Characterizing the endocrine, digestive and morphological adjustments of the intestine in response to food deprivation and torpor in a cold-adapted teleost, *Tautogolabrus adspersus*

by:

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A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree of Master of Science

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May, 2014

St. John's, NL Newfoundland

Abstract

The cunner, *Tautogolabrus adspersus*, is a marine teleost endemic to the cold waters of the Northwest Atlantic Ocean. The cunner is non-migratory and is known for its remarkable ability to endure the freezing winter months with little to no food. During this period, the cunner seeks refuge in the same rocky shelters it inhabits during the summer months and enters a torpid/dormant state. This response is characterized by a cessation of feeding, sluggish movements and a lowering of metabolic rate. Such a response calls for strict control of energy balance, and might use in part regulatory mechanisms present within the gut. To evaluate the physiological strategies employed by the cunner's intestinal tract to withstand food deprivation, experimental fasting trials were conducted. Cunner were sampled for their stomachless digestive tract after a four-week period of acute food deprivation in July and August during their summer (active/feeding) state, and during their natural overwinter fasting in March. Digestive enzyme activities were assessed by biochemical assay. Specific activities for trypsin, alkaline phosphatase and lipase were all reduced in both 4-week fasted and torpid fish, whereas aminopeptidase-N was only lowered in 4-week fasted fish. Neither summer nor winter fasting caused significant changes in the intestinal mRNA expressions of digestive enzymes, with the exception of a decrease in aminopeptidase-N expression during torpor. Transcript expression in the gastrointestinal tract was also quantified for four putative appetite regulators. Orexin, the mechanistic target of rapamycin and cholecystokinin expressions were all reduced in torpid cunner, but not in summer fasting whereas apelin expression was reduced in summer fasting, but not in torpid fish. This work contributes to the overall understanding of energy balance in fish and provides novel insights into the intestine's endocrine contribution to appetite regulation and digestive function in cunner during natural fasting and acute food deprivation.

Acknowledgements

I greatly appreciate the guidance, patience and trust of my supervisor, Dr. Hélène Volkoff, and for all of the opportunities she allowed me during this project, academic and otherwise. To Sarah Tuziak for her support and friendship. To Dr. Robert Hooper, Dennis Rumbolt and Allison Eaton for their tremendous assistance with sampling and animal care at the Bonne Bay Marine Station. I am grateful for the advice and support from my committee members, Dawn Marshall and Chris Parish. And, to my new colleagues in Newfoundland and around the world for all of their encouragement.

This project was funded by Natural Sciences and Engineering 699 Research Council Discovery (DG) and Research Tools and Infrastructure 700 (RTI) grants.

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List of Abbreviations

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ANPEP	Alanine aminopeptidase
as	Atlantic salmon
BAL	Bile salt-activated lipase
bb	Brandt's bat
bt	Bluefin tuna
c	Bunner
CCK	Cholecystokinin
CDS	Coding DNA Sequence
ch	Chicken
DI	Distal intestine
es	European seabass
fr	Fugu rubripes
g	Goldfish
gc	Grass carp
GIT	Gastrointestinal tract
gs	Gilthead seabream
GSI	Gonadosomatic index
h	Human
HSI	Hepatosomatic index
ICV	Intracerebroventricular
IP	Intraperitoneal
lyc	Large yellow croaker
MI	Mid intestine
mTOR	Mechanistic target of rapamycin
nt	Nile tilapia
osg	Orange spotted grouper
PCR	Polymerase chain reaction
PI	Proximal intestine
qPCR	Quantitative reverse transcription polymerase chain reaction
r	Rat
RACE	Rapid Amplification of cDNA Ends
sgp	Spotted green pufferfish

ssb	Spotted sand bass
wcf	Western clawed frog
wf	Winter flounder
wlg	Western lowland gorilla
z	Zebrafish

1 Introduction

1.1 Cunner

1.1.1 Habitat and lifestyle

Cunner, *Tautogolabrus adspersus*, is a cold-water marine fish of the family Labridae and order Perciformes. Labrid fish, commonly known as wrasses, consist of many fish species that inhabit tropical and temperate ocean reefs globally (Cowman et al., 2009; Tuya et al., 2009). Cunner is one of the most northern species of labrid and is found in coastal waters of the North Atlantic Ocean from Chesapeake Bay, PA, USA to the Gulf of St. Lawrence and around Newfoundland, Canada.

Of interest to scientific research is cunner's overwintering strategy. Cunner is non-migratory and must overcome the challenges of overwintering in the North Atlantic where water temperatures drop below freezing (-1ºC). Some controversy exists as to whether cunner occupy deeper habitats during the winter months or remain at their usual summer depths of 2-10 m (Christel et al., 2007; Gibbs et al., 1973; Green and Farwell, 1971). Several documented observations of cunner indicate that they enter a dormant torpid state during the winter which appears to be induced by a drop in water temperature below 5ºC (Green and Farwell, 1971; Haugaard and Irving, 1943; Naya et al., 2009; Raybould et al., 2006).

1.1.2 Overwinter torpor

Torpor is a decrease in an organism's metabolic rate and is a useful strategy for energy savings for species that face environmental challenges such as cold temperatures, a lack of food, low oxygen levels or dry conditions (Geiser, 2004). Factors that induce torpor and physiological changes that accompany torpor can vary from species to species, but the common goal is a reduction in energy expenditure to survive the period of stress.

Metabolic suppression, including torpor, is observed in a diverse range of phyla. One of the most extreme cases of metabolic suppression is the *Artemia* cyst, capable of suppressing metabolism to nearly undetectable levels and surviving over 1.5 years in anoxic conditions (Clegg, 1993). The Andean toad enters a cold-induced hibernation during which its metabolism is reduced to 7.8% of summer rates (Naya et al., 2009). Ground squirrels hibernate and rely on fat stores for 5 months during the winter (Carey et al., 2001). The lungfish estivates in response to dryness and heat, a condition marked by burrowing behaviour and a reduction in oxygen consumption (Delaney et al., 1974). There is a range of survival strategies used by cold-water overwintering fish. Some species remain active in the winter through the use of biochemical adaptations, while others enter dormancy such as torpor or hibernation. It is hypothesized that cold water fish species which utilize dormancy have relatively recently evolved from warm water relatives and fish that remain active during the winter have developed specialized adaptations to accommodate for the cold temperatures (Crawshaw, 1984). One such example of the harsh cold climate as a selection pressure is the evolution of plasma antifreeze proteins in cunner (Hobbs et al., 2011). Additionally, cunner are thought to have recently adapted to a cold water environment due to the disproportionate amount of antifreeze protein expressed in its epithelium (Hobbs and Fletcher, 2013).

1.1.3 Diet and lack of stomach

Cunner are a member of the carnivorous family of fish Labridae and feed on small mussels, crustaceans and urchins during the spring, summer and fall months when water temperatures are above 5ºC (Chao, 1973). Carnivorous fish are typically characterized by a relatively short gastrointestinal tract (GIT) compared to omnivorous or herbivorous fish of similar size (Al-Hussaini, 1949) and usually have a morphologically and physiologically distinct stomach with low pH and peptic digestion to break down ingested proteins (Wilson and Castro, 2010). The acidic phase of digestion is indispensable as it accounts for the initial denaturation and hydrolysis of food proteins in their native structure. Only once proteins have undergone an initial acidic hydrolysis can they be further broken down by enzymes such as trypsin and chymotrypsin that act at higher pH levels in the intestine. However, some carnivore fish have a neutral GIT pH, which is attributable to their diet (Lobel, 1981) composed mainly of mollusks and crustaceans that contain large amounts of calcium carbonate in their shells. The ingested calcium carbonate has a buffering effect and rises the pH within the GIT (Lobel, 1981). The resulting shift towards a neutral or slightly basic pH does not allow for an efficient acidic phase of digestion with the enzyme pepsin. For example, the pH throughout the entire intestine of cunner ranges between 7 and 8, thus providing no opportunity for acid-peptic digestion (Chao, 1973). Cunner along with some other species that lack acid-peptic digestion, such as crayfish, overcome this challenge with a specialized form of trypsin that is capable of hydrolyzing proteins in their native structure (Simpson and Haard, 1985).

The inability to utilize peptic digestion in labrids that consume a high amount of calcium carbonate, which neutralizes stomach acid, may be a driving force behind the

evolutionary loss of a stomach in this family. The loss of a stomach in most stomachless fish species is accompanied by a switch in digestive strategies. Whereas the portion of the GIT immediately following the stomach is most commonly associated with nutrient absorption in traditional GIT systems with distinct stomachs, in most stomachless fish, such as the stout longtom, *Tylosurus gavialoides* (Manjakasy et al., 2009), the distal intestine or rectum are the primary sites of digestion. It appears that stomachless carnivores pass ingested food to their highly distensible distal intestine, just anterior to the ileorectal valve (if present), where it is digested and absorbed (Manjakasy et al., 2009).

Cunner provide a useful experimental model for studying intestinal physiology because they have a simplified stomachless gastrointestinal tract (GIT) and can withstand long periods of food deprivation (Chao, 1973; Green and Farwell, 1971). In particular, evaluating digestive capacity can be simplified for stomachless fish given that there is only a single phase of digestion and no compartmentalization of the GIT other than a separate non-absorptive rectum (Manjakasy et al., 2009). As mentioned above, nutrient absorption in stomachless fish appears to primarily take place in the distal intestine, thus digestive function in cunner can be most effectively evaluated by studying this region.

1.2 Food deprivation and dormancy

Almost all animals experience periods of food deprivation. What varies is the length of these periods and the extent to which physiological mechanisms are adjusted to accommodate the lack of food intake. There are many factors controlling the length of time an animal may go without food. Some species, such as humans, will eat periodically throughout the day. Other species, like shrews, eat almost constantly while others will typically eat every few hours when active. Many species have also adapted to undergo

long periods of fasting as a response to food availability or harsh environmental conditions such as extreme cold, heat or dryness.

Animals capable of hibernation, estivation or those with naturally low metabolic rates (*e.g.* ectotherms) can sustain themselves for months without food (Secor, 2005). For example, snakes may fast for months as a consequence of their "sit and wait" hunting strategy or during gestation and egg incubation (Secor and Diamond, 1995). Many northern fish overwinter without any food (T. Wang et al., 2006) as a consequence of limited food availability (Pottinger et al., 2003). Species adapted for surviving these periods have a number of unique endocrine, metabolic and other physiological regulatory strategies to conserve and store energy and make efficient use of their fuel stores (Guppy and Withers, 1999).

GIT hormones are important contributors to the endocrine response to food deprivation (Kirchgessner, 2002; Wren and Bloom, 2007). Classical gastrointestinal hormones such as cholecystokinin (CCK) and gastrin have long been implicated in appetite regulation (Wren and Bloom, 2007). Other hormones, such as orexin (Kirchgessner and Liu, 1999), initially thought to act only in the central nervous system to regulate feeding, are now thought to be produced in and affect peripheral tissues such as the GIT and other organs related to the regulation of digestion and appetite.

1.2.1 GIT and metabolic down-regulation

Species adapted to long-term food deprivation show an intestinal response characterized by a combination of physiological (Ferraris and Diamond, 1989) and morphological

adjustments (Carey, 1990; Secor and Lignot, 2010), the most common of which is a decrease in digestive enzyme activities. Food deprived Atlantic salmon show a decrease in maltase and leucine aminopeptidase specific activities in both pyloric caeca and distal intestine (Krogdahl and Bakke-McKellep, 2005). Snakes, such as the Burmese python, show marked decreases in aminopeptidase-N (ANPEP) and maltase activities between meals and can withstand months of fasting (Secor, 2008). European glass eels, capable of long distance migration with little to no food, respond to fasting with a decrease in the digestive enzymes trypsin, amylase, alkaline phosphatase (ALP) and leucine aminopeptidase (Gisbert et al., 2011).

However, not all cases of food deprivation result in a decrease in enzyme activities. Brush border leucine aminopeptidase activity increases with fasting in Atlantic salmon (Krogdahl and Bakke-McKellep, 2005). House sparrows increase the digestive enzymes sucrase-isomaltase and aminopeptidase-N during fasting (Chediack et al., 2012). The metabolic enzyme citrate synthase activity is up-regulated in fasted Atlantic cod pyloric caeca (Bélanger et al., 2002). European sea bass transcript expressions for intestinal brush border ANPEP and maltase are increased with fasting (Hakim et al., 2009). Such increases in digestive enzyme activities would seem useless and energetically costly, as no food is ingested. The up-regulation of digestive function during fasting may be an anticipatory strategy to make efficient use of nutrients from the next available meal. Unlike species that undergo seasonal fasting, the species noted above that demonstrate certain forms of GIT up-regulation when fasted are not adapted to food deprivation.

In addition to a down-regulation of digestive capacity, many species adapted for food deprivation make use of a whole body or a tissue-specific reduction in metabolic

rate. Tissue-specific metabolic depression allows for metabolic savings during stress while not compromising key tissue function and has been demonstrated in many species. For example, the cichlid fish *Astronotus crassipinnis* uses different metabolic strategies in heart and skeletal muscle when exposed to hypoxia as demonstrated by changes in metabolic enzyme activities such as lactate dehydrogenase and pyruvate kinase (Chippari-Gomes et al., 2005). As the GIT serves no purpose during food deprivation and is metabolically costly to maintain, it is logical that many species that have adapted to fasting exploit the GIT as a means for energy savings by restructuring of the epithelial morphology and/or regulating digestive physiology on a cellular level (Gas and Noailliac-Depeyre, 1976; Naya et al., 2009; Ott and Secor, 2007; Secor and Lignot, 2010).

Often, metabolic rate depression in ectotherms is passive and due to a change in body temperature. Torpid cunner have been shown to actively (beyond the effect of temperature alone) reduce their metabolic rate in response to cold. Early metabolic rate experiments measuring the oxygen consumption of cunner exposed to various water temperatures found that whole animal metabolic rate, as determined by respirometry, is decreased at overwintering temperatures (0 to 5ºC) to about one quarter of the summer (15 to 25ºC) rates (Haugaard and Irving, 1943). These findings only account for a metabolic reduction consistent with a Q10 effect and thus provide little to no evidence for active metabolic depression or torpor in cunner. More recent metabolic measurements employing more sensitive experimental techniques showed that cunner actively depress their routine metabolic rate in response to environmental temperature (Costa et al., 2013). These adjustments are associated with a metabolic rate well below that which can be accounted for by a Q10 effect and are indicative of a true overwinter torpor (Costa et al., 2013). Cunner's physiological and behavioural adjustments include a reduction in protein

synthesis, lowered metabolic rate and reduced locomotion (Costa et al., 2013; Lewis and Driedzic, 2007; Olla et al., 1975). Cold exposure depresses cunner protein synthesis in white muscle, brain, heart and gill (Lewis and Driedzic, 2007) although it does not affect protein synthesis rates in whole liver and gill mitochondria, indicative of a tissue-specific response to cold exposure (Lewis and Driedzic, 2010; 2007). However, not all species that exhibit overwinter dormancy demonstrate this same metabolic switch. For example, neither brown bullhead nor largemouth bass show a cold-induced active depression of metabolic rate (Crawshaw, 1984). Thus far, no work has explored the potential for the intestine to contribute to the overwintering response.

Given the overwinter cessation of feeding and metabolic depression in many tissues studied, it is logical that the unneeded intestine would be a candidate for energy savings during dormancy and fasting in general. Snakes and anurans that do not make use of fasting or estivation show relatively little GIT modulation in response to long-term food deprivation compared with fasting-adapted conspecifics (Christel et al., 2007). However, it is possible that, in cunner, the GIT plays an important role in the adaptation to long-term fasting and overwintering.

1.2.2 GIT morphology

In addition to down-regulation of digestive capacity by manipulation of biochemical processes, tissue structure can be exploited for energy savings. The intermittent feeding Burmese python reduces villus and microvillus height and enterocyte (the absorptive cells of the intestine) size between meals (Lignot and LeMaho, 2012). In the fasting and dormant Andean toad, there is a reduction in villus height, enterocyte height and intestinal diameter. These structural modifications are accompanied by physiological and

biochemical changes (Naya et al., 2009). In the teleost traíra, intestinal length and pyloric caeca thickness are reduced after 30 and 150 days of fasting, respectively (Rios et al., 2004) and juvenile *Tinca tinca* enterocytes show proteolysis with 48 h of starvation (Ostaszewska et al., 2005). It is possible that morphological plasticity also occurs in the GIT of cunner in response to fasting or dormancy.

1.3 Digestive enzymes

1.3.1 Lipase

Lipases in the GIT catalyze the hydrolysis of ingested triacylglycerols, mainly forming 2-monoacylglycerol and two free fatty acids. The composition of fatty acids in the diets of fish is different from other vertebrates. In mammals, the most prominent lipase is pancreatic lipase. In fish, however, there is a higher proportion of unsaturated fatty acid in the diet for which pancreatic lipase is not optimal. Other lipases such as bile salt-activated lipase (BAL) and neutral bile-salt independent lipase are considered to be the main form of lipase used in lipid digestion in fish (Costa et al., 2013; Murray et al., 2003; Patton et al., 1975).

The effect of diet on lipase activity has been studied from several angles. In turbot, the proportion of lipids in the diet does not seem to have an effect on the activity of BAL (Chao, 1973; Hoehne-Reitan et al., 2001). On the other hand, fasting does cause a decrease in lipase activity in sturgeon and trout (Chao, 1973; Furné et al., 2008). These contrasting results suggest that mechanisms controlling lipase levels in the GIT are

sensitive to several factors and may not be responsive to lipid nutrient levels, but rather the presence of food in the GIT.

1.3.2 Aminopeptidase-N (ANPEP)

Aminopeptidase-N is produced in a variety of tissues such as kidney, intestine, and the respiratory system (in mammals) (Luan and Xu, 2007). Following proteolysis by secreted proteases such as trypsin and chymotrypsin, food protein in the intestine is further digested by ANPEP and other aminopeptidases as the final digestive step before absorption (Bakke et al., 2010). ANPEP cleaves amino acids from peptides at the N-terminus with highest affinity for terminal alanine. Aminopeptidases with other preferential amino acid targets also exist, such as aminopeptidase-A, leucine aminopeptidase and aminopeptidase B. ANPEP in the GIT is bound to the plasma membrane of enterocytes. By measuring ANPEP enzymatic activity in concert with secreted protease, trypsin, pepsin or chymotrypsin, it is possible to infer an individuals digestive capacity for proteins of an individual (Gawlicka et al., 2000; Harpaz and Uni, 1999).

The effect of food deprivation on the activity of ANPEP is unclear. Five species of python show a decrease in proximal intestine ANPEP activity following 30 days of fasting, typically experienced by these species between meals (Ott and Secor, 2007; Secor, 2008). Like pythons, Gila monsters experience lowered ANPEP activity during fasting between meals and up-regulate their intestinal capacity upon feeding (Christel et al., 2007). In Andean toads, a species that undergoes seasonal metabolic depression, ANPEP activity decreases with fasting and more so with hibernation (Naya et al., 2009).

In contrast, house sparrows exhibit high distal intestine ANPEP activity when fasted for 31-34 h, which is explained as an anticipatory strategy to maximize nutrient utilization upon re-feeding (Chediack et al., 2012). This again illustrates differing GIT regulatory strategies between species who are adapted for food deprivation, such as the snake and toad, and those who are not, such as the sparrow.

1.3.3 Alkaline phosphatase (ALP)

ALP is widely distributed throughout the body in tissues such as liver, spleen, intestine, bone, kidney and intestine and is responsible for catalyzing dephosphorylation reactions. The intestinal form of ALP has numerous roles, including absorption of lipids, protection against microbes and pH regulation via regulation of bicarbonate release (Lallès, 2010). Unlike secreted digestive enzymes, ALP is bound to the enterocytes of the brush border epithelium. The localization of ALP in the absorptive membrane of the intestine makes ALP activity a logical proxy for various brush border condition factors such as villus height, cell proliferation, and surface area and can provide an overall estimate of absorptive function. One of the roles of ALP in the intestine, however, is not completely understood. In ALP knockout mice, there is an increase in lipid absorption suggesting a possible rate-limiting function (Narisawa et al., 2003). This finding challenges the accepted function of ALP in digestion and absorption of fatty acids (Buchet et al., 2013; Swarup et al., 1981).

The extent of the ALP activity decrease with food deprivation is variable and dependent on the species. In juvenile roach, short-term (1 or 2 weeks), but not long-term (3 weeks), fasting causes a decrease in GIT ALP activity (Abolfathi et al., 2012; Taheri et al., 2002). Glass eels show a decrease in ALP activity after five days of fasting, and re-

feeding following a 40 day fast elicits a compensatory increase in activity to levels higher than fed individuals. This shows an ability to quickly recover digestive capacity when food becomes available (Gisbert et al., 2011).

1.3.4 Trypsin

Trypsin is an endopeptidase responsible for digesting proteins into small peptides for absorption in the intestine. It hydrolyzes nonterminal peptide bonds located C-terminally to lysine or arginine residues. Trypsin is synthesized as its proenzyme, trypsinogen, in the pancreas of most vertebrates. Trypsinogen is released into the intestine in response to CCK stimulation after a meal and subsequently cleaved by enteropeptidase to form trypsin. Trypsin is capable of auto-catalyzing further activation of trypsinogen (Olsen et al., 2004; Taheri et al., 2002).

In most organisms, protein digestion begins in the stomach. This peptic phase of digestion is characterized by an acidic pH as low as 2 and enzymatic degradation of proteins by pepsin. In GITs that lack a stomach, such as that of cunner, there is no opportunity for peptic digestion, thus, in addition to some mechanical digestion, enzymatic protein digestion must occur entirely in the intestine via the enzymes trypsin and chymotrypsin. However, trypsin and chymotrypsin are not well suited to breaking down native proteins and work optimally on proteins that have been denatured or partially digested by stomach acid and pepsin (Behnke, 1974). In cunner, there appears to be a true trypsin and another trypsin-like enzyme capable of hydrolyzing phenylalanine-methionine peptide bonds which traditional trypsin is not able to do (Simpson and Haard, 1985). In the present study, trypsin activity accounts for the actions of these enzymes together and trypsin transcript expression refers to only true trypsin transcript expression. The

"simplified system" in cunner makes the activity of trypsin a powerful indicator of proteolytic digestive capacity, as the system is not dependent on gastric pH and pepsin activity. The thermal optimum of cunner trypsin activity remains similar to warm water species making it inefficient at cold temperatures, which is hypothesized to be a driving factor influencing cunner's overwinter cessation of feeding (Simpson and Haard, 1985). Fish that do feed in the winter typically express cold-adapted enzymes to optimize digestion. One example is the Greenland cod, in which trypsin activity remains relatively high even in freezing conditions (Simpson and Haard, 1984).

1.3.5 Enzyme regulation in cunner

Cunner represents a valuable model for further expanding the knowledge about digestive enzyme regulation and food deprivation in fish and can provide insights into the digestive function in other vertebrates. The present study utilizes a comparative approach to evaluate the change in digestive enzyme activities in the GIT of cunner during acute food deprivation in the active summer months and natural overwinter fasting. Two opposite strategies for intestinal regulation during fasting have been proposed. A maintenance strategy is exemplified by sparrows and other animals that do not usually experience periods of fasting in which digestive enzymes are maintained at a similar level or higher compared to fed individuals in preparation for food to become available, thus maximizing eventual nutrient utilization (Chao, 1973; Chediack et al., 2012; Green and Farwell, 1971). This strategy is metabolically expensive since energy is required to produce enzymes and maintain the GIT. On the other hand, animals that typically experience periods of fasting have adapted a down-regulatory strategy in which they reduce digestive function by lowering enzyme production. This is commonly seen in snakes with a sit-and-wait foraging strategy (Manjakasy et al., 2009; Secor, 2005). Cunner is a candidate for

either one of these strategies. Cunner feed regularly during the summer, but do not feed at all in the winter when they enter a torpid state. This study aims to identify which model of digestive regulation cunner utilizes in both summer and winter states to cope with food deprivation.

1.4 Appetite and energy balance

1.4.1 Control of energy balance

The regulation of food intake is characterized by a balance between the metabolic requirements of an individual and its energy intake. Endocrine factors regulating food intake can have orexigenic (appetite stimulating) or anorexigenic (appetite inhibiting) effects. Many of these factors such as neuropeptide-Y (NPY), cholecystokinin (CCK), ghrelin, cocaine- and amphetamine- related transcript (CART), are produced within the brain, especially within the hypothalamus (Volkoff et al., 2005). These act in response to neural or endocrine signals from various central and peripheral organs such as the brain, gonad, kidney, pancreas, liver, gut and pituitary (Volkoff et al., 2009). Peripheral tissues are also extremely important with respect to their contribution to appetite-regulating endocrine systems. Many appetite regulating factors, *e.g*. orexin, apelin and CCK are expressed in the gut. Keystone pathway intermediates, such as the mechanistic target of rapamycin (mTOR), exist to integrate and relay information about intracellular energy status to pathways that influence energy intake and fuel mobilization to restore energy balance (Hay and Sonenberg, 2004; Yang and Guan, 2007).

Many of the mechanisms regulating mammalian food intake have been studied in fish and are found to be similar with regards to the structures and actions of their

components. Thus, fish are a useful experimental model for understanding the broader context of appetite regulation and energy balance in vertebrates (Volkoff et al., 2009).

In addition to possessing functionally conserved appetite regulatory systems, fish are also capable of withstanding harsher periods of nutritional availability such as the overwinter drop in food availability. This provides an opportunity to explore endocrine mechanisms at their limit and can provide novel information about their function that would go unnoticed in traditional models.

This study will examine the endocrine contribution of the gut from cunner in different metabolic and nutritional states. It will expand the understanding of four appetite regulators and/or energy sensors expressed in the gut: CCK, orexin, mTOR and apelin.

1.4.2 Orexin

Orexins (or hypocretins), discovered in 1998 in rats, were identified as stimulators of food consumption localized to the lateral and posterior hypothalamic areas of the brain (Sakurai et al., 1998). Previous studies had shown that the ablation of the lateral hypothalamic area elicited a decrease in feeding behaviour, suggesting that this brain region, and potentially the neuropeptides associated with it, were implicated in appetite control (Bernardis and Bellinger, 1993). Studies in rats (Sakurai et al., 1998) and fish (Volkoff et al., 1999) have shown that orexin increased appetite. However, a study comparing the effect of orexin administration on appetite with that of NPY, a potent stimulator of food intake, found that orexin plays a relatively small part in the greater picture of appetite regulation (Edwards et al., 1999), suggesting that orexin may have

physiological roles other than appetite regulation such as control of wakefulness and locomotor activity.

In addition to the hypothalamus, the gut is critically important in maintaining energy balance via nutrient sensing and indirect control of appetite. In agreement with this role was the discovery of orexin-like immunoreactivity in neurons of the gut in rats (Kirchgessner and Liu, 1999). Peripheral orexins also influence gut motility (Näslund et al., 2002) and may stimulate intestinal fluid secretion in guinea pigs (Kirchgessner, 2002), suggesting that peripheral orexins may prepare the gut for digestive processes. Glucose sensitive and orexin immunoreactive neurons co-localize in the lateral hypothalamic area (Kirchgessner, 2002; Oomura et al., 1974) and orexin neuron electrical activity increases in response to low glucose concentration and decreases when glucose concentration is high (Yamanaka et al., 2003), suggesting that the control of orexin expression may also be influenced by blood-glucose levels.

Proper control of the sleep-wake cycle is important in the regulation of energy balance, especially in its relationship to foraging behaviour. The involvement of orexin in controlling wakefulness is evidenced by studies showing that a mutation in the orexin receptor Hctr2 causes narcolepsy (a neurological disease characterized by an inability to regulate sleep-wake cycles for which the cause is unknown) in dogs (Lin et al., 1999) and orexin knockout mice exhibit symptoms of narcolepsy (Chemelli et al., 1999). Furthermore, seven out of nine narcoleptic humans had no detectable brain orexin expression (Nishino et al., 2000).

In cunner, orexin has been partially cloned and its transcript expression detected in the brain and throughout the body in the gill, gut, liver, spleen, kidney, ovary and heart (Babichuk and Volkoff, 2013). In cunner, hypothalamic levels of orexin are reduced following three weeks of fasting and drop further during overwinter torpor (Babichuk and Volkoff, 2013). Little is known about the peripheral action of orexin, especially in fish, and cunner presents a useful model to expand on the current knowledge.

1.4.3 Cholecystokinin (CCK)

CCK is released by enteroendocrine cells of the GIT following a meal in response to lipid and protein digestion products. Early studies in rats established CCK as a satiety signal, as intraperitoneal (IP) CCK injection reduced food intake (Gibbs et al., 1973). The actions of CCK are well conserved across species; it decreases gastric emptying, increases fluid secretion and increases motility (Raybould and Tache, 1988).

In 1994, CCK was isolated in fish and its mRNA expression was found in brain and gut (Himick and Peter, 1994). CCK decreases feeding when administered by IP or intracerebroventricular (ICV) injection in goldfish (Himick and Peter, 1994) or by IP injection in catfish (Silverstein and Plisetskaya, 2000). In addition, both summer fasting and overwintering induce decreases in CCK gut expression in winter flounder (MacDonald and Volkoff, 2009). Similarly, yellowtail tuna fasted for 72 h have lowered CCK transcript expression in the anterior intestine (Murashita et al., 2006). There also seems to be a seasonal effect on CCK expression in winter flounder, where expression is much higher during the summer (MacDonald and Volkoff, 2009). CCK in goldfish is also influenced by season and gender, where females have higher CCK transcript expression

than males at certain times of year depending on the brain region (Peyon et al., 1999). However, no changes are seen in CCK expression between fed and fasted winter skate (MacDonald and Volkoff, 2009). Although CCK is generally accepted as a satiety signal following meal consumption, there is increasing evidence suggesting that the basal level of CCK may be implicated in controlling seasonality and the long-term regulation of food intake and energy balance.

1.4.4 Apelin

Apelin was first identified as the ligand for the APJ receptor in 1998, initiating a search for its potential role in brain signaling (Tatemoto et al., 1998). In rats, apelin was found to have widespread distribution in tissues including mammary gland, lung, heart, adipose tissue, brain and ovary. Apelin injections in Wistar rats elicit a drop in blood pressure when administered intravenously and an acute increase (1 hour) in drinking behaviour (Kidoya and Takakura, 2012; Lee et al., 2000; Taheri et al., 2002).

The hypothalamic distribution of apelin is consistent with those areas responsible for the regulation of food intake. This prompted investigation into the potential for apelin to regulate energy balance and food consumption. ICV injection of apelin in rats elicits an increase in water consumption (Lee et al., 2000; Taheri et al., 2002). To date, the effects of apelin on feeding are not clear. Apelin has a limited and time-specific orexigenic effect in fasted rats 2-4 hours post injection (Taheri et al., 2002). ICV injection of apelin at dusk has inhibitory effects on nocturnal feeding in rats, but high dose daytime injections in fed rats stimulates appetite, while no effect on water consumption is observed (O'Shea et al., 2003). Apelin administered by ICV injection also reduced appetite in Wistar rats in either a fed or fasted state; fed rats showed a reduction in feeding after 8 hours and fasted after

24 hours (Sunter et al., 2003). These results suggest a food-state specific and time-specific role for apelin and potentially implicate it in the control of energy balance, not simply feeding behaviour.

Given the evidence for apelin as an appetite regulator and its observed presence in the gastrointestinal tract, its role in this tissue may play a part in energy balance. Apelin expression has been observed in the stomach and intestine of rats, the latter having relatively lower expression (G. Wang et al., 2004). Gastric cell proliferation is also promoted by apelin *in vitro* and apelin stimulates the secretion of CCK in enteroendocrine cell preparations (G. Wang et al., 2004).

In fish, the effects of apelin on feeding have only been assessed in goldfish, in which ICV or IP injections stimulate food intake and fasting increases apelin brain transcript expression (Volkoff and Wyatt, 2009) and cavefish, in which apelin IP injections increase food intake (Penney and Volkoff, 2014). Additionally, apelin has been further implicated as an orexigenic factor in fish by recent work with spexin, a novel peptide identified as a satiety factor in goldfish. Following spexin brain injection in goldfish, known orexigenic factors NPY and agouti-related peptide as well as apelin show reduced transcript expression. In contrast, transcript expression levels for anorexigenic factors (CCK, CART, proopiomelanocortin, melanin-concentrating hormone and corticotropin releasing hormone) are increased (Wong et al., 2013). This supports an orexigenic role of apelin in goldfish. Studies examining different species at various levels of food status may help to uncover new roles of apelin and the mechanisms that lie behind its action.

1.4.5 Mechanistic target of rapamycin (mTOR)

mTOR is a keystone peptide to several pathways that are capable of receiving diverse inputs and is potentially implicated in the development of cancer, diabetes and obesity (Sarbassov et al., 2005). Notably, mTOR detects cell energy status via the AMP:ATP ratio and AMPK signaling and contributes to the regulation of metabolism, cell autophagy and ribosome production (Berthoud and Morrison, 2008; Woods et al., 2008). More specifically, mTOR has an intricate role in regulating protein synthesis, is capable of directly sensing amino acid availability (Berthoud and Morrison, 2008) and is also involved in the regulation of food intake (Martins et al., 2012).

In Sprague-Dawley rats, hypothalamic mTOR is sensitive to leucine, that upon administration, causes a decrease in food intake (Cota et al., 2006). Incorporation of leucine in mouse diets has been shown to increase uncoupling protein 3 expression resulting in elevated metabolic rate, decrease in weight gain and lowered adiposity (Zhang et al., 2006). It is thought that mTOR may be implicated in this system to mediate the control of protein synthesis in response to leucine levels (Woods et al., 2008). Providing evidence for this theory are the findings that hypothalamic mTOR signalling is not regulated following central leucine administration as determined by increased phosphorylation of downstream targets of mTOR: S6 kinase 1 and S6 ribosomal protein (Cota et al., 2006). These findings provide convincing evidence that mTOR is connected with hypothalamic cell energy status and signalling. This signalling has been linked to, and can mediate, the action of other appetite and energy status related peptides. In rats, leptin treatment by ICV injection increases mTOR signalling, and the inhibition of mTOR

by rapamycin (a well-known inhibitor of mTOR) diminishes this effect (Cota et al., 2006), suggesting that mTOR is an intermediate peptide requisite to the action of leptin.

Peripheral actions of mTOR are less clear. Although some papers allude to a peripheral role of mTOR in synthesizing cell energy status and nutritional signals with hormonal signals (Cota et al., 2006), little evidence has been provided in vertebrates and many of these claims are based on knowledge obtained from unicellular organisms (Sabatini et al., 1994; Schmelzle et al., 2004). However, there is a reasonably comprehensive understanding of mTOR's upstream inputs and downstream cellular effects. Thus, studying the role of mTOR in peripheral systems will help develop a more comprehensive picture of appetite regulation and whole organism energy balance.

1.5 Thesis objectives

I aim to identify the molecular and biochemical changes in the gastrointestinal tract (GIT) of the cunner that contribute to it's ability to withstand overwinter torpor by evaluating digestive enzyme activity, transcript expression of appetite- and energy status-related peptides, and changes intestinal morphology. In addition, I aim to draw comparisons and outline differences between these GIT modifications during summer fasting and overwinter torpor. Finally, by establishing new data on appetite regulating peptides during an extreme case of food deprivation, I hope to provide new insights for future research into the mechanism of these often complicated and multifunctional endocrine and regulatory systems.

Three overarching techniques define this work. The quantification of enzyme activity was assessed by enzymatic assay. Molecular cloning and quantitative real-time

PCR was used to identify novel gene sequences, construct phylogenies and evaluate transcript expression for the digestive enzymes in question as well as putative appetite regulating peptides. Finally, histological examination of the intestine is used to shed light on the possibility of structural modifications to the intestine during over-winter food deprivation.

2 Materials and methods

2.1 Animals

Fish were caught with baited passive fish pots off the shore in Norris Point, Newfoundland (NL) during the fall of 2011 (to be used for torpor experiments) and the spring of 2012 (to be used for summer food deprivation experiments). The water temperature at the time of catch was between 10 and 15ºC. Traps were set off the wharf where the water depth ranges from 3 to 6 m. Fish were housed at the Bonne Bay Marine Station (Norris Point, NL) in 1 m^2 (400 L) stock tanks. For the summer experiment, fish were subsequently transferred to 0.65 m^2 (170 L) tanks at a density of 20-30 fish per tank. Tanks were supplied with flow through seawater. Natural seawater temperature and photoperiod were used for all experiments. Dissolved oxygen and water temperature were monitored to ensure adequate water recycling (PT4 Tracker, Point Four Systems, Coquitlam, BC, Canada). All tank environments were enriched with rock shelters to provide refuge for fish. All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines.

2.2 Tissue collection

Fish were netted by hand from their tanks and killed with an overdose of MS-222 (tricaine methanesulfonate, Syndel Laboratories, Vancouver, BC, Canada) followed by a cervical transection of the spinal cord. Body mass and standard length were recorded for each individual. The peritoneal cavity was dissected for liver, gonad and intestine. Gonad and liver were weighed to calculate gonadosomatic index [GSI = (gonad mass · body $mass^{-1}$) × 100] and hepatosomatic index [HSI = (liver mass · body $mass^{-1}$) × 100]. Intestines were emptied of contents and divided into thirds lengthwise (proximal, middle

and distal intestine). Each intestinal region was transferred into a 2 mL tared and labeled round bottom Eppendorf tube and immediately frozen in liquid nitrogen. Tissues were kept in a liquid nitrogen Dewar for the duration of the experimental trials and during transport back to Memorial University of Newfoundland (total time approximately 6 weeks). Upon returning to the lab, tissues were transferred to a -80ºC freezer.

2.3 Experimental design

All experiments were carried out at the Bonne Bay Marine Station (Norris Point, NL). Torpid fish were sampled from their original holding tank in March 2012 (from fish caught in fall 2011, Table 1). Food deprivation, effect of food type, and periprandial studies were conducted in July and August 2012 (from fish caught in spring 2012). For these experiments, fish were transferred from stock tanks to experimental tanks. Six tanks were used for food deprivation trials (Table 1), one tank was used for periprandial studies and one for food type.

2.3.1 Torpor

Fish in overwinter torpor were sampled on March 28, 2012, and had been in torpor since December 2011. The water temperature was 1ºC at the time of sampling. Nine fish were sampled for use in enzyme assays and quantitative reverse transcription polymerase chain reaction (qPCR) experiments. Fish did not accept food while in torpor.

2.3.2 Food deprivation

The summer food deprivation experiment was conducted in July and August, 2012, when the water temperature ranged from 13 to 18ºC. Thirty fish were transferred to each of six tanks (Table 1). Large numbers of fish per tank were used to reduce stress. Fish were acclimated under standard conditions for one week, during which they were all fed to satiety with cut up frozen squid 3 times per week between 5 and 7 pm. After the acclimation period, fish in 3 control tanks continued to be fed to satiety on the same 3 times per week schedule. Three tanks were not fed for a period of 4 weeks followed by 1 week of re-feeding. Nine fish (3 from each triplicate tank) were sampled after 4 weeks for both control and fasted fish. Nine fish (3 fish per tank) were sampled following the one-week re-feeding period.

Table 1. Experimental design overview for fed, fasted, re-fed and torpor fish used in fasting and torpor studies. Holding tank was 400 L, all others (A-F) were 170 L. All fish tissues were used for enzyme assays and qPCR unless noted otherwise (for histology).

Tank(s)	Experimental condition	# fish sampled
March 2012		
Holding tank	Torpor	9
Holding tank	Torpor *(for histology)*	3
July-August 2012		
A, B, C	Fed	3 fish/tank (n=9)
A, B, C	Fed *(for histology)*	1 fish/tank (n=3)
D, E, F	Fasted 4 weeks	3 fish/tank (n=9)
D, E, F (following 4 weeks fasting)	Refed 1 week	3 fish/tank (n=9)

2.3.3 Periprandial study

Fish in one tank were fed on the same schedule as described above for fed fish and acclimated for 2 weeks. Fish were fasted for 2 days prior to sampling. On the day of the experiment, 9 fish were sampled immediately before feeding. Fish were then fed to satiety with cut up squid. One hour after feeding, another 9 fish were sampled. Photoperiod and temperature were the same as for the food deprivation experiment as the experiments were conducted at similar times.

2.3.4 Effect of food type

Twenty fish housed in one tank were fed to satiety with a commercially available pellet diet (4 mm pellet size, Marine Finfish diet, Skretting, Vancouver, BC, Canada) 3 times per week for 5 weeks. The feeding schedule was the same as for the squid fed group in the fed/fasted experiment. Nine fish were sampled on a non-feeding day five weeks after the diet began. Nine squid-fed fish were sampled at the same time from the control tank for the fed/fasted experiment. Photoperiod and temperature were the same as for the food deprivation experiment.

2.4 Enzyme assays

Intestinal tissue destined for enzyme assays was homogenized in five volumes (w/v) of 50 mM Tris/HCl Buffer (pH 7.8) with 0.2% NaCl using a PowerGen 125 (Thermo Fisher Scientific, Ottawa, ON, Canada) homogenizer for 3x10 s bursts with 30 s pauses on ice to allow tissue to cool. Following homogenization, samples were centrifuged at 8000 × g at 4ºC for five minutes and the supernatants were transferred to a new tube by micropipette. All assays were monitored in a Biotek Powerwave XS microplate spectrophotometer (Biotek, Winooski, VT, USA). Enzyme V_{max} values were determined using a linear

regression algorithm built into the Biotek Gen5 plate reader software package. Samples were run in duplicate and averages were taken. Enzyme activity was expressed as U/mg protein.

Assays for the effect of food type and periprandial studies were performed with tissue from the mid intestine and they took place before all intestinal regions had been evaluated by enzyme assay. Subsequently obtained preliminary data evaluating the enzyme activities in proximal, mid and distal intestinal regions suggested a more significant impact of fasting on enzyme activity in the distal intestine, so this regions was chosen for the food deprivation (fasting and re-feeding) and torpor experiments.

2.4.1 Trypsin

Trypsin activity was assayed by a modified protocol developed by Erlanger (Erlanger et al., 1961). 15 μL of undiluted homogenate was combined with 35 μL assay buffer (100 mM ammonium bicarbonate) and monitored for 5 minutes at 405 nm to establish a steady state before beginning the assay. 50 μL of substrate in solution was then added to the reaction (1 mM BAPNA (a-N-benzoyl-l-arginine-p-nitroanilide hydrochloride) in 100 mM ammonium bicarbonate buffer). The absorbance at 405 nm was monitored for 40 minutes at 25ºC.

2.4.2 Lipase

Lipase activity was assayed as previously described for Atlantic halibut and winter flounder (Gawlicka et al., 2000; Murray et al., 2003). Intestinal homogenate was reacted with 0.4 mM p-nitrophenyl myristate as substrate in buffer containing 0.5% Triton X-100

and 100 mM ammonium bicarbonate. Reactions were monitored at 405 nm for 30 minutes at 25ºC.

2.4.3 ALP

ALP activity was assayed as previously described for Atlantic halibut (Gawlicka et al., 2000). 4 mM p-nitrophenyl phosphate was used as substrate in 55 mM ammonium bicarbonate and 0.6 mM $MgCl_2$. Reactions were monitored at 405 nm for 10 minutes at 25ºC.

2.4.4 ANPEP

ANPEP activity was measured as previously described for pig kidney (Pfleiderer, 1970). 1.66 mM L-alanine p-nitroanilide hydrochloride was used as substrate in 60 mM potassium phosphate, pH 7.2. Reactions were monitored at 405 nm for 10 minutes at 25ºC.

2.4.5 Protein quantification (Bradford method)

Bradford assays were used to quantify homogenate total protein content. The Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific) was used as per the manufacturer's instructions. Reactions were performed in duplicate and protein concentrations were interpolated by comparison with a standard curve of BSA protein dilutions prepared fresh each day of Bradford assays. End-point absorbance at 595 nm was measured using a Powerwave XS microplate reading spectrophotometer (Biotek). Standard curve analysis (plotting and interpolation) of protein content for unknown homogenate samples were constructed using Gen5 software (Biotek) with a 4^{th} degree polynomial regression as recommended by the manufacturer (Thermo Fisher Scientific).

2.5 Molecular cloning and sequencing

2.5.1 RNA isolation

Approximately 30 mg of intestinal tissue was homogenized in TRI reagent (Sigma-Aldrich, Oakville, ON, Canada) and RNA was extracted according to the manufacturer's protocol. Isolated RNA was quantified by spectrophotometry on a NanoDrop 2000 UV-Vis Spectrophotometer (NanoDrop, Wilmington, DE, USA). RNA quality was evaluated by the 260/280 nm and 260/230 nm spectrophotometric ratios from the NanoDrop readings and by gel electrophoresis (1.5% w/v agarose gel, 80V for 30 minutes). Finally, each sample was cleaned using the Thermo Scientific GeneJET RNA Cleanup Kit according to the manufacturer's protocol (Thermo Fisher Scientific). Isolated RNA samples were stored at -80ºC in 1.5 mL nuclease-free Eppendorf tubes.

2.5.2 First strand cDNA synthesis

1 μg of RNA from each sample was used in synthesizing cDNA for downstream use in cloning and qPCR. cDNA was synthesized using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) using both the supplied anchored oligo-dT primers and random hexamers as primers for first strand synthesis. A genomic DNA wipe-out step was performed before reverse transcription using the supplied DNase. cDNA products were stored at -20ºC.

2.5.3 Primer design for cloning

Primers used to amplify genes of interest for cloning were designed based on Basic Local Alignment Search Tool (BLAST) alignments of gene sequences from several species

(Altschul et al., 1990). Nucleotide BLAST searches and alignments were performed using the gene sequence of interest from a closely related species. Sequence regions conserved among species were selected as primer target regions and degenerate primers were designed using Primer 3 software (Koressaar and Remm, 2007). Primer sequences for cloning are presented in Table 2.

2.5.4 5' rapid amplification of cDNA ends (5' RACE)

5' RACE was performed to extend the sequenced region for apelin. First-strand cDNA synthesis was performed using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) with an apelin gene specific primer. cDNA was then purified with Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA, USA) and tailed using terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, CA, USA). Two PCRs using overlapping gene specific primers and adaptor primers designed to bind the synthesized poly-A tail were performed to amplify the 5' sequence. Gene specific and adaptor primers used for 5' RACE are presented in Table 3.

2.5.5 cDNA PCR and cloning

PCR was performed with primers designed to target genes of interest using GoTaq Green Master Mix (Promega, Madison, WI, USA). PCR products were separated by gel electrophoresis (1.5% agarose gel in 1x TAE buffer, 120V for 25 minutes) and bands of appropriate size for each primer pair were excised and purified using the GenElute Gel Extraction Kit (Sigma-Aldrich).

Extracted PCR products were ligated, transformed and grown in recombinant *E. coli* using the pGEM®-T Easy Vector System (Promega) per the manufacturer's protocol.

Plasmids were isolated from *E. coli* cultures with the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The presence of an insert in the cloning vector was verified in purified plasmid products using an EcoRI digest followed by agarose gel electrophoresis. Plasmids containing an insert were sent for Sanger sequencing at The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, ON, Canada).

2.5.6 Tissue distribution

cDNA was synthesized by reverse transcription as described above from several tissues: proximal intestine, mid intestine, distal intestine, liver, spleen, kidney, heart, brain, gill and skin. Equal amounts of total RNA from each tissue were used to standardize the amount of cDNA synthesized. PCR products were separated by gel electrophoresis (1.5% agarose in TAE buffer) for 30 min at 120 V. Images were captured using an Epichemi Darkroom Bioimaging System (UVP, Upland, California, USA) and analyzed with LabWorks 4.0 software. Negative controls (no cDNA) were performed for each gene of interest.

2.6 Real-time quantitative PCR

qPCR primers were designed for cunner genes of interest using a combination of Primer 3 (Koressaar and Remm, 2007), RNASPL (Softberry, Mount Kisco, NY, USA), BLAST (Altschul et al., 1990) and Clustal Omega alignments (Sievers et al., 2011). Primers were designed to span an exon-exon junction or flank an intron sequence greater than 100 bases long that would be too large to amplify during the 15 s elongation step of qPCR. Several sets of primers for each gene of interest were optimized and those with the highest efficiency and linearity when amplifying four serially diluted samples of cDNA were chosen. Primer sequences used in qPCR are presented in Table 4.

qPCR was performed using the KAPA SYBR FAST qPCR Kit Master Mix (Kapa Biosystems, Boston, MA, USA). cDNA from reverse transcription reactions was diluted 1:4 for all qPCR reactions. Duplicate (10 μL) reactions were made up of 5 μL KAPA Master Mix, 2 μL cDNA, 0.2 μL 10 μM sense primer, 0.2 μL 10 μM antisense primer and 2.6 μL molecular grade water. Plates were loaded with an epMotion 5070 automated pipetting system (Eppendorf, Mississauga, ON, Canada). qPCR assays were monitored using a Mastercycler ep realplex2 thermocycler (Eppendorf). Drift correction was performed for all quantifications. Relative quantification was performed based on ubiquitin (reference gene) expression and calculated with Realplex software (Eppendorf). After testing several candidate genes (elongation factor 1α, glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein L37) as reference genes, ubiquitin was found to have the lowest variability and most stable expression across treatment groups.

Table 2. Primers used in RT-PCR cloning of genes of interest designed from multiple sequence alignments of target genes in other species

Target Gene	Orientation	Primer sequence (5'-3')
ALP	Sense	GACACACGTGGAGATGGACA
	Antisense	ACTGACTTGCCTGCGTCTTT
BAL	Sense	ATYCGYTCATTTGGAGGWGA
	Antisense	GCAGCCGWGTAGGAAC
ANPEP	Sense	AGATGCAGCCCACAGAYGC
	Antisense	GCCACARGTCATTCCACCAC
Apelin	Sense	CCTCCACCGGAGCATRGCAAAG
	Antisense	TAGAATGGCATTGGCCCCTTRTG
mTOR	Sense	AGCTGAGTGAAGACCAGCAG
	Antisense	CACTGCTGAGCCAATGAAAA

IUPAC degenerate nucleotide codes used: Y (C or T), W (A or T) and R (A or G)

Table 3. Primer sequences used for 5’ RACE PCR

Primer name	Primer sequence (5’-3’)
Apelin gene specific primer 1	TATGGGAAAGACGTGGCCGTG
Apelin gene specific primer 2	CTCTTCCAGCCGGCTGGTCTG
Oligo-dT adapter primer	GGCCACGCGTCGACTAGTAC(T_{17})
Adapter primer	GGCCACGCGTCGACTAGTAC

Table 4. Primer sequences used for qPCR

Target Gene	Orientation	Primer sequence (5'-3')	GenBank ID
ALP	Sense	TCTCCACTATCGACTACCAGGA	KF924763
	Antisense	AGCCACATCCTCTCCTCCAT	
BAL	Sense	CTGAGGAGATTGCCCTGAAG	KF924765
	Antisense	GACTGCCAGCCAAGGTAAGA	
ANPEP	Sense	CCATTGATGGAGTTTTGATCC	KF924764
	Antisense	GGATGGGACCAGTTTTGTTG	
Trypsin	Sense	TACGTCAAGACTGTGGCTCT	AY496969
	Antisense	GGATGGGGATGTTCAAGCAC	
Orexin	Sense	CACCCTCCATCTTGTCCTGC	JX126914
	Antisense	CCAGGGTGATTGTTTTGCCC	
CCK	Sense	TCCAGGAAAGGTTCTGTGCG	JX126917
	Antisense	CCGAAACCCATCCATCCCAA	
Apelin	Sense	GTGAAGATCCTGACGCTGGT	KC895876
	Antisense	GTAGGCGCATCTTCAAGCTC	
mTOR	Sense	GGGAGAATGGGGCCAGTTG	KC791688
	Antisense	CAGCCATTCGAGCCATCTTG	
Ubiquitin	Sense	CACCCTCCATCTTGTCCTGC	JX218022
	Antisense	CCAGGGTGATTGTTTTGCCC	

2.7 Sequence analysis and phylogeny construction

Nucleotide sequences were translated and open reading frames were determined using ExPASy online software (Artimo et al., 2012) in combination with BLAST protein alignment (Altschul et al., 1990). Signal and mature peptide sequences were predicted with SignalP (Petersen et al., 2011) and Clustal Omega (Sievers et al., 2011) alignment with annotated sequences from other species. Peptide alignments were performed with sequences from multiple species using Clustal Omega online software (Sievers et al., 2011). Phylogenies were built with MEGA5 (Tamura et al., 2011) based on Clustal Omega peptide alignments. Evolutionary relationships were predicted, and trees constructed, using the Neighbour-joining algorithm. Bootstrap analysis was performed with 100 replicates to assess confidence.

2.8 Histology

Three cunner intestines each from both fed and torpid fish were fixed in Bouin's solution. Intestines were embedded in paraffin wax, cross-sectioned with a microtome (10 μm) and mounted on silane prepared slides (Sigma-Aldrich, Oakville, ON, Canada). Tissues were stained with haematoxylin and eosin and visualized on a compound light microscope. Images were captured with a Moticam digital microscope camera and accompanying software (Motic, Richmond, BC, Canada).

2.9 Statistical analysis

Statistical analysis was performed with Prism 6 (GraphPad Software, San Diego, CA, USA). Transcript expression and enzyme activities for fasting and torpor experiments were compared using one-way ANOVAs followed by Tukey's post-hoc tests. Where indicated for mTOR analysis, a Student's t-test was also performed to compare treatment

groups. qPCR results from transcript expression studies were expressed as percentages relative to a control group (fed summer fish). Significance was inferred at $p<0.05$.

3 Results

Results from this thesis have been published in:

Hayes, J., Volkoff, H., 2014. Characterization of the endocrine, digestive and morphological adjustments of the intestine in response to food deprivation and torpor in cunner, *Tautogolabrus adspersus*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 170, 49-59.

3.1 Molecular cloning, sequencing and phylogenetic analysis

3.1.1 Enzymes

The nucleotide sequence corresponding to a 163 amino acid fragment of cunner alkaline phosphatase (ALP) mature peptide was cloned (Figure 1). Compared with other species by BLAST alignment (Figure 2), amino acid sequence identities are 78% for zebrafish, 78% for goldfish, 41% for western clawed frog, 41% for rat and 39% for human (Figure 3).

A cDNA coding for a 202 amino acid section of the mature aminopeptidase-N (ANPEP) peptide was cloned for cunner (Figure 4). When aligned by BLAST with other species (Figure 5), the amino acid sequence identities are 89% for European seabass, 84% for northern bluefin tuna, 84% for winter flounder, 84% for spotted sand bass, 76% for zebrafish, 75% for grass carp, 66% for chicken, 64% for rat, 64% for human and 63% for western clawed frog (Figure 6).

A cDNA coding for a 221 amino acid portion of the mature bile salt-activated lipase (BAL) peptide was cloned (Figure 7). BLAST alignment with other species (Figure 8) gives amino acid sequence identities of 84% for European seabass, 82% for winter flounder, 81% for *Takifugu rubripes*, 74% for Nile tilapia, 65% for zebrafish, 63% for orange spotted grouper, 55% for rat, 55% for Brandt's bat, 54% for western clawed frog and 52% for western lowland gorilla (Figure 9).

caccacgagggcaaggccaagcaggctctgcatgaaactgtggagatggacagagccatc
H H E G K A K Q A L H E T V E M D R A I
gggcgggcgggtctcctgaccagcatccacgatacattgaccatagtgacagctgaccat
G R A G L L T S I H D T L T I V T A D H
tctcacgtcttcaactttggaggctacacacgccgaggaaacacaatatttggtctggcc
S H V F N F G G Y T R R G N T I F G L A
ccgatcctgagtgatgtcgaccagaagccctttaccgccatcttatacagcaacggacca
P I L S D V D Q K P F T A I L Y S N G P
ggttacaaacttgttaatggtgcaagagagaatgtctccactatcgactaccaggaaaac
G Y K L V N G A R E N V S T I D Y Q E N
aactaccaggcccaatcagctgtaccaatgagcatggagactcatggaggagaggatgtg
N Y Q A Q S A V P M S M E T H G G E D V
gctgtgtttgctaaaggtcccctggcccatctgctccacagggtccacgagcagaac
A V F A K G P L A H L L H R V H E Q N

Figure 1. Partial coding sequence and translated amino acid sequence for cunner alkaline phosphatase (ALP). Underlined amino acids indicate the mature peptide.

```
wcf       KNPKGFFLFVEGGRIDHGHHDGNAKQSLTEALEFDKAVLRGGQLTQENETLTVVTADHSH    392
h         KNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVEMDRAIGQAGSLTSSEDTLTVVTADHSH    198
r         KNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVEMDEAIGKAGTMTSQKDTLTVVTADHSH    381
z         KNERGFFLLVEGGRIDHGHHEGKAKQALHEAVEMDRAITRAGLLTSEYDTLTVVTADHSH    417
c         ------------------HHEGKAKQALHETVEMDRAIGRAGLLTSIHDTLTIVTADHSH    66
as        KNPRGFYLLVEGGRIDHGHHEGKAKQALHEAVEMDRAIGRAGLITSIYDTMTVVTADHSH    395
lyc       KNPNGFYLLVEGGRIDHGHHEGKAKQALYEAVEMDRAIGRADLMTSIHDTLTIVTADHSH    400
es        RNPSGFYLLVEGGRIDHGHHEGKAKQALHEAVEMDRAIGRADLMTSIHDTLTIVTADHSH    384
gs        KNPSGFYLLVEGGRIDHGHHEGKAKQALHEAVEMDRAIGRASLLTSIHDTLTIVTADHSH    385
fr        KNPNGFYLLVEGGRIDHGHHEGKAKQALYEAVEMDRAISRAGLMTSIHDTLTIVTADHSH    384
sgp       KNPNGFYLLVEGGRIDHGHHEGKAKQALYEAVEMDRAIQRAGLLTSVHDTLTIVTADHSH    384
                 :  ...    **:*:***:* *::*:*.*: :.  :*.  :*:*:*******

wcf       VFTFGGYTDRGNSIFGLAPSLA-SDRKPYTSLLYGNGPGYPLPDAIRANITGVNTGSNSY    451
h         VFTFGGYTPRGNSIFGLAPMLSDTDKKPFTAILYGNGPGYKVVGGERENVSMVDYAHNNY    258
r         VFTFGGYTPRGNSIFGLAPMVSDTDKKPFTAILYGNGPGYKVVDGERENVSMVDYAHNNY    441
z         VFSFGGYTPRGNSIFGLAPTLSDVDQKPFTAILYGNGPGFKLVNGARENVSTVDYQQNNY    477
c         VFNFGGYTRRGNTIFGLAPILSDVDQKPFTAILYSNGPGYKLVNGARENVSTIDYQENNY    126
as        MFNFGGYTPRGNTIFGLAPMLSDVDQKPFTAILYGNGPGFKVINGLRENVSTLDYQGNNY    455
lyc       VFSFGGYTSRGNTIFGLAPMLSDVDQKPFTSILYGNGPGYKLVNGARENVSTIDYQENNY    460
es        VFNFGGYTGRGNTIFGLAPMLSDIDQKPFTSILYGNGPGYKIVKGARENVSTIDYQENNY    444
gs        MFNFGGYTPRGNTIFGLAPMLSDADQKPFTSILYGNGPGYKLVNGARENVSTIDYQENNY    445
fr        VFNFGGYTHRGNTIFGLAPAVSDVDQKPFTAILYGNGPGYKLVNGGRENVSAIDYQENNY    444
sgp       VFNFGGYTHRGNPIFGLAPAVSDVDQKPFTAILYGNGPGYKLVNGGRENVSTYNYQENNY    444
          :*.***** *** ****** ::  *:**:*::**.****: :  . * *::  :   *.*

wcf       LQQAAVPVVSETHGGEDVAIMAKGPQAHLFHGFHEQSYIAHVMAYAACLEPYT-ECPPNG    510
h         QAQSAVPLRHETHGGEDVAVFSKGPMAHLLHGVHEQNYVPHVMAYAACIGANLGHCAPAS    318
r         QAQSAVPLRHETHGGEDVAVFAKGPMAHLLHGVHEQNYIPHVMAYASCIGANLDHCAWAS    501
z         QAQSAVPLRMETHGGEDVAIFSKGPMAHLLHGVQEQHYIPHVMAYAACIGQNKDHCRTNS    537
c         QAQSAVPMSMETHGGEDVAVFAKGPLAHLLHRVHEQN----------------------    163
as        QAQSAVPLRMETHGGEDVAVFAKGPMAHLLHGVHEQNYIPHVMAYAACIGQNRDHCMSSG    515
lyc       QAQSAVPLSMETHGGEDVAVFAKGPLAHLLHGVHEQNYIPHVMAYAGCIGQNREHCKLNN    520
es        QAQSAVPLSMETHGGEDVAVFAKGPLAHLLHGVHEQNYIPHVMAYAGCIGQNREHCRSRS    504
gs        QAQAAVPLSMETHGGEDVAVFAKGPMAHLLHGVHEQNYIPHVMAYAGCIGQNREHCLSRS    505
fr        QAQAAVPLSMETHGGEDVAVFAKGPMAHLLHGVHEQNYIPHVMAYASCIGQNRQHCAGHN    504
sgp       QAQAAVPLSSETHGGEDVAVFAKGPMAHLLHGVHEQNYIPHVMAYASCIGQNRQHCLQHN    504
            *:***:  *********:::*** ***:* .:**
```

Figure 2. Alkaline phosphatase (ALP) amino acid sequence alignment for western clawed frog (wcf; NP_001116946.1), human (h; BAA88367.1), rat (r; NP_037191.1), zebrafish (z; AFU97155.1), cunner (c, **bold**), Atlantic salmon (as; NP_001133448.1), large yellow croaker (lyc; AEL33276.1), European seabass (es; CBN80701.1), gilthead seabream (gs; AAP04486.1), *Takifugu rubripes* (fr; NP_001027823.1) and spotted green pufferfish (sgp; AAN64271.1).

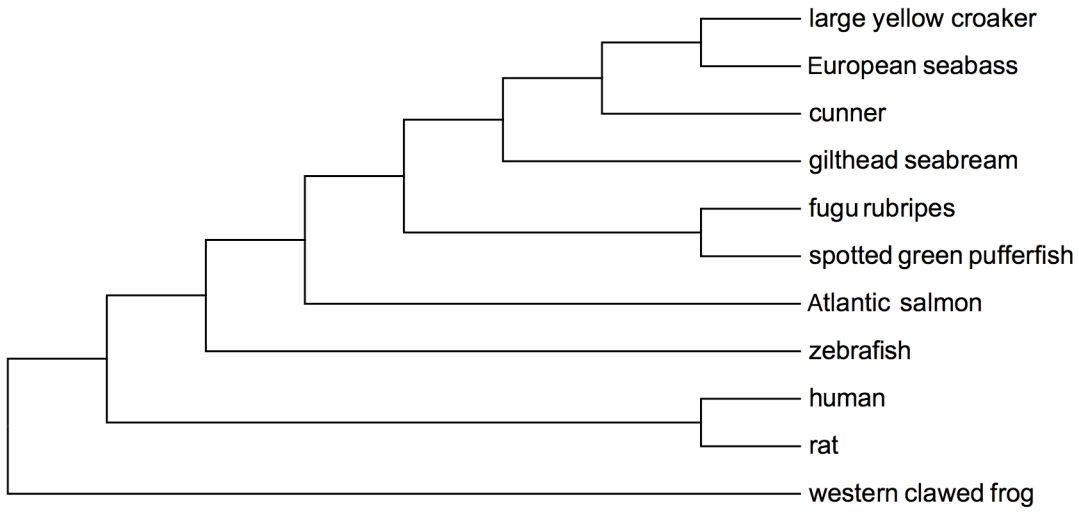


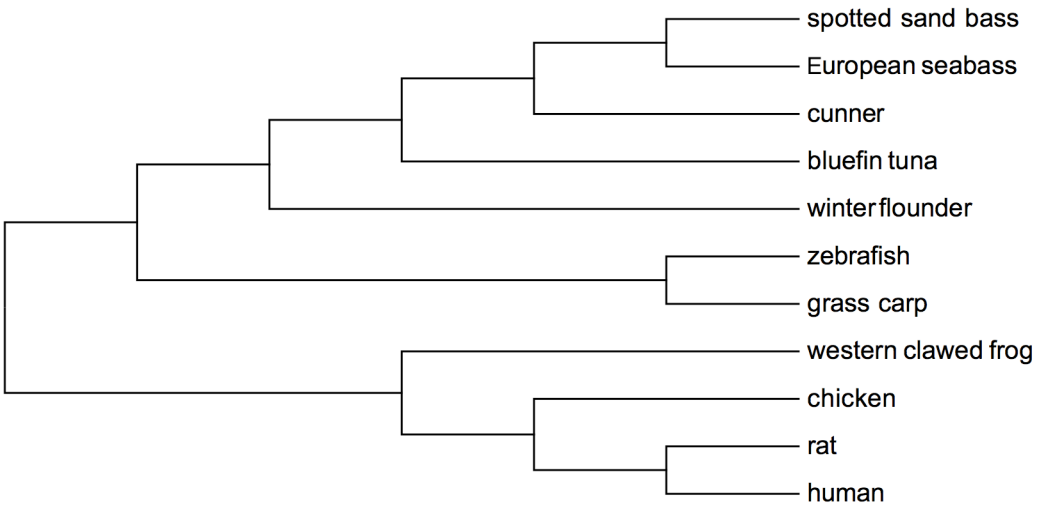
Figure 3. Phylogenetic tree constructed using MEGA5 based on Clustal Omega alignment of alkaline phosphatase (ALP) amino acid sequences. Evolutionary history inferred by the Neighbour-Joining method.

```
atgcagcccacagatgccaggaaggctttcccctgtttcgatgaacctgctatgaaagct
 M  Q  P  T  D  A  R  K  A  F  P  C  F  D  E  P  A  M  K  A
attttccacatcactctgatccaccagaaccgaactgtagccctgtccaatggggaagaa
 I  F  H  I  T  L  I  H  Q  N  R  T  V  A  L  S  N  G  E  E
aaggagtcaggccatgtcaacatggacggtcaggttgtgcagaggactgtttttgagcca
 K  E  S  G  H  V  N  M  D  G  Q  V  V  Q  R  T  V  F  E  P
actgagaaaatgtccacctacttgttggcattcattgtcagtgactttggattcatcaat
 T  E  K  M  S  T  Y  L  L  A  F  I  V  S  D  F  G  F  I  N
caaaccattgatggagttttgatccgtatctttgcacggaagccggctattgatgctggg
 Q  T  I  D  G  V  L  I  R  I  F  A  R  K  P  A  I  D  A  G
caaggagagtacgccctcaacaaaactggtcccatcctcaagttctttgagaaatattat
 Q  G  E  Y  A  L  N  K  T  G  P  I  L  K  F  F  E  K  Y  Y
aattccaaataccctctgccaaagtccgatcagatagctttaccagacttcaacgctgga
 N  S  K  Y  P  L  P  K  S  D  Q  I  A  L  P  D  F  N  A  G
gccatggagaactggggtcttatcacatacagagagacagcactgctctatgatgaagag
 A  M  E  N  W  G  L  I  T  Y  R  E  T  A  L  L  Y  D  E  E
tactcatccaactccaacaaggagagaattgcctccattatcgctcatgaactggctcac
 Y  S  S  N  S  N  K  E  R  I  A  S  I  I  A  H  E  L  A  H
atgtggtttggtaatctggtgaccctgaggtggtggaatgacctgtggca
 M  W  F  G  N  L  V  T  L  R  W  W  N  D  L  W
```

Figure 4. Partial coding sequence and translated amino acid sequence for cunner aminopeptidase-N (ANPEP). Underlined amino acids indicate the mature peptide.

```
ch       ----------------------GGFYRSEN-TEGNVTKVVATTQMQAPDARKAFPCFDEP    37
r        SLVKGHQYEMDSEFQGELADDLAGFYRSEY-MEGGNKKVVATTQMQAADARKSFPCFDEP    227
h        SLVKDSQYEMDSEFEGELADDLAGFYRSEY-MEGNVRKVVATTQMQAADARKSFPCFDEP    227
wcf      NLVPGKQYGLYTEFVGELADDLAGFYRSEY-IEDGVTKIIATTQMQAPDARKAFPCFDEP    234
z        ELTAGESYELYTEFVGELSDDLGGFYRSEYYDENGVLKVVATTQMQATDARKAFPCFDEP    227
gc       KLTAGESYELYTKFVGELADDLGGFYRSEY-EENGVKKVVATTQMQATDARKAFPCFDEP    215
wf       KLLKDQWYHLFTDFTGELADDLGGFYRSVY-MENGQRKVVATTQMQPTDARKAFPCFDEP    235
c        ----------------------------------------------MQPTDARKAFPCFDEP    16
ssb      ------------------------------------------------------FPCYDEP    7
es       ---------------------------SGY-MENGVKKVVPTTQMQPTDARKAFPCFDEP    32
bt       -------------------------------ENGVRKVVATTQMQPTDARKAFPCFDEP    28
                                                                ***:***

ch       AMKAVFTVTMIHPSDHTAISNMPVHSTYQLQMDGQSWNVTQFDPTPRMSTYLLAFIVSQF    97
r        AMKASFNITLIHPNNLTALSNMLPKDS-RTLQEDPSWNVTEFHPTPKMSTYLLAYIVSEF    286
h        AMKAEFNITLIHPKDLTALSNMLPKGPSTPLPEDPNWNVTEFHTTPKMSTYLLAFIVSEF    287
wcf      AMKATFNITLKYRQPYKAMSNMREIEKSTVTEDGQQWTVSKFDKTPKMSSYLVAFIVSEF    294
z        AMKAVFNIVLLHDPGTVALSNGVVIEEIPVTVDGISLTKTTFAPTEKMSTYLLAFIVSEF    287
gc       AMKAVFHITLLHDRGTVALSNGAVKDKVNITEDGALLTKTTFEPTEVMSTYLLAFIVSDF    275
wf       AMKATFNITLIHDPETVALSNGAQRESKPVTIDGKNLKQTDFEQTEKMSTYLLAFIVSEF    295
c        AMKAIFHITLIHQNRTVALSNGEEKESGHVNMDGQVVQRTVFEPTEKMSTYLLAFIVSDF    76
ssb      AMKAVFYITLIHDHGTVALSNGKQRDSINTNTDGHSVLKTTFEPTEKMSTYLLAFIVSDF    67
es       AMKAIFHITLIHDLGTVALSNGEEKESSNVNIEGHDVQKTVFQPTEKMSTYLLAFIVSDF    92
bt       AMKAIFHISLIHDSDKVALSNSEEKESSSITIQNKNLQKTVFKPTKKMSTYLLAFIVSDY    88
         **** * : : :     *:**           :      : *  *  **:**:*:***::

ch       DYVENN-TGK-VQIRIWGRPAAI-AEGQGEYALEKTGPILSFFERHYNTAYPLPKSDQVG    154
r        KYVEAVSPNR-VQIRIWARPSAI-DEGHGDYALQVTGPILNFFAQHYNTAYPLEKSDQIA    344
h        DYVEKQASNG-VLIRIWARPSAI-AAGHGDYALNVTGPILNFFAGHYDTPYPLPKSDQIG    345
wcf      EAVGDPGNATVTGVQIWGRKKAIQDENQGEYALSVTKPILDFFAEYYRTPYPLPKSDQVA    354
z        TYIEQKLDDL--QIRIFARKEAI-DANQGEYALSVTGKILRFFEEYYNSSYPLPKSDQIA    344
gc       DYIEQIDEKL--QIRIYARQEAI-KAGQGEYALNVTGPILRFFEDYYRVPYPLPKSDQIA    332
wf       TSINNTVDNV--LIRIFARKPAI-DAGQGAYALSKTGPILKFFEGYYNSSYPLPKSDQIA    352
c        GFINQTIDGV--LIRIFARKPAI-DAGQGEYALNKTGPILKFFEKYYNSKYPLPKSDQIA    133
ssb      DFINNTIDGV--LIRIFARKPAI-AAGQGQYALNKTGPILKFFEKYYNSSYPLPKSDPIA    124
es       SFINNTIDGV--LIRIFARTPAI-AAGQGDYALNKTGPILKFFEKYYNSSYPLPKSDQIA    149
bt       AFINNTIDGV--LIRIFARRPAI-AAGQGQYALNITGPILKFFENYYNSTYPLPKSDQIA    145
           :          ::*:.*  **    :* ***. *  ** **  :*   *** *** :.

ch       LPDFNAGAMENWGLVTYRENSLLYDNAYSSIGNKERVVTVIAHELAHQWFGNLVTLRWWN    214
r        LPDFNAGAMENWGLVTYRESALVFDPQSSSISNKERVVTVIAHELAHQWFGNLVTVDWWN    404
h        LPDFNAGAMENWGLVTYRENSLLFDPLSSSSSNKERVVTVIAHELAHQWFGNLVTIEWWN    405
wcf      LPDFSAGAMENWGLVTYRETALLFDDQVSSIGNKERVVTVIAHELAHQWFGNLVTIRWWN    414
z        LPDFNAGAMENWGLITYRETALLYDEEMSSNGNKERVVTVIAHELAHQWFGNLVTIRWWN    404
gc       LPDFNAGAMENWGLITYRETALLYDNEISSNANKERIVTVIAHELAHQWFGNLVTIRWWN    392
wf       LPDFNAGAMENWGLITYRETALLYDEAVSSNSNKERIATIIAHELAHMWFGNLVTLRWWN    412
c        LPDFNAGAMENWGLITYRETALLYDEEYSSNSNKERIASIIAHELAHMWFGNLVTLRWWN    193
ssb      LPDFNAGAMENWGLITYRETALLYDEEFSSNSNKQRIATIIAHELAHMWFGNLVT-----    179
es       LPDFNAGAMENWGLITYRETALLYDETFSSNSNKQRIATIIAHELAHMWFGNLVTLRWWN    209
bt       LPDFNAGAMENWGLITYRETALLYDEEFASNSNKERIATIIAHELAHMWFGNLVTLRWWN    205
         ****.*********:****.:*::*   :* .**:*:.::******* *******

ch       DLWLNEGFASYVEYLGADSAEPTWDIKDLMVLNELYTVMATDALTTSHPLTFREDEINTP    274
r        DLWLNEGFASYVEFLGADYAEPTWNLKDLIVLNDVYRVMAVDALASSHPLSSPANEVNTP    464
h        DLWLNEGFASYVEYLGADYAEPTWNLKDLMVLNDVYRVMAVDALASSHPLSTPASEINTP    465
wcf      DLWLNEGFASYVEYLGADKAEPNWNIKDLIVLNDVHRVMAVDALASSHPLTSREDEVNSP    474
z        DLWLNEGFASYVEYLGADEAEPLWNIKDLIVLNDVHRVFAIDALASSHPLSSKEEDVQRP    464
gc       DLWLNEGFASYVEYLGADEAESKWNIKDLIVLNDVHRVFAIDALASSHPLSSKEEDIQRP    452
wf       DLWLNEGFASYVEYLGADVAEPDWNIKDLIVLNDVHRVFAVDALASSHPLSAKEDDIQRP    472
c        DLW-------------------------------------------------------    202
ssb      -------------------------------------------------------      179
es       DLWLNEGFASYVEYLGAHEAEPEWNVKDLIVLSDVHRVFAVDALASSHPLSSKEEDIQKP    269
bt       DLWLNEGFASYVEYLGADKAEPNWNVKDLIVLNDVHRVFAVDALASSHPLSSKEEDIQKP    265
```

Figure 5. Aminopeptidase-N (ANPEP) amino acid sequence alignment for chicken (ch; CAB52136.1), rat (r; NP_112274.1), human (h; AAA51719.1), western clawed frog (wcf; XP_002932303.2), zebrafish (z; NP_001082794.1), grass carp (gc; AET36828.1), winter flounder (wf; AAC32807.1), cunner (c, **bold**), spotted sand bass (ssb; ABQ95349.1), European seabass (es; ACO92321.1) and bluefin tuna (bt; CAX33862.1).



.

Figure 6. Phylogenetic tree constructed using MEGA5 based on Clustal Omega alignment of aminopeptidase-N (ANPEP) amino acid sequences. Evolutionary history inferred by the Neighbour-Joining method.

```
attcgttcatttggaggagaccccgacaacatcaccctctttggagagtctgcaggggga
 I  R  S  F  G  G  D  P  D  N  I  T  L  F  G  E  S  A  G  G
gctagtgtcagcttccagactctcactccacacaacaaaggaatgatcaggagagccatc
 A  S  V  S  F  Q  T  L  T  P  H  N  K  G  M  I  R  R  A  I
tctcagagcggggttgctacctgcccctgggctatcaacaaaaaccccgcagatatgct
 S  Q  S  G  V  A  T  C  P  W  A  I  N  K  N  P  R  R  Y  A
gaggagattgccctgaaggtcaactgccccaccgatgagtctatggctgcatgtttgaag
 E  E  I  A  L  K  V  N  C  P  T  D  E  S  M  A  A  C  L  K
atgactgatcctgtgactcttaccttggctggcagtctcagtatcgtcagttcacctgat
 M  T  D  P  V  T  L  T  L  A  G  S  L  S  I  V  S  S  P  D
aaccccattgtgttcaacctggacctatctcctgtggtcgacggcgacttcctgcctgat
 N  P  I  V  F  N  L  D  L  S  P  V  V  D  G  D  F  L  P  D
gagcctcataagctgttccacaatgcggctgagattgactacatcgctggagtcaatgac
 E  P  H  K  L  F  H  N  A  A  E  I  D  Y  I  A  G  V  N  D
atggacggacacctcttcactggtttggatgttccttcaatcaactcccacctggtggac
 M  D  G  H  L  F  T  G  L  D  V  P  S  I  N  S  H  L  V  D
acacctgttgatgatgtaaagaggctcctggcttcatatgttaaggatggtggccctctg
 T  P  V  D  D  V  K  R  L  L  A  S  Y  V  K  D  G  G  P  L
gctctagacaatgctttctccacatatacctcaacctggggatcaaatcctaacaaggag
 A  L  D  N  A  F  S  T  Y  T  S  T  W  G  S  N  P  N  K  E
acaatcaagaaaactgttgtggatattggaacagactacatcttcctggttcctacacag
 T  I  K  K  T  V  V  D  I  G  T  D  Y  I  F  L  V  P  T  Q
gctgc
 A
```

Figure 7. Partial coding sequence and translated amino acid sequence for cunner bile salt-activated lipase (BAL). Underlined amino acids indicate the mature peptide.

```
z       TGDADAPGNYGLWDQHAAISWVHRNIKAFGGNPDNITIFGESAGSTSVNFQIISPKNKGL  230
osg     TGDSRLPGNYGLWDQHAAIAWVHRNIRSFGGDPDNIIIFGESAGGVSVSFQTLSPHNKGL  143
nt      TGDSDLPGNYGLWDQQAAIAWVHRNIRSFGGDPDNITIFGESAGGVSVSFQTLTPHNKGI  222
fr      TGDSSLPGNYGLWDQQAAIAWVHRNIRSFGGDPDNITLFGESAGGASVSFQTLTPHNKGT  229
c       ------------------------IRSFGGDPDNITLFGESAGGASVSFQTLTPHNKGM  35
es      TGDSGLPGNYGLWDQHTAIAWVHRNIRSFGGDPDKITIFGESAGGASVSFQTLTPHNKGL  73
wf      TGDSSMPGNYGLWDQQAAIAWVHRNIRSFGGDPDNVTLFGESAGAASVSFQTLTPHNKGL  132
bb      RGQGPRDSNYGLRDQHMAIAWVKRNIAAFGGDPNNITIFGESAGGASVSLQTLSPYSKGL  232
wcf     TGDSNLPGNYGLWDQHMAIAWVKRNIAAFGGNPDNITIFGESAGGASVSLQTLSPYNKGL  233
r       TGDANLPGNFGLRDQHMAIAWVKRNIAAFGGDPDNITIFGESAGAASVSLQTLSPYNKGL  232
wlg     TGDANLPGNYGLRDQHMAIAWVKRNIAAFGGDPNNITLFGESAGGASVSLQTLSPYNKGL  235
                               * :***:*::: :******..**.:* ::* .**

z       IRRAISQSGVALCPWAINRNPRKFAEEIAKKVGCPTDS--GMVACLRRTDPKAVTLAGKV  288
osg     FKRAISQSGVALCPWGLNRNPRKVAEEVAVNVGCPTDD--RMVACLKSTDAKNLTMAAPR  201
nt      IRRAISQSGVALCPWALNRNPRRFAEEVARKVNCPTDS--RMASCLKMTDPGTLTKAGTV  280
fr      IRRAISQSGVALCPWAVNRNPRRFAEEVALKVNCPTDE--KMAACLKMTDPELLTLAGSL  287
c       IRRAISQSGVATCPWAINKNPRRYAEEIALKVNCPTDE--SMAACLKMTDPVTLTLAGSL  93
es      YKRAISQSGVALCPWAINKNPRRFAEEVALKVNCPTDQ--SMAACLKMTDPALLTLAGSL  131
wf      FKRAISQSGVALCPWAINKNPRRFAEEIALKVNCPTDQ--SMAACLKMTDPALLTLAGSL  190
bb      IRRAISQSGVALTPWAIQKNPLSWAKGIAKKVGCPLDDTARMAKCLKVTDPRALTLAYKM  292
wcf     IRRAISQSGVGMSPWALQSNPLFWTTKVAEKVGCPVHDTAAMANCLKISDPKAVTLAYKL  293
r       IRRAISQSGVALSPWAIQENPLFWAKTIAKKVGCPTEDTAKMAGCLKITDPRALTLAYRL  292
wlg     IRRAISQSGVALSPWVIQKNPLFWAEKVAEKVGCPVGDAARMAQCLKVTDPRALTLAYKV  295
         :********.  ** :: **   :  :* :* **  .   *. **: :*   :* *

z       RLATSATEPIVHNLYLSPVIDGDFIPDEPDTLFGNAADIDYIAGVNDMDGHIFATLDIPS  348
osg     VKFGTPDYPAVKYLVLSPVADGDFLPDQPENLFHNAADIDYLVGVNNMDGHLFSSKDIPN  261
nt      NLLSSPDQPLVLNLEIAPVIDGDFLPDDPSNLFHNAAEIDYIAGVNNMDGHMFTNFDVPS  340
fr      KMSGSPDNPLVSNLVLSPVIDGDFLPDEPYNLFHNAADIDYIAGVNDMDGHLFTTFDIPS  347
c       SIVSSPDNPIVFNLDLSPVVDGDFLPDEPHKLFHNAAEIDYIAGVNDMDGHLFTGLDVPS  153
es      SISSSPDHPLVNNLVLSPVIDGDFLPDEPSNLFHNAADIDYIAGVNDMDGHLFTALDVPS  191
wf      SLTSSPDSPILDNLLLSPVIDGDFLPDDPSNLFPNAADIDYIAGINDMDGHLFTGLDVPS  250
bb      PLL-GRKYPFLHYLGLIPVVDGDFIPDDPINLFANAADIDYIAGTNDMDGHLFASVDVPA  351
wcf     DPA-LMDYPAVYYLGISPVIDGDFIPDEPRNLFANAADVDYLAGVNNMDAHLFAGIDMPV  352
r       PLK-SQEYPIVHYLAFIPVVDGDFIPDDPINLYDNAADIDYLAGINDMDGHLFATVDVPA  351
wlg     PLA-GLEYPMLHYVGFIPVIDGDFIPDDPVNLYANAADIDYIAGTNNMDGHIFASIDMPA  354
                * :  : : ** ****:**:* .*: ***::**:.* *:**.*:*:  *:*

z       INNALATTTEEEVQALATALSKDRGQDAGIATFQEYTVNWGSKPNKEDIKKTVVEMETDY  408
osg     FNNKNQEIPVEDVKRLLAAYTKEKGQAGFETAFAEYSSNWESTPSQISIKRTAVDIGTDY  321
nt      VNLDLVVTPTNDVRRLLAALTKEKGESGLNNAYSTYTSNWGSFPTWDTIKKTIVDIETDY  400
fr      INSQLVDTPVDEMKRLLRSYTKEKGAAAAEIGFSTYTLNWGSNPNRETIKKTVVDVGTDY  407
c       INSHLVDTPVDDVKRLLASYVKDGGPLALDNAFSTYTSTWGSNPNKETIKKTVVDIGTDY  213
es      VNSHLVDTPIEDVKRLLGTYTKEKGKAGLENAYSTYTSTWGSNPNKETIKKTVVEIGTDY  251
wf      INSPLVDTHVEDMKRLLGSYTKEKGKAGLDNAYSTYTSTWGSNPSREINKRTVVDIGTDY  310
bb      INVNLLTVTGEDFFKLVSQVTIAKGPRGANATFDLYTASWAEDSSQEAKKKTVVDFETDV  411
wcf     INKPLQKISEADVHNLVQGLTLTKISSALETAYNLYSANWGPNPEQENMKRTVIDLETDY  412
r       IDKAKQDVTEEDFYRLVSGHTVAKGLKGTQATFDIYTESWAQDPSQENMKKTVVAFETDI  411
wlg     INKGNKKVTEEDFYKLVSEFTFTKGLRGAKTTFDVYTESWAQDPSQENKKKTVVDLETDV  414
        .:         :.  *           .    :  *: .*         *:* : . **

z       IFLVPTQTALYLHSDNAKSARTYSYLFSESSRIP----IFPLWMGADHADELQYVFGKPF  464
osg     MFLVPTQTAIYLHAANARSGRTYSYLMSEPSLLARPGKPYQ-------------------  362
nt      IFLVPTQAALYLHADHATTGRTYSYLFSEPNLLGGPLLPYPSWMGADHADDLQYVFGKPF  460
fr      IFLVATQAALYLHAAHAKTRRTYSYMFSEPSRLGGIAKPYPSWMGADHADDLFYVFGKPF  467
c       IFLVPTQA-------------------------------------------------------  221
es      IFLVPTQAALYLHAANATTGRTYSYLFSQPNRMGGIGRPYPSWMGADQADDLQYVFGKPF  311
wf      IFLVPTQAALYLHAANAKTGRTYSYVFSQPNRMGGIGRPYPSWMGADHADDLQYVFGKPF  370
bb      YFLMPTKMAVAQHRAKAKSARTYTYLFSEPSRIP----LYPSWVGADHTDDLQYVFGKPF  467
wcf     LFLVPTQEALALHSMNARSGRTYNYVFSLPTRMP----IYPSWVGADHADDLQYVFGKPF  468
r       LFLIPTEMALAQHRAHAKSAKTYSYLFSHPSRMP----IYPKWMGADHADDLQYVFGKPF  467
wlg     LFLVPTEIALAQHRANAKSAKTYTYLFSHPSRMP----VYPKWVGADHADDIQYVFGKPF  470
         **: *:
```

Figure 8. Bile salt-activated lipase (BAL) amino acid sequence alignment for zebrafish (z; XP_005161146.1), orange spotted grouper (osg; ACH53597.1), Nile tilapia (nt; XP_003440274.1), *Takifugu rubripes* (fr; XP_003978424.1), cunner (c, **bold**), European seabass (es; ACD10792.1), winter flounder (wf; AAN63868.1), Brandt's bat (bb; EPQ11548.1), western clawed frog (wcf; XP_002936238.2), rat (r; NP_058693.2) and western lowland gorilla (wlg; XP_004048842.1).

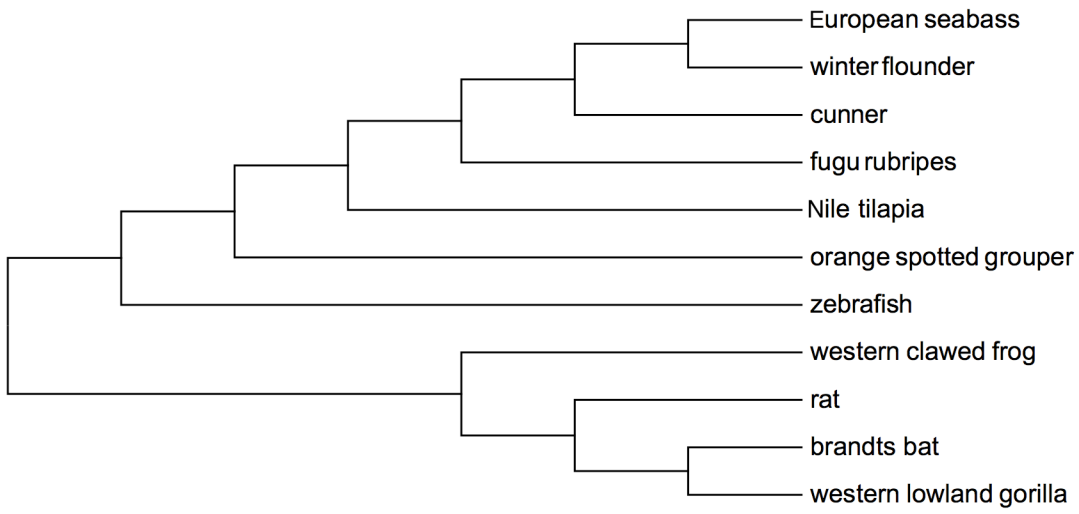


Figure 9. Phylogenetic tree constructed using MEGA5 based on Clustal Omega alignment of bile salt-activated lipase (BAL) amino acid sequences. Evolutionary history inferred by the Neighbour-Joining method.

3.1.2 Appetite regulators

The complete 156 nucleotide long coding sequence (CDS) for apelin was determined, and found to code for a 77 amino acid long apelin precursor (Figure 10). Clustal Omega alignment with other species indicates that a 35 amino acid sequence at the C-terminus of this peptide codes for the mature apelin peptide. The first 22 amino acids from the N-terminus make up the signal peptide as determined by alignment with multiple species and SignalP software. The biologically active apelin-13 fragment is present (PRPRLSHKGPMPF). Sequence identities are 78% for zebrafish, 78% for goldfish, 41% for western clawed frog, 41% for rat and 39% for human as determined by BLAST alignment with cunner apelin amino acid sequence (Figure 11 and Figure 12).

A partial CDS was determined for the mechanistic target of rapamycin (mTOR) coding for a 234 amino acid region of the mTOR peptide (Figure 13). Sequence identities are 99% for zebrafish, 99% for Nile tilapia, 94% for rat, 94% for human and 91% for western clawed frog (Figure 14 and Figure 15).

```
atgaatgtgaagatcctgacgctggtgattgtgctcttggtgtctctgctgtgttctgcc
 M  N  V  K  I  L  T  L  V  I  V  L  L  V  S  L  L  C  S  A
agtgctggtcccatggcctccacagagcatggcagagagcttgaagatgcgcctacagtg
 S  A  G  P  M  A  S  T  E  H  G  R  E  L  E  D  A  P  T  V
agaaaaatggtccagcaaaaccctgcaaggggtggtcagtcccacagaccagccggctgg
 R  K  M  V  Q  Q  N  P  A  R  G  G  Q  S  H  R  P  A  G  W
aagaggagacgcccacggccacgtctttcccataaggggccaatgccattc
 K  R  R  R  P  R  P  R  L  S  H  K  G  P  M  P  F
```

Figure 10. Complete CDS nucleotide and translated amino acid sequence for cunner apelin. The predicted signal peptide sequence is highlighted in grey and the mature peptide is underlined. Italics indicate the apelin-13 fragment.

```
c         MNVKILTLVIVLLVSLLCSASAGPMASTEHGRELEDAPTVRKMVQQNPARGGQSHRPAGW    60
g         MNVKILTLVIVLVVSLLCSASAGPMASTEHSKELEEVGSMRTPLRQNPARAGRSQRPSGW    60
z         MNVKILTLVIVLVVSLLCSASAGPMASTEHSKEIEEVGSMRTPLRQNPARAGRSQRPAGW    60
wcf       MNLRLWALALLLFILTLTSAFGAPLAEGSDR-NDEE-QNIRTLVNPKMVRNPAPQRQANW    58
r         MNLSFCVQALLLLWLSLTAVCGVPLMLPPDGKGLEE-GNMRYLVKPRTSRTGPGAWQGGR    59
h         MNLRLCVQALLLLWLSLTAVCGGSLMPLPDGNGLED-GNVRHLVQPRGSRNGPGPWQGGR    59
          **: : . .::*.   * :. .  :    .    *:  .:*  :. .  *       .

c         KR-RRPRPRLSHKGPMPF      77
g         RR-RRPRPRLSHKGPMPF      77
z         RR-RRPRPRLSHKGPMPF      77
wcf       RKIRRQRPRLSHKGPMPF      76
r         RKFRRQRPRLSHKGPMPF      77
h         RKFRRQRPRLSHKGPMPF      77
          :: ** ************
```

Figure 11. Apelin amino acid sequence alignment for cunner (c, **bold**; KC895876), goldfish (g; ACT76163.1), zebrafish (z; NP_001159596.1), western clawed frog (wcf; NP_001165146.1), rat (r; NP_113800.1) and human (h; NP_059109.3).

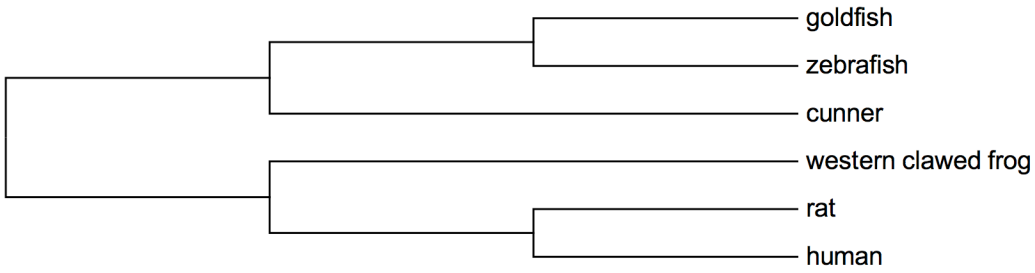


Figure 12. Phylogenetic tree constructed using MEGA5 based on Clustal Omega alignment of apelin amino acid sequences. Evolutionary history inferred by the Neighbour-Joining method.

```
ctgagtgaagaccagcaggatgagctcatccgcagcattgagctagctctgacctctcaa
 L  S  E  D  Q  Q  D  E  L  I  R  S  I  E  L  A  L  T  S  Q
gacatcgctgaggtcacgcagactctgctcaacctggcggagttcatggagcacagtgac
 D  I  A  E  V  T  Q  T  L  L  N  L  A  E  F  M  E  H  S  D
aagggccctctgcccctcagagatgataacggcattgtgctgcttggcgagagagctgcg
 K  G  P  L  P  L  R  D  D  N  G  I  V  L  L  G  E  R  A  A
aaatgtcgtgcctacgccaaggctctacattacaaagagctagagttccagaaaggtgct
 K  C  R  A  Y  A  K  A  L  H  Y  K  E  L  E  F  Q  K  G  A
tctcctctcatcctggagtctcttatcagcatcaacaataaattgcagcagccagaagca
 S  P  L  I  L  E  S  L  I  S  I  N  N  K  L  Q  Q  P  E  A
gcatcaggggtgctggagtacgccatgaaacatttcggtgaactggaaatccaagccacc
 A  S  G  V  L  E  Y  A  M  K  H  F  G  E  L  E  I  Q  A  T
tggtatgagaagctccatgagtgggaggatgccctggtagcatatgacaagaaaatcgac
 W  Y  E  K  L  H  E  W  E  D  A  L  V  A  Y  D  K  K  I  D
atgaacaaagaggatccggagctcatcctgggcagaatgcgctgcttggaggccctggga
 M  N  K  E  D  P  E  L  I  L  G  R  M  R  C  L  E  A  L  G
gaatggggccagttgcaccagcagtgctgtgaagagtggacactggtgagcgaagaaacc
 E  W  G  Q  L  H  Q  Q  C  C  E  E  W  T  L  V  S  E  E  T
caggccaagatggctcgaatggctgctgctgcctggggactagggcactgggacagc
 Q  A  K  M  A  R  M  A  A  A  A  A  W  G  L  G  H  W  D  S
atggaggagtacacatgtatgataccaagaggcacacatgatggagcgttttatcgagca
 M  E  E  Y  T  C  M  I  P  R  G  T  H  D  G  A  F  Y  R  A
gttctagcgctgcatcaggacctcttttcattggctcagcag
 V  L  A  L  H  Q  D  L  F  S  L  A  Q  Q
```

Figure 13. Partial CDS nucleotide and translated amino acid sequences for cunner mTOR. The mature peptide sequence is underlined.

```
wcf      RDLFNAAFLSCWSELNEDQQDELIRSIELALTSQDIAEVTQTLLNLAEFMEHSDKGPLPL    1367
h        RDLFNAAFVSCWSELNEDQQDELIRSIELALTSQDIAEVTQTLLNLAEFMEHSDKGPLPL    1374
r        RDLFNAAFVSCWSELNEDQQDELIRSIELALTSQDIAEVTQTLLNLAEFMEHSDKGPLPL    1374
nt       RDLFNAAFLSCWSELSEDQQDELIRSIELALTSQDIAEVTQTLLNLAEFMEHSDKGPLPL    1358
c        --------------LSEDQQDELIRSIELALTSQDIAEVTQTLLNLAEFMEHSDKGPLPL    46
z        RDLFNAAFLSCWSELSEDQQDELIRSIELALTSQDIAEVTQTLLNLAEFMEHSDKGPLPL    1357
                     *.****************************************

wcf      RDDNGVVLLGERASKCRAYAKALHYKELEFQKGPSPAILESLISINNKLQQPEAASGVLE    1427
h        RDDNGIVLLGERAAKCRAYAKALHYKELEFQKGPTPAILESLISINNKLQQPEAAAGVLE    1434
r        RDDNGIVLLGERAAKCRAYAKALHYKELEFQKGPTPAILESLISINNKLQQPEAASGVLE    1434
nt       RDDNGIVLLGERAAKCRAYAKALHYKELEFQKGPTPLILESLISINNKLQQPEAASGVLE    1418
c        RDDNGIVLLGERAAKCRAYAKALHYKELEFQKGASPLILESLISINNKLQQPEAASGVLE    106
z        RDDNGIVLLGERAAKCRAYAKALHYKELEFQKGASPLILESLISINNKLQQPEAASGVLE    1417
         *****:*******:****************** :* ******************:****

wcf      FAMKHYGEMEIQATWYEKLHEWEDALVAYDKKIEVNKDDSELILGRMRCLEALGEWGQLH    1487
h        YAMKHFGELEIQATWYEKLHEWEDALVAYDKKMDTNKDDPELMLGRMRCLEALGEWGQLH    1494
r        YAMKHFGELEIQATWYEKLHEWEDALVAYDKKMDTNKDDPELMLGRMRCLEALGEWGQLH    1494
nt       YAMKHFGELEIQATWYEKLHEWEDALVAYDKKIDMNKEDPELILGRMRCLEALGEWGQLH    1478
c        YAMKHFGELEIQATWYEKLHEWEDALVAYDKKIDMNKEDPELILGRMRCLEALGEWGQLH    166
z        YAMKHFGELEIQATWYEKLHEWEDALVAYDKKIDMNKDDPELILGRMRCLEALGEWGQLH    1477
         :****:**:************************:: **:* **:******************

wcf      QQCCENWTNVNEDARAKMARMAAAAAWGLEQWDSMEEYTCLIPRDTHDGAFYRAVLALHQ    1547
h        QQCCEKWTLVNDETQAKMARMAAAAAWGLGQWDSMEEYTCMIPRDTHDGAFYRAVLALHQ    1554
r        QQCCEKWTLVNDETQAKMARMAAAAAWGLGQWDSMEEYTCMIPRDTHDGAFYRAVLALHQ    1554
nt       QQCCEEWTLVSEETQAKMARMAAAAAWGLGHWDSMEEYTCMIPRDTHDGAFYRAVLALHQ    1538
c        QQCCEEWTLVSEETQAKMARMAAAAAWGLGHWDSMEEYTCMIPRGTHDGAFYRAVLALHQ    226
z        QQCCEEWTLVSEETQAKMARMAAAAAWGLGHWDSMEEYTCMIPRDTHDGAFYRAVLALHQ    1537
         *****:** *.::::************** :**********:*** ***************

wcf      DLFSLAQQCIDKARDMLDAELTAMAGESYSRAYGAMVTCQMLSELEEVIQYKLVPERREI    1607
h        DLFSLAQQCIDKARDLLDAELTAMAGESYSRAYGAMVSCHMLSELEEVIQYKLVPERREI    1614
r        DLFSLAQQCIDKARDLLDAELTAMAGESYSRAYGAMVSCHMLSELEEVIQYKLVPERREI    1614
nt       DLFSLAQQCIDKARDLLDAELTAMAGESYSRAYGAMVSCQMLSELEEVIQYKLVPERRDI    1598
c        DLFSLAQQ--------------------------------------------------      234
z        DLFSLAQQCIDKARDLLDAELTAMAGESYSRAYGAMVSCQMLSELEEVIQYKLVPERRDI    1597
         ********
```

Figure 14. mTOR amino acid sequence alignment for western clawed frog (wcf; XP_004916088.1), human (h; NP_004949.1), rat (r; NP_063971.1), Nile tilapia (nt; XP_003449179.1), cunner (c, **bold**; KC791688) and zebrafish (z; NP_001070679.2).

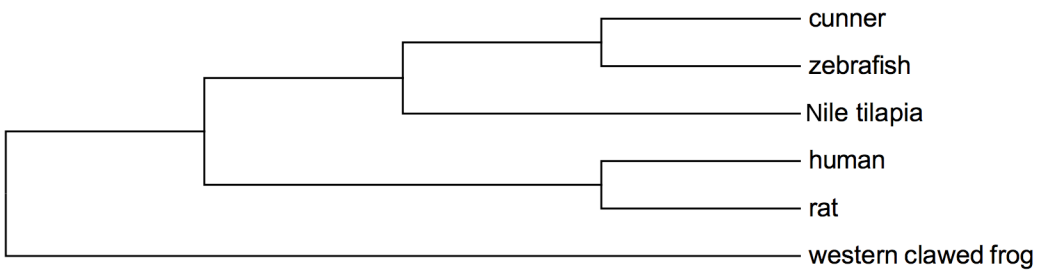


Figure 15. Phylogenetic tree constructed using MEGA5 based on Clustal Omega alignment of mTOR amino acid sequences. Evolutionary history inferred by the Neighbour-Joining method.

3.1.3 Tissue distribution of apelin, CCK, mTOR, orexin and ubiquitin

Tissue distributions were conducted for orexin, CCK, apelin, mTOR and the reference gene, ubiquitin (Figure 16). Orexin was expressed in all tissues, *i.e*. proximal intestine, mid intestine, distal intestine, liver, spleen, kidney, heart, brain, gill and skin. CCK was expressed in all tissues with only slight expression apparent in kidney and gill. Minimal CCK expression was seen for liver. Apelin was seen in all tissues with relatively lower expression in the intestine, particularly the mid intestine. mTOR was expressed in all tissues. The reference gene ubiquitin was consistently expressed across all tissues.

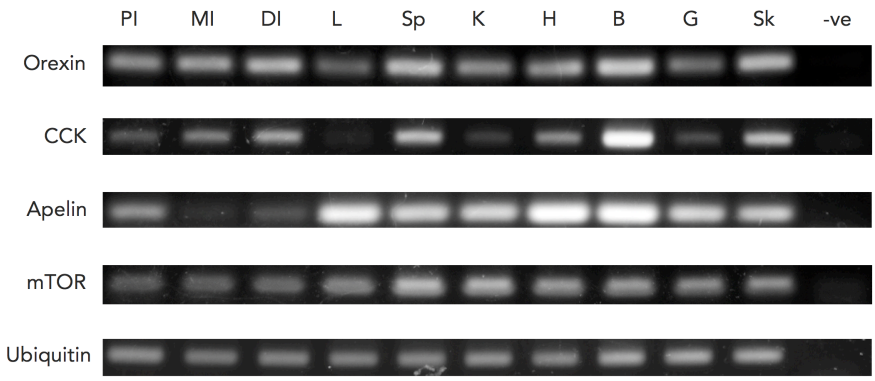


Figure 16. Tissue distribution of orexin, CCK, apelin, mTOR and ubiquitin in cunner proximal intestine (PI), mid intestine (MI), distal intestine (DI), liver (L), spleen (Sp), kidney (k), heart (H), brain (B), gill (G) and skin (Sk). "–ve" indicates the negative (no cDNA) control.

3.2 Enzyme activities

3.2.1 Fasting and torpor

Digestive enzyme activities were measured in distal intestine homogenates from fish subjected to four treatments: fed (summer), fasted 4 weeks (summer), re-fed 1 week after 4 weeks fasting (summer) and torpor (winter) (Figure 23). Specific enzyme activities are expressed in U/mg protein, where U represents the amount of enzyme required to turnover 1 μmol of substrate into product in 1 min (U = 1 μmol substrate / minute). Lipase, ANPEP, ALP and trypsin specific activities decreased following 4 weeks of food deprivation. In re-fed fish, lipase, ALP and trypsin activities were similar to those of fed fish and ANPEP activity was similar to that of fasted fish. Torpid fish displayed a significant decrease in lipase, trypsin and ALP specific activities compared to fed fish. ANPEP activities were similar to fed fish. There were no significant differences in lipase, trypsin and ALP activities between torpid and 4 week fasted individuals.

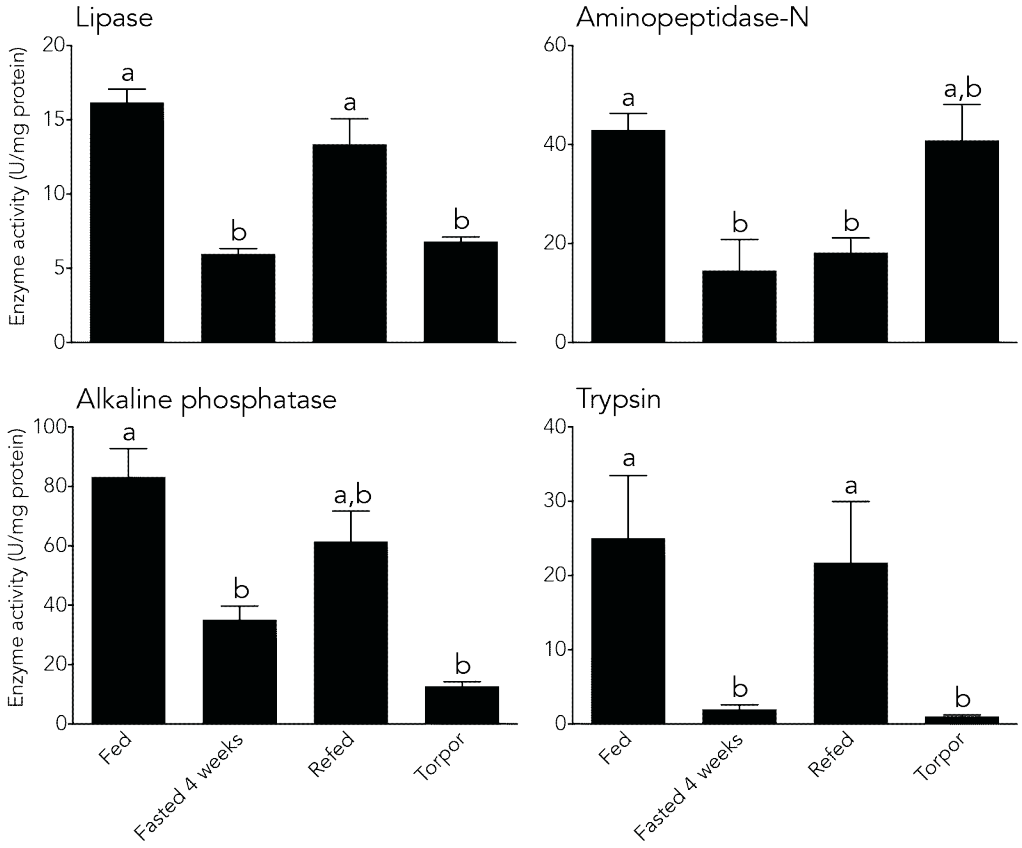


Figure 17. Mean specific enzyme activities for lipase, aminopeptidase-N, alkaline phosphatase and trypsin in distal intestine homogenates from cunner subjected to four treatments of food availability. Fed, n=8; fasted 4 weeks, n=8; re-fed 1 week after 4 weeks fasting, n=9; torpor, n=9. Data are presented as means+SEM and different letters indicate significance (one-way ANOVA followed with Tukey's multiple comparison post-hoc test; $p<0.05$).

3.2.2 Periprandial feeding and effect of food type

After 5 weeks, fish that were fed squid had significantly higher lipase activity compared to fish fed commercially available fishmeal pellets (Skretting marine feed). No effect of diet was seen for ALP activity. Lipase activity did not change between fish sampled immediately before and 1 h after feeding. ALP activity decreased 1h after feeding (Figure 18).

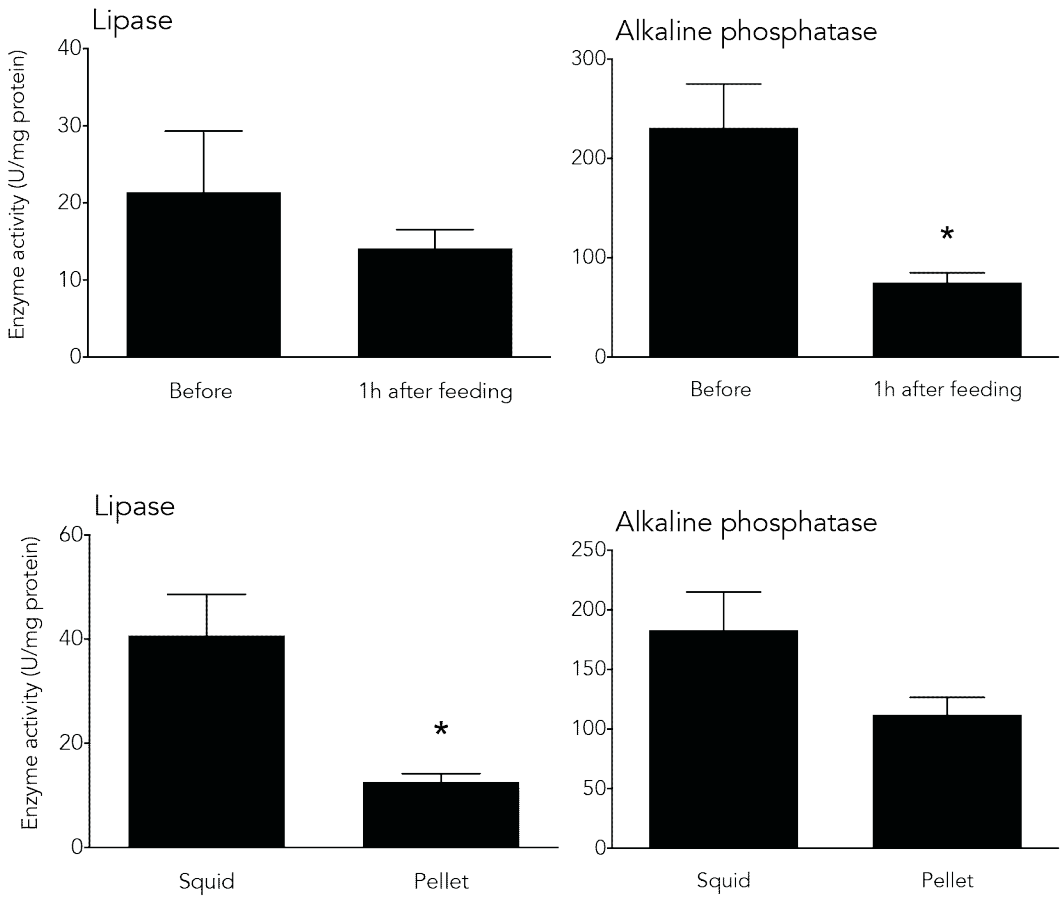


Figure 18. Lipase and alkaline phosphatase activities (expressed relative to total protein content (U/mg protein)) for fish immediately before and 1 hour after feeding as well as fish fed a frozen squid or pellet diet. An asterisk indicates significance (Student's t-test; $p<0.05$).

3.3 Effect of fasting on hepatosomatic (HSI) and gonadosomatic (GSI) indices

Fish fasted for 4 weeks showed a decrease in HSI [HSI = (liver mass · body mass^{-1}) × 100], but no significant change in GSI [GSI = (gonad mass · body mass^{-1}) × 100] relative to control fish sampled on the same day (Figure 19).

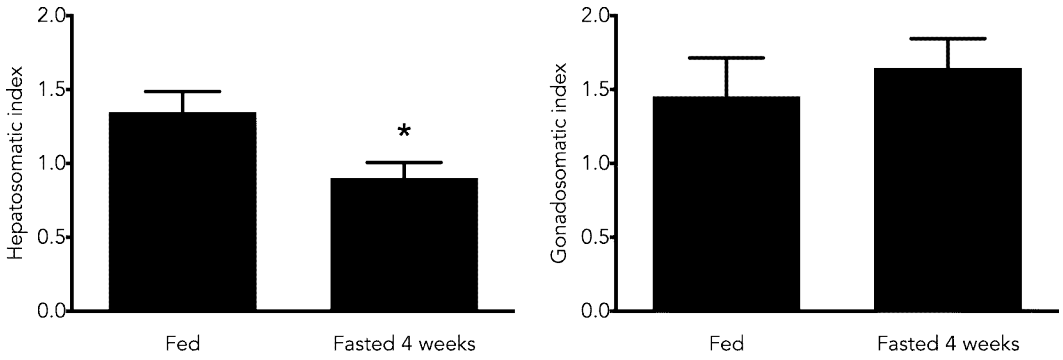


Figure 19. Hepatosomatic (HSI) and gonadosomatic (GSI) indices for fed and 4 week fasted fish. An asterisk indicates significance (Student's *t*-test; $p<0.05$).

3.4 Transcript expression

3.4.1 Enzyme precursor transcripts

No significant differences in transcript levels were seen for either trypsin, ALP or BAL for either 4 week fasted or torpid fish when compared to control fed fish. A significant decrease in ANPEP activity was seen for torpid fish, but not fasted fish (Figure 20).

3.4.2 Appetite regulating peptide transcripts

Distal intestine transcript expression levels of four putative appetite and/or energy status regulators, CCK, apelin, orexin and mTOR were quantified by qPCR (Figure 21). For both orexin and CCK, mRNA was lower in torpid fish than in fed fish and there were no significant differences between fasted fish and either torpid or fed fish. Apelin expression in 4 week fasted fish was significantly lower than that of fed fish, but similar to that of torpid fish. There were no significant differences in apelin levels between torpid and fed fish. mTOR mRNA levels in 4 week fasted fish was not significantly different to that of either fed fish or torpid fish. mTOR expression in torpid fish was lower than that of fed fish (Student's t-test; $p<0.05$).

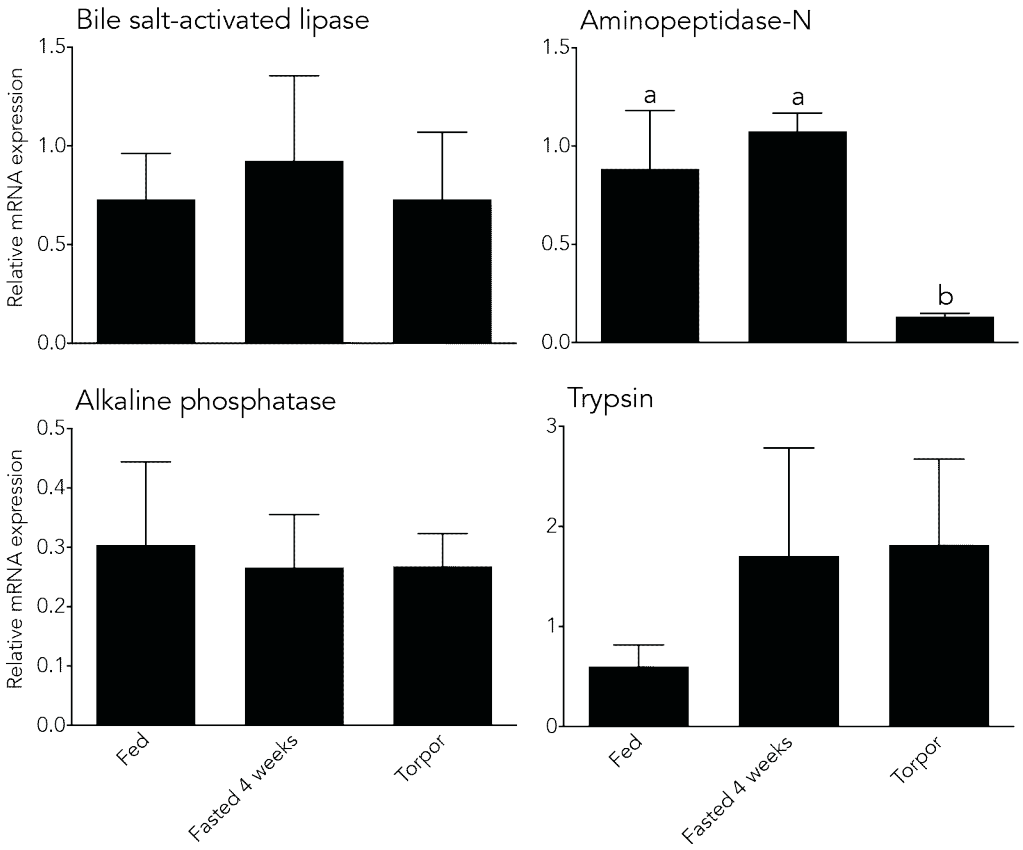


Figure 20. Mean relative mRNA expression levels for digestive enzyme transcripts (bile salt-activated lipase (BAL), alkaline phosphatase (ALP), aminopeptidase-N (ANPEP) and trypsin) in cunner distal intestine subjected to three treatments of food availability (n=7 for each treatment). Ubiquitin was used as an endogenous control gene. Data are presented as means +SEM. Different letters indicate significance (one-way ANOVA followed with Tukey's multiple comparison post-hoc test; $p<0.05$).

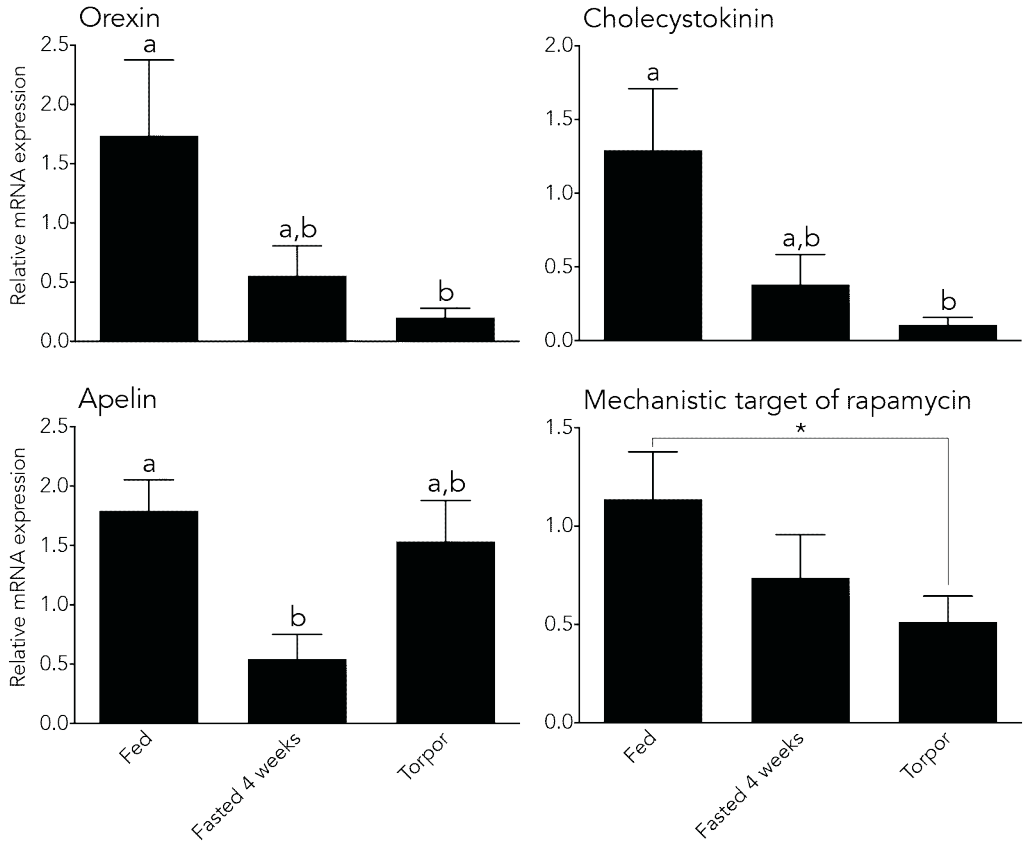


Figure 21. Mean relative mRNA expression levels for appetite regulating peptide transcripts (orexin, apelin, cholecystokinin (CCK) and mechanistic target of rapamycin (mTOR)) in cunner distal intestine subjected to three treatments of food availability (n=7 for each treatment). Ubiquitin was used as an endogenous control gene. Data are presented as means +SEM. Different letters (one-way ANOVA followed with Tukey's multiple comparison post-hoc test; $p<0.05$) or an asterisk (Student's *t*-test) indicate significance.

3.5 Intestinal morphology

Cunner intestinal morphology, specifically villus height, did not change between active summer fish and torpid winter fish (Figure 22).

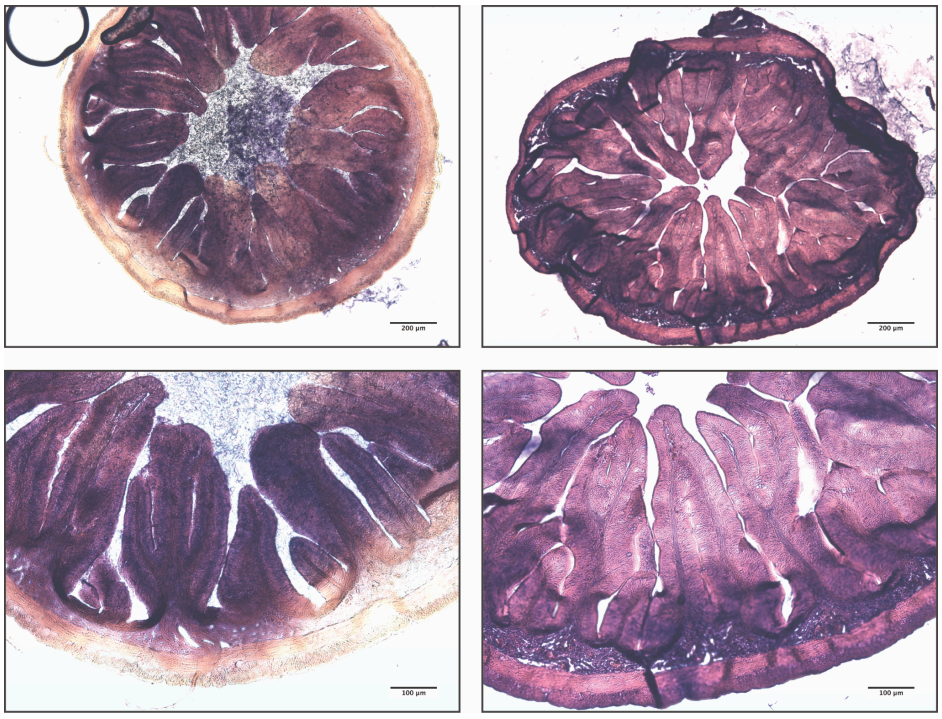


Figure 22. Structure of cunner intestine for fed (left) and torpid (right) fish viewed at 50× (top) and 100× (bottom). Scale bars indicate 200 μm (top) and 100 μm (bottom). Tissues prepared from paraffin sections with hematoxylin and eosin staining.

4 Discussion

4.1 Sequence analysis

Previously undocumented partial nucleotide and predicted amino acid sequences were cloned and sequenced for cunner digestive enzymes ALP, ANPEP and BAL. All three amino acid sequences showed between 65 and 89% similarity to other fish species and between 39 and 55% when compared to other vertebrates. ANPEP demonstrated the highest sequence similarity to other species, but this may be attributed to the region of the cDNA that was cloned, which is one of the more highly conserved portions of the full sequence.

ALP and ANPEP phylogenies show the closest relationships between cunner and other Perciformes, to which cunner is a member, such as European seabass, large yellow croaker, gilt-head seabream, Nile tilapia, spotted sea bass, orange-spotted grouper and Atlantic bluefin tuna. This pattern was not seen for BAL, however, fish from other orders show higher sequence identity and closer relationships with cunner than other Perciformes did.

Substantial variation was seen for ALP, ANPEP and BAL amino acid sequences among all species examined as well as among fish, even within the Perciformes. The enzyme amino acid sequences obtained were within the predicted mature peptide for ALP, ANPEP and BAL. Therefore, any amino acid substitutions represent potential functional changes in the enzyme. Enzymes have narrow windows for optimal activity and their efficiencies can be dramatically affected by factors such as temperature, salinity and pH. For instance, ALP from cod shows different kinetic parameters than ALP from

calves, which might be indicative of the cold adaptation for the fish enzyme (Ásgeirsson et al., 1995). High amino acid sequence diversity and lack of congruence of phylogenies with expected relationships suggest that there is significant selection pressure on the enzymes investigated here, and that evolutionary changes in enzyme structure and function enable species to adapt to their environments.

The complete coding sequence for cunner apelin was cloned and sequenced. The corresponding amino acid sequence has a 78% similarity with both zebrafish and goldfish apelin. Apelin amino acid sequence identities to other vertebrates are low (39-41%). Despite low sequence similarities between fish apelin and other vertebrates, the C-terminal apelin-13 peptide, which is responsible for binding to and activating the apelin receptor (APJ) is conserved in all species considered, with the exception of one amino acid [(Q/P) RPRLSHKGPMPF].

A partial sequence of cunner mTOR was cloned and shows high amino acid sequence identity ranging from 91 to 99% across all fish and vertebrates examined. The cloned region represents a part of a much larger (~2500 amino acid (Fingar and Blenis, 2004)) protein. mTOR is highly conserved across vertebrates and is found in all eukaryotes studied to date (Wullschleger et al., 2006). The high sequence identities for apelin and mTOR amongst species illustrate the power that lower vertebrate models provide in elucidating the function of appetite and energy balance related peptides.

4.2 Fasting and torpor

Fasting and torpor trials were conducted in order to identify the GIT response to food deprivation in these two very different seasonal states as well as draw comparisons and

point out discrepancies between the responses to both. Digestive enzyme activities and their respective transcript expression were quantified (trypsin, lipase/bile salt-activated lipase, ANPEP and ALP). Appetite and energy balance-related peptide transcript expression levels were also quantified (orexin, apelin, mTOR and CCK).

4.2.1 Enzyme activities and transcript expression

4.2.1.1 Lipase

Lipase is secreted into the lumen of the intestine where it is responsible for the hydrolysis of triacylglycerols. Lipase activity decreased in 4-week fasted cunner. This result is consistent with the trend for decreasing lipase activity found for starved sturgeon and rainbow trout (Furné et al., 2008). However, cunner shows reduced lipase activity after 4 weeks of starvation whereas sturgeon and trout show a decrease only after 72 days of starvation (Furné et al., 2008). Cunner re-fed for 1 week quickly regained lipase activity. This contrasts with sturgeon and rainbow trout, which do not show an increase in lipase activity after 10 days of re-feeding, but do after 2 months (Furné et al., 2008). Thus, the results from the present study suggest more plasticity and responsiveness of lipase levels in cunner GIT undergoing starvation or re-feeding when examined alongside conspecifics with similar diets.

The present results, along with other studies in fish, seem to indicate a reduction in lipase levels with starvation. However, the regulatory mechanism for lipase activity in fish remains to be elucidated. In turbot, lipase activity is unchanged when diet lipid levels are altered, but whole diet energy content is kept constant (Hoehne-Reitan et al., 2001), suggesting that substrate (lipid) sensing is not an important contributor to the control of

lipase levels in this species and possibly fish in general. Cunner appears to have highly responsive GIT lipase levels when starved and thus makes a good candidate model for further exploration of the dietary signal influencing lipase activity in fish and potentially other vertebrates.

Torpid cunner had low lipase levels similar to levels of 4-week fasted individuals. The modulation of lipase activity in cunner GIT appears to play an important role in the acute (4 weeks) starvation response, but might not further implicated in the response to torpor.

4.2.1.2 ANPEP

ANPEP is responsible for the final absorption of proteins that have been broken down into dipeptides and tripeptides by other proteases such as trypsin and chymotrypsin. ANPEP is a good indicator of the intestine's capacity to take up protein digestion products since it is localized on the enterocyte membrane. ANPEP activity was significantly lowered following 4 weeks of fasting. This matching of enzyme production with nutrient availability provides further evidence for cunner's ability to regulate their digestive function during the summer in response to food deprivation. However, after 1 week of re-feeding ANPEP activity had levels similar to those seen for fasted cunner, suggesting a slow response time to feeding status. Further studies comparing ANPEP activity at several time points during fasting might provide clues on the timeframe at which production is blunted, and help understand its regulatory mechanism.

During torpor, ANPEP activity was maintained at control levels. Similarly, sparrows fasted for 31 hours display no changes in ANPEP activity compared to fed birds, while there is a simultaneous decrease in other enzymes (Chediack et al., 2012).

Periods of 31 h for birds and 4 months for fish can both be considered prolonged food deprivation, so that the elevation of ANPEP activity may allow both cunner and house sparrows to efficiently digest and utilize proteins from the diet for energy or protein synthesis as soon as they become available. This process would allow these animals to maintain protein synthesis in specific essential tissues, even when other less valuable organs are being down regulated or shut down. For example, it has been shown that cunner maintain protein synthesis in the gill, an organ essential for gas and ion exchange, but not in the heart, during acute cold exposure (Lewis and Driedzic, 2010). Although the GIT might appear to have little energetic value during torpor, preservation of ANPEP activity in torpid compared to fasted cunner suggests that mechanisms in place in the GIT differ between fasting and torpid fish. It is possible that the presence of ANPEP in the GIT during torpor may help to utilize or reutilize any protein that may have entered the intestine passively. One such source of peptides may be the intestinal epithelia itself, as cell turnover is constantly occurring in the intestine and degraded tissue might be recycled rather than wasted.

4.2.1.3 ALP

Also located on the enterocyte membrane, ALP regulates fatty acid absorption and its activity can approximate brush border absorptive capacity and various digestive parameters related to enterocyte maturity and density (Infante and Torrissen, 2001). Four-week fasted and torpid cunner both showed similarly lowered ALP activity. These results are in line with studies in carp, which show a comparable decline in ALP activity during fasting (Gas and Noailliac-Depeyre, 1976). The decrease in ALP activity could be indicative of compromised fatty acid absorption by enterocytes in part due to a reduction in the microvillus structure in enterocyte plasma membranes, as seen in snakes (Ott and

Secor, 2007; Secor, 2008). The relationship between enterocyte structure and ALP activity is also evidenced by a measurable spike in ALP activity during sea bass, red drum (Infante and Torrissen, 2001) and yellow croaker (Ma et al., 2005) larval development when enterocyte maturation is most profound. Electron microscope histological examination of torpid and fed cunner intestinal brush border performed in parallel with ALP activity assays would provide evidence for or against such a change in cunner GIT during food deprivation.

ALP is not exclusively responsible for digestion and has been shown to also contribute to the biochemical defense function of the intestine against microbes and other pathogens (Lallès, 2010). One cost of torpor, especially in mammalian species, is a weakened immune response (Carey et al., 2003). Several species, such as bats, utilize periodic rewarming or arousal patterns during torpor. Although the main function of these rewarming events is unclear, it is thought that they play a role in protecting torpid animals against pathogens since torpid individuals are highly susceptible to infection (Bouma et al., 2010). Low, but not nil ALP activity during fasting and torpor may suggest that ALP participates in the immune defense of cunner submitted to starvation.

4.2.1.4 Trypsin

A limitation of the quantification of trypsin activity in higher vertebrates is the separation of pancreas and intestine, as the accurate assessment of total trypsin levels requires that both tissues be considered. In most fish, including cunner, the pancreas is diffuse along the GIT (Chao, 1973). This allows for good estimation of total trypsin content in a single assay, despite some variation caused by uneven pancreatic distribution along the GIT.

Trypsin is a proteolytic enzyme responsible for protein digestion into smaller di- and tri-peptides, which are later absorbed by intestinal brush border enzymes. Cunner is stomachless and lacks acid-peptic digestion (Chao, 1973), so trypsin is one of few peptidases responsible for the initial denaturation and breakdown of proteins from food. 4-week fasted and torpid cunner both show similarly low levels of trypsin compared to fed fish. Similar reductions in trypsin activity during torpor, which represents a much more extreme case of food deprivation, suggest that trypsin is exclusively influenced by food availability and does not respond to other seasonal cues that accompany torpor, such as temperature.

After 1 week of re-feeding, trypsin levels returned to those of control fish. Considering that 1 week earlier, trypsin activity was reduced to levels similar to those experienced during dormancy, cunner appears to be well suited to adjusting its trypsin production to match feeding status. This is consistent with studies on python which increase trypsin activity 12 fold 3 days following a meal (Cox and Secor, 2008). As trypsin is a major secreted proteolytic enzyme, the drastic reduction in its activity with food deprivation may account for substantial energy savings and contribute to a reduction in GIT tissue specific metabolic rate such as that seen in other tissues in torpid cunner (Lewis and Driedzic, 2007).

4.2.1.5 Enzyme precursor transcripts

mRNA expression levels were also evaluated for trypsin, ALP, ANPEP and bile-salt activated lipase (BAL). Whereas the lipase enzyme assay was non-specific, qPCR is specific to particular transcripts. Thus, for lipase transcript quantification BAL was selected since it is a key lipolytic enzyme in fish. No significant differences were seen for

precursor transcript expression for trypsin, ALP or lipase between fed, 4-week fasted and torpid cunner. These results suggest that there might be a dysregulation between the transcript expression of an enzyme and its activity, suggesting the presence of a regulatory step between transcript expression and the translation of the active peptide or post-translational modifications of that peptide for lipase, trypsin and ALP.

ANPEP transcript expression decreased for torpid cunner, which is contradictory to its increase in activity. This further supports the dysregulation between the transcript expression of an enzyme and its actual activity. These opposite effects raise questions about the production of ANPEP in the intestine and potential mechanisms for increased ANPEP activity that cannot be accounted for by transcript expression.

It must be noted that high variation was seen in enzyme transcript expression levels in the GIT, which might have biased my results. This variation might have been due to an uneven distribution of enzyme producing cells or to the small amount of tissue used in RNA extraction (as most tissue was destined for enzyme assays).

4.2.1.6 Enzyme activities in the mid intestine

In addition to distal intestine, preliminary enzyme assays for fed and fasted individuals were performed for mid intestine. Similar, but less significant, trends were noted (data not shown), suggesting that mid intestine is less important in the control of digestive capacity than the distal intestine. These results are consistent with the fact that in most stomachless fish, such as a carnivorous needlefish, *Strongylura krefftii,* and cunner, protein digestion and nutrient absorption are primarily associated with the distal intestine (Chao, 1973; Day et al., 2011). The greater contribution of the distal intestine to digestive function and its

regulation in cunner is also in agreement with theories proposed by Karasov (Karasov and Martínez del Rio, 2007) which predict a distally biased digestive tract in carnivorous fish.

4.2.2 Appetite regulators

4.2.2.1 Orexin

Orexin transcript expression levels in the GIT decreased in torpid cunner and had intermediate values after 4 weeks of fasting. This presents an interesting question about the role of orexin in long-term food deprivation. Considering orexin is an orexigenic hormone, an increase in its expression following 4 weeks of summertime food deprivation was hypothesized. Previous studies have shown that orexin mRNA expression levels also decrease in the brain of fasted and torpid cunner (Babichuk and Volkoff, 2013). The decrease in both brain and intestinal orexin mRNA levels in fasted and torpid cunner raises the possibility that the role of orexin may extend to regulatory systems other than appetite stimulation, such as digestion and perhaps wakefulness.

Indeed, in mammals, orexin regulates wakefulness and activity patterns and is implicated in the mechanisms behind narcolepsy (Chemelli et al., 1999; Lin et al., 1999; Nishino et al., 2000). Although detailed and standardized recordings of locomotor activity have not been performed in my study, cunner appear to reduce their locomotor behaviour and arousal when fasted (personal observations and N. Babichuk, personal communication, 2012), a response that might be linked with the reduction in orexin transcript expression levels during fasting. Expression data from the present study show that cunner orexin does not to respond to food deprivation as an orexigenic hormone, which would typically be up-regulated in response to energy demands. Rather, cunner

may be well suited to cope with food deprivation during the summer as well as the winter by reducing their energy expenditure in a state of low activity or dormancy, which I suggest may be potentiated by lowered arousal via the depressed expression of GIT and brain orexin. In addition, although the proximal factor causing cunner to enter torpor seems to be water temperatures less than about 5ºC (Green and Farwell, 1971), metabolic rate assessments on fasted cunner in summer water temperatures would potentially uncover an ability to metabolically down-regulate in response to food deprivation without the temperature cue. Further longer term studies may explore the possibility of a role of orexin in the initiation of torpor, which may be extendable to other vertebrate models.

4.2.2.2 CCK

CCK is a potent satiety factor that inhibits feeding behaviour following a meal and increases GIT secretions and motility to facilitate digestion (Koven and Schulte, 2012; Murashita et al., 2006). Tissue distributions in the present study show that CCK is most highly expressed in the distal intestine compared to other GIT regions, whereas yellowtail have higher CCK expression in the proximal intestine than the distal intestine (Murashita et al., 2006). The distribution of CCK in yellowtail and cunner GIT is consistent with the regions most responsible for nutrient absorption in these species: distal intestine for stomachless fish and proximal intestine for those with stomachs (Bakke et al., 2010; Manjakasy et al., 2009). This may be related to enteroendocrine cell density and/or PepT1 distribution, responsible for CCK secretion and digested protein product sensing respectively, in the intestines of species with varying absorptive strategies (Liou et al., 2011).

Torpid cunner had reduced CCK transcript expression in the distal intestine compared to fed cunner in the summer, whereas 4-week summer-fasted cunner showed intermediate CCK expression, with levels between those seen for summer-fed and torpid fish. These findings are consistent with previously published data, showing decreases in hypothalamic and telencephalic CCK transcript expression in both torpid and one-week fasted cunner (Babichuk and Volkoff, 2013). Several studies in mammals and fish have shown that CCK levels rapidly decrease within days or weeks of food deprivation and remain consistently low as fasting continues (Koven and Schulte, 2012; Murashita et al., 2006). In line with these studies, my results show a non-significant decrease in 4-week fasted cunner, but a significant decrease in torpid cunner, suggesting a continuous reduction in CCK as food deprivation extends from weeks to months. As CCK is involved in maintaining a digestive tone and implicated in long term energy balance (Matson and Ritter, 1999), the reduction of CCK levels in torpid cunner suggests that CCK digestive tone is reduced in torpid individuals which thus have no "expectation" for a return of food availability. Further investigation into the role of CCK in the control of basal level digestive secretions and motility would help understand the longer term implications of CCK expression, or lack thereof.

4.2.2.3 Apelin

In cunner, apelin transcript expression in the distal intestine was significantly reduced following 4 weeks of summertime fasting and remained unchanged in torpid fish. In both goldfish (Volkoff and Wyatt, 2009), and cavefish (Penney and Volkoff, 2014), injections of apelin induces a significant increase in food intake, and in goldfish hypothalamic and telencephalic apelin transcript expression is elevated in 5 day fasted compared to fed

individuals (Volkoff and Wyatt, 2009), suggesting that apelin has an orexigenic role in goldfish. In rats (Carpéné et al., 2007; Lv et al., 2013), contradictory results have been reported and apelin has been identified as both an orexigenic and anorexigenic factor. My results for summer fasting show that apelin in cunner distal intestine follows an expression pattern typical of an anorexigenic factor, which contrasts with results in fasting goldfish (Volkoff and Wyatt, 2009). These findings raise the possibility of tissue-specific roles of apelin given its reported orexigenic role in the brain of goldfish (Volkoff and Wyatt, 2009) and an anorexigenic expression pattern in cunner GIT (this study).

The lack of change in apelin expression in torpid cunner suggests a possible seasonal or environmental influence on apelin expression beyond that of feeding status during the winter. In favour of this hypotheses, studies have shown that, in mouse adipocytes, clock (a "circadian gene") and apelin transcript expression respond similarly to melanin treatment, a well-known controller of seasonal and circadian cycles (Li et al., 2013). Future work examining the apelin response at several time points throughout the summer and winter in brain and GIT of cunner might shed some light on the contributions of apelin to the control of seasonality and torpor, as well as a tissue specific response. Apelin has also been shown to stimulate rat gastric cell proliferation *in vitro* (G. Wang et al., 2004), which, in combination with my findings, suggest that one implication of increased apelin expression in the GIT of torpid cunner may be related to tissue restructuring. Apelin also stimulates CCK release in rodent enteroendocrine cells (Lv et al., 2013; G. Wang et al., 2004). If this effect exists in fish, the trend for lowered CCK expression in fasted cunner would occur in parallel to a decrease in apelin, but a further reduction of CCK expression in torpid cunner cannot be explained by apelin.

The work here suggests that apelin is implicated in the GIT response to food deprivation, but other confounding factors such as season and water temperature may be responsible for the conflicting apelin responses between summer fasting and winter torpor.

4.2.2.4 mTOR

mTOR transcript expression was decreased in the GIT of torpid cunner. My results are consistent with previous studies in zebrafish showing that fish fasted for three weeks show decreased hepatic mTOR transcript expression (Craig and Moon, 2011). These results suggest that mTOR expression is affected by food deprivation and has a critical role in energy sensing in fish. In rats, an energy sensing role for mTOR has been shown and mTOR expression in the arcuate nucleus co-localizes with that of appetite regulating peptides such as neuropeptide-Y and proopiomelanocortin (Cota et al., 2006). mTOR provides information on cell energy status to regulatory mechanisms controlling the rate of protein synthesis by either direct or indirect phosphorylation. For example, mTOR inactivates the eukaryotic initiation factor 4E-binding protein resulting in an increase in mRNA transcription and activates S6 kinase resulting in an increase in ribosomal protein translation (Hay and Sonenberg, 2004). It is probable that in torpid cunner, the decrease in mTOR expression induces changes in transcription factors/proteins, perhaps resulting in an overall decrease in mRNA synthesis.

mTOR has also been implicated in the regulation of autophagy and in the balance of cell growth and breakdown (Jung et al., 2010). In cunner, a reduction of mTOR GIT expression during overwinter torpor could result in the inhibition of protein synthesis,

potentially leading to intestinal atrophy. Although no evidence is provided for intestinal atrophy in cunner, snakes and other long-term fasting organisms have been shown to drastically reduce the structure of their intestine when dormant or between meals (Secor, 2005; Secor and Lignot, 2010). In mammals, the mTOR/Akt pathway is implicated in the control of skeletal muscle hypertrophy and can prevent muscle atrophy (Bodine et al., 2001). In summary, my data suggest that mTOR may play an important role in energy sensing and control of growth in the GIT of fish, particularly in dormancy.

4.3 Effect of food type on ALP and lipase

Cunner fed a pellet diet showed significantly lower lipase activity than squid-fed fish, demonstrating a reduction in digestive capacity for energy-rich lipids. No changes were noted for ALP activity between the pellet and squid-fed fish. Thus, cunner GIT lipase, but not ALP, activity appears to respond to food type. Since an enzyme's activity is related to the availability of its substrate (Ferraris and Diamond, 1989) and ALP performs many other functions in the GIT other than digestion, it is logical that its activity remains high despite alterations in food type. Lipase, on the other hand, is solely involved in the digestion of lipids, thus a change in diet would have a more pronounced effect on the concentration of its substrates. In addition, ALP activity is correlated with several brush border membrane digestive parameters and it is likely that no major changes of the absorptive surface of the intestine occurred after 5 weeks of consuming either a pellet or squid diet. These results may have also been confounded by feed preferences.

4.4 Periprandial intestinal enzyme activities

To evaluate changes in digestive enzyme activities after a meal, cunner were sampled prior to feeding and 1 h after feeding. No change was noted for lipase activity, which

contrasts with results in southern catfish showing that peaks in lipase activity in both the proximal and middle intestinal segments occur at 16 h and 24 h post-feeding (Zeng et al., 2012). The variation in results between studies might be due to differences in sampling times and changes in lipase activities might be seen in cunner had sampling occurred beyond 1h post feeding. ALP activity decreased 1h after feeding, which, as for the lack in change in lipase activity, is not consistent with the expected rise in digestive enzymes upon feeding. It has been suggested that ALP may regulate lipid uptake in the intestine as a rate-limiting step, as ALP knockout mice show a marked increase in fatty acid uptake in the absence of ALP (Narisawa et al., 2003). Such a rate-limiting role may be true for ALP in cunner given the decrease in activity accompanying feeding. Additionally, increased protein concentration in the GIT from digestion products may have skewed the specific activity values for ALP (expressed as U/mg protein) resulting in an artificially low value for the same amount of ALP. Further experiments including more postprandial time points would help to fully evaluate the change in ALP activity.

4.5 Intestinal morphology

No obvious evidence of morphological changes was seen between fed and torpid cunner. The outer muscular layer, villus height, villus number and lumen size all appeared similar between treatments. My preliminary investigation into the morphology of cunner intestinal epithelium during torpor does not provide evidence for any structural changes during this period of extreme nutrient deprivation and the associated partial loss of digestive function (reduction in enzyme activities). It is possible that further electron microscopy analyses may reveal similar microvillus changes such as those seen in pythons (Lignot et al., 2005).

4.6 Conclusions

Through a combination of biochemical and molecular approaches I have characterized the response of cunner intestine to acute summer fasting and overwinter torpor. A decrease in the activities of trypsin, ALP and lipase for both fasted and torpid fish illustrates a remarkable ability to match digestive function to food availability, which may provide significant energy savings during food deprivation. ANPEP appears to play a significant role in the GIT response to torpor, which may be related to the reuptake of small peptides. Orexin expression is lowered during torpor and may be more involved in the control of wakefulness than appetite in cunner. Apelin expression follows an anorexigenic expression pattern for 4-week fasted cunner and is maintained at fed levels during torpor, implicating it in the torpor response. CCK expression is lowered in torpid cunner, but not 4-week fasted cunner, demonstrating a slow long-term decline in expression, which may indicate a role in maintaining a basal digestive tone between meals. mTOR expression is lowered during torpor which may have an influence on the control of protein synthesis during the winter.

The effect of food type study showed higher lipase activity in squid-fed fish than pellet-fed fish. This may be indicative of food preference and/or a digestive response to the nutrient content of the feed. Fish sampled 1 h after feeding had measurably decreased ALP activity, thus raising important questions about the role of ALP in the regulation of digestion and if ALP may indeed act in a rate-limiting manner.

I have identified novel food deprivation responses in the cunner. Without such an extreme model for fasting, the inconsistencies I have discovered between short and long-term food deprivation would not have been explored. The intestinal epithelia of food-deprived cunner should be examined further, perhaps by electron microscopy, to fully

understand its role in food deprivation. Future work must ask why such differential responses, such as those seen for ANPEP and apelin, occur when food deprivation is prolonged over an entire season, but not during short-term fasting. The role of the intestine in the regulation of energy balance is not fully understood and digging deeper into the mechanisms allowing extreme food deprivation has the potential to shed new light on other animal systems, including humans.

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