrained to eat at a specific time of day and exhibited FAA.

There were no significant differences observed in OX mRNA expression between fed and fasted fish at any sampling interval, which contrasts from previous studies in which forebrain OX mRNA expression has been shown to increase after a fast or food restriction period, such as in goldfish fasted for 2 or 6 days (Nakamachi *et al.*, 2006), winter skate (*Leucoraja ocellata*) fasted for 4 weeks (MacDonald and Volkoff, 2010) and Atlantic cod fed low rations for 4 weeks (Xu and Volkoff, 2007). The duration of a fast or food restriction period appears to determine if mRNA expression changes occur, as in zebrafish which must be fasted for longer than two days in order to observe an increase in whole brain OX expression (Novak *et al.*, 2005). In the present study, a 4 day fast may not have been long enough to up-regulate OX mRNA expression. Indeed, there was even a trend for higher expression in fed fish than in fasted fish after mealtime and for the rest of the light period. It is possible that higher OX levels in fed fish are due to fish actively eating, with higher locomotor activity as they foraged for food. Additionally, as cod swimming speed is known to be reduced during starvation (Bjornsson, 1993), it is possible that lower OX levels in fasted fish may be indicative of decreased locomotor activity.

The present study shows that NPY mRNA expression fluctuated significantly around mealtime in fasted cod, but not in fed cod. High preprandial NPY mRNA expression in this study contrast with low forebrain NPY levels two hours before the meal shown in a previous study (Kehoe and Volkoff, 2007). However, the use of the forebrain in the

previous study may have generated different NPY expression profiles. In addition, the previous study used a 1% body weight ration of food, whereas my cod were fed a 2% ration, and as ration size has been shown to affect periprandial fluctuations of other appetite regulators, such as OX mRNA expression in cod (Xu and Volkoff, 2007), the same may be true for NPY.

A preprandial increase in expression was not observed in fasted cod, suggesting that the preprandial peak observed in regularly fed cod was not sustained when food was consistently withheld for four days. After the scheduled meal, NPY mRNA expression in both fed and fasted cod tended to decline until reaching baseline levels at 13:00, which is consistent with observations in rats (Kalra *et al.*, 1991) and goldfish (Narnaware *et al.*, 2000), where hypothalamic NPY secretion and mRNA expression, respectively, is highest before the daily meal and declines over the course of the meal. Aside from a preprandial peak in NPY mRNA expression in fed, but not fasted, no other differences in expression were observed at any given sampling interval between fed and fasted fish, which differs from observations in goldfish, in which fasting has been shown to increase hypothalamic NPY mRNA expression in a time-dependent fashion (Narnaware *et al.*, 2000). However, other studies suggest that fasting does not always increase NPY expression, as in Atlantic salmon whole brain (Murashita *et al.*, 2009) and Atlantic cod forebrain (Kehoe and Volkoff, 2007) where a 6 or 7 day fast does not affect NPY mRNA expression, as compared to their fed counterparts. Perhaps a longer fasting period is needed before a change in NPY levels can be observed, as fasting for several weeks has been shown to be necesasary to increase NPY mRNA expression in Chinook (*Oncorhynchus tshawytscha*)

and coho salmon (*Oncorhynchus kisutch*) hypothalami (Silverstein *et al.*, 1998). In addition, NPY expression increases in the telencephalon of winter skate, an elasmobranch, after two weeks of fasting, but is unaltered in the hypothalamus (MacDonald and Volkoff, 2009b), suggesting that distinct brain regions may respond differently to fasting.

2.4 Summary

This study shows, for the first time in goldfish and Atlantic cod, daily mRNA expression profiles of circadian regulatory proteins and appetite-related neuropeptides in the hypothalamus of the brain. In goldfish held under a light-dark cycle, both Clock and Per1 are transcribed cyclically over the day in antiphase of one another, while in cod, Clock transcription is cyclical, but Per2 is constitutively transcribed. The evidence presented in this study suggests that cyclical transcription of Clock might be common to the teleost mode of circadian regulation.

Accompanied by daily changes in circadian gene transcription are daily variations in physiology. Cycles of food intake and sleep-wake/activity-rest cycles are inherently linked to circadian rhythms. In the present study, this is exemplified by fluctuating expression levels of two neuropeptides involved in these processes, OX and NPY. In goldfish and cod, the daily mRNA expression of OX supports the proposed roles of this neuropeptide in promoting wakefulness and appetite stimulation, as periprandial changes and low nighttime levels were observed. In addition, in the goldfish, but not in cod, OX levels increased prior to lights off possibly due to a role of OX in initiating the nightly production of melatonin. As for NPY, expression levels did not significantly vary over the day in goldfish held under a light-dark cycle, but in cod, NPY levels reflected expected changes in appetite, with a peak in expression before mealtime, when fish are expected to be hungriest, and declining expression after mealtime, when fish are satiated.

In some cases, the act of feeding appeared to affect daily mRNA expression. In goldfish, Per1 mRNA expression was higher at mealtime in fed fish, as opposed to those who were not offered food, providing further evidence that Per expression can be altered by nutritional cues. In cod, fasting for four days affected the profile of NPY, as periprandial differences in expression were observed between fed and fasted fish.

In goldfish, constant lighting had strong effects on daily mRNA expression profiles, as Per1 mRNA expression was up-regulated and both Clock and OX rhythmic mRNA expression patterns were abolished. Disruption of rhythms might be a result of desynchronization between cellular oscillators within the hypothalamus in the absence of a light-dark cycle. NPY expression appeared to entrain to cycles of food availability under constant light, with a peak in expression observed prior to the daily scheduled meal.

Differences in mRNA expression profiles between goldfish and cod observed in this study are likely species-specific. These two fish models are quite different in terms of life history, ecological niche and physiology. For example, different digestive physiologies between the goldfish, a stomachless omnivore, and the cod, a carnivore with a well-developed stomach, may be expected to contribute to differences in appetite-related hormone mRNA expressions between the two. Additionally, the importance of a light-dark cycle in determining expression profiles likely differs between the two species, as they occupy very different habitats, especially with regards to environmental lighting. In addition, differential susceptibility to stress may affect patterns of mRNA expression, as cod are especially prone to stress. My sampling procedure required the netting of fish

every few hours, so it is likely that these fish displayed higher levels of stress, as compared to goldfish which tend to be more tolerant to stress.

3.0 The effects of long-term administration of NPY and CCK on food intake and mRNA expression of Orexin, CART and Amylin in goldfish

3.1 Introduction

Appetite in fish, as in other vertebrates, is regulated by a number of peripheral and central hormones, which act on feeding centres of the brain to stimulate or inhibit feeding (Volkoff *et al.*, 2005). One of the most potent stimulators of appetite known, at least in mammals (Beck, 2006), is Neuropeptide Y (NPY). The effects of peripheral administration of NPY on food intake have not been examined in mammals and have received little attention in fish (Lopez-Patino *et al.*, 1999; Carpio *et al.*, 2006; Kiris *et al.*, 2007) where no or little effect has been found. On the other hand, central administration of NPY in fish, as in mammals (Clark *et al.*, 1984; Kalra *et al.*, 1988), induces pronounced increases in food-seeking behaviour and food intake in several species, including goldfish (*Carassius auratus*; Lopez-Patino *et al.*, 1999), channel catfish (*Ictalurus punctatus*; Silverstein and Plisetskaya, 2000) and rainbow trout (*Oncorhynchus mykiss*; Aldegunde and Mancebo, 2006), suggesting that NPY stimulates appetite mostly by a central mode of action.

Another important appetite regulatory factor is cholecystokinin (CCK), a peptide hormone secreted by both the gut and brain in response to food consumption (McLaughlin *et al.*, 1985; Peyon *et al.*, 1999; Murashita *et al.*, 2007; Dockray, 2009). Evidence suggests that in mammals (Beinfeld *et al.*, 1981; Moran *et al.*, 1990; Wang *et al.*, 1998; Berthoud *et al.*, 2004; Whited *et al.*, 2006), and fish (Himick and Peter, 1994;

Himick *et al.*, 1996; Silverstein and Plisetskaya, 2000; Rubio *et al.*, 2008), CCK induces satiety by actions both in the periphery, in particular the gastrointestinal (GI) tract and associated organs, and in feeding centers of the brain. In goldfish, CCK expression and binding sites are distributed throughout the brain and the gut and food consumption decreases significantly following both acute peripheral and central injections of CCK (Himick and Peter, 1994; Himick *et al.*, 1996; Peyon *et al.*, 1998). In addition, food intake decreases in channel catfish after central injections of CCK (Silverstein and Plisetskaya, 2000) and in European sea bass (*Dicentrarchus labrax*) after oral administration of capsules containing CCK (Rubio *et al.*, 2008). Together, this evidence suggests that CCK may inhibit appetite in fish by acting on both the brain and GI tract.

Other peptides that have been implicated in the regulation of appetite in fish include orexin (OX), Cocaine- and amphetamine-regulated transcript (CART) and amylin (Volkoff, 2006). Both central and peripheral OX treatments increase appetite (Volkoff *et al.*, 1999; Novak *et al.*, 2005; Nakamachi *et al.*, 2006; Xu and Volkoff, 2007; Facciolo *et al.*, 2009) and wakefulness (Volkoff *et al.*, 1999; Volkoff and Peter, 2000; Prober *et al.*, 2006; Facciolo *et al.*, 2009; Naumann *et al.*, 2010) in several teleost species, including goldfish, zebrafish (*Danio rerio*), Atlantic cod (*Gadus morhua*) and ornate wrasse (*Thalassoma pavo*). Central injections of CART decrease food intake in goldfish (Volkoff and Peter, 2000) and rats (Kristensen *et al.*, 1998), likely by acting at the level of the hypothalamus, and both acute peripheral and central injections of amylin reduce food intake in goldfish (Thavanathan and Volkoff, 2006) and rats (Chance *et al.*, 1991; Rushing *et al.*, 2000; Lutz *et al.*, 2001; Mollet *et al.*, 2004).

In goldfish, the appetitive effects of NPY and CCK have been described thus far in terms of the effects of acute, single bolus administration on food intake (Himick and Peter, 1994; Lopez-Patino *et al.*, 1999), which only reveals effects of these peptides in short-term appetite regulation. The effects of peptides in long-term regulation of feeding can be assessed by use of osmotic minipumps. These pumps can be implanted in animals to release a test solution continuously over an extended period of time, which ensures chronic infusion of the test solution with no handling stress during the infusion period.

Osmotic minipumps have been used effectively in rodents to chronically infuse NPY into the third ventricle of the brain (intracerebroventricularly or ICV) via a cannula. Continuous ICV infusion of NPY for a period ranging from three days to two weeks has been shown to increase total daily food intake in rats and mice (Beck *et al.*, 1990; Zarjevski *et al.*, 1993; McMinn *et al.*, 1998; Raposinho *et al.*, 2004; Henry *et al.*, 2005; Fuzesi *et al.*, 2007). To date, the effects of chronic peripheral infusion of NPY have not been examined in any species.

CCK has been chronically administered in rodents using osmotic minipumps to infuse CCK into the peritoneal cavity (intraperitoneal or IP), blood vessels or the third ventricle of the brain. Continuous IP infusion of CCK for a period ranging from two to four weeks does not affect total daily food intake in rats (Crawley and Beinfeld, 1983; Covasa et al., 2001). Rodents appear to build up a tolerance to the satiating effects of peripherally infused CCK, as chronic infusions of CCK into the jugular vein of rats decrease food intake over the first four days of administration before returning to control levels (Lukaszewski and Praissman, 1988). However, continuous ICV infusions of CCK over an

eight day period in rats decrease food intake (Schick *et al.*, 1988), which suggests possible differential effects of CCK on food intake when administered peripherally as compared to centrally.

Osmotic minipumps have been successfully used in fish, including Atlantic cod, common carp (*Cyprinus carpio*) and rainbow trout to study various aspects of physiology, including thyroid metabolism, osmoregulation and growth related metabolism using IP implantation (Comeau and Campana, 2003; Metz *et al.*, 2003; Very and Sheridan, 2007), and in goldfish and rainbow trout in which test solutions were delivered to the cranial cavity (Schmidt and Shashoua, 1988; Levy and Baker, 1997; Schmidt and Schachner, 1998; Bernier and Craig, 2005).

In the present study, the effects of long-term treatment of NPY and CCK on feeding in goldfish were assessed using osmotic minipumps to chronically infuse these peptides by central and peripheral routes of administration. Fish were submitted to IP and ICV long-term NPY or CCK treatment and food intake was measured daily during the entire infusion period. To assess if changes in feeding could be mediated by other peptides, hypothalamic mRNA expressions of appetite-related neuropeptides were measured following this chronic infusion. The neuropeptides examined which include OX, CART and amylin, were chosen based on previous observations of interactions with the NPY and CCK systems (see Section 1.4).

3.2 Materials and methods

Experimental animals

A mixture of 120 male and female goldfish (*Carassius auratus*) was purchased from Ozark Fisheries (Martinsville, IN, USA). For IP experiments, fish with an average weight of 75 g were housed in 12 aquaria (25 gal) which were subdivided into two compartments using a mesh screen. This allowed the flow-through of water but confined individual fish and their food pellets to a single compartment. For ICV experiments, fish with an average weight of 20 g were housed in 12 aquaria (25 gal) which were subdivided into three compartments using mesh screens. The difference in the number of compartments per tank for IP and ICV experiments was necessary to accommodate the size of fish. Aquaria contained fresh water that was constantly aerated and filtered. Fish were maintained at 20°C under a photoperiod of 16 hours light: 8 hours dark (16L:8D). A single daily meal was administered at 12:00 and consisted of an approximately 2% wet body weight (BW) ration of commercially prepared trout pellets. Fish were acclimated to these conditions for 2 weeks prior to the experiment. All fish were in the same reproductive stage (gonadal recrudescent). All experiments were conducted according to the principles published in the Canadian Council on Animal Care's guidelines on the Care and Use of Fish in Research, Teaching and Testing.

Peptides

Porcine NPY and cholecystokinin octapeptide (CCK-8) were purchased from American Peptide Company (Sunnyvale, CA, USA). Stock solutions were made in water, aliquoted

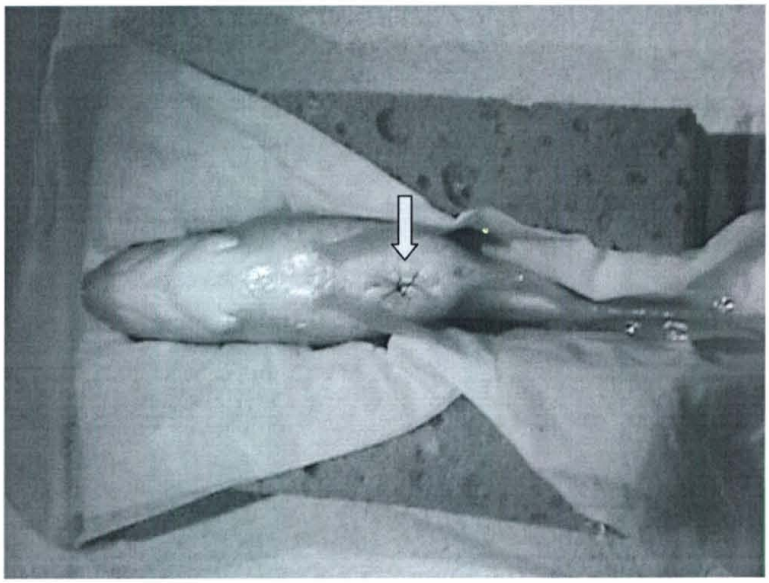
and stored at −20 °C. Aliquots were subsequently thawed and diluted in fish physiological saline (NaCl 5.9 g/L; KCl 0.25 g/L; $CaCl_2$ 0.28 g/L; $NaHCO_3$ 2.1 g/L; KH_2PO_4 1.6 g/L; $MgSO_4 \cdot 7H_2O$ 0.29 g/L; glucose 2.0 g/L; Burnstock, 1958) to obtain weight-specific doses for individual fish prior to use.

Long-term IP administration

Goldfish were chronically infused with either NPY, CCK or saline (n = 6 to 8 per treatment) for two weeks using osmotic minipumps (Alzet, Model 1002, DURECT Corp., Cupertino, CA, USA) that were prepared according to the manufacturer's protocol. The dose of CCK used in this study was chosen based on a previous study reporting a 50 ng/g BW to be effective in suppressing food intake when administered acutely by IP injection in goldfish (Himick and Peter, 1994).

There is no published report on a peripheral orexigenic effect for NPY in goldfish, so the effective peripheral dose of NPY necessary to elicit increased food intake in goldfish has not been determined. As a general rule, the effective IP dose for a peptide is approximately 10 fold that of the effective ICV dose (Makovec *et al.*, 1986). As the effective ICV dose of NPY in goldfish has been shown to be between 0.5 to 5 ng/g BW (Narnaware *et al.*, 2000), I chose an IP dose of NPY at 50 ng/g BW. For both CCK and NPY, the concentration of the treatment solutions were prepared such that 50 ng/g BW was administered over a period of 24 hours. This dose was infused continuously for 2 weeks.

Fish were not fed on the day of surgery to avoid gut distension which could interfere with surgery within the peritoneum. Fish were deeply anaesthetized in a concentrated solution of tricaine methanesulfonate. A 1 cm incision was made close to the ventral midline of the body posterior to the pelvic fins and a minipump was inserted into the peritoneal cavity. The incision was closed using two sutures and antibiotic ointment was applied to the incision area (Fig. 3.1). Following surgery, fish were returned to their compartments and allowed to recover from anaesthesia. An antibiotic solution was added to the tank water as a prophylactic against infection (Melafix, Aquarium Pharmaceuticals, Mars Fishcare North America Inc., Chalfont, PA, USA). Two tankmate fish were always administered the same test solution to avoid possible effects from pump leakage. All minipump preparations and surgeries were performed using sterile equipment and techniques.



1.9 cm

Figure 3.1 Goldfish model of intraperitoneal minipump implantation for long-term peptide infusion. The arrow indicates the site of pump implantion. Scale: 1 cm = 1.9 cm

Acute IP administration of NPY

Only two studies have examined the effects of acute peripheral administration of NPY on food intake in fish (Lopez-Patino *et al.*, 1999; Kiris *et al.*, 2007). In tilapia (*Oreochromis* spp.), IP injections of NPY at a dose of 0.6 μg/g BW increase food intake within 10 hours post-injection (Kiris *et al.*, 2007). In goldfish, satiated fish were injected with only two doses of NPY (100μg/g and 330μg/g BW) and neither of the doses affected food intake (Lopez-Patino *et al.*, 1999). In order to determine if NPY had an effect on feeding when injected peripherally and to determine a putative effective dose of NPY necessary to elicit increased food intake, I sought to establish a dose-response curve for acute NPY IP injection. Individual fish were weighed and food intake was monitored for 1 week prior to the experiment by administering a known number of food pellets at the daily feeding time (12:00) and removing and counting the uneaten pellets remaining after 1 hour. The average food intake over this period was the untreated control group. For acute experiments, just prior to the daily feeding time, fish were lightly anaesthetized in tricaine methanesulfonate and injected IP with 100 μL of NPY at a dose of 50, 100, 200, 300 or 500 ng/g BW (n = 5 to 6 per dose). IP injections were performed using a 26 gauge needle attached to a 250 μL Hamilton syringe close to the ventral midline posterior to the pelvic fins. All injections were performed using sterile techniques. Fish were then returned to their tanks and after a brief recovery period (approximately 10 minutes), food pellets were administered. After 1 hour, the remaining uneaten pellets were removed and counted. Food consumption was converted to milligrams of food consumed/wet BW/time feeding based on the mean pellet weight fed to fish (approximately 50 mg/pellet).

Long-term ICV administration

Goldfish were chronically infused with either NPY, CCK or saline (n = 4 to 8 per treatment) for ten days by ICV infusion using osmotic minipumps (Alzet, Model 1002) attached to a brain infusion apparatus (Brain infusion kit 2, Alzet, DURECT Corp., Cupertino, CA, USA). As this infusion apparatus was designed for use in rodents, the technique had to be developed for use in goldfish. The ICV apparatus was assembled based on the manufacturer's protocol for use in mice. Briefly, a cannula pedestal was attached to a length of catheter tubing which was, in turn, attached to a minipump (Fig. 3.2A). Minipumps were filled with a peptide solution or saline according to the manufacturer's protocol. These components were held together using an instant adhesive (Loctite 454, Mississauga, ON, Canada) and the entire apparatus was incubated in sterile water for several days to ensure the minipump solution was flowing through the apparatus.

Fish were deeply anaesthetized by immersing in a concentrated solution of tricaine methanesulfonate and wrapped in a wet towel to prevent damage to the scales. A small patch of the cranial surface was scraped to remove the tissue and expose the underlying bone. A dentist drill equipped with a small drill bit (approximately 0.25 cm in diameter) was used to drill a hole through the cranium to expose the brain below. A combination of instant adhesive and tissue adhesive (Loctite 454; Vetbond, 3M Animal Care Products, St. Paul, MN, USA) was applied to the base of the cannula pedestal of the brain infusion apparatus and the cranial surface was blotted dry to allow better adhesion. Using known stereotaxic coordinates (+1.0, M, D 1.2) taken from the stereotaxic atlas of the goldfish