The influence of nutritional status and environmental factors on growth, feeding behaviour and appetite-related peptide transcript expression in Atlantic cod (*Gadus morhua*).

by

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Abstract

Appetite regulation and energy homeostasis are critical processes for vertebrate survival that are regulated by neuroendocrine mechanisms. Understanding how appetite-regulating hormones are influenced by intrinsic and extrinsic cues is essential for the comprehension of how vertebrates maintain an appropriate energy balance. This thesis focuses on some important appetite regulators [melanin-concentrating hormone (MCH), orexin, neuropeptide Y (NPY), gonadotrophin-releasing hormone (GnRH) and cocaine- and amphetamine-regulated transcript (CART)] in a commercially important fish species, Atlantic cod (*Gadus morhua*). To begin my analyses of these important appetite-regulating hormones in cod, I identified for the first time cDNA sequences for MCH and GnRH mRNAs, while orexin, NPY and CART transcripts were previously isolated in cod. MCH and GnRH hormones were shown to be expressed in regions of the brain and peripheral tissues (*i.e.* gastrointestinal tract) related to food intake regulation. MCH, but not GnRHs, transcript expression was higher in fed compared with fasted fish, indicating that MCH may play an important role in appetite regulation in cod. Then, I examined how diet (*i.e.* plant-based feed, *Camelina sativa*) and environment (*i.e.* background colour) influence feeding behaviour and appetite-related peptide transcript expression. Camelina meal-supplemented feed reduced food intake and growth, and increased orexigenic peptide mRNA expression (*i.e.* MCH, orexin and NPY), but did not affect CART expression, suggesting fish are still "hungry" and that MCH, orexin and NPY peptides could be involved in other feeding behaviours and metabolic processes, such as nutrient sensing and food searching behaviour. Finally, I addressed the effects of background

colour and fasting on Atlantic cod feeding and swimming behaviour, as well as appetite-related transcript expression. Atlantic cod subjected to fasting and different background colours displayed reduced locomotion. Furthermore, a decrease in MCH and orexin mRNA expressions in fasted fish was observed and might possibly be correlated to a reduction in locomotion and feeding activity rather than indicative of anorexigenic effects. Finally, fasted fish had paler skin colour compared to fed fish in white backgrounds. These results suggest that MCH, orexin and NPY may play important roles in various appetite-related behaviours and physiological processes, whereas CART and GnRH may not have significant roles in food intake regulation in Atlantic cod. Understanding feeding regulation and how various cues can influence food intake is fundamental for the development of sustainable aquaculture industries.

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ACC: acetyl-CoA carboxylase

ACOA: Atlantic Canada Opportunities Agency

ADC: apparent digestibility coefficients

AgRP: agouti-related peptide

AIF: Atlantic Innovation Fund

ALA: α-linolenic acid

AMPK: AMP-activated protein kinase

ANF: anti-nutritional factor

ANOVA: analysis of variance

arpc4: actin-related protein complex 4

°C: Celsius

C: cerebellum

CART: cocaine- and amphetamine-regulated transcript

CCK: cholecystokinin

cDNA: complementary deoxyribonucleic acid

CF: condition factor

cfGnRH: catfish-GnRH

cGnRH: chicken-GnRH

chr: chromosome

CM: camelina meal

CO: camelina oil

COSEFM: camelina oil, solvent extracted fish meal

CPT: carnitine palmitoyltransferase

CRF: corticotrophin-releasing factor

C_T: cycle threshold

D: dark photocycle

DEPC: diethylpyrocarbonate

DFO-SABS: Department of Fisheries and Oceans – St. Andrew's Biological Station

DPA: docosahexaenoic acid

dpf: days post-fertilization

dph: days post-hatch

DNase: deoxyribonuclease

E: amplification efficiencies

EDTA: ethylenediaminetetraacetic acid

EPA: eicosapentaenoic acid

FAA: food anticipatory action

FACS: fatty acyl-CoA synthase

FAS: fatty acid synthase

FB: faba bean

FBSB: faba bean/soybean

FCR: food/feed conversion ratio

FO: fish oil

FSH: follicle-stimulating hormone

g: grams

GAL: galanin

GI: gastrointestinal

GnRH: gonadotrophin-releasing hormone

GOI: gene of interest

GRP: gastrin-releasing peptide

H: hypothalamus

h: hour

hcrt: hypocretin

He: heart

HKG: housekeeping gene

HV: Helene Volkoff

ICV: intracerebroventricular

IP: intraperitoneal

ir: immunoreactive

IRC: inter-run calibrator

JBARB: Joe Brown Aquatic Research Building

K: Fulton's condition factor

L: ladder

L: light photocycle

LA: linoleic acid

LED: light emitting diode

LH: luteinising hormone

Li: liver

LVR: lateral ventricular recess

M: muscle

m: metres

MCD: malonyl-CoA decarboxylase

MCH: melanin-concentrating hormone

mdGnRH: medaka-GnRH

Mg: midgut

mGnRH: mammalian-GnRH

mL: millilitres

MLYCD: malonyl-CoA dehydrogenase

MMLV: Moloney murine leukemia virus

MO: medulla oblongata

mRNA: messenger ribonucleic acid

MS-222: tricaine methanesulfonate

NCBI: National Center for Biotechnology Information

NLT: nucleus tuberis lateralis

NPY: neuropeptide Y

NSERC: Natural Sciences and Engineering Council of Canada

NTC: no template control

O: ovaries

OEA: Oleoylethanolamide

OSC: Ocean Sciences Centre

OT: optic tectum/thalamus

OX: orexin

OX-: orexin neuron-ablated mouse

P: pituitary gland

PA: phytic acid

Pc: pyloric caecum

PCR: polymerase chain reaction

pmch: pro-melanin-concentrating hormone

POA: pre-optic area

PUFA: polyunsaturated fatty acids

QC: quality control

qPCR: quantitative real-time polymerase chain reaction

RDC: Research and Development Corporation

RNA: ribonucleic acid

RPS9: ribosomal protein s9

RQ: relative quantities

RT: reverse transcription negative control

RT-PCR: reverse transcription polymerase chain reaction

S: skin

SB: soybean

sbGnRH: seabream-GnRH

SBM: soybean meal

SC: spinal cord

SD: standard deviation

SEFM: solvent extracted fish meal

SEM: standard error of mean

sGnRH: salmon-GnRH

SGR: specific growth rate

SGR_M: mass specific growth rate

SL: standard length

SMT: Sarah Maria Tuziak

sp.: species

Sp; spleen

St: stomach

T: telencephalon/preoptic area

T_3: triiodothyronine

T_4: thyroxine

TAE: tris-acetic acid-EDTA

Te: testis

TMS: tricaine methanesulfonate

TRH: thyroid-releasing hormone

TSH: thyroid stimulating hormone

uba52: ubiquitin A-52 residue ribosomal protein fusion product 1

UBI: ubiquitin

UCP: uncoupling protein

UI: urotensin I

µl: microlitres

USA: United States of America

ω3: omega 3

VTA: ventral tegmental area

1.1. Background information

1.1.1. Overview of appetite regulation

In vertebrates, the neuroendocrine control of food intake is an intricate process involving hormones and other biotic and/or abiotic cues. These hormones can be synthesized in and released from the brain or peripheral tissues, including the gastrointestinal (GI) tract and liver (Figure 1.1A,B). The primary site for peptide production within the brain is the hypothalamus located in the diencephalon (Figure 1.1A). In all vertebrates, the hypothalamus is divided into nuclei among which the arcuate (Joly-Amado *et al.* 2014) and paraventricular nuclei (Williams and Elmquist 2012) are primarily responsible for synthesis of appetite-regulating factors. Peripheral food intake-related signals can be received directly by the hypothalamus or indirectly relayed to the hypothalamus via the brainstem (Hussain and Bloom 2013). Similar to the mammalian model, fish appetite-related hormone synthesis occurs mainly within the hypothalamic nuclei (Lin *et al.* 2000; Volkoff *et al.* 2005). However in vertebrates, these appetite-related hormones are also produced in other brain regions, such as the telencephalon (including the preoptic area), mesencephalon (optic tectum and thalamus), and the pituitary gland (Figure 1.1B) (Volkoff *et al.* 2005; Lin *et al.* 2014). In both fish and mammals, several studies provide evidence of a cross-talk between these regions during appetite regulation, and an intricate web of signals throughout the vertebrate brain (Miranda *et al.* 2013; Perez Sirkin *et al.* 2013; Suzuki and Yamamoto 2013; Akieda-Asai *et al.* 2014).

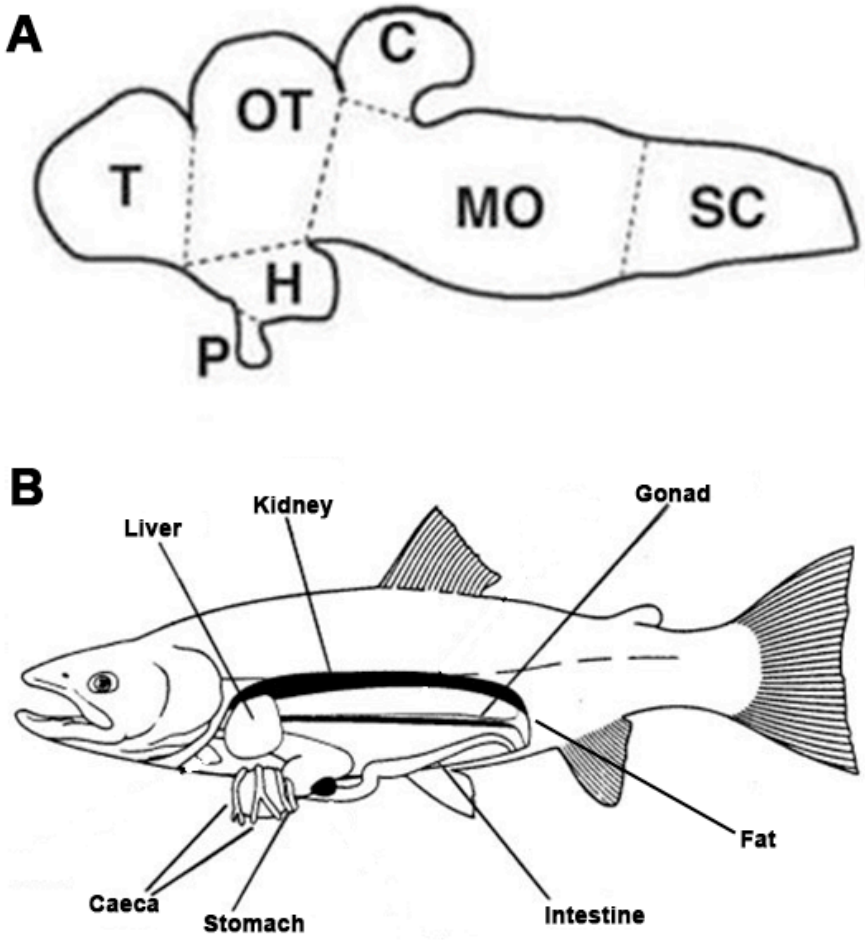

Figure 1.1. A) General/unspecific model of fish brain showing main anatomical regions. T: telencephalon/preoptic area, OT: optic tectum/thalamus, H: hypothalamus, P: pituitary gland, C: cerebellum, MO: medulla oblongata, and SC: spinal cord. B) Example of some peripheral tissues involved in the synthesis and release of hormones that are implicated in food intake regulation in fish.

Synthesis of appetite-related hormones can be localized or disparate (*i.e.* a single nucleus within the brain, multiple brain regions and/or in the brain as well as peripheral tissues) and peptides released act on local (autocrine or paracrine regulation) or distant (endocrine regulation) receptors. For example, in mammals, orexin neurons are found within the lateral hypothalamic area (LHA) and their axons synapse on other neurons within the LHA or extend to other hypothalamic nuclei, such as the arcuate nucleus, for paracrine, and possibly autocrine, regulation of energy balance (Peyron *et al.* 1998). Furthermore, orexin axons extending from the LHA synapse on neurons within hindbrain nuclei, which receive signals from peripheral tissues, such as the GI tract (Grabauskas and Moises 2003) and liver (Stanley *et al.* 2010).

Hormones involved in appetite regulation can either be food intake stimulators (orexigenic) or suppressors (anorexigenic). Both types of factors work to regulate food intake and feeding behaviour in animals. In mammals, orexigenic factors include, but are not limited to, melanin-concentrating hormone (MCH), orexin, neuropeptide Y (NPY) and ghrelin, while gonadotrophin-releasing hormone (GnRH), cocaine- and amphetamine regulated transcript (CART) and cholecystokinin (CCK) act as a few of many appetite inhibitors [reviewed in (Neary *et al.* 2004; Arora and Anubhuti 2006)]. For the most part, the functions of appetite-related hormones appear to be conserved throughout the animal kingdom, however some group/species-specific differences have been demonstrated in fish and are discussed in more detail below (Table 1.1) [reviewed in (Lin *et al.* 2000; Volkoff *et al.* 2005)].

This thesis will focus on evaluating the roles of some known "classical" orexigenic (MCH, orexin and NPY) and anorexigenic (GnRH and CART) factors in the

Table 1.1. Major known appetite-regulating hormones in fish

Hormone and effect on feeding	Major producing site	Fish species and references
Orexigenic		
Agouti-related peptide (AgRP)	Brain	Goldfish (Cerda-Reverter and Peter 2003); zebrafish (Drew *et al.* 2008); Ya-fish (Wei *et al.* 2013b); sea bass (Agulleiro *et al.* 2014); common carp (Bernier *et al.* 2012; Zhong *et al.* 2013)
Apelin	GI tract/brain	Goldfish (Volkoff and Wyatt 2009); Ya-fish (Lin *et al.* 2014); Mexican blind cavefish (Penney and Volkoff 2014); red bellied piranha (Volkoff 2014)
Galanin (GAL)	Brain	Goldfish (de Pedro *et al.* 1995; Volkoff and Peter 2001b; Unniappan *et al.* 2004b)
Ghrelin	GI tract	Goldfish (Unniappan *et al.* 2004a); sea bass (Terova *et al.* 2008); zebrafish (Amole and Unniappan 2009); Atlantic salmon (Hevroy *et al.* 2011); grass carp (Feng *et al.* 2013); Ya-fish (Wei *et al.* 2013a); Mexican blind cavefish (Penney and Volkoff 2014); brown trout (Tinoco *et al.* 2014b); *Schizothorax davidi* (Zhou *et al.* 2014)
Growth hormone (GH)	Pituitary gland	Rainbow trout (Johnsson and Bjornsson 1994; Cleveland *et al.* 2009); channel catfish (Peterson *et al.* 2009); fine flounder (Fuentes *et al.* 2012); Coho salmon (Shimizu *et al.* 2009); Nile tilapia (Fox *et al.* 2010)

Melanin-concentrating hormone (MCH)	Brain	Starry flounder (Kang and Kim 2013a); winter flounder (Tuziak and Volkoff 2012); zebrafish (Berman *et al.* 2009)
Neuropeptide Y (NPY)	Brain/GI tract	Ya-fish (Wei *et al.* 2014); yellowtail (Hosomi *et al.* 2014b); zebrafish (Yokobori *et al.* 2012); orange-spotted grouper (Tang *et al.* 2013); grass carp (Zhou *et al.* 2013); snakeskin gourami (Boonanuntanasarn *et al.* 2012); Japanese eel (Li *et al.* 2012); channel catfish (Peterson *et al.* 2012); tiger puffer (Kamijo *et al.* 2011); goldfish (Narnaware *et al.* 2000); winter flounder (MacDonald and Volkoff 2010)
Orexin	Brain	Red-bellied piranha (Volkoff 2014); Mexican blind cavefish (Penney and Volkoff 2014); African cichlid (Grone *et al.* 2012); zebrafish (Yokobori *et al.* 2011); barfin flounder (Amiya *et al.* 2012b); winter flounder (Buckley *et al.* 2010); goldfish (Abbott and Volkoff 2011)
Thyrotrophin-releasing hormone (TRH)	Brain	Winter flounder (Buckley *et al.* 2010); goldfish (Abbott and Volkoff 2011)
Anorexigenic		
Cocaine- and amphetamine-regulated transcript (CART)	Brain	Red-bellied piranha (Volkoff 2014); channel catfish (Peterson *et al.* 2012); goldfish (Abbott and Volkoff 2011); zebrafish (Akash *et al.* 2014); common carp (Wan *et al.* 2012); Atlantic salmon (Valen *et al.* 2011); African sharp tooth catfish (Subhedar *et al.* 2011); medaka (Murashita and Kurokawa 2011)

Cholecystokinin (CCK)	GI tract	Mexican blind cavefish (Penney and Volkoff 2014); Ya-fish (Yuan *et al.* 2014); white sea bream (Micale *et al.* 2012); zebrafish (Koven and Schulte 2012); grass carp (Feng *et al.* 2012); channel catfish (Peterson *et al.* 2012); Atlantic salmon (Valen *et al.* 2011); winter flounder (MacDonald and Volkoff 2010); goldfish (Himick and Peter 1994)
Corticotrophin-releasing factor (CRF)	Brain	Ya-fish (Wang *et al.* 2014b); goldfish (Bernier and Peter 2001)
Cortisol	Interrenal	Nile tilapia (Janzen *et al.* 2012); rainbow trout (Gregory and Wood 1999)
Gastrin-releasing peptide (GRP)	GI tract	Zebrafish (Koven and Schulte 2012); goldfish (Himick and Peter 1994)
Gonadotrophin-releasing hormone (GnRH)	Brain	Ya-fish (Wang *et al.* 2014a); winter flounder (Tuziak and Volkoff 2013); African cichlid (Grone *et al.* 2012); zebrafish (Nishiguchi *et al.* 2012); goldfish (Hoskins *et al.* 2008)
Insulin	Brockmann bodies within pancreatic tissue	Rainbow trout (Soengas and Aldegunde 2004; Caruso and Sheridan 2012); Coho salmon (Shimizu *et al.* 2009)
Insulin-like growth factor (IGF)	Liver	Coho salmon (Shimizu *et al.* 2009; Yamamoto *et al.* 2011); Atlantic salmon (Hevroy *et al.* 2011); Nile tilapia (Fox *et al.* 2009; Fox *et al.* 2010); grass carp (Yuan *et al.* 2011); rainbow trout (Cleveland *et al.* 2009); channel catfish (Peterson *et al.* 2009)
Leptin	Liver	Rainbow trout (Salmeron *et al.* 2015); Ya-fish (Yuan *et al.* 2014); striped bass (Won *et al.* 2012); Atlantic salmon (Trombley *et al.*

		2012); goldfish (Vivas *et al.* 2011); grass carp (Li *et al.* 2010)
Melatonin	Pineal gland	Zebrafish (Piccinetti *et al.* 2010); goldfish (Lopez-Olmeda *et al.* 2006)
Proopiomelanocortin (POMC)	Brain	Snakeskin gourami (Boonanuntanasarn *et al.* 2015); common carp (Bernier *et al.* 2012); Atlantic salmon (Valen *et al.* 2011)
Urotensin I (UI)	Brain	Goldfish (Bernier and Peter 2001)

regulation of feeding in cod and how this regulation might be affected by environmental factors, such as background colour and daily rhythms.

1.1.2. "Classical" key appetite-regulating hormones in fish

1.1.2.1. Melanin-concentrating hormone

In fish, MCH has been identified as the primary hormone responsible for colour change through its actions on melanin granules within melanocytes (Kawauchi *et al.* 1983). Studies showing the presence of MCH mRNA throughout the brain – especially in the hypothalamus, where most MCH neurons are found in appetite-regulating centres projecting to the pituitary gland, optic tectum and thalamus (Amano *et al.* 2003; Pandolfi *et al.* 2003; Berman *et al.* 2009; Kang and Kim 2013a; Mizusawa *et al.* 2014) – as well as peripherally, in organs involved in metabolism and feeding (*i.e.* liver and GI tract, including the pyloric caeca)(Tuziak and Volkoff 2012; Geiger *et al.* 2013), suggest that MCH is a likely candidate as an appetite regulator in fish. In mammals, MCH plays a clear role in stimulating food intake (Qu *et al.* 1996; Rossi *et al.* 1997). In fish, the role of MCH in the regulation of feeding is still unclear; whereas in most fish, MCH acts as an appetite stimulator (Amiya *et al.* 2008; Berman *et al.* 2009; Kang and Kim 2013a), it appears to have appetite-inhibiting effects in goldfish (*Carassius auratus*) (Matsuda *et al.* 2009).

Studies involving MCH in fish are more complicated by the fact that up to two

transcripts (paralogues: two or more genes that arise from a common ancestral gene via gene duplication events) have been identified in the transcriptomes of several fish species and these may have undergone subfunctionalization or neofunctionalization. For example, two MCH paralogues have been identified (MCH1 and MCH2) in the transcriptomes of flatfish [*i.e.* winter flounder (*Pseudopleuronectes americanus*) (Tuziak and Volkoff 2012), starry flounder (*Platichthys stellatus*) (Kang and Kim 2013a) and barfin flounder (*Verasper moseri*) (Amiya *et al.* 2008)] and zebrafish (*Danio rerio*) (Berman *et al.* 2009). Studies suggest that in flatfish, MCH1 might function as both an orexigenic factor in appetite regulation and a melanosome aggregator in skin colour change, while MCH2 may not play a role in food intake regulation and only mediate skin colour change (Tuziak and Volkoff 2012; Kang and Kim 2013a; Mizusawa *et al.* 2014). In contrast, in zebrafish, MCH1 appears to be involved in skin colour adaptation, whereas MCH2 primarily functions as an appetite stimulator (Berman *et al.* 2009). Although the roles of each MCH isoform in appetite regulation and skin colour adaptation appear to be species-specific, the MCH system likely plays a key function in the regulation of fish energy homeostasis. However, further studies in multiple fish species are needed to better understand this functional variability (*i.e.* orexigenic *vs*. anorexigenic) in fish appetite regulation.

Few studies have examined the early ontogeny of MCH in fish, an important tool to help identify relationships between hormone functions and localization beginning during early life history, as well as to determine whether transcripts have diverged based on expression profiles. For example, in an African cichlid (*Cichlasoma dimerus*), MCH-immunoreactive (ir) perikarya are only expressed in the brain after hatch in brain regions

(*i.e.* hypothalamus) associated with feeding regulation (Pandolfi *et al.* 2003). Expression of MCH mRNA during early feeding stages is a key indication that MCH may play a significant role in food intake control throughout fish life cycles. Additional studies examining MCH transcript expression during early ontogeny in fish are essential to understand the function MCH plays in larval fish development.

1.1.2.2. Orexin

Orexin (synonym: hypocretin) is a major appetite regulating peptide in vertebrates and its function as an appetite stimulator has been demonstrated in mammals (Edwards *et al.* 1999; Rodgers *et al.* 2002). Pre-pro-orexin (the precursor protein containing all orexin subunits) consists of two orexin peptides (orexin-A and –B or hypocretin-1 and -2) arising from post-translational cleavage of the common precursor (Sakurai *et al.* 1998), unlike the case of MCH isoforms that arise from different genes.

In fish, orexin mRNAs and ir cells are highly abundant within the central nervous system, with a marked presence in peripheral tissues. The orexin-expressing cells are primarily located within the hypothalamus as well as the pituitary gland in barfin flounder (Amiya *et al.* 2012a), cunner (*Tautogolabrus adspersus*) (Babichuk and Volkoff 2013), African cichlid (Perez Sirkin *et al.* 2013), Mexican blind cavefish (*Astyanax fasciatus mexicanus*) (Wall and Volkoff 2013) and red-bellied piranha (*Pygocentrus nattereri*) (Volkoff 2014). Peripherally, orexin has relatively ubiquitous expression, but most notably it is present within the GI tract and liver of fish (Amiya *et al.* 2012b; Babichuk and Volkoff 2013; Perez Sirkin *et al.* 2013; Wall and Volkoff 2013; Volkoff 2014). The

presence of orexin mRNA within key appetite-regulating centres in the brain, as well as its presence in peripheral tissues related to digestion and metabolism, suggests that orexin plays a role in fish food intake regulation. In the majority of fish examined, orexin functions as an appetite-stimulating peptide, although some species-specific differences have come to light in recent years. Both central [goldfish (Volkoff and Peter 2001b; Nakamachi *et al.* 2006), zebrafish (Yokobori *et al.* 2011)] and peripheral [goldfish (Nakamachi *et al.* 2006; Facciolo *et al.* 2010), ornate wrasse (*Thalassoma pavo*) (Facciolo *et al.* 2009), Mexican blind cavefish (Penney and Volkoff 2014)] injections of orexin increase feeding and locomotion. In red-bellied piranha (Volkoff 2014), Mexican blind cavefish (Wall and Volkoff 2013), goldfish (Hoskins and Volkoff 2012b), African cichlid (Grone *et al.* 2012) and barfin flounder (Amiya *et al.* 2012b), increases in brain orexin mRNA expression are seen during fasting conditions, and/or before mealtime (when fish are presumably "hungry"). Similarly, hypothalamic orexin transcript expression is highest at mealtime compared to before or after a meal in Atlantic cod (*Gadus morhua*), suggestive of appetite-stimulating actions (Xu and Volkoff 2007). In cunner - a fish which undergoes a seasonal torpor during the winter months - hypothalamic and gastrointestinal orexin mRNA expression decreases during both summer-induced and natural torpor fasting, which may indicate that orexin has either true anorexigenic effects, may play a more important role in regulating sleep/wakefulness and locomotor activity or that cunner shut down their orexin system during winter (Babichuk and Volkoff 2013; Hayes and Volkoff 2014).

Only a few studies have examined the early developmental expression of orexin in fish. In the species examined to date, orexin mRNA is initially detected as early as the

cleavage stage and is up-regulated until late segmentation [orange-spotted grouper (*Ephinephelus coioides*) (Yan *et al.* 2011)] or the neurula stage [Atlantic cod (Xu and Volkoff 2007)] peaking at hatch in Atlantic cod (Xu and Volkoff 2007). It has been hypothesized that orexin might regulate energy distribution and metabolism through these high energy-consuming processes (*i.e.* neural plate formation and hatching) in fish (Xu and Volkoff 2007; Kortner *et al.* 2011; Yan *et al.* 2011).

In fish, the appetite-stimulating role of orexin in food intake regulation, as well as molecular characterization of the orexin transcripts, have been widely studied. However, few studies have examined how interactions between biotic and abiotic factors affect the function of orexin in fish feeding, which is critical to comprehend how orexin works to regulate appetite and other physiological processes.

1.1.2.3. Neuropeptide Y

NPY is part of a family of peptides, which include peptide Y, peptide YY (PYY) and pancreatic polypeptide (PPP), that are structurally homologous, but have functionally distinct roles in appetite regulation (Cerda-Reverter and Larhammar 2000). Whereas both PYY and PPP are mostly present in peripheral tissues (*i.e.* pancreas and GI tract) (Kimmel *et al.* 1986; Conlon *et al.* 1991b) and have predominant roles in digestion (Montpetit *et al.* 2005; Gonzalez and Unniappan 2010) and cardiovascular regulation (Conlon *et al.* 1991a; Bjenning *et al.* 1993), NPY displays a more central distribution and its major function appears to be food intake regulation (Kamijo *et al.* 2011; Boonanuntanasarn *et al.* 2012; Hosomi *et al.* 2014a).

In fish, NPY is found primarily in telencephalic/preoptic area nuclei (Kamijo *et al.* 2011; Boonanuntanasarn *et al.* 2012; Perez Sirkin *et al.* 2013; Hosomi *et al.* 2014b; Saha *et al.* 2014). Fibres from NPY neurons in the telencephalon/preoptic area extend into the hypothalamus and synapse on neurons in nuclei related to appetite regulation (Kamijo *et al.* 2011; Perez Sirkin *et al.* 2013; Saha *et al.* 2014). NPY cells have also been identified in the hypothalamus with fibres extending into thalamus, olfactory bulbs and pituitary gland (Kamijo *et al.* 2011; Boonanuntanasarn *et al.* 2012; Yokobori *et al.* 2012; Perez Sirkin *et al.* 2013; Saha *et al.* 2014). Peripherally, NPY is widely-distributed throughout the body, specifically in tissues related to energy homeostasis (*i.e.* liver, adipose tissue, GI tract, including the pyloric caeca) and reproduction *(i.e.* ovaries and testis) (Boonanuntanasarn *et al.* 2012; Wu *et al.* 2012; Xu *et al.* 2012; Zhou *et al.* 2012; Babichuk and Volkoff 2013; Ma *et al.* 2013; Perez Sirkin *et al.* 2013; Hosomi *et al.* 2014b; Ping *et al.* 2014; Wei *et al.* 2014).

In both fish and mammals, NPY has been recognized as the most powerful appetite-stimulator to date (Kalra *et al.* 1999; Kageyama *et al.* 2012; Wu *et al.* 2012; Yokobori *et al.* 2012). Intracerebroventricular (ICV) injections clearly demonstrate an orexigenic role for NPY (Wu *et al.* 2012; Yokobori *et al.* 2012). Several studies have shown that fasting usually up-regulates NPY mRNA in fish telencephalon and hypothalamus, including tiger puffer (*Takifugu rubripes*) (Kamijo *et al.* 2011), Brazilian flounder (*Paralichthys orbignyanus*) (Campos *et al.* 2012), channel catfish (*Ictalurus punctatus*), zebrafish (Yokobori *et al.* 2012), orange-spotted grouper (Tang *et al.* 2013), yellowtail (*Seriola quinqueradiata*) (Hosomi *et al.* 2014a) and Ya-fish (*Schizothorax prenanti*) (Wei *et al.* 2014). However, species-specific differences are seen, perhaps

owing to the adaptation of fish to their environments. For example, during periods of torpor and fasting, NPY mRNA expression is significantly decreased in cunner, suggesting that, in order to conserve energy, this fish actively shuts down feeding regulating pathways and decreases metabolism during periods of low food availability (Babichuk and Volkoff 2013). The post-prandial expression of NPY transcripts is usually lower compared with pre-prandial and at mealtime expression levels in fish, such as tiger puffer (Kamijo *et al.* 2011), Atlantic salmon (*Salmo salar*) (Valen *et al.* 2011), snakeskin gourami (*Trichogaster pectoralis*) (Boonanuntanasarn *et al.* 2012), Brazilian flounder (Campos *et al.* 2012), channel catfish (Peterson *et al.* 2012) and Ya-fish (Wei *et al.* 2014). Interestingly, although goldfish exposed to a 16L:8D photoperiod do not display a peri-prandial cycle in NPY mRNA expression (Hoskins and Volkoff 2012b), a peri-prandial increase in expression is seen when fish are exposed to constant light (Vera *et al.* 2007; Hoskins and Volkoff 2012b) suggesting that a scheduled meal and food anticipatory behaviour can entrain hypothalamic NPY mRNA expression in the absence of photoperiod cues.

With regards to development, early NPY transcript up-regulation is relatively consistent in fish, where expression is first observed at the blastula stage [*e.g.* blunt snout bream (*Megalobrama amblycephala*) (Ping *et al.* 2014) and Ya-fish (Wei *et al.* 2014)]. However, highest NPY mRNA expression is typically observed post-hatch and, for example, can climax up to two times that seen in pre-hatch expression for some species, including Atlantic cod (Kortner *et al.* 2011), blunt snout sea bream (Ping *et al.* 2014) and Ya-fish (Wei *et al.* 2014), coinciding with changes from endogenous (yolk) to exogenous feeding.

1.1.2.4. Gonadotrophin-releasing hormone

In vertebrates, GnRH was first identified as the major hypothalamic hormone responsible for regulating reproduction. Up to three different paralogous GnRH genes (GnRH1, GnRH2, GnRH3) can be found within the genome of diploid vertebrates. In mammals, two forms (GnRH1 and GnRH2) can be present. GnRH1 [synonym: mammalian-GnRH (mGnRH)], acts as a hypophysiotropic hormone on the gonadotrophs in the anterior pituitary gland, causing the release of gonadotrophins, follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Millar 2005). GnRH2 [synonym: chicken-GnRH (cGnRH)] is described in mammals as an anorexigenic factor that integrates the reproductive and energy homeostasis systems (Temple and Rissman 2000; Kauffman and Rissman 2004). GnRH3 [synonym: salmon-GnRH (sGnRH)] is only found in fish (Shahjahan *et al.* 2014).

Most vertebrates possess two, and some teleosts have three, forms of GnRH (Shahjahan *et al.* 2014). Since fish genomes have undergone up to three whole genome duplication events (Jaillon *et al.* 2004) [or more in the unique cases of salmonids (Allendorf and Thorgaard 1984) and sturgeons (Ludwig *et al.* 2001)], more than 16 different GnRH isoforms have been isolated in this lineage. The most divergent form is GnRH1 accounting for the majority of homologues found in fish [*i.e.* seabream-GnRH (sbGnRH), medaka-GnRH (mdGnRH) and catfish-GnRH (cfGnRH)] (Okubo *et al.* 2002). Both GnRH2 and the fish-specific GnRH3 are highly conserved functionally and structurally, with GnRH2 being the most prevalent form existing in fish genomes (Millar 2005; Kah *et al.* 2007). Characteristically, if only two GnRH paralogues are present in the

fish genome, one of them will compensate for the functional loss of the third absent GnRH form (Okubo and Nagahama 2008). For example, in teleost fish, the functions, brain cellular distributions and embryonic origins of GnRH1 and GnRH3 are similar and somewhat redundant (Kah *et al.* 2007; Okubo and Nagahama 2008). For fish that have lost GnRH1, it is mainly GnRH3, not GnRH2, which substitutes for reproductive functions. GnRH2 plays a minor role in reproduction and has a greater function in neuromodulation of other hormones compared to other GnRH forms (Chen and Fernald 2008).

The GnRH system of hormones has a distinctive distribution in fish brains. Typically, GnRH1 is the hypophysiotropic hormone in fish and thus GnRH1 cells are specifically isolated within the mediobasal hypothalamus - the hypothesized reproductive control centre in fish - with fibres extending into the pituitary to regulate the gonadotrophs secreting gonadotrophins (Lepretre *et al.* 1993; Karigo *et al.* 2012; Kawabata *et al.* 2012; Gomes *et al.* 2013; Sukhan *et al.* 2013; Karigo *et al.* 2014; Zmora *et al.* 2014). However, GnRH1 cells have also been identified in the olfactory nerves and bulbs, as well as the ventral telencephalon and preoptic area (Karigo *et al.* 2012; Kawabata *et al.* 2012; Gomes *et al.* 2013; Sukhan *et al.* 2013). The highest abundance of GnRH2 neurons is found in the midbrain tegmentum (Lepretre *et al.* 1993; Kawabata *et al.* 2012; Gomes *et al.* 2013; Sukhan *et al.* 2013). Finally, GnRH3 cell bodies tend to be distributed throughout the olfactory system (*i.e.* olfactory nerve, terminal nerve and olfactory bulbs), ventral telencephalon and thalamus (Kawabata *et al.* 2012; Tubert *et al.* 2012; Gomes *et al.* 2013; Sukhan *et al.* 2013).

The peripheral distributions of GnRH transcripts vary among fish species. For

example, in Chinese sturgeon (*Acipenser sinensis*), GnRH1 mRNA is found in the liver, spleen, testes and ovaries (Yue *et al.* 2013), while in flatfish, GnRH1 transcripts have a broader distribution, including in the skin, heart, kidney, liver, GI tract, testes and ovaries (Xu *et al.* 2012; Tuziak and Volkoff 2013). As for GnRH2, its transcripts are not detected at all in sturgeon peripheral tissues (Yue *et al.* 2013), and a wide distribution is observed in flatfish (skin, muscle, heart, liver, gall bladder, testis) (Xu *et al.* 2012; Tuziak and Volkoff 2013). Finally, GnRH3 transcripts have only been identified in skin, heart and gonads (Xu *et al.* 2012; Tuziak and Volkoff 2013).

The development of the GnRH system is not very well described during early life history in fish. In larval fish, GnRH1 neuron populations have been identified in three separate brain regions: telencephalon, preoptic area, and hypothalamus, with cell bodies originating in the olfactory system, telencephalon and hypothalamus, respectively, with fibres extending to the pituitary, confirming the hypophysiotropic function of GnRH1 in some fish (Pandolfi *et al.* 2002; Okubo *et al.* 2006). Up-regulation of GnRH1 transcripts occurs at variable times throughout larval development (embryonic: 22-somite stage and pre-juvenile stage) suggesting that functions during early ontogeny may be species-specific (Pandolfi *et al.* 2002; Okubo *et al.* 2006). In cichlids, GnRH2 mRNA expression can initially be observed as late as post-hatch at the mouth-opening stage and suggests an involvement in early endocrine appetite regulation (Pandolfi *et al.* 2002). GnRH3 mRNA expression has been found within unfertilized eggs in half-smooth tongue sole (*Cynoglossus semilaevis*) (Zhou *et al.* 2012) and medaka (*Oryzias latipes*) (Okubo *et al.* 2006) and GnRH3 is thought to function as a factor involved in oocyte maturation. GnRH3 mRNA and protein is consistently up-regulated throughout the mid- to late stages

of embryonic development - such as gastrulation and segmentation - the latter coinciding with brain and olfactory system differentiation, suggesting that GnRH3 possibly functions as a neuromodulator (Pandolfi *et al.* 2002; Zhou *et al.* 2012). The role of GnRH3s in neuromodulation is observed in the African cichlid, when the migration of GnRH3 neurons is traced from the olfactory placode through the olfactory nerves to their final destination in the POA and extend fibres into the pituitary to regulate gonadotrophs (Pandolfi *et al.* 2002).

Most functional studies on GnRH in fish focus on reproduction as opposed to food intake regulation. Arguably, since energy status is a key proponent as to whether an individual will undergo mating and related sexual behaviours, understanding the correlations between GnRH, appetite regulation and reproduction would be useful in understanding the endocrine regulation of feeding in fish and other vertebrates. Studies in mammals have shown that GnRH2 has a role in linking food intake and reproductive behaviours (Temple and Rissman 2000; Kauffman and Rissman 2004) and this dual function has also been shown in some fish, such as winter flounder (Tuziak and Volkoff 2013) and zebrafish (Nishiguchi *et al.* 2012). In cichlids, GnRH1 may also play a significant role in correlating reproductive states and feeding, as female African mouth brooding cichlids (*Astatotilapia burtoni*) have lower GnRH1 mRNA expression compared with those that are gravid (and not brooding eggs) and fed females have higher GnRH1 transcript expression compared with fasted fish (Grone *et al.* 2012).

Although some data are available for the distribution and early developmental expression of GnRHs in fish, the role GnRH isoforms play in fish feeding is still far from understood and a more rigorous examination in more fish species is necessary to obtain a

clear picture of their function in appetite regulation.

1.1.2.5. Cocaine- and amphetamine-regulated transcript

In mammals, CART was first implicated in the reward system, as early studies demonstrated an increase in CART mRNA expression following psychomotor drug injections, such as cocaine and amphetamine (Douglass *et al.* 1995). Later studies revealed that CART neuronal projections led from the nucleus accumbens and substantia nigra into the ventral tegmental area (VTA) and the lateral hypothalamus, both known reward centres with the latter also being implicated in appetite regulation (Kuhar *et al.* 2002). CART is now also known for its appetite-inhibiting roles in fish (Volkoff *et al.* 2005) and mammals (Kuhar *et al.* 2002).

In goldfish and carp (*Cyprinus carpio*), two paralogous forms of CART exist, with CART1 being the more active form in food intake regulation than CART2 (Volkoff and Peter 2000; Wan *et al.* 2012). However, some fish genomes have retained extra copies of CART genes, including four paralogous copies in zebrafish (Akash *et al.* 2014) and six paralogous copies in medaka (Murashita and Kurokawa 2011), which are partially a result of the teleost fish whole genome duplication events and could give rise to subfunctionalization (*i.e.* distributing one or many functions among gene variants) or neofunctionalization (*i.e.* a gene variant takes on a new function) of these genes in fish.

The distribution of CART in the brain appears to be form- and species-specific. In zebrafish, CART1 and CART3 have localized expressions in the hypothalamus (Akash *et al.* 2014), whereas CART2 and CART4 have similar, widespread distributions in the

brain in areas including, but not limited to, the telencephalon, thalamus, hypothalamus, optic tectum, hindbrain and spinal cord. Two forms of CART (CART1 and CART2) are present in goldfish, where CART1 is present in the olfactory bulb, hypothalamus and extracerebrally in the pituitary gland and CART2 is localized to the optic tectum and pituitary gland (Volkoff and Peter 2001a). Similarly, common carp transcriptomes contain two copies of CART (CART1 and CART2), both with central expressions, however specific regions are not yet known (Wan *et al.* 2012). In Atlantic cod, CART mRNA is detected in all regions of the brain, except the cerebellum, with highest apparent expression in the forebrain, hypothalamus and medulla (Kehoe and Volkoff 2007).

Peripherally, CART mRNA has been detected in most fish tissues examined, including the eye, gill, skin, muscle, liver, kidney, gut, and gonads (Wan *et al.* 2012; Babichuk and Volkoff 2013; Volkoff 2014). In medaka, the six paralogous CART transcript variants have unique peripheral distributions (Murashita and Kurokawa 2011). Highest expression for all medaka CART transcripts are within the brain and eye, while in other peripheral tissues, including the gill, skin, muscle, liver, and gut, only four forms (named according to the chromosome they appear on: 3, 6, 9, 22) are present. Both goldfish CART transcripts have widespread distributions in the periphery, with expression in the eye, gill, kidney and gonads (Volkoff and Peter 2001a). In common carp, CART1 is the only transcript expressed peripherally and is found in the ovary and eye (Wan *et al.* 2012). Similarly, Atlantic cod CART peripheral mRNA expression is limited to the ovary (Kehoe and Volkoff 2007). CART has been implicated in a variety of physiological roles, including sleep, reproduction, development and feeding behaviour, which is consistent with its widespread tissue distributions in fish (Subhedar *et al.* 2014).

CART transcripts are up-regulated early in fish development, even maternally deposited in some species. For example, CART mRNA is maternally deposited in Atlantic salmon, but the function of this mRNA in the oocyte is still unknown (Moen *et al.* 2010). Later in development, CART mRNA expression becomes more prominent in the head region with highest expression during the feeding stages (*i.e.* after onset of exogenous feeding), likely playing a key role in food intake regulation at this early stage. In zebrafish, transcripts are detected at various time points in development, with the earliest at the pharyngula stage (CART3) and latest at the protruding mouth stage (CART1) in the hypothalamus and the diencephalon (Akash *et al.* 2014). The location of these transcripts in the hypothalamus and the diencephalon and the increased mRNA expression during feeding stages indicates that CART could be an important appetite regulator early in the fish life cycle (Moen *et al.* 2010; Nishio *et al.* 2012; Akash *et al.* 2014).

As previously mentioned, CART is believed to act as an anorexigenic factor in fish. ICV injection of CART and the consequent reduction of food intake in fish evidently indicate the anorexigenic effects of CART peptide on appetite (Volkoff and Peter 2000). Further evidence of an anorexigenic role of CART in fish, is provided by a decrease in CART mRNA expression in fasted fish compared with fed fish [*i.e.* cunner (Babichuk and Volkoff 2013), red-bellied piranha (Volkoff 2014) and common carp (Wan *et al.* 2012)]. In addition, post-feeding CART mRNA expression is high and can remain elevated for up to four hours following feeding while fish are digesting their food (Valen *et al.* 2011; Peterson *et al.* 2012; Elisio *et al.* 2014).

When multiple isoforms are present, there seems to be a specialization of certain

forms for the regulation of food intake. For example, in zebrafish, CART2 and CART4 transcript levels are significantly diminished in hypothalamic nuclei following fasting, whereas no differences in mRNA expression are observed for CART1 or CART3 (Akash *et al.* 2014). A similar pattern is exhibited in medaka, where only CART chr3, but none of the remaining five mRNAs, has significantly reduced transcript expression levels in fasted fish compared with fed fish (Murashita and Kurokawa 2011).

The widespread roles of CART in fish physiology and early development still remain largely unknown. The genetic architecture (*i.e.* multiple variants within a species) renders the analyses more difficult, and few fish species have been studied. Identifying variants and their respective functions in different species would help elucidate the role CART plays in feeding behaviour in fish.

1.1.3. Factors affecting the action of appetite regulators

1.1.3.1. Environmental cues

MCH is classically known for its role in fish skin colour adaptation in changing environments. The aggregation of melanin granules within melanosomes in fish skin is controlled by MCH and causes the skin pallor (Kawauchi 2006). For example, *in vitro* exposure of barfin flounder chromatophores [*e.g.* melanophores (black colouration) and xanthophores (yellow colouration)], to either MCH1 or MCH2 causes pigment concentration and demonstrates a clear relationship between these hormones and skin pallor (Mizusawa *et al.* 2014).

Environmental cues, including background colour, play an important function in skin colour change to ensure that fish are camouflaged to better avoid predation (Ryer *et al.* 2008). Early studies in fish focused on how environmental background contributed to physiological colour changes (*i.e.* rapid colour change) and the endocrine mechanisms by which these changes occurred. For example in Arctic charr (*Salvelinus alpinus*) (Hoglund *et al.* 2002), olive flounder (*Paralichthys olivaceus*) (Kang and Kim 2013b) and starry flounder (Kang and Kim 2013a) prolonged exposure to light backgrounds induce skin pallor and concomitant increases in MCH hypothalamic mRNA expression (Suzuki *et al.* 1995; Kang and Kim 2013b, a), peptide synthesis (Baker and Bird 1992) and plasma MCH protein levels (Lyon and Baker 1993). Differences in background colour, which may be perceived as a stress in certain fish, has also been shown to cause changes in skin colour and in levels of MCH and stress-related hormones. For example, in rainbow trout (*Oncorhynchus mykiss*) maintained in black backgrounds, cortisol levels are markedly increased (Gilham and Baker 1984), fish are less tolerant to short-term stress (Gilham and Baker 1984), incorporate less radiolabelled methionine (Baker and Bird 1992) – an amino acid used in the production of MCH and empirically used to demonstrate MCH protein synthesis – and have lower MCH mRNA expression in the hypothalamus (Suzuki *et al.* 1995) compared with white-adapted fish (Gilham and Baker 1984; Baker and Bird 1992). This stress response and interaction with background colour is likely species-specific, since in olive flounder, white backgrounds induce high levels of cortisol compared to black backgrounds under conditions of increased stress (Kang and Kim 2013b). It is important to examine how fish behave under different coloured backgrounds, as some colours may induce stress, changes in feeding behaviour and thus changes in appetite

regulator transcript expression. Conversely, some background colours might have neutral or positive effects on fish, increasing growth and general health.

Most studies on skin colour change pertain to flatfish, specifically flounders, because of their unique morphology (bilaterally asymmetrical and flattened), physiology and ecology [habitat and surrounding environment (*i.e.* camouflaged within the sand)]. The "eyed-side" of the flatfish faces up and is generally pigmented to facilitate camouflage with the substrate, while the "non-eyed side" faces the substratum and is unpigmented. Typical studies have included manipulation of background colour (*i.e.* light *vs.* dark, variable colours) to determine the impacts on skin colouration. In some flounder, light backgrounds induce a paling of the skin with concomitant high levels of MCH transcript and protein production (Kang and Kim 2013a, b; Mizusawa *et al.* 2014). Interestingly, some flounder undergo a process termed hypermelanosis where the "non-eyed side" becomes pigmented to varying degrees. In this case, MCH mRNA levels tend to be lower in skin on the non-eyed side compared with ordinary flounder indicating that the phenomenon of hypermelanosis could be a result of changes in MCH hormone regulation (Kang and Kim 2013a, b). Flounder housed in tanks with a higher density of conspecifics tend to have an increased prevalence of hypermelanosis, which has been shown to be a result of changes in stress-related hormone production, specifically with increases in cortisol levels (Kang and Kim 2013b).

A recent study by Amiya *et al.* (2008) in barfin flounder examined the effects of background colour on MCH and GnRH simultaneously. Results show that brain MCH protein levels are higher in fish exposed to white background compared to those under a black background whereas an increase in GnRH2, but not GnRH1 or GnRH3, protein

levels are observed in black background-adapted fish, suggesting an antagonism between the two hormone families. Furthermore, the study reports neuronal connections between the two systems, where GnRH2-ir fibres are in direct contact with MCH-ir cell bodies in the hypothalamus, suggesting a neuromodulatory role of GnRH2 on MCH (Amiya *et al.* 2008).

Photoperiod and background colour have also been shown to affect MCH production and skin colouration in fish, but effects are species-specific. Daily rhythms for MCH production have been demonstrated in trout exposed to white backgrounds under natural photoperiods, where both MCH plasma concentrations and hypothalamic mRNA expression show increases throughout the day to a peak around 16:00h (*i.e.* middle of the photophase) and a decrease to a trough around 04:00h (*i.e.* dark period) (Lyon and Baker 1993; Suzuki *et al.* 1995). Interestingly, when white background-adapted fish acclimated to a long photoperiod (*i.e.* 18L:6D) with changing onset of light conditions (*i.e.* delayed or early dawn or sunrise) were switched to a constant long photoperiod (*i.e.* 18L:6D without delays or early onset), the rhythm in MCH levels was abolished (Lyon and Baker 1993). Conversely, when fish were adapted to black backgrounds, overall MCH plasma concentrations were markedly decreased compared to fish in white backgrounds and daily patterns were abolished suggesting that white backgrounds and the "turning-on" of lights stimulate MCH synthesis and release (Lyon and Baker 1993). In contrast, the number of MCH-ir cells in the hypothalamus is similar in olive flounder exposed to either natural and 24h light cycles, but abnormal pigment patterns (*i.e.* ambicolouration and pseudoalbinism) were observed (Shin *et al.* 2011), suggesting that another endocrine system (*i.e.* stress axis) may be playing a larger role in skin colour adaptation during

photoperiod manipulation in these fish.

Photoperiod may also impact GnRH regulation in fish, and this process may be species- and season-specific. In goldfish subjected to multiple photoperiods [*i.e.* short (10L:14D), normal (12L:12D) and long (14L:10D)], hypothalamic GnRH2 and GnRH3 mRNAs were significantly increased under longer photocycles compared with shorter ones (Shin *et al.* 2014b), showing that GnRHs are responsive to light. However, in a study by Hildahl *et al.* (2011), cod were exposed to either natural (NL) or constant light (LL) photoperiods throughout an entire reproductive season and overall brain GnRH2 and GnRH3 mRNA expressions were not affected by photoperiod (Hildahl *et al.* 2013). Although no overall effects were observed, at specific time points throughout the cycle, such as spawning season, LL conditions inhibited sexual development and the spawning-related increase of GnRH3 mRNA expression (Hildahl *et al.* 2011). Furthermore, fish exposed to LL conditions during rearing and then switched to a NL photoperiod during spawning, had delayed increases in GnRH3 transcript expression and spawning, suggesting that GnRH3 and spawning, but not GnRH2, may be under photoperiod control in cod.

Although correlations between photoperiod and background colour with MCH and GnRHs are not apparent among the species examined (*i.e*. background colour and photoperiod either increase or decrease their expression patterns in fish brain), it is likely that there is some neuromodulatory action occurring between GnRH and MCH cells. In the example of barfin flounder cited previously, GnRHs are likely fine-tuning MCH activities through neuronal connections in the brain.

The colour of light also impacts both MCH and GnRH mRNA expressions. In

goldfish, light emitting diode (LED) colour affects MCH mRNA expression, as fish exposed to shorter wavelengths (*e.g.* purple and green) have higher hypothalamic MCH transcript expression compared with fish under longer wavelengths (*e.g.* red) and white light (Shin and Choi 2014). Hypothalamic GnRH2 and GnRH3 mRNA expression are significantly increased under both long wave (*i.e.* red) and short wave (*i.e.* green) LED lights compared with white light (Shin *et al.* 2014a). It is noteworthy that female goldfish became reproductively mature at a quicker rate under green lighting compared with red and white lights (Shin *et al.* 2014a). As MCH, GnRH2 and GnRH3 mRNA expression levels are all higher under green lights, this could suggest that the hormones work together for reproduction-related endocrine regulation, but possibly have opposite effects in the regulation of other processes, such as food intake regulation and background colour adaptation.

The length of exposure and acclimation to backgrounds and photoperiods may influence the regulation of MCH and skin colour changes. For example, over a shorter period of time (60 days *vs.* 120 days), olive flounder skin colour was significantly lighter and MCH mRNA expression was greater in white backgrounds compared to dark backgrounds (Kang and Kim 2013b). However, after 120 days, there were no differences in skin colour between fish exposed to white and black backgrounds and MCH transcript expression was significantly lower in the white background compared with the dark background. Furthermore, fish exposed to both backgrounds had significantly lower MCH mRNA expression after 120 days (Kang and Kim 2013b). The changes in MCH expression over short and long periods suggest that MCH transcript expression may equilibrate over time and "adapt" to changing background colours.

Orexin regulation may also be influenced by light cycles. For example, in Atlantic cod, zebrafish, goldfish and orange-spotted grouper, similar orexin expression profiles (mRNA and number of synapses) have been demonstrated: orexin levels are highest during the "lights on" phase and decrease during the "lights off" phase suggesting that orexin transcript is affected by photoperiod and may play a role in regulating fish swimming activity and sleep/wake cycles (Appelbaum *et al.* 2010; Yan *et al.* 2011; Hoskins and Volkoff 2012a, b). It is also possible that orexin regulation is entrained in fish, since zebrafish and goldfish exposed to a 24h dark or light cycle, respectively, exhibit similar, but less pronounced, expression patterns to fish under a "lights on/lights off" photoperiod, and both the number of orexin synapses and orexin mRNA levels decrease during the dark phase and increased during the perceived light phase (Appelbaum *et al.* 2010; Hoskins and Volkoff 2012b).

Social hierarchies among individuals within fish populations have also been shown to influence skin colour. In cases where social hierarchies develop, as seen in red porgy (*Pagrus pagrus*) and Arctic charr, dominance might drive changes in fish colouration where subordinate fish are significantly darker than dominant fish regardless of tank colour (Hoglund *et al.* 2002; Rotllant *et al.* 2003). Social hierarchies can also affect the expression of appetite regulators: in male zebrafish, dominant fish have higher orexin mRNA levels and display increased swimming behaviour, compared to subordinate, fish suggesting roles for orexin in social status as well as locomotion (Pavlidis *et al.* 2011).

In summary, in fish, MCH, GnRH orexin and, to a lesser extent, NPY appear to play roles in multiple physiological processes (*i.e.* skin colour adaptation, locomotion).

However, few studies have examined the effects of abiotic factors (*i.e.* background colour, photoperiod, social hierarchies) on these peptides. Furthermore, a broader range of species must be examined to build an accurate model of how external cues affect the expression of these peptides and to determine if species-specific divergences exist.

1.1.3.2. Daily rhythms and swimming activity

Recent studies in mammals have demonstrated that, in addition to appetite regulation, orexins are involved in the control of other physiological processes, such as locomotion and sleep/wake cycles, which might be correlated to feeding behaviour (Thorpe and Kotz 2005; Li *et al.* 2009; de Lecea and Huerta 2014).

In fish, orexin has been shown to regulate swimming activity. IP injections of orexin in ornate wrasse stimulate locomotor activity via activation of orexin receptors in feeding and arousal centres within the hypothalamus, similar to the mammalian model (Facciolo *et al.* 2009). Furthermore, in goldfish, ICV injections promote increased swimming behaviour and could be a consequence of food seeking behaviour induced by orexin's appetite-stimulating properties (Nakamachi *et al.* 2006; Nisembaum *et al.* 2014). Daily orexin administration entrains a 24 h rhythm of locomotor activity under constant light and fasting conditions and these rhythms are maintained for two days after the last injection, suggesting that orexin might mediate the locomotor food anticipatory activity in goldfish (Nisembaum *et al.* 2014).

Transcriptional studies have also identified relationships between orexin, feeding and locomotion. Peaks of orexin expression have been reported before or around

mealtime, when fish feed and are active [*e.g.* goldfish (Hoskins and Volkoff 2012b), cod (Hoskins and Volkoff 2012a) and orange-spotted grouper (Yan *et al.* 2011)]. In both cod and goldfish, higher levels of activity during the day (due to increased food availability and searching), are correlated with high brain orexin transcript expression levels (Hoskins and Volkoff 2012a, b)

Orexin has also been implicated in the regulation of circadian rhythms that control food intake and activity in both fish and mammals. Transgenic orexin neuron-ablated (OX-) mice maintain activity cycles, food intake quantities and *mper2*, a crucial circadian oscillator gene, mRNA expression levels similar to wild-type mice during both *ab libitum* feeding and feed restriction, suggesting that orexin is not responsible for entraining food intake-dependent circadian clocks (Mieda *et al.* 2004). However, orexin is responsible for the locomotor activity associated with food anticipatory actions (FAA) during both light and dark phases: (OX-) mice are awake for less time and have reduced activity during hours prior to feeding compared with wild-type mice (Mieda *et al.* 2004).

Daily rhythms for orexin have also been described in few fish. For example, in goldfish, normal photoperiods induce a peak in orexin mRNA expression around feeding (FAA) and a second peak is observed 12 h later during the evening dark phase, suggesting that fish may be becoming more active in expectation of a meal (Hoskins and Volkoff 2012b). However, this rhythm is abolished in fish under a 24 h light cycle and not present during fasting for either photoperiod, suggesting that orexin plays a key role in regulating food-entrained circadian rhythms in fish under natural photoperiods. Similarly, in ornate wrasse, orexin IP injections stimulate feeding and activity of fish maintained under natural photoperiods, but have no or a lesser effect under dark or light cycles (Facciolo *et*

al. 2009), indicating that the actions of orexin are influenced by lighting conditions.

Daily rhythms have also been reported for GnRHs in fish. In orange-spotted grouper (Chai *et al.* 2013), European sea bass (*Dicentrarchus labrax*) (Servili *et al.* 2013) and medaka (Karigo *et al.* 2012), GnRH1, GnRH2 and GnRH3 mRNA expressions are significantly higher during the day compared to the evening, which is a pattern opposite to that of orexin. It appears that interactions between orexin and GnRHs exist in the control of feeding and reproduction, as ICV injections of orexin in goldfish brain decrease brain GnRH2 mRNA expression and spawning behaviour, and GnRH2 injections inhibit feeding and decrease brain orexin expression (Hoskins *et al.* 2008). It is thus possible that the opposite daily patterns might be indicative of the antagonistic and coordinating effects of these two systems with regards to feeding, reproduction and circadian rhythms.

In fish, whether or not appetite-related hormones undergo daily rhythms is not well understood. Few studies have examined how orexin and GnRH regulation and consequent feeding behaviours are affected by the perception of external (*i.e.* background colour, photoperiod) and internal (*i.e.* feeding status) cues by fish.

1.1.3.3. The influence of diet and metabolites

Recent evidence show that appetite regulators affect metabolism and that diet might affect the expression/production profiles of appetite regulators in fish. For example, both NPY and CART play a role in fatty acid metabolism. The fatty acid synthase (FAS) complex is a multienzyme complex that catalyzes the synthesis of fatty acids from acetyl CoA. FAS inhibitors hinder food intake, and up- and down-regulate hypothalamic

orexigenic (*i.e.* NPY) and anorexigenic (*i.e.* CART) hormones, respectively, in fasted animals (Loftus *et al.* 2000; Shimokawa *et al.* 2002). In fish, studies relating fatty acid metabolism and metabolites with food intake regulators are still novel and the literature is sparse. In orange-spotted grouper, peaks in NPY and CPT1a – an isoform of CPT, carnitine palmitoyltransferase (CPT), the enzyme involved in the rate-limiting step in fatty acid oxidation, ultimately promoting lipogenesis (see Figure 1.2) – mRNA expression are observed at mealtime followed by drops in expression after the meal (Tang *et al.* 2013). Grouper hypothalami exposed to NPY have increased CPT1a and UCP2, but not ACC2 or FAS (UCP: uncoupling protein is an enzyme that reduces fatty acid anion quantities in the mitochondria, ACC: acetyl-CoA carboxylase is an inhibitor of fatty acid metabolism, promoting lipogenesis; see Figure 1.2), mRNA expression levels, indicating that NPY may play a strong role in regulating fatty acid anabolic processes.

Oleoylethanolamide (OEA) is a fatty acid present in peripheral tissues and is regulated by nutrient status. Recently, OEA has been shown to promote lipolysis and inhibit lipogenesis in the liver, adipose, muscle and gut of rats (Thabuis *et al.* 2008; Serrano *et al.* 2011). In mice, OEA IP injections cause a significant decline in both food intake and hypothalamic CART mRNA expression in fasted compared with fed animals, as well as an increase in NPY mRNA expression (Serrano *et al.* 2011). Similarly, goldfish IP-injected with OEA have reduced levels of circulating triglycerides and short-term reductions in food intake and locomotor activity as well as decreased GI tract ghrelin

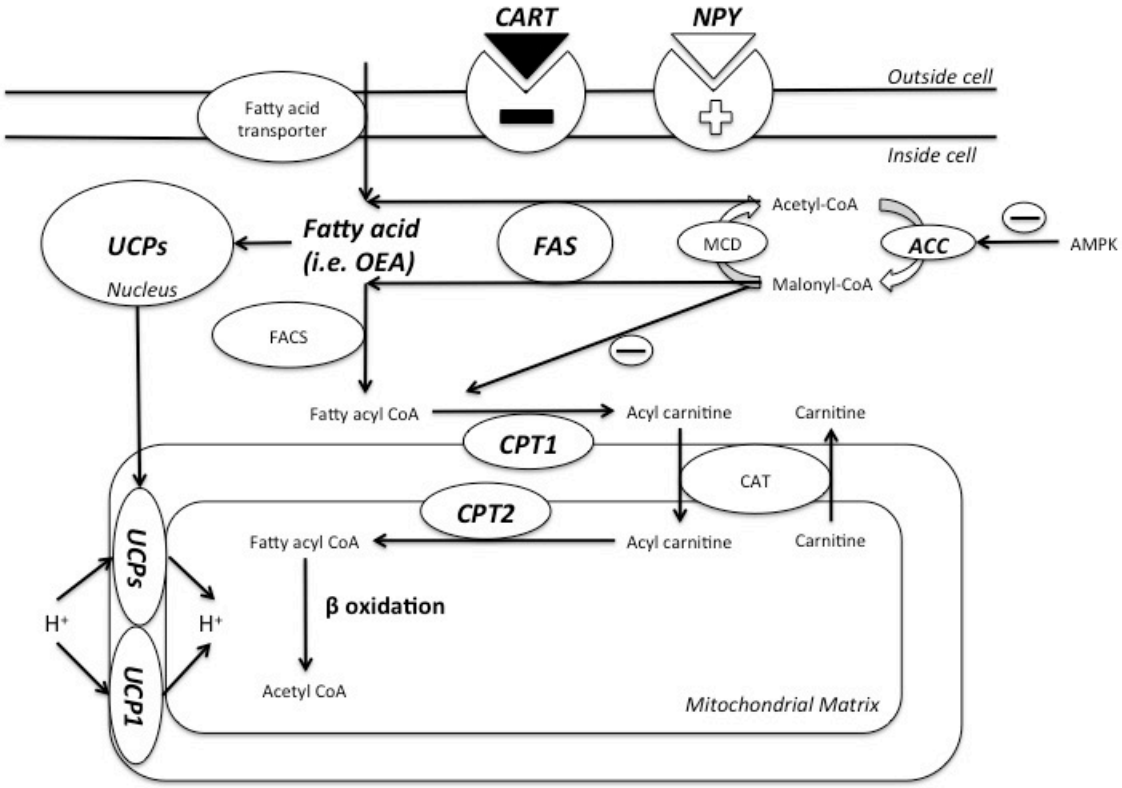

Figure 1.2. A simplified model of vertebrate fatty acid metabolism (adapted from: Fillmore *et al*. 2011). Bold and italicized enzymes (capitalized), substrates and hormones are referenced within chapter, all other enzymes in diagram are noted for a comprehensive model, but not discussed in detail. ACC: acetyl-CoA carboxylase, AMPK: AMP-activated protein kinase, CART: cocaine and amphetamine regulated transcript, CAT: carnitine translocase, CPT: carnitine palmitoyltransferase, FACS: fatty acyl-CoA synthase, FAS: fatty acid synthase, MCD: malonyl-CoA decarboxylase, NPY: neuropeptide Y, OEA: oleoylethanolamide, UCP: uncoupling protein

(appetite stimulator) mRNA expression, indicating that OEA induces the utilisation of fat as an energy source in fish using a mechanism similar to mammals and has appetite-suppressing effects, possibly by modulating peripheral appetite hormone production (Tinoco *et al.* 2014a). These results are not surprising, as OEA is a metabolic derivative of oleic acid, which also contributes to CARTs anorexigenic effects and modulates fatty acid sensing as seen in rainbow trout (Libran-Perez *et al.* 2014).

Furthermore, ICV injections of oleate and octanoate in rainbow trout decrease food intake, hypothalamic and plasma fatty acid and triglyceride levels and mRNA expression of CPT1c and CPT1d, while decreasing FAS and CPT1 enzyme activity (Libran-Perez *et al.* 2014). Conversely, in grass carp (Zhou *et al.* 2013), NPY ICV injections did not change UCP1 mRNA expression after 4 h, which could be related to the response time for UCP1 to sense the presence of fatty acids, which takes longer than 4 h, as seen in the 6 h delay following octanoate injection in rainbow trout (Libran-Perez *et al.* 2014).

Neuropeptides are also affected by oleate and octanoate injections in rainbow trout: NPY mRNA expression is diminished with oleate and octanoate treatment after 2 h, but not 6 h, compared with control saline groups, suggesting a role for NPY early in fatty acid metabolism (Libran-Perez *et al.* 2014). However, six hours post-injection, two different responses occur for oleate and octanoate, where a recovery in NPY transcript expression is observed for oleate, but a further decrease in NPY mRNA expression is seen with octanoate injections. The effects of injections of either oleate or octanoate on CART mRNA expression are opposite to those seen for NPY, *i.e.* an increase in CART transcript expression compared to the control after 2 h, but a decrease 6 hours post-

injection compared with CART mRNA levels at 2 h post-injection (Libran-Perez *et al.* 2014). These results are consistent with the known actions of CART (anorexigenic) and NPY (orexigenic) in the regulation of food intake.

Understanding the effects of diet on fatty acid metabolism and food intake regulators has been a major concern in the aquaculture industry with the introduction of plant-based diets and the need to maximize growth at an efficient cost. Ratios of protein, fat and starch have marked effects on feeding, growth and fatty acid metabolism. For example, in rainbow trout, diets containing gelatinized starch as the main energy source significantly reduce food intake and induce high hepatic UCP2 mRNA expression after 6 weeks and hypothalamic NPY and CART mRNA expression after 0 and 1 h, respectively, compared with diets using fat as the main source of energy (Figueiredo-Silva *et al.* 2012), suggesting that the hypothalamus senses changes in different energy sources and fatty acid levels in the body and adjusts NPY and CART transcription levels to optimize food/energy intake.

Furthermore, plant-based diets, such as duckweed (*Lemna minor*), have become a major area of research in aquaculture due to the decline of some baitfish stocks and a need for a fish oil and meal protein supplement with equivalent levels of fatty acid content and nutrient value. Recent studies on grass carp have examined the effects of duckweed-supplemented diets on food intake regulation and fatty acid metabolism. Hypothalamic and liver NPY mRNA levels are significantly higher in the plant-based diet compared with a control animal-based [*i.e.* zooplankton (*Chironomus tentans*)] diet, suggesting that either fish are still "hungry" or are not receiving adequate nutrition (He *et al.* 2013). Furthermore, decreases in liver and intestinal lipase activities and mRNA

expressions are detected in duckweed-fed fish compared with the control, suggesting that duckweed may have lower fat levels than animal-based diets as fat, carbohydrates and protein levels were neither measured nor consistent between duckweed and animal-based diets (He *et al.* 2013). The nutrient-sensing capabilities of NPY cells in fish is likely species-specific, as suggested by studies in cobia (*Rachycentron canadum*) where changes in appetite regulators, NPY and CCK, were not observed peri-prandially when diets differing in arginine and lysine ratios were used (Nguyen *et al.* 2013).

A major pitfall in the use of plant-based diets is the presence of high levels of anti-nutritional factors (ANFs) in plant meal, which can affect digestibility and palatability of feed and inhibit food intake. One common ANF is phytic acid (PA; myoinositol hexaphosphate) or phytate, which is the major phosphate-storing component in plants. PA is known to chelate with cations, and its association with amino acids, protein, starch and lipids, reduces the availability of nutrients for absorption and digestion in fish (Papatryphon *et al.* 1999; Bakke-McKellep and Refstie 2008; Laining *et al.* 2010; Plaipetch and Yakupitiyage 2014). In grass carp, PA-supplementation greatly decreases food intake, food utilization and growth with concomitant increases in brain CART and CCK, NPY and ghrelin mRNA expressions (Liu *et al.* 2014).

Although many studies examine the phenotypic effects (*i.e.* growth) of diet in fish, few studies have assessed the effects of diets on appetite-related hormone transcript expression, which is essential to determine if changes in appetite cause the observed phenotypic changes. A wide range of fish and diets must be explored to have a complete understanding of the interactions between appetite regulating factors, diet and metabolism in fish.

1.1.4. Study species: Atlantic cod, Gadus morhua

Atlantic cod is a cold water, demersal fish species that can reside in shallow, coastal (5 metres in depth) to deep, continental shelf (600 metres in depth) waters. The North American population spans from east and west of Greenland to Atlantic Canadian shores, along the United States Atlantic seaboard and ending in Cape Hatteras, North Carolina, USA (Department of Fisheries and Oceans 2013). Studies have shown that these populations have a variety of migratory modes, where fish can either remain residents of a specific locale or migrate various distances throughout the year. For example, in the Canadian Gulf of St. Lawrence cod population both resident and migratory individuals have been identified, each displaying different preferences for water depths and temperature (Le Bris *et al.* 2013). Migratory cod overwinter in warm (5°C), deep (300 - 500 m) waters moving south along the coast of western Newfoundland, but move back to the more northern habitat during summer months. The resident cod prefer cooler (1.5°C), shallow waters (<100 m) off the coast of western Newfoundland and are relatively immobile. Conversely, migratory cod display a variety of alternate diurnal migrations during the summer which could be reflective of spawning and feeding behaviours (Le Bris *et al.* 2013).

Atlantic cod is a commercially important species in Canada (Newfoundland and Labrador), the northeastern United States and northwestern Europe. However, overfishing resulted in the collapse in the North American fishery during the early 1990s, leading to a moratorium on the Atlantic northwest cod fishery (geographical location: Newfoundland and Labrador). Although some of the wild stocks have since recovered, the species is still

at risk and sustainable aquaculture might help to reduce fishing pressure on wild fish and ensure that all wild cod stocks are able to recover to their previous capacities. Therefore, developing a sustainable aquaculture industry may aid in a quicker recovery of the wild populations by reducing fishing pressure to what was seen pre-moratorium.

Successful Atlantic cod aquaculture industries had been developed in North-western Europe (namely in Norway, the United Kingdom and Iceland), where in 2009, 20 000 tonnes of cod were being produced annually, thus removing pressure from and allowing the European stocks to recover (FAO Fisheries Statistics, 2016). However, North America (Canada and the eastern seaboard of the United States) is yet to develop an effective commercial farming industry for cod. In the past, attempts have been made to raise farmed Atlantic cod in Atlantic Canadian waters to remove pressure from the depleted wild stocks, but these attempts at commercially raising cod have remained largely unsuccessful.

However, in order for a cod aquaculture program to be developed and successful, the biology of cod, especially its feeding behaviour, must be ascertained to reduce costs and make the process as efficient as possible. Multiple studies have identified cod feeding habits and behaviour (von der Decken and Lied 1993; Tibbetts *et al.* 2006; Hoskins and Volkoff 2012b; Morais *et al.* 2012) and expression patterns of transcripts involved in food intake during varied environmental conditions [*i.e.* turbidity (Meager *et al.* 2011), temperature (Treberg *et al.* 2005; Kehoe and Volkoff 2008)]. Furthermore, fasting studies have provided valuable information on the molecular mechanisms underlying appetite regulation in Atlantic cod (Kehoe and Volkoff 2007; Xu and Volkoff 2007). Finally, understanding how feeding-related transcript expression develops throughout cod life

history is important to comprehend how larval fish regulate appetite during sensitive and critical developmental stages (Webb and Ronnestad 2011).

1.1.5. Concluding remarks

Hormones have evolved to be multifunctional in fish, making research in the field quite challenging. Both the environment which the fish inhabits and its internal milieu are ever changing and naturally affect multiple systems due to interactions between endocrine processes. In order to hypothesize how these natural changes cause behavioural changes in the organism, a model integrating these aspects must be formed by first determining how each of these systems works separately and then together. With regards to how fish regulate feeding, a general model for the role each hormone plays in food intake regulation and other physiological processes is necessary to set a baseline for future research initiatives. These baselines give a starting point for fish species where no prior knowledge regarding food intake regulators is known. For example, the general consensus is that, in fish, MCH, orexin and NPY are potent orexigenic factors, while GnRH and CART are anorexigenic peptides, and this information can be used to set a criterion for experiments. Needless to say, deviations from the norm are seen in multiple fish models with respect to appetite regulation, not only among species, but also within species, since external factors (*i.e.* different environments and perception of cues/stressors) and species-specific genetic differences (*i.e.* MCH gene/transcript copies and related functions) can play a large role in affecting food intake regulation and peptides involved. Therefore, understanding how external cues affect peptide regulation

and potential interactions between endocrine systems is necessary for creating sustainable aquaculture industries and expanding our general knowledge within the field of comparative fish endocrinology.

1.2. Research objectives

1.2.1. Overall objectives

The overall objective of my thesis was to characterize appetite-related hormones (*i.e.* MCH, orexin, NPY, GnRH and CART) in Atlantic cod and understand how background colour, feed type and internal (*i.e.* feeding status, life stage) factors influence food intake-related hormone transcript regulation and behaviour in Atlantic cod.

1.2.2. Chapter two objectives, rationale and hypotheses

The first objective was to determine in which brain regions and peripheral tissues MCH and GnRH cDNAs are present in Atlantic cod and how their transcript expressions change throughout development and following fasting to give us some insight into their functions in fish. MCH is classically known for its roles in skin colour regulation and appetite regulation in lower vertebrates, thus it was expected that MCH would be prominent in brain regions (*i.e.* telencephalon/preoptic area, optic tectum/thalamus, hypothalamus) and extra-cerebrally in the the pituitary gland, which play roles in regulating food intake and colour change. Furthermore, peripheral regulation of skin

colour and food intake regulation would suggest that MCH transcripts are present in the skin and possibly the GI tract of Atlantic cod. GnRH is typically recognised for its function in reproduction, as well as food intake regulation, and mRNAs would be found throughout the forebrain and midbrain, as well as the pituitary gland, and peripherally in the gonads and GI tract.

The localization of mRNAs allowed me to examine transcript expression in targeted tissues under specific conditions. I first examined changes during development, as MCH and GnRH could be integral hormones during early life history, especially during gastrointestinal tract and brain development.

I then examined transcripts during fasting to determine the roles of MCH and GnRH in food intake regulation. Since in fish, MCH typically stimulates food intake, while GnRH tends to inhibit appetite, I predicted an increase in expression in MCH and decrease in expression of GnRHs in fasted Atlantic cod.

1.2.3. Chapter three objectives, rationale and hypotheses

I wanted to determine if a plant-based diet, namely camelina (*Camelina sativa*)-supplemented feed, affected growth, feed intake and the expression levels of appetite-related factors (*i.e.* MCH, orexin, NPY and CART) in Atlantic cod. I assessed how two different camelina meal-supplemented diets (15 and 30%) affected growth rates and feed consumption. Atlantic cod are carnivores thus feeding them a plant-based diet may have adverse effects on their digestive physiology, including effects of anti-nutritional factors (*i.e.* phytates and other chemicals known to affect digestion) consequently reducing

growth. Furthermore, plant-supplemented diets may not be as appealing to carnivorous fish thus reducing feed intake. My hypothesis was that fish fed camelina diets would have lower feed intake and growth.

I also quantified appetite-related transcripts (MCH, orexin, NPY and CART) in several brain regions (telencephalon/preoptic area, optic tectum/thalamus and hypothalamus) in fish fed either camelina meal diets or a control fish meal diet, to determine if these diets affected appetite-regulating hormones. If camelina meal diets reduce feed intake then it would be expected that orexigenic factors, such as MCH, orexin and NPY, would have higher transcript expression in fish fed these diets compared to the control fish. Conversely, the mRNA expression of anorexigenic peptides would be reduced in fish fed camelina meal-supplemented diets, since fish may still be feeling "hungry".

1.2.4. Chapter four objectives, rationale and hypotheses

I determined whether background colour and feeding status affected feeding and locomotor behaviours, skin colour and appetite-related hormone expressions, including MCH, orexin and GnRHs. Since MCH putatively induces skin pallor and stimulates food intake, it was expected that under light coloured backgrounds, cod would have lighter skin and eat more due to higher brain MCH transcript expression. Since MCH and GnRH appear to have opposite effects on feeding, one could assume that fish placed in light backgrounds, which eat more and have higher MCH levels, might have lower GnRH mRNA expression. To date, no studies have examined the effects of background colour

on orexin mRNA expression in fish. This study is the first to determine if background colour affects orexin transcript expression in fish, specifically Atlantic cod.

I further looked at the interactions between background colour and feeding regime (fed and fasted) to determine if background colour and feeding status can impact feeding, locomotion and hormone expression. The absence of an external feed supply usually causes fish to reduce their swimming activity due to limited energy stores. Therefore, I expected fasted fish to display reduced locomotion compared with fed fish. It has also been suggested that light coloured backgrounds cause increases in swimming activity and the same would be expected in Atlantic cod. Since MCH and orexin are known for their orexigenic effects in fish, I hypothesized that fasted Atlantic cod would have higher MCH and orexin mRNA expression levels compared with fed fish. Conversely, GnRH transcript expressions would be reduced in fasted cod compared with fed fish, since GnRHs are putative appetite-inhibitors in fish

1.2.5. Contributions to science

The results of these studies further contribute to understanding the roles MCH, GnRH, orexin, NPY and CART play in fish food intake regulation. They specifically present information to which internal (*i.e.* feeding status, life stage) or external factors (*i.e.* background colour, feed type) affect the expression and action of these peptides in Atlantic cod. The knowledge acquired in this thesis might be applied to Atlantic cod aquaculture practices and, more generally, provide insights on endocrine mechanisms involved in the regulation of feeding of fish and help establish the model described

earlier.

1.3. Co-authorship statement

Sarah M. Tuziak and Dr. Hélène Volkoff designed the project. A NSERC Discovery grant (H. Volkoff), a NSERC Canadian Graduate Student scholarship (S. Tuziak) and a RDC Ocean Industries Student award (S. Tuziak) funded laboratory, fieldwork and travel completed by S. Tuziak. Fieldwork included experimental design and sampling of fish at the end of all experiments [Oceans Sciences Centre (OSC) and Department of Fisheries and Oceans Canada – St. Andrew's Biological Station (DFO-SABS)].

In chapter four, Dr. Edward Trippel helped in the experimental design and editing of the manuscript and Janelle Arsenault assisted with sampling. For chapter three, Drs. Matthew Rise and Derek Anderson, along with the Camelina Project scientific management team aided with the design of the experiment. In chapter three, H. Volkoff and Isabel Costa both helped with sampling.

S. Tuziak completed the RNA extractions, cDNA synthesis, polymerase chain reactions (PCR) and gel electrophoresis for tissue distributions, early ontogeny study, and quantitative real-time PCR (qPCR) analyses. All data were analysed by S. Tuziak. Dr. David Schneider was involved with the implementation of data analyses used in Chapter four.

Chapters two and three are published in peer-reviewed scientific journals (*Peptides* and *Gene*, respectively), while chapter four has been submitted to a journal (*Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*) for publication.

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Chapter 2: Melanin-concentrating hormone (MCH) and gonadotrophin-releasing hormones (GnRH) in Atlantic cod, *Gadus morhua*: Early ontogeny, tissue distributions and effects of fasting.

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Abstract

Melanin-concentrating hormone (MCH) is classically known for its role in regulating teleost fish skin colour change for environmental adaptation. Recent evidence suggests that MCH also has appetite-stimulating properties. The gonadotrophin-releasing hormone (GnRH) peptide family has dual roles in endocrine control of reproduction and energy status in fish. Atlantic cod (*Gadus morhua*) are a commercially important aquaculture species inhabiting the shores and deeper waters of Atlantic Canada. In this study, we examine MCH and GnRH transcript expression profiles during early development as well as in central and peripheral tissues, and quantify juvenile Atlantic cod MCH and GnRH hypothalamic mRNA expressions following food deprivation. MCH and GnRH3 cDNAs are maternally deposited into cod eggs, while MCH has variable expression throughout early development. GnRH2 and GnRH3 mRNAs "turn-on" during mid-segmentation once the brain is fully developed. For both MCH and GnRH, highest expression appears during the exogenous feeding stages, perhaps supporting their functions as appetite regulators during early development. MCH and GnRH transcripts are found in brain regions related to appetite regulation (telencephalon/preoptic area, optic tectum/thalamus, hypothalamus), as well as the pituitary gland and the stomach, suggesting a peripheral function in food intake regulation. Atlantic cod MCH mRNA is up-regulated during fasting, while GnRH2 and GnRH3 transcripts do not appear to be influenced by food deprivation. In conclusion, MCH might be involved in stimulating food intake in

juvenile Atlantic cod, while GnRHs may play a more significant role in appetite regulation during early development.

2.1. Introduction

Appetite regulation in fish depends on a complex system of interacting hormones that integrate signals from within the brain and peripheral tissues or from external abiotic factors (*i.e.* photoperiod, temperature). These hormones can either be orexigenic (appetite-stimulating) or anorexigenic (appetite-inhibiting). Orexigenic factors include orexin (Volkoff *et al*. 1999; Xu and Volkoff 2007; Yokobori *et al*. 2011) and neuropeptide Y (Aldegunde and Mancebo 2006; Narnaware and Peter 2001a; Narnaware and Peter 2001b; Silverstein *et al*. 1998), while anorexigenic peptides include cholecystokinin (Himick and Peter 1994; MacDonald and Volkoff 2009; Murashita *et al*. 2006) and cocaine- and amphetamine-regulated transcript (CART) (Kehoe and Volkoff 2007; Murashita *et al*. 2009; Volkoff and Peter 2000). Appetite-related peptides are synthesized and released from feeding-related centers in the brain and/or peripheral tissues, including the gastrointestinal tract, to regulate food intake behaviour in fish (Volkoff and Peter 2006).

Melanin-concentrating hormone (MCH) is primarily known for inducing skin colour pallor in teleosts through melanosome aggregation (Kawauchi *et al*. 1983, Oshima *et al*. 1986). Although, in mammals, MCH is recognized as an orexigenic peptide, its role in fish food intake regulation is not yet clear (Matsuda *et al*. 2009; Qu *et al*. 1996; Shimakura *et al*. 2008; Tuziak and Volkoff 2012). In goldfish (*Carassius auratus*), inhibition of appetite and a reduction in MCH-immunoreactive (ir) cells have been observed following intracerebroventricular (ICV) MCH injections and fasting, respectively (Matsuda *et al*. 2006; Matsuda *et al*. 2007; Shimakura *et al*. 2008). However, in winter flounder

(*Pseudopleuronectes americanus*), barfin flounder (*Verasper moseri*) and zebrafish (*Danio rerio*), increased MCH mRNA and -ir cells are seen in fasted fish compared to fed fish (Berman *et al*. 2009; Takahashi *et al*. 2004; Tuziak and Volkoff 2012).

Three gonadotrophin-releasing hormone (GnRH) lineages (GnRH1, GnRH2 or GnRH3) have been identified in teleost fish and at least two GnRH forms are present in a fish transcriptome (Kah *et al*. 2007; Parhar *et al*. 1996; White *et al*. 1995). Characteristically, GnRHs are known for their roles in reproduction either through direct innervation of the gonadotrophs in the pituitary, thus regulating follicle stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and release, or through hypothalamic neuromodulation (Chang *et al*. 2009; Kawai *et al*. 2009). It has been suggested that GnRH2 plays a role in food intake regulation in fish, as ICV injections of GnRH2 in both goldfish and zebrafish induce decreases in food intake (Hoskins *et al*. 2008; Nishiguchi *et al*. 2012) and fasting winter flounder and zebrafish display reduced GnRH2 mRNA expression, indicative of a possible anorexigenic action (Nishiguchi *et al*. 2012; Tuziak and Volkoff 2013).

GnRH1 and GnRH3 have been identified as the main reproductive hormones in fish (Sherwood 1987). These peptides are primarily responsible for regulating various aspects of reproduction, such as nest building behaviours, sex reversal and ovulation (Garber *et al*. 2009; Kuramochi *et al*. 2011). In winter flounder, GnRH3 mRNA appears to be down-regulated during fasting, suggestive of an appetite-inhibiting response (Tuziak and Volkoff 2013). However, ICV injections of GnRH3 in goldfish do not affect feeding behaviour,

suggesting that the role of GnRH3s in regulating food intake might be species-specific (Matsuda *et al*. 2008).

Atlantic cod (*Gadus morhua*) are cold-water, commercially exploited fish off the Grand Banks. Both juvenile and adult cod undergo a period of fasting or reduced food intake during the winter months. During this time of reduced feeding, adults are preparing to spawn and devote energy to gonadal development instead of foraging, whereas juveniles are subjected to reduced prey availability due to lowered temperatures (Fordham and Trippel 1998; Hawkins *et al*. 1985). This period of natural, seasonal fasting makes cod a good model species to study hormonal mechanisms regulating food intake.

The purpose of this study was to characterize MCH and GnRH transcripts with regards to feeding in Atlantic cod. We assessed the role of these transcripts during early development by analyzing a comprehensive developmental profile of MCH, GnRH2 and GnRH3 – but not GnRH1 as it is known to be a non-functional pseudogene (Hildahl *et al*. 2011) in cod – mRNAs from unfertilized eggs to the *Artemia* sp. second feeding stage. We also examined the expression of these transcripts in Atlantic cod central and peripheral tissues. Finally, to ascertain if these peptides play a putative role in Atlantic cod food intake regulation, we quantified hypothalamic MCH, GnRH2 and GnRH3 mRNA expression in juveniles fish following a 14-day food deprivation.

2.2. Materials and Methods

2.2.1. Animals

2.2.1.1. Early ontogeny experiment

Larval Atlantic cod were maintained and collected from the Joe Brown Aquatic Research Building (JBARB, Logy Bay, Newfoundland, Canada). Fish were fed in excess to ensure all fish were receiving adequate nutrition. Fish were maintained in a constant 16L:8D photoperiod at ambient temperature (11°C). Various stages were sampled, including unfertilized eggs, cleavage: 128-cell stage [1-day post fertilization (dpf)], blastula sphere stage (2 dpf), early gastrulation: 10% - 25% epiboly (3 dpf), late gastrulation: 75% epiboly (4 dpf), early segmentation: 20-somite stage (6 dpf), mid-segmentation: 40-somite stage (8 dpf), hindgut development stage (13 dpf), fully developed jaw (14 dpf), hatch, algal feeding [1 day post hatch (dph)], 1st feeding: rotifers (*Brachionus plicatilis*) supplemented with algae (7 dph), 1st feeding: rotifers (20 dph), 2nd feeding: *Artemia* sp. supplemented with rotifers (35 dph) and 2nd feeding: *Artemia* sp. (40 dph). Two 1.5 mL tubes of approximately 250 µl of eggs and/or larvae were collected and stored in RNAlater (Qiagen, Mississauga, Ontario, Canada) at -20°C until further use. Stages were determined based on previously annotated early Atlantic cod developmental stages (Hall *et al*. 2004).

2.2.1.2. Tissue distributions

Adult Atlantic cod were maintained at ambient temperature (11°C) in 1000 L flow-through tanks under a 16L:8D photoperiod and fed EWOS marine diet pellets (EWOS Canada, Surrey British Columbia, Canada) to satiation daily at 10:00. On the day of sacrifice, 4 fish were anaesthetized in 0.05% tricaine methanesulfonate (MS-222, Syndel Laboratories, Vancouver, BC, Canada) and brain and peripheral tissues, including skin, muscle, heart, liver, spleen, stomach, pyloric caeca, midgut, ovaries and testes were dissected and stored in RNAlater at -20°C until further use. Tissues from one male and gonads from one female were used for the tissue distributions.

2.2.1.3. Feeding status experiment

Four 0.5m^3 flow-through tanks containing ~ 30/tank mixed sex juvenile Atlantic cod (mean weight: 4.23 ± 0.05 g) were acclimated for two weeks on a 16L:8D photoperiod at ambient temperature (11°C) and fed a commercial pellet diet daily to satiation at 12:00. Following the acclimation period, two tanks were fasted for two weeks, while the others continued to be fed daily to satiation. The fish used in this experiment were young-of-year juveniles, therefore we expected that an acute state of food deprivation would significantly alter appetite-related peptides during this period of critical growth and survival (Campana 1996). Furthermore, other marine fish (i.e. winter flounder) have demonstrated significant

differences in the specific hormones evaluated in this study (MCH and GnRHs) following a two-week fast (Tuziak and Volkoff 2012, Tuziak and Volkoff 2013). Fish were randomly selected from the tanks for sampling. Once sampled, , fish were anaesthetized in 0.05% tricaine methanesulfonate, sacrificed and brains dissected. Tissues were and stored in RNAlater at -20°C until further use. All experiments followed the Canadian Council on Animal Care and Memorial University's Animal Care Council guides to ensure proper animal care and treatment.

2.2.2. RNA extraction and cDNA synthesis

2.2.2.1. Early ontogeny

Cod eggs and larvae RNA was extracted using the TRIreagent/chloroform (BioShop, Burlington, Canada) method as per the manufacturer's instructions. Final RNA concentrations, 260/280 (ratio > 1.8) and 260/230 (ratio > 1.8) ratios were obtained using Nanodrop (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) spectrophotometry. RNAs were run on 1.15% agarose gel electrophoresis to assess their quality in congruence with the Nanodrop results. Samples were then DNase-treated (Sigma, St. Louis, Missouri, USA) and column purified using the Fermentas RNA clean up and concentration kit (Fermentas/Thermo-Fisher) as per the manufacturer's protocol. Sample quality was then

analyzed with the Nanodrop and run on gel electrophoresis, as previously described, to ensure these procedures did not cause any RNA degradation.

cDNA synthesis was completed with the Superscript II cDNA synthesis kit (Invitrogen, Carlsbad, California, USA) using 1 µg total purified RNA and 250 ng random primers (Promega, Fitchburg, Wisconsin, USA) as per the manufacturers protocol, with the exception that the RNaseOUT step was removed since > 50 ng•µl^{-1} of RNA was used.

2.2.2.2. Tissue distribution

Cod brains were dissected into distinct regions, including preoptic area/telencephalon, optic tectum/thalamus, hypothalamus, cerebellum, medulla oblongata and spinal cord, as well as the pituitary gland for the central tissue distribution. Peripheral tissue distributions were completed using skin, muscle, heart, liver, spleen, stomach, pyloric caeca, midgut, ovaries and testes. RNA extractions, quality control and cDNA synthesis were performed as previously described.

2.2.2.3. Feeding status experiment

Hypothalami were dissected from each cod brain for mRNA quantification. RNA extraction and quality control were completed as previously described. cDNA was synthesized using the RealMasterScript ^{TM} SuperMix Kit (5 Prime Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol.

2.2.3. Expression of MCH, GnRH2 and GnRH3 transcripts in early development of Atlantic cod

The early developmental transcript expression was assessed using qualitative reverse transcription polymerase chain reaction (RT-PCR). Primers for housekeeping genes (HKG), actin-related protein 2/3 complex subunit 4 (*arpc4*) and ubiquitin (UBI) were used from the Olsvik *et al*. (2008) study (Table 2.1) (Olsvik *et al*., 2008). Two HKG were used since early mRNA expression (unfertilized eggs to 2 dpf) was not evident with UBI and unstable expression was seen with *arpc4*. It is important to note that these are simply qualitative experiments being used to determine presence or absence of mRNA and not to quantify actual cDNA expression. Primers for MCH (Ensembl: **ENSGMOT00000010147**), GnRH2 (NCBI: **GU332294**) and GnRH3 (NCBI: **GU332295**) were designed based on previously annotated sequences (Eurofins MWG Operon, Huntsville, Alabama, USA). A 25-µL reaction consisted of: 2X GoTaq Green Master Mix, 125 ng cDNA and 10 µM of each forward and reverse primer. Preliminary primer optimization reactions were run, where a gradient polymerase chain reaction (PCR) profile was used [95°C for 5 minutes (min), 95°C for 30 seconds, 53-62°C for 30 s, 72°C for 20-60 s for 30-35 cycles and a final extension of 72°C for 5 min] on the Bio-Rad C1000 Touch Thermocycler (Bio-Rad, Mississauga, Ontario, Canada). All subsequent reactions were run with the following program: 95°C for 5 min, 95°C for 30 s, 58°C for 30 s, 72°C for 17-40 s and a final extension of 72°C for 5 min.

Table 2.1. Atlantic cod (*Gadus morhua*) specific primers and associated transcripts.

Experiment and Transcript	Forward primer (5' → 3')	Reverse primer (5' → 3')
Reverse transcription polymerase chain reaction		
Actin-related protein complex 4	TGA TCC TCC ACG ACG ATG AG	CAG GGC CTT GGC GAA GA
Ubiquitin	GGC CGC AAA GAT GCA GAT	CTG GGC TCG ACC TCA AGA GT
Melanin-concentrating hormone	GAC CGA GGG CTG TCC TAC TAC	CTC ATG GTG TCC CGC TTG
Gonadotrophin-releasing hormone 2	GCC TAT ACA CGT GTT CTG ACT GC	CCT CAC ACA GCT TGA TCT CG
Gonadotrophin-releasing hormone 3	AGA GCA GCA GCA AAG TGA CG	TCT GTC AGT CGT GTA GCA ACC

Quantitative real-time polymerase chain reaction		
Ubiquitin	GGC CGC AAA GAT GCA GAT	CTG GGC TCG ACC TCA AGA GT
Melanin-concentrating hormone	GAC CGA GGG CTG TCC TAC TAC	CTC ATG GTG TCC CGC TTG
Gonadotrophin-releasing hormone 2	GTC TCA TGG CTG GTA CCC TGG	GCC TCA GGT AGC TAC ACT CC
Gonadotrophin-releasing hormone 3	AGA GCT GGA AGC CAC CAT CAG G	CTG GTC GTT GAC CAG ACT GTA TGG

2.2.4. Expression of MCH, GnRH2 and GnRH3 transcripts in central and peripheral Atlantic cod tissues

Distributions for central and peripheral tissues were assessed using qualitative RT-PCR. UBI was used as the control HKG since relatively stable transcript expression was observed between samples. Primers for MCH, GnRH2 and GnRH3 were the same as previously mentioned (Table 2.1). Primer optimization and final PCR reactions and profiles were the same as preceding sections.

2.2.5. Effects of fasting on Atlantic cod hypothalamic MCH, GnRH2 and GnRH3 transcript expression

Primers were designed based on the sequences described in section 2.2.3 (Table 2.1). Primer optimization was completed with 1:2 cDNA dilution using a 6-point standard curve with a 10 µl reaction containing 2X KAPA SYBR Fast master mix (Kapa Biosystems, Inc., Woburn, MA, USA), 1.25 ng cDNA (1:4 dilution) and 10 µM of each forward and reverse primers on an Eppendorf Mastercycler ep *realplex* 2s (Eppendorf, Hamburg, Germany). The qPCR cycling parameters used for primer optimization was: 95°C for 3 min, 95°C for 30 s, 56°C for 15 s, 72°c for 20 s repeated for 40 cycles, followed by a melting curve: 95°C for 15 s, then a temperature ramp for 20 min ranging from 55 - 95°C for 15 s increasing at 0.1°C s^{-1}. Primer efficiencies (UBI: 1.04, MCH: 0.96, GnRH2: 1.07, GnRH3: 1.09), R^2 values ($0.991 \leq R^2 \leq 0.997$) and single peaks in the melting curve (no primer dimers) were determined. Once primers passed the quality

control measures, qPCRs for each target were run under the same conditions for the experimental samples using 1.25 ng cDNA using UBI as the normalizer. Since multiple plates were used for a given study (1 HKG + 2 target transcripts), a linker individual sample was used to ensure inter-assay reproducibility was maintained. Relative mRNA expression was analyzed using the Pffafl method (Pfaffl 2001). The normalizer was determined based on the individual with the lowest relative expression for each transcript.

2.2.6. Statistical analyses

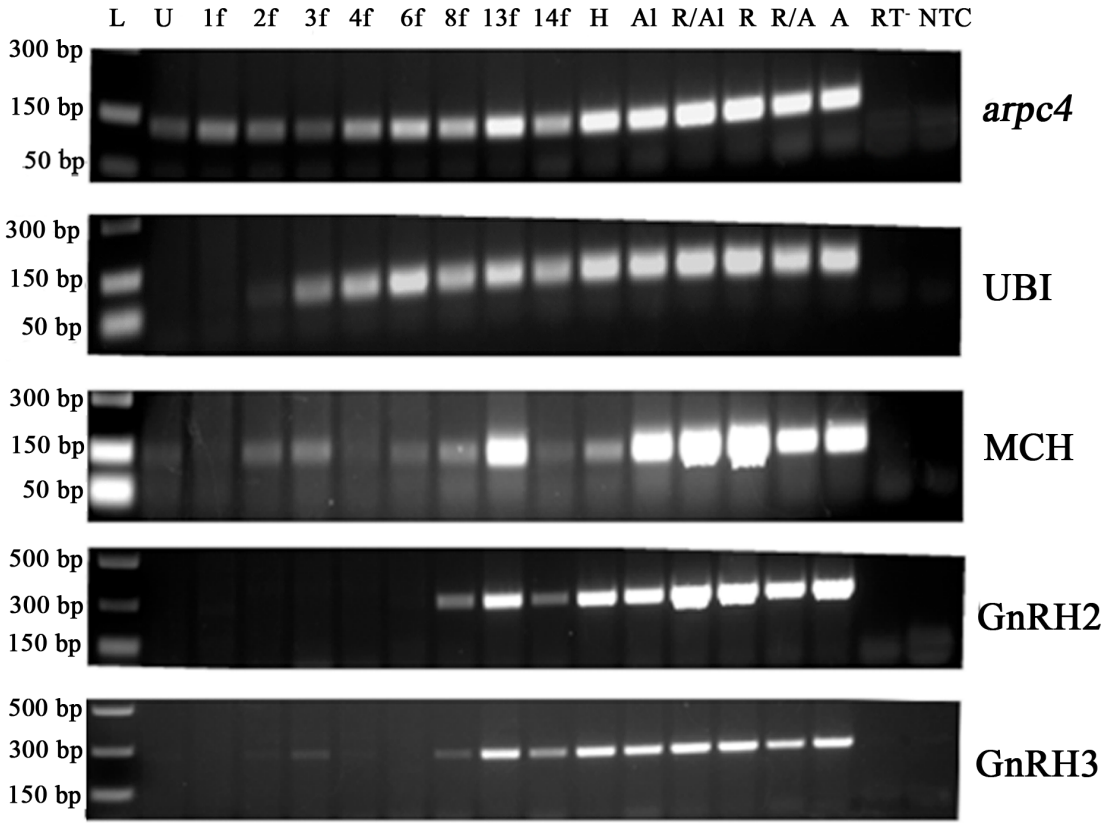
Statistical analyses and figures were generated using GraphPad prism v6.0c (GraphPad Inc., San Diego, California, USA). Student's unpaired, one-tailed *t*-test assuming equal standard deviation and a p-value < 0.05 were used for parametric analyses. When residuals were non-normal, the non-parametric Mann-Whitney unpaired, one-tailed *t*-test was used with a p-value < 0.05. Means are given with ± standard error.

2.3. Results

2.3.1. Early ontogeny

Two reference genes, UBI and *arpc4*, were used for the early ontogeny tissue distribution as stability could not obtained for each individual gene (Figure 2.1): UBI had relatively stable expression after 3 dpf (early gastrulation) but was not expressed in

Figure 2.1. Actin-related protein complex 4 (*arpc4*), ubiquitin (UBI), melanin-concentrating hormone (MCH), gonadotrophin-releasing hormone 2 (GnRH2) and GnRH3 reverse-transcriptase polymerase chain reaction (RT-PCR) early developmental profile in Atlantic cod (*Gadus morhua*). L: ladder, U: unfertilized eggs, 1f: cleavage: 128-cell stage [1 day post fertilization (dpf)], 2f: blastula sphere stage (2 dpf), 3f: early gastrulation: 10% - 25% epiboly (3 dpf), 4f: late gastrulation: 75% epiboly (4 dpf), 6: early segmentation: 20-somite stage (6 dpf), 8f: mid-segmentation: 40-somite stage (8 dpf), 13f: hindgut development stage (13 dpf), 14f: fully developed jaw (14 dpf), H: hatch, Al: algal feeding stage, 1 day post hatch (dph), R/Al: 1st feeding: rotifers (*Brachionus plicatilis*) supplemented with algae (7 dph), R: 1st feeding: rotifers (20 dph), R/A: 2nd feeding: *Artemia* sp. supplemented with rotifers (35 dph), A:2nd feeding: *Artemia* sp. (40 dph), RT^{-}: reverse transcription negative control and NTC: RT-PCR no template control.


L U 1f 2f 3f 4f 6f 8f 13f 14f H Al R/Al R R/A A RT- NTC
300 bp
150 bp
50 bp
arpc4
300 bp
150 bp
50 bp
UBI
300 bp
150 bp
50 bp
MCH
500 bp
300 bp
150 bp
GnRH2
500 bp
300 bp
150 bp
GnRH3

unfertilized and 1 dpf eggs whereas *aprc4* was expressed as early as the unfertilized eggs, but its expression was not stable during early life history stages. The combined expression profiles of the two reference genes show that RNA was present in all samples examined. It is important to note that from hereon in that gene specific "apparent" transcript expressions (i.e. up or down regulated) are relative to the housekeeping genes (UBI and *arpc4*) expression profiles.

MCH mRNA appears to "turn-on" and "turn-off" multiple times throughout early larval development (Figure 2.1). MCH transcript expression is detected early in development in the unfertilized eggs, with no detection of MCH mRNA at 1 dpf (cleavage). MCH transcripts then appear to be present during days 2 and 3 post-fertilization (blastula and early epiboly). Low MCH mRNA is seen at 6 dpf (early segmentation). From 8 dpf (mid-segmentation) until the final *Artemia* sp. feeding stage, MCH cDNA expression appears to fluctuate, but is present in all stages.

Strong GnRH2 mRNA expression is seen at mid-segmentation (8 dpf) and throughout the rest of larval development (Figure 2.1). Low GnRH3 mRNA expression is seen in unfertilized eggs and again at 3 dpf (early gastrulation). GnRH3 transcripts are absent from days 4 to 6 post-fertilization, when transcripts are once again detected at 8 dpf (mid-segmentation) and remain "turned-on" throughout the rest of development.

2.3.2. Tissue distribution

Central tissues. MCH transcripts are expressed throughout the brain, with apparent low mRNA expression in the telencephalon/preoptic area, optic

tectum/thalamus, cerebellum and spinal cord and relatively higher cDNA expression in the hypothalamus and medulla oblongata, as well as in the pituitary gland (Figure 2.2). GnRH2 mRNA is expressed ubiquitously throughout the brain, with apparent lower transcript expression in the medulla oblongata (Figure 2.2). GnRH3 cDNA is detected in the telencephalon/preoptic area, optic tectum/thalamus, hypothalamus, spinal cord, as well as the pituitary gland, but not in the cerebellum or medulla oblongata (Figure 2.2).

Peripheral tissues. MCH mRNA is found in multiple tissues throughout the Atlantic cod periphery, including skin, heart, liver, spleen, stomach, pyloric caeca, ovaries and testes, but not in muscle or midgut (Figure 2.3). GnRH2 cDNA expression is detected at apparent low levels in the skin, stomach and ovaries, while higher transcript expression is seen in the spleen and testes (Figure 2.3). GnRH3 mRNA is seen at apparent very low levels in the heart and testes, but not in the ovaries or other feeding-related tissues (stomach, pyloric caeca, midgut) (Figure 2.3).

2.3.3. Effects of fasting on Atlantic cod MCH, GnRH2 and GnRH3 mRNA expression

Final weights and lengths for the fed fish (n = 20) were 9.20 ± 0.58 g and 10.43 ± 0.43 cm, and for fasted fish (n = 20) 7.15 ± 0.36 g and 10.16 ± 0.19 cm, respectively. A significant difference in weights, but not lengths, was observed between fed and fasted fish (p = 0.0023).

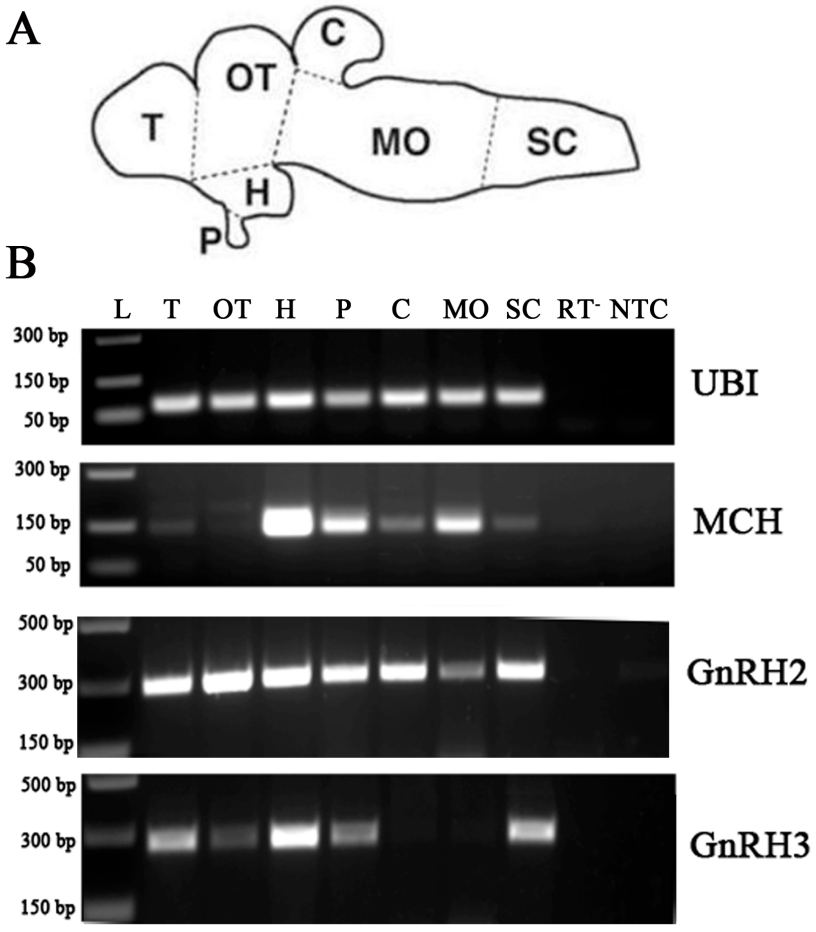


Figure 2.2. A) Atlantic cod (*Gadus morhua*) brain schematic, T: telencephalon/preoptic area, OT: optic tectum/thalamus, H: hypothalamus, P: pituitary, C: cerebellum, MO: medulla oblongata, and SC: spinal cord. B) Atlantic cod central tissue distribution (n = 1) for ubiquitin (UBI), melanin-concentrating hormone (MCH), gonadotrophin-releasing hormone 2 (GnRH2) and GnRH3 mRNAs using reverse-transcriptase polymerase chain reaction (RT-PCR). L: ladder, T: telencephalon/preoptic area, OT: optic tectum/thalamus, H: hypothalamus, P: pituitary, C: cerebellum, MO: medulla oblongata, SC: spinal cord, RT^-: reverse-transcription negative control and NTC: RT-PCR no template control.

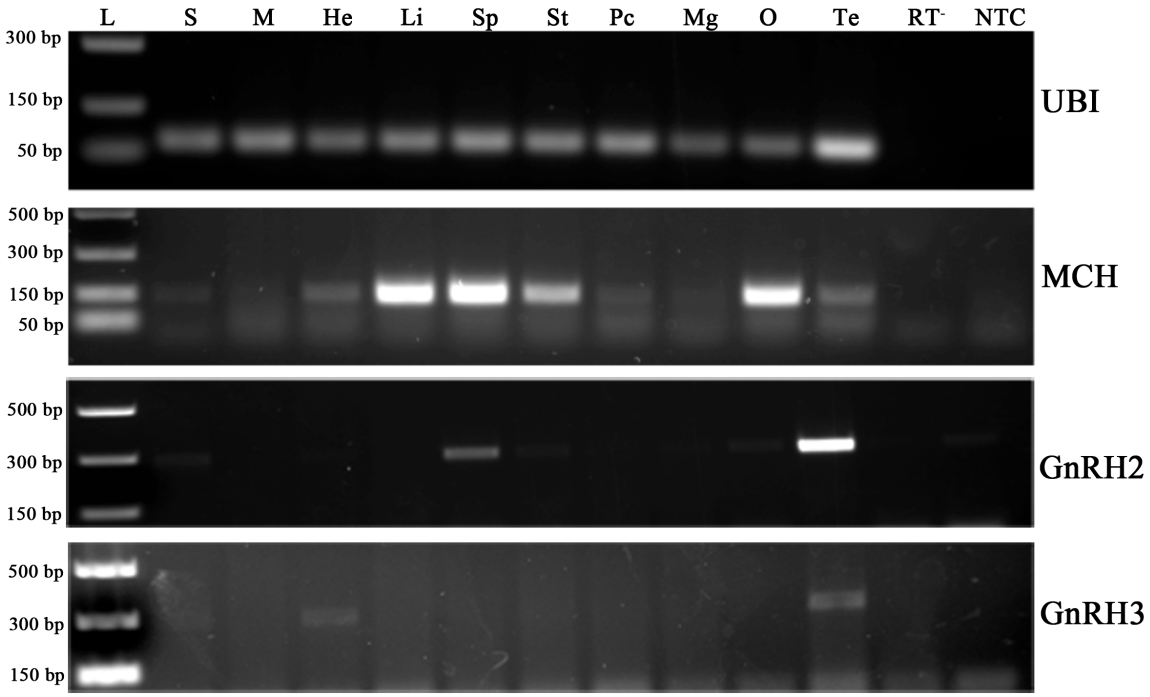


Figure 2.3. Atlantic cod (*Gadus morhua*) peripheral tissue distribution (n = 1) for ubiquitin (UBI), melanin-concentrating hormone (MCH), gonadotrophin-releasing hormone 2 (GnRH2) and GnRH3 mRNAs using reverse-transcriptase polymerase chain reaction (RT-PCR). L: ladder, S: skin, M: muscle, He: heart, Li: liver, Sp: spleen, St: stomach, Pc: pyloric caecum, Mg: midgut, O: ovaries, Te: testis, RT^-: reverse transcription negative control and NTC: RT-PCR no template control.

MCH mRNA expression was significantly different between fed and fasted groups ($p = 0.0383$), where fasted fish had 1.7-fold higher expression than fed fish (Figure 2.4A). No differences in transcript expression for either GnRH2 or GnRH3 were observed between fed (mean GnRH2 expression: 4.000 ± 0.9434, mean GnRH3 expression: 10.69 ± 3.698) and fasted cod (mean GnRH2 expression: 3.870 ± 1.006, mean GnRH3 expression: 12.79 ± 1.708) (Figures 2.4B and C).

2.4. Discussion

2.4.1. Early developmental profiles of MCH, GnRH2 and GnRH3 mRNA expression in Atlantic cod

UBI and *arpc4* are reference genes that are involved in proteolysis and cell structure (cytoskeleton), respectively. Our results suggest that, in Atlantic cod, *arpc4* is maternally transferred to the zygote, while UBI is actively transcribed by 2 dpf. Similar to Atlantic cod, in zebrafish, UBI is not up-regulated until the late blastula stage (Mathavan *et al*. 2005). Increased UBI presence at the blastula stage is likely representative of proteolysis of maternal gene products in the zygote (Mathavan *et al*. 2005). Although *arpc4*s function has not been examined in fish, in other eukaryotes it is responsible for structural development of actin filaments in cells and is crucial for embryonic development at early ontogenetic stages, hence its maternal deposition (Millard *et al*. 2004).

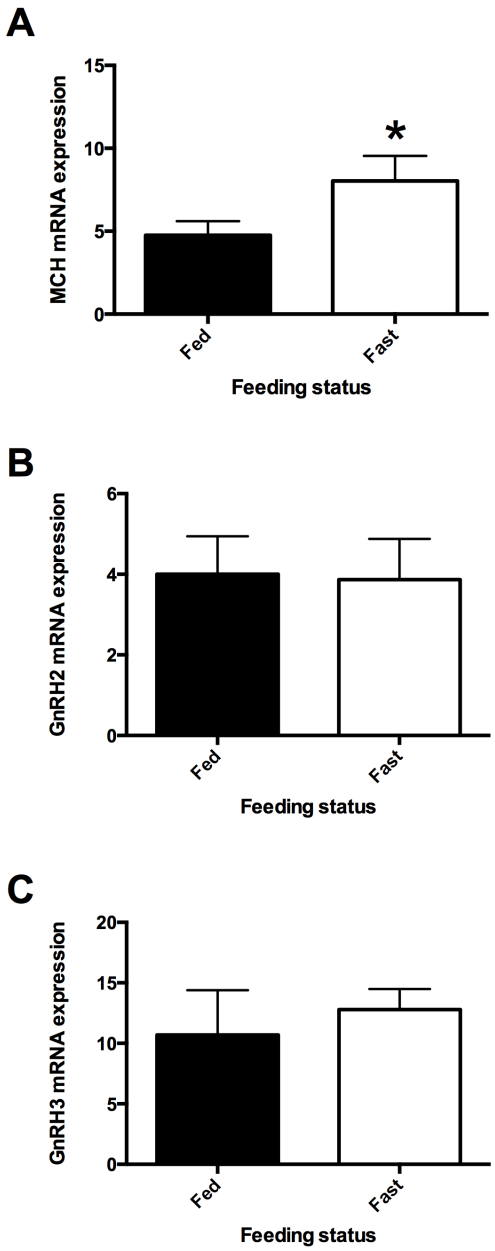


Figure 2.4. Quantification of real-time polymerase chain reaction (qPCR) results for 14-day juvenile Atlantic cod (*Gadus morhua*) hypothalami fasting experiment. All transcripts are relative to ubiquitin. A) Melanin-concentrating hormone (MCH), B) gonadotrophin-releasing hormone 2 (GnRH2), and C) GnRH3 mRNA expression for fed and fasted. Significance is denoted at a p-value < 0.05 and is identified with an *.

MCH mRNA transcription levels appear to vary throughout early Atlantic cod development. Levels are initially detected in unfertilized eggs, suggesting that there is maternal transfer of MCH mRNA to the embryo. MCH mRNA levels are not detected during cleavage, which might be explained by the up-regulation of ubiquitin at this stage: The increase in embryonic ubiquitin mRNAs at the cleavage stage suggests an increase in proteolytic activity that might remove maternally deposited MCH transcripts in preparation for zygotic MCH transcription (Mathavan *et al*. 2005). Low MCH expression is again observed during the blastula sphere stage [2 days post fertilization (dpf)] until 10-25% epiboly (3 dpf). Since it is at the early stages of epiboly that cod larvae begin to transcribe their own mRNA (Hall *et al*. 2004), MCH transcript expression seen at early epiboly and onwards could be a product of the zygote's own genetic material. A down-regulation is seen again at 75% epiboly (4 dpf) until early segmentation (20 somite stage; 6 dpf), when a progressively higher expression profile is observed until the development of the hindgut (13 dpf). The increase in MCH mRNA during gastrulation is not surprising as this stage corresponds to the development of forebrain and midbrain (telencephalon/preoptic area, optic tectum/thalamus and hypothalamus) as well as that of the gastrointestinal tract. Furthermore, pigment cells become visible during the late stages of epiboly in Atlantic cod, which could also coincide with the increased MCH transcript expression during early segmentation (Adoff 1986). A drop in MCH mRNA expression is seen just prior to hatch at 14 dpf when the jaw is completely developed and the larvae are ready to feed on exogenous prey. Finally, high MCH transcript expression is observed during all of the feeding stages (from hatching onwards), which could be indicative of MCHs appetite-stimulation action in larvae feeding on exogenous prey.

In barfin flounder yolk-sac larvae, MCH-ir somata and fibres are first observed in the hypothalamus and pituitary, respectively, just prior to mouth opening [7 days post-hatch (dph)] [see Table 2.2 for references used to determine embryonic and larval staging of other fish species to compare with Atlantic cod embryogenesis when life stages were not defined in original manuscript (*i.e.* cleavage, blastula, gastrulation, etc.)] (Amano *et al*. 2003). The pre-flexion, open mouth larva (14 dph) stage is characterized by a period of increased growth and corresponds to when the onset of exogenous feeding occurs. At this time, MCH-ir somata migrate towards the nucleus tuberis lateralis (NLT) and the lateral ventricular recess (LVR) - key feeding centers in the hypothalamus (Amano *et al*. 2003). Similarly, in alevin rainbow trout, MCH and its salmonid paralogue, MCH2, are detected in the NLT and LVR regions of the hypothalamus 7 days before hatching, with an intensification of the signal at hatch and 28 dph when the larvae have begun exogenous feeding (Suzuki *et al*. 1997). In the *Cichlasoma dimerus*, MCH-ir somata are first detected at the open mouth stage (4 dph) in the hypothalamus and MCH-ir cells are present in the NLT and LVR by the pre-juvenile stage (30 dph) when the larvae begin exogenous feeding (Pandolfi *et al*. 2003). The apparent increase in Atlantic cod MCH mRNA prior to hatch and onwards might correspond to the formation of new neural connections in the hypothalamus in preparation for exogenous feeding.

In both barfin flounder and Atlantic cod, increases in MCH mRNA and -ir cells are observed when melanophore development is at its highest (Amano *et al*. 2003). MCH-ir sensory neuromasts are observed in the dorsal and ventral skin of embryonic *Cichlasoma dimerus* heads in proximity to melanocytes and blood vessels, suggesting that

Table 2.2. Fish species and references used to stage embryonic and larval development.

Fish species	Reference(s)
Burtons mouth-brooding cichlid, *Haplochromis burtoni*	(Fujimura and Okada 2007; Morrison *et al*., 2001)
Zebrafish, *Danio rerio*	(Hisaoka and Battle 1958; Holmberg *et al*., 2004)
Barfin flounder, *Verasper moseri*	(Aritaki *et al*. 2000)
Gilthead seabream, *Sparus aurata*	(Firat *et al*. 2005; Kamaci *et al*. 2005; Ronnestad *et al*. 1994)
Atlantic cod, *Gadus morhua*	(Adoff 1986)

skin-produced MCH could affect colour changes in early developmental stages (Pandolfi *et al*. 2003).

Atlantic cod have two functional GnRHs in their transcriptome, GnRH2 and GnRH3, GnRH1 being present as a non-functional pseudogene (Hildahl *et al*. 2011). Our study shows that Atlantic cod GnRH2 mRNA appears during mid-segmentation (8 dpf) and remains present until second *Artemia* feeding stage. Similar to MCH, apparent higher GnRH2 transcript levels during mid-segmentation are not surprising since this is the final stage of the brain development. Although GnRH2 expression levels are variable among stages, highest expression levels appear to occur following hatching - 1 dph and throughout the different feeding phases – whereas lowest levels occur just prior to hatch when the jaw is fully developed (14 dpf). Increased levels of GnRH2 during the feeding stages could be linked to an anorexigenic role of GnRH2, as described in other adult fish species (see section 4.3). In zebrafish, GnRH2 mRNA is first detected at 24 hpf when the optic cup is formed (20 somites) in the lateral regions of the midbrain (optic tectum/thalamus), similar to the stage when GnRH2 mRNA is first detected in Atlantic cod (Palevitch *et al*. 2007). By first feeding (5 dpf), when the yolk sac is almost entirely absorbed and viscera fully developed, GnRH2 cells are localized in the median midbrain tegmentum and GnRH2 mRNA is found in the forebrain region (telencephalon/preoptic area). In African catfish (*Clarias gariepinus*), GnRH2-ir perikarya are observed in the midbrain tegmentum (optic tectum/thalamus) as early as 7 dph, when the larvae are beginning to feed on *Artemia*, but present at highest concentrations at 14 dph, when the larvae are well-adapted to exogenous feeding (Dubois *et al*. 2001). In the *Cichlasoma dimerus*, GnRH2 transcripts are detected in the midbrain during late segmentation (4 dpf),

somewhat later than Atlantic cod. Once the cichlid embryo has hatched and exogenous feeding commences (14 dpf), GnHR2-ir fibres maintain a broad distribution in the midbrain, olfactory bulbs and hindbrain, possibly relaying appetite regulatory signals and sensory cues (*i.e.* olfaction and vision) (Pandolfi *et al*. 2002). In the gilthead seabream (*Sparus aurata*), GnRH2 mRNA cells are first expressed at 1.5 dpf within the hindbrain of hatched embryo with ~70% formed gut (Wong *et al*. 2004). However, the hindbrain GnRH neuron population is not detected at the onset of exogenous feeding (10 dpf), suggesting that GnRH2 may have other unknown functions in early development. Like *Cichlasoma dimerus* and Atlantic cod, when the gilthead seabream larvae yolk-sac is resorbing (2 dpf), GnRH2 transcripts are observed in the midbrain and might be involved in early food intake regulation.

Atlantic cod GnRH3 mRNA is maternally deposited into oocytes since GnRH3 transcripts are present in unfertilized eggs, similar to what is seen in half-smooth tongue sole (*Cynoglossus semilaevis*), medaka and gilthead seabream (Okubo *et al*. 2006; Wong *et al*. 2004; Zhou *et al*. 2012). In medaka and gilthead seabream, it has been suggested that GnRH3 maternal deposition and early transcript expression could be related to the presence of gonadotrophin mRNA [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] in oocytes and functions as an early regulator of oocyte maturation. A similar role could be expected for Atlantic cod GnRH3 mRNAs in oocyte and embryonic development (Okubo *et al*. 2006; Wong and Zohar, 2004).

The absence of GnRH3 mRNA on days 1 and 2 post fertilization might indicate that, as seen for MCH, maternally transferred transcripts are degraded by ubiquitin and that the embryo begins to transcribe its own GnRH3 mRNAs at 3 dpf (Mathavan *et al*.

2005). Similarly to GnRH2, GnRH3 mRNAs are detected at mid-segmentation (8 dpf), once the cod brain is fully developed. In zebrafish, GnRH3 mRNA is detected as early as 1 dpf (4-8 cell stage) in clusters anterior to the olfactory region (Palevitch *et al*. 2007; Vosges *et al*. 2010). This GnRH3 cell population then migrates ventrally to the telencephalon/preoptic area, where GnRH3-ir fibres extend further into the telencephalon/preoptic area and the optic tectum/thalamus of the embryonic zebrafish brain until first feeding (1 dph). The presence of Atlantic cod GnRH3 mRNA at early segmentation could be related to the formation of the telencephalic cell population and extending GnRH3 fibres into the midbrain, suggesting a relay of sensory cues from the olfactory bulbs to appetite-centers in the optic tectum.

Similar to Atlantic cod, moderate GnRH3 transcript levels are observed prior to hatching in sole embryo and then highest concentrations from hatch onwards when the larvae are feeding on exogenous prey (Zhou *et al*., 2012). In Masu salmon (*Oncorhynchus masou*), GnRH3-ir fibres are first seen during somitogenesis and the pigmented eye stage (36 dpf), around the olfactory placode and along the olfactory nerve when the gastrointestinal tract, teeth and eyes are developing (60 dpf), and extend to the olfactory bulb and throughout the brain and pituitary in the alevin, yolk-sac stage (80 dpf) (Amano *et al*. 1998). The presence of GnRH3 mRNAs and -ir cells in the olfactory region and telencephalon/preoptic area with fibres extending to regions of the brain related to food intake (*i.e.* optic tectum), concomitant high levels of GnRH3 mRNA with gastrointestinal and eye development, as well as commencement of exogenous feeding could indicate an early role for GnRH3 in food sensing (*i.e.* olfaction and visual cues) and relaying these signals to control food intake in Atlantic cod, salmon, sole and zebrafish.

2.4.2. MCH, GnRH2 and GnRH3 mRNAs are expressed in brain regions related to appetite regulation and in the gastrointestinal tract

2.4.2.1. Central Atlantic cod tissue distributions

MCH mRNA was present in all adult brain tissues examined, with variable expression levels between regions. Highest transcript concentrations appeared to be present in the hypothalamus, medulla oblongata and extra-cerebrally in the pituitary, while lower expression levels were observed throughout the remaining regions (telencephalon/preoptic area, optic tectum/thalamus, cerebellum and spinal cord). In line with our results, in the winter flounder, highest MCH mRNA expression is seen in the hypothalamus, with varied expression throughout the rest of the brain (Tuziak and Volkoff 2013). In zebrafish, goldfish, barfin flounder, medaka (*Oryzias latipes*), *Cichlasoma dimerus* and the more ancestral bonnethead shark (*Sphyma lewini*), MCH-immunoreactive (ir) cells are found in hypothalamic nuclei such as the nucleus tuberis lateralis (NLT) and the lateral ventricular recess (LVR), and MCH-ir fibres occur throughout the brain and pituitary (Amiya *et al*. 2007; Amiya *et al*. 2008; Berman *et al*. 2009; Matsuda *et al*. 2009; Mizusawa *et al*. 2012; Pandolfi *et al*. 2003). In our study, high MCH mRNA expression levels in the hypothalamus might indicate the presence of cell bodies of densely populated MCH-producing cells in this region and that of tracking fibres with lower transcript expression throughout the brain. Future immunohistochemical or *in situ* hybridization studies will determine the exact distribution of MCH perikarya and fibres in Atlantic cod. MCH mRNA has also been detected in both brain and pituitary

of starry flounder (*Platichthys stellatus*) (Kang and Kim 2013). MCH-ir fibres have been detected in the pituitary of the red-bellied piranha (*Pygocentrus nattereri*), *Cichlasoma dimerus* and goldfish (Perez Sirkin *et al*. 2012; Suzuki and Yamamoto 2013; Tanaka *et al*. 2009), but not in the scalloped hammerhead shark (*Sphyrna lewini*) (Mizusawa *et al*. 2012), suggesting that the proliferation of MCH cells into the pituitary might be a recent evolutionary event.

In the Atlantic cod, GnRH2 transcript expression is observed throughout the brain, with apparent similar mRNA levels in all regions, except medulla oblongata. In fish, GnRH2 mRNA and ir-perikarya are most commonly found at high levels in the optic tectum/thalamus with lower expression in other regions of the brain, such as the hypothalamus, as seen in the winter flounder, European eel (*Anguilla anguilla*), mummichog (*Fundulus heteroclitus*), chub mackerel (*Scomber japonicus*) and African catfish (Dubois *et al*. 2001; Ohkubo *et al*. 2010; Penaranda *et al*. 2010; Selvaraj *et al*. 2009; Tuziak and Volkoff 2013). Teleost-specific distribution patterns have been shown: for example, medaka appears to have highest GnRH2 mRNA expression in the telencephalon/preoptic area and none in the optic tectum and hindbrain (cerebellum, medulla oblongata and spinal cord) (Kawabata *et al*. 2012), whereas GnRH2-ir perikarya are predominantly found in the midbrain tegmentum (optic tectum/thalamus) proximal to the third ventricle in Siberian sturgeon (*Acipenser baeri*) and chub mackerel, with fibres extending to the telencephalon/preoptic area, other regions of the optic tectum/thalamus, hindbrain and pituitary gland (Lepretre *et al*. 1993; Selvaraj *et al*. 2009). It is possible that the ubiquitous expression of GnRH2 mRNA in the cod brain might be due to the presence of GnRH2 fibres extending throughout the brain or might be representative of a cod-

specific distribution. Overall, the broad distribution of GnRH2 within fish brain suggests various regulatory functions, including food intake and reproduction.

In Atlantic cod, GnRH3 cDNA is present in the telencephalon/preoptic area, hypothalamus, optic tectum/thalamus, spinal cord and pituitary gland. In fish, high concentrations of GnRH3 mRNA, protein and ir-perikarya are typically found in the telencephalon/preoptic area, more specifically the terminal nerve ganglia, with lower levels distributed in other regions of the brain, as seen in winter flounder, Masu salmon, Mozambique tilapia (*Oreochromis mossambicus*), bonnethead sharks (*Sphyma tiburo*), chub mackerel and sockeye salmon (*Oncorhynchus nerka*) (Amano *et al*. 1998; Fukaya *et al*. 2013; Kuramochi *et al*. 2011; Moeller and Meredith 2010; Selvaraj *et al*. 2009; Tuziak and Volkoff 2013). A broader brain distribution of GnRH3 mRNA expression has been reported in mummichog, with highest levels in the ventral region, in particular the olfactory bulbs (Ohkubo *et al*. 2010). However, like GnRH2 mRNA expression, the GnRH3 mRNA found in the ventral region of the brain might correspond to other Atlantic cod-specific populations of GnRH3 cells and some fibres, as those seen in histological experiments in sockeye salmon (Amano *et al*. 1998). The presence of GnRH3 expression in the telencephalic and olfactory regions could be suggestive of a food and or pheromone sensing function.

2.4.2.2. Peripheral Atlantic cod tissue distributions

MCH mRNA was observed in several Atlantic cod peripheral tissues, including skin, heart, liver, spleen, stomach, pyloric caeca, ovaries and testes, but not muscle or

midgut. Apparent highest MCH cDNA expression was seen in the ovaries, liver, spleen and stomach. A wide peripheral MCH mRNA distribution has previously been reported for winter flounder, where expression is seen in most tissues, except for the heart and liver (Tuziak and Volkoff 2012), in goldfish, where MCH is present in testes, ovary, intestine and skin (Cerda-Reverter *et al*. 2006), and starry flounder, where cDNA is only detected in gills and testes (Kang and Kim 2013). The presence of MCH mRNA in the Atlantic cod stomach and gonads suggests a role in peripheral appetite regulation, digestive processes and/or reproduction.

In Atlantic cod, only skin, spleen, stomach, ovaries and testes express the GnRH2 transcript, with highest apparent concentrations present in the testes and spleen. GnRH2 expression has been shown in peripheral tissues such as foregut, intestine, ovaries and testes in other fish species, such as winter flounder, mummichog, medaka, zebrafish and Japanese eel (Kuo *et al*. 2005; Ohkubo *et al*. 2010; Okubo *et al*. 2006; Okubo *et al*. 1999; Tuziak and Volkoff 2013), although in the pejerrey GnRH2 is only present in the brain and not in peripheral tissues (Guilgur *et al*. 2007). Interestingly, in contrast to our results, another study using female Atlantic cod, only detected GnRH2 mRNA in the ovaries (Hildahl *et al*. 2011). The presence of GnRH2 in gut and gonads of Atlantic cod suggests that it may play a role in regulation of reproduction and food intake/digestion.

In our study, GnRH3 cDNA was only detected in the heart and testes in Atlantic cod, with no expression in the ovaries, stomach or pyloric caeca. In contrast to our results, GnRH3 mRNA has previously been reported in the ovaries of female Atlantic cod (Hildahl *et al*. 2011), winter flounder (Tuziak and Volkoff 2013) and spotted halibut (*Verasper variegates*) (Xu *et al*. 2012). In winter flounder, GnRH3 is expressed at low

levels in the skin, heart and gonads (Tuziak and Volkoff 2013) whereas in the smooth-tongued sole, GnRH3 transcripts are only found in the gonads, with higher mRNA expression in the ovaries than the testes, suggestive of a sex-bias (Zhou *et al*. 2012).

2.4.3. Food restriction increases MCH mRNA, but does not affect GnRH2 and GnRH3 transcript expression

Fasted fish had significantly lower masses than fed fish, but total lengths did not differ, suggesting that fasted fish diverted energy into somatic and visceral growth. Studies in fasted cod have shown that although fish loose weight, they produce constant quantities of protein in most tissues and organs (and thus continue to grow in length) (Houlihan *et al*. 1988). Thus, the loss of weight may be indicative of energy stores from the liver being converted into alternate organ growth and of a reduction in fat reserves (Foster *et al*. 1993).

MCH mRNA expression was significantly increased in fasted animals, suggesting that MCH might have orexigenic actions in Atlantic cod. Our results are in line with other studies showing that fasting induces increases in MCH brain expression, as seen in winter flounder, starry flounder and zebrafish (Berman *et al*. 2009; Kang and Kim 2013; Tuziak and Volkoff 2012), but contrast with studies in goldfish showing that MCH injections have anorexigenic effects (Matsuda *et al*. 2006; Shimakura *et al*. 2008).

In Atlantic cod, feeding status did not influence hypothalamic GnRH2 expression. Similar to our results, in Burton's mouthfeeder cichlid, whole brain GnRH2 mRNA expression is not affected by either mouth-brooding (a natural fast) or acute food

deprivation (Grone *et al*. 2012). However, in both winter flounder and zebrafish, a decrease in hypothalamic GnRH2 transcript expression is observed in fasted fish (Hoskins *et al*. 2008; Nishiguchi *et al*. 2012; Tuziak and Volkoff 2013). The lack of effects of fasting on GnRH2 in cod seems to contradict its proposed anorexigenic properties in winter flounder, zebrafish, and in goldfish, for which GnRH2 ICV injections inhibit feeding (Hoskins *et al*. 2008; Nishiguchi *et al*. 2012; Tuziak and Volkoff 2013). In fish, GnRH forms tend to be specific to a brain region, where GnRH1 is typically found in the hypothalamus, GnRH2 in the optic tectum/thalamus and GnRH3 in the telencephalon/preoptic area and olfactory region (Oka 2009; Sherwood and Adams 2005). In our study we chose to examine the expression in the hypothalamus because previous studies in fish show that it is the primary feeding center in the brain and most appetite-related hormones are differentially regulated in this region when studying the effects of nutritional status (Volkoff and Peter 2006). As GnRH2 mRNA transcripts are abundant in several brain regions, including optic tectum/thalamus, it is possible that fasting-induced changes in GnRH2 expression might have been detected in extra-hypothalamic areas.

Similar to GnRH2, no significant differences in hypothalamic GnRH3 mRNA expression were observed between fed and fasted cod. Similar results have been reported in Burton's mouthfeeder cichlid where fasting did not alter brain GnRH3 transcript expression (Grone *et al*. 2012; Matsuda *et al*. 2008). Winter flounder exhibit a decrease in telencephalic, but not hypothalamic, GnRH3 mRNA during fasting (Tuziak and Volkoff 2013), indicating that fasting might induce region-specific effects. As in the case of GnRH2, fasting-induced changes in GnRH3 expression might have been detected in extra-hypothalamic areas.

It is noteworthy that a high level of variation in relative GnRH3 mRNA expression was observed in both fed and fasted Atlantic cod hypothalami. This variability might have been due to sex-related differences in GnRH3 mRNA expression, as observed in some species. For example, in medaka, GnRH3 expression is higher in female than male brains (Kawabata *et al*. 2012) and in Mozambique tilapia, males have more GnRH3-ir neurons in the terminal ganglion than females, demonstrating sexual dimorphic nuclei as well as species-specific expression patterns (Kuramochi *et al*. 2011). In this study we used juvenile Atlantic cod and were unable to identify sexes, therefore a sex bias could have been introduced with regards to GnRH3 expression.

Although juvenile fish are not actively reproducing, GnRH3 and subsequent reproduction-related transcription factors have been shown to be similarly expressed in immature and mature fish, but also may have sex biases at an early stage. For example, in cichlids, no differences in GnRH3 mRNA expression is seen between mature and immature fish, indicating that GnRH3 may play a role in regulating sex-specific secondary reproductive behaviours, like male nest-building and/or sex reversals observed in other fish, but not gonadal maturation (Kuramochi *et al*. 2011; Parhar *et al*. 2003). Atlantic cod transcript *dmrt1*, a sex-determining factor, is higher in immature male cod compared with mature males and females of both reproductive states. Increases in *dmrt1* mRNA is positively correlated with GnRH-analog ICV injections, suggesting that GnRH3 could play a role in regulating sex-specific *dmrt1* expression in immature cod (Shin *et al*. 2009).

2.5. Conclusions

In this study, we identified the time at which MCH, GnRH2 and GnRH3 transcripts are first expressed during early development. MCH and GnRH3, but not GnRH2, are maternally deposited in unfertilized eggs and could play roles in regulating early development. All three transcripts are highly expressed from hatching onwards, when larvae are feeding on exogenous prey, which could indicate roles in the regulation of early food intake (MCH and GnRH2) and food sensing (GnRH3). The presence of MCH and both GnRHs in areas of the forebrain - telencephalon/preoptic area, optic tectum/thalamus and hypothalamus - previously implicated in appetite regulation and feeding behaviour - as well as the presence of MCH and GnRH2 mRNAs in the gastrointestinal tract suggests that these peptides might be involved in the central and peripheral regulation of food intake or other digestive processes. This regulatory role is further suggested for MCH by the fact that MCH is affected by nutritional status and might have orexigenic effects in fasted fish. However, the fact that Atlantic cod GnRH2 and GnRH3 expressions are not affected by fasting and that GnRH transcripts are present in gonads suggests that GnRHs might have a more prominent role in reproduction than in feeding.

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Chapter 3: An investigation of appetite-related peptide transcript expression in Atlantic cod (*Gadus morhua*) brain following a *Camelina sativa* meal-supplemented feeding trial

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Abstract

Camelina sativa is a hardy oilseed crop with seeds that contain high levels of ω3 polyunsaturated fatty acids and protein, which are critical components of fish feed. Camelina might thus be used as a cheaper and more sustainable supplement to fish-based products in aquaculture. Atlantic cod, *Gadus morhua*, is a species of interest in the aquaculture industry due to a decrease in wild populations and subsequent collapse of some cod fisheries. As cod are carnivorous fish, it is necessary to determine how this species physiologically tolerates plant-based diets. In this study, juvenile Atlantic cod were subjected to 13 weeks of either 15 or 30% camelina meal (CM)-supplemented diets or a control fish meal feed. Growth and feed intake were evaluated and the mRNA expression of appetite-related hormones [pro-melanin-concentrating hormone (*pmch*), hypocretin (synonym: orexin, *hcrt*), neuropeptide Y (*npy*) and cocaine- and amphetamine-regulated transcript (*cart*)] were assessed using quantitative real-time PCR in brain regions related to food intake regulation (telencephalon/preoptic area, optic tectum/thalamus and hypothalamus). CM inclusion diets caused decreases in both growth and feed intake in Atlantic cod. Optic tectum *pmch* transcript expression was significantly higher in fish fed the 30% CM diet compared to fish fed the 15% CM diet. In the hypothalamus, compared to fish fed the control diet, *hcrt* expression was significantly higher in fish fed the 30% CM diet, while *npy* transcript expression was significantly higher in fish fed the 15% CM diet. *cart* mRNA expression was not affected by diet in any brain region. Further studies are needed to determine which factors (*e.g.* anti-

nutritional factors, palatability and nutritional deficits) contribute to reduced feed intake and growth, as well as the maximum CM inclusion level that does not negatively influence feed intake, growth rate and the transcript expression of appetite-related factors in Atlantic cod.

3.1. Introduction

In recent years, *Camelina sativa* (henceforth referred to as camelina), a hardy oilseed crop commonly known as false flax or gold-of-pleasure, has made its way into the livestock agricultural community as a feed alternative to less sustainable fish products. The US Food and Drug Administration has approved camelina meal (CM) for use in cattle, broiler chicken and laying hen feeds, but studies and approval for fish are still underway (Montana Department of Agriculture 2012). Unlike other plants used (*e.g.* soybean and linseed) in agriculture feeds, camelina seeds contain high levels of medium chain polyunsaturated fatty acids (PUFA), including linoleic acid (LA; 18:2ω6) and α-linolenic acid (ALA; 18:3ω3), which are important fish feed constituents because they are precursors for long chain ω3 fatty acids, such as eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DPA; 22:6ω3) (Zubr 1997; Ni Eidhin *et al.* 2003). Furthermore, over-fishing of some baitfish (*e.g.* herring) (Department of Fisheries and Oceans 2013), commonly used for meals and/or oils in commercial fish feed, has made aquaculturalists aware that more sustainable alternatives to fish oil and/or meal are needed to maintain aquaculture programs of marine and anadromous fish species, such as Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*).

CM may be a suitable supplement to fish meal and other protein-rich aquafeed ingredients, given that it contains components that are beneficial for both fish and human health, including high protein levels (30-45%), about 13% fibre, about 5% minerals, ω3 fatty acids, in particular α-linolenic acid, antioxidants, tocopherols and an appealing

amino acid content (e.g. arginine, cysteine, glycine, lysine, methionine and threonine) (Zubr 1993; Ni Eidhin *et al*. 2003; Frame *et al*. 2007; Aziza *et al*. 2010). However, CM is also known to contain many anti-nutritional factors (ANFs), such as glucosinolates and their metabolites, saponins, phytates and non-starch polysaccharides, which have been shown to reduce food intake, digestibility and growth in poultry (Lange *et al*. 1995; Zubr 1997; Acamovic *et al*. 1999; Francis *et al*. 2001; Quezada and Cherian 2012). Phytates lower growth rates through reduced feed digestibility and protein availability by formation of phytic acid-protein complexes, diminish mineral availability through chelation and decrease nutrient absorption in the intestine due to epithelial hypertrophy (Spinelli *et al*. 1983; Richardson *et al*. 1985; Hossain and Jauncey 1991). In the fish thyroid gland, both phytates and glucosinolates cause morphological and hormonal disturbances (*e.g.* high thyroid hormone levels), which result in decreased feed consumption/utilization and growth rates (Higgs *et al*. 1982; Richardson *et al*. 1985). In the salmonid distal intestine - a region functionally important in nutrient absorption - saponins have been shown to cause damage to the intestinal epithelium and depress mucosal enzyme activity, which consequently lowered food intake (Bureau *et al*. 1998). Taken together, these results suggest that the presence of ANFs in CM may have negative impacts on the physiology of fish (including Atlantic cod) fed the CM diets, potentially leading to reduced feed intake and growth.

While some studies have suggested that camelina oil (CO)-supplemented fish feeds are tolerated in Atlantic cod, as these do not affect either growth or food intake parameters (Morais *et al*. 2012; Hixson *et al*. 2013), one study has reported negative effects of CO replacement diets on cod growth and food consumption (Hixson and

Parrish 2014). The study of Hixson and Parrish (2014) involved fish that were fed a control diet (i.e. no camelina products) or one of three CO-containing test diets [100% of fish oil (FO) replaced by CO (100CO); 100CO with solvent-extracted fish meal (100COSEFM); or 100CO with 15% CM (100CO15CM)]. Fish fed the 100CO15CM diet had the lowest feed intake and growth of all CO-supplemented diets included in Hixson and Parrish (2014). The current study uses the same control diet as Hixson and Parrish (2014); however, while Hixson and Parrish (2014) used CO-containing diets and focused on tissue lipid analyses, this study uses CM-supplemented diets and focuses on their effects on food intake and brain appetite-related transcript expression. In light of the results in Hixson and Parrish (2014), and more specifically the detrimental effects of some camelina-supplemented diets on food intake and growth, it would be valuable to determine if the effects of CM-containing diets on feeding and growth occur via changes in the brain expression levels of appetite-related transcripts.

Appetite can either be repressed (anorexigenic) or induced (orexigenic) by centrally (*i.e.* brain) or peripherally (*i.e.* gut) synthesized peptides and can be further regulated by the nature of the diets, and factors such as ANFs, food palatability and nutritional value. In mammals, centrally-acting orexigenic peptides include pro-melanin-concentrating hormone (PMCH) (Rossi *et al*. 1997; Shimada *et al*. 1998; Edwards *et al*. 1999), hypocretin (synonym: orexin, HCRT) (Edwards *et al*. 1999), and neuropeptide Y (NPY) (Edwards *et al*. 1999), while cocaine- and amphetamine-regulated transcript (CART) is anorexigenic (Stanley *et al*. 2001). For the most part, the functions of these peptides are conserved in mammals and fish, with some species-specific exceptions. For example, PMCH has been suggested to be orexigenic in most fish species, such as

Atlantic cod (Tuziak and Volkoff 2013 – Chapter 2), zebrafish (*Danio rerio*) (Berman *et al*. 2009), winter flounder (*Pseudopleuronectes americanus*) (Tuziak and Volkoff 2012) and barfin flounder (*Verasper moseri*) (Takahashi *et al*. 2004), whereas in goldfish (*Carassius auratus*), it appears to be anorexigenic (Matsuda *et al*. 2006; Matsuda *et al*. 2007). Both HCRT and NPY are appetite stimulators in fish, including Mexican blind cavefish (*Astyanax fasciatus mexicanus*) (Wall and Volkoff 2013; Penney and Volkoff 2014), orange-spotted grouper (*Epinephelus coioides*) (Tang *et al*. 2013), and zebrafish (Yokobori *et al*. 2011; Yokobori *et al*. 2012). The appetite-inhibitory function of CART is conserved throughout vertebrates and is established in teleosts, including goldfish (Volkoff and Peter 2000; Volkoff and Peter 2001), Atlantic cod (Kehoe and Volkoff 2007), Atlantic salmon (Murashita *et al*. 2009), winter skate (*Raja ocellata*) (MacDonald and Volkoff 2009b) and channel catfish (*Ictalurus punctatus*) (Kobayashi *et al*. 2008).

It is well-known that the hypothalamus is the main site in the mammalian brain where endocrine control of food intake occurs. However, in fish, a more diverse functional distribution of appetite regulating peptide transcripts throughout the brain is observed. For example, in fasted winter flounder, *pmch* transcript expression is seen at high levels in the hypothalamus - but not in the telencephalon/preoptic area or optic tectum/thalamus - compared to fed fish (Tuziak and Volkoff 2012). Likewise, increased hypothalamic NPY-like-immunoreactivity (ir) is observed in fasted zebrafish and tiger pufferfish (*Takifugu rubripes*) compared with fed fish, but no difference in NPY-like-ir is detected in the telencephalon/preoptic area (Kamijo *et al*. 2011; Yokobori *et al*. 2012). Similarly, *hcrt* mRNA expression is higher in fasted winter skate hypothalami compared to fed fish, but not in the telencephalon/preoptic area (MacDonald and Volkoff 2010). In

fasted goldfish, *cart* mRNA has a lower abundance in the telencephalon/preoptic area, hypothalamus, olfactory bulbs, but not in the optic tectum/thalamus, when compared with fed fish (Volkoff and Peter 2001). Analysis of all brain regions associated with food intake regulation, not just the hypothalamus, is necessary to ensure that species-specific and region-specific differences in transcript expression are not missed.

Atlantic cod is a cold-water fish species inhabiting the coast of Newfoundland and Labrador. As native North American cod populations are still recovering from the wild fisheries collapse and the European fisheries might be at risk (Lilly *et al*. 2013), the development of a sustainable aquaculture program including plant-based diets may be necessary to meet the current demand for cod.

Since Atlantic cod are carnivores, understanding how a CM-supplemented diet affects their physiology is crucial if these feeds are to be efficiently used in aquaculture. The negative palatability and presence of ANFs associated with plant-based diets remain a problem in aquaculture as both limit feed intake and thus growth (Paloheimo and Dickie 1966; Mackie and Mitchell 1985; Toften *et al*. 1995; Francis *et al*. 2001). By determining if CM-supplemented diets affect food intake and growth, we can then assess if there are associations among growth, feed intake and appetite-related transcripts to help us better understand if Atlantic cod can tolerate plant-based diets and the extent of supplementation of plant materials in their diet.

In this study, we examined whether CM-supplemented fish feed impacts growth, feeding and brain appetite regulating peptide transcript expression in Atlantic cod. Fish were subjected to either a control fish meal diet or CM-containing diets, and effects on growth and feed intake were assessed. We then examined the expression of transcripts

encoding four major appetite-related peptides (PMCH, HCRT, NPY and CART) in various brain regions (telencephalon/preoptic area, optic tectum/thalamus and hypothalamus) at the end of the feeding trial.

3.2. Materials and Methods

3.2.1. Camelina meal preparation and diets

Isonitrogenous and iso-energetic - similar nitrogen and caloric levels, respectively - diets were formulated at the Faculty of Agriculture, Dalhousie University, to meet the nutritional requirements of gadoids, based on previous studies (Tibbetts *et al*. 2004; Tibbetts *et al*. 2006) (Table 3.1). Diets used in the feeding trial are as follows: control (FO/meal-containing) diet, 100% FO replaced by CO (100CO); 100CO with solvent-extracted fish meal (100COSEFM); 100CO with 15% CM (100CO15CM); 15% CM, 30% CM, and 40% CM. This study focused on cod fed the 15% and 30% CM-containing diets. A meal cake was formed by pressing CM through a hammer mill (screen size 8 mm) then solvent extracted with 3 mL $\bullet$ g^{-1} petroleum ether to remove all lipids at the Faculty of Agriculture, Dalhousie University. Diets were steam pelleted using a laboratory pelleting mill (California Pellet Mill, San

Table 3.1. Percent diet formulations for camelina meal-supplemented feed experiment in juvenile Atlantic cod

Ingredient	Control	15% camelina meal	30% camelina meal
Fish oil	5.43	5.43	6.55
Herring meal	50.49	45.08	39.66
Wheat middlings	27.13	16.98	6.83
Wheat gluten meal	5	5	5
Whey powder	5	5	5
Krill hydrolysate	2.5	2.5	2.5
Corn starch (pre-gelatinized)	0.25	0.25	0.25
Vitamin premix[a]	1.95	1.95	1.95
Mineral mix[b]	1.95	1.95	1.95
Choline chloride	0.3	0.3	0.3
Camelina meal	0	15	30
Camelina oil	0	0.56*	0
Total	100	100	100
Proximate composition			
Dry matter	89.1	88.1	88.9
Ash	2.81	4.72	7.02
Crude protein	46.8	46.1	46.5
Lipid	8.9	12.2	13.1

Note: Requirements for optimal growth: 48.4% crude protein and 12.1% lipid (Tibbets *et al*. 2006)

[a] Vitamin Premix contains per kg: Vitamin A 0.9 g, Vitamin D3 8.0 g, Vitamin E 50.0 g, Vitamin K 3.0 g, Thiamine 2.8 g, Riboflavin 4.0 g, Pantothenic acid 24.0 g, Biotin 0.1 g, Folic acid 26.7 g, Vitamin B12 0.03 g, Niacin 15.1 g, Pyridoxine 3.3 g, Ascorbic acid

10.8 g, Wheat middlings (carrier) 851.3 g

[b] Mineral Premix contains per kg: Manganese oxide 12.3 g, Zinc oxide 20.6 g, Copper sulphate 6.1 g, Iodine 15.8 g, Wheat middlings (carrier) 954.2 g.

* Camelina oil is added to the 15% CM diet to balance the lipid content, while maintaining constant fish oil concentrations across diets. No significant differences in growth and feed intake were observed in cod fed 80% CO (Hixson *et al*. 2013), and thus CO inclusion is not expected to affect food intake and growth in this study.

Francisco, USA) with an initial pellet size of 4.0 mm increasing to 6.0 mm as the fish grew larger. Diets were stored at -20°C. Further details on formulation, proximate and fatty acid diet composition of the control fish meal diet are reported in Hixson and Parrish (2014).

3.2.2. Animals

Male and female juvenile Atlantic cod (mean initial mass ± SD: 14.4 ± 1.6 g fish $^{-1}$; mean initial length ± SD: 11.3 ± 0.4 cm fish $^{-1}$) were grown and maintained at the Ocean Sciences Centre, Memorial University of Newfoundland (Logy Bay, Newfoundland, Canada) in 500 L flow-through tanks (1 µm filtration; flow rate: 8 L • min $^{-1}$) from May - September 2012. Fish were randomly distributed at the beginning of the experiment among tanks {~ 70 fish/tank, 3 tanks per treatment (diets), number of treatments = 7 [although only 2 CM treatments (15% and 30%) and the control diet were included in the current gene expression analysis]}. The CM diets were chosen because they demonstrated the most significant effect in feeding and growth. Although, examining transcript expression in all diets for all brain regions would have been ideal, time and financial constraints were taken into consideration. Water temperatures ranged from 9.5 to 11.5˚C, dissolved oxygen was 10 mg • L^{-1} and a 12L:12D photoperiod was used. During a one-week acclimation period, fish were fed a commercial pellet diet (Europa 15, 2 mm, Skretting, New Brunswick, Canada) twice daily to satiation at 09:00 h and 15:00 h. All procedures were carried out in accordance with the Canadian Council on Animal Care

and Memorial University's Animal Care Council guidelines.

3.2.3. Camelina supplemented diet experimental protocol

Fish were acclimated to the control diet (fish oil/fish meal) for one week prior to sampling as per Hixson and Parrish (2014). At week 0 (immediately after first sampling), fish were gradually transitioned over 3 days from the control diet to their respective CM experimental diet or with control fish remaining on the control diet. Diets used in the present experiment were: control (full fish meal, no camelina products), 15% CM inclusion (partial replacement of fish meal), and 30% CM inclusion (partial replacement of fish meal) (Table 3.1). Three replicate tanks were used for each experimental diet, including the control diet. Fish were fed to apparent satiation and feed consumption for each tank was determined each week by measuring the difference in the amount of food weighed prior to feeding and the amount of food that remained after satiation was reached. Fish were fed carefully by hand to ensure no pellets were wasted and fish consumed all pellets that entered the tank. Further details on fish rearing conditions are described in Hixson and Parrish (2014).

Fish were fasted for at least 24 hours prior to sampling to ensure food was digested and metabolised, which was necessary for analyses in other studies (Hixson and Parrish, 2014). Sampling took place at weeks 0 (1 day prior to the experimental diets being fed), 1, 6 and 13. Fish were sacrificed by an overdose (400 mg $\bullet$ L^{-1}) of tricaine methane sulfonate (TMS, Syndel Laboratories Ltd., Nanaimo, British Columbia, Canada)

followed by a spinal cord sever. At each sampling time, total length and mass were measured and whole brains were dissected from three fish/tank/treatment (n = 9 per treatment) and stored in RNAlater [11.012 M ammonium acetate, 91.053 M sodium citrate dihydrate, 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 7.0] at -20˚C until processing. Some brains (used in the hypothalamic study, see below) were flash-frozen in liquid nitrogen and stored at -80°C until further use. A separate, flash-frozen set of samples was used for this experiment because initial RNA from hypothalami stored in RNAlater was lost following RNA extraction. All tissues were sampled at the same time, from the same experimental fish, however the hypothalami were from a separate sub-sample of brains [tissues from fish used in Hixson and Parrish (2014) study]. In this study, we focused on the control diet, 15% CM and 30% CM diets (Table 3.1) and the week 13 final time point. These diets were chosen based on the long-term growth and food consumption data, which showed that growth and food intake were reduced as fish were fed a greater percentage of CM in diets.

3.2.4. Growth and food consumption data analyses

Mass specific growth rates {SGR_M; [*ln*(final mass) - *ln*(initial mass) / number of days fed] • 100} were calculated based on average masses for fish from weeks 0 (3 tanks • diet $^{-1}$, 9 fish • diet $^{-1}$) and week 13 (3 tanks • diet $^{-1}$, 86-124 fish • diet $^{-1}$) [n = 3 (tank means) for SGR_M mean] as per Hixson and Parrish (2014). Variability in fish sample numbers for week 13 analyses is due to the demand for fish used in multiple studies [*i.e.*

Hixson and Parrish (2014) and this present study] within this camelina feeding trial.

Fulton's condition factor {*K*; [mass (g) / length ^{3} (cm^{3})] x 100} was calculated for each individual from each of the treatments for all samplings (3 tanks • diet $^{-1}$, 86-124 fish • diet $^{-1}$).

Since fish were kept in large groups and individual feed consumption was not obtainable in this study, an average feed consumption per tank was determined as described in section 3.3. The average feed consumption was then calculated per fish [g • fish $^{-1}$; mass of feed given to fish (g) / number of fish in tank], under the assumption that all fish in each tank were consuming equal amounts of feed and no competition was occurring. Only feed consumption data for week 13 are shown, since this is the week on which we focused all other analyses.

Feed conversion ratio [FCR; feed intake (g • fish $^{-1}$) / weight gain (g • fish $^{-1}$)] was calculated using tank averages over the 13-week trial.

3.2.5. RNA extraction and cDNA synthesis of Atlantic cod tissues

Telencephalon/preoptic area and optic tectum/thalamus were dissected from whole brains based on Delfini and Diagne (1985) and stored in RNAlater at -20°C until further use. Hypothalami were dissected from flash-frozen brains that were transitioned into RNAlater-*ICE* (Ambion, Austin, Texas, USA) as per the manufacturer's protocol. Dissected samples were then washed in 500 µl of ice-cold diethylpyrocarbonate (DEPC) water and RNA was extracted using the TriReagent (BioShop, Burlington, Ontario,

Canada) method as per the manufacturer's protocol. RNA concentration and purity were analyzed using A260/A280 and A260/A230 readings with a NanoDrop spectrophotometer (NanoDrop, Wilmington, North Carolina, USA). Samples were then electrophoretically separated on a 1.15% agarose gel at 80 V for 30 minutes in 1X tris-acetic acid-EDTA (TAE) buffer and visualized with ethidium bromide to identify 18S and 28S rRNA bands to check RNA integrity. Samples were then DNaseI digested (6.8 Kunitz units) at room temperature for 10 min using the QIAGEN RNase-free DNaseI kit using the manufacturer's protocol (Mississauga, Ontario, Canada) to ensure any residual genomic DNA was removed. RNA was column-purified using the RNeasy MinElute clean-up kit (QIAGEN) as per the manufacturer's protocol to remove any impurities. Finally, purified RNA samples were analyzed with the NanoDrop for A260/A230 (accepted ratio: > 1.8) and A260/A280 (accepted ratio: > 2.0) and electrophoretically separated on a 1.15% agarose gel as described above to ensure that genomic DNA and impurities were removed and that RNA was not degraded during the purification process.

cDNA was synthesized from 1 μg purified RNA using the Moloney murine leukemia virus (MMLV) reverse transcription (RT) system (Invitrogen, Burlington, Ontario, Canada) with random primers (250 ng) at 25°C for 10 minutes, 37ºC for 50 min and 72°C for 15 min following the manufacturer's protocol (the optional RNaseOut step was excluded).

3.2.6. Quantification of appetite-related transcripts in Atlantic cod fed camelina-supplemented diets

qPCR transcript-specific primers were designed based on previously sequenced mRNAs (*pmch*: ENSGMOT00000010147, *hcrt*: DQ486137, *npy*: AY822596, and *cart*: DQ167209) available in the National Center for Biotechnology Information (NCBI) and Ensembl databases (Table 3.2). Primers were designed to have similar melting temperatures, yield amplicons with lengths between 80-150 bp, and have each primer for a given gene of interest on two separate exons spanning an intron to avoid genomic DNA amplification. Preliminary assays were run to determine the most effective normalizer gene for this experiment using actin-related protein 2/3 complex, subunit 4 (*arpc4*), ubiquitin A-52 residue ribosomal protein fusion product 1 (*uba52*) and ribosomal protein s9 (*rps9*). Primer sequences and information for the normalizer transcripts were taken from the Olsvik *et al*. (2008) study. These three reference genes were selected based on the analysis in Olsvik *et al*. (2008), showing they are the best normalizers for brain tissue giving the most stable expression profiles using wild fish from different populations and contaminated habitats. Preliminary qPCRs were run using three to five 1:8 diluted optic tectum/thalamus cDNA samples in triplicate from each of the groups (control, 15% CM and 30% CM) to determine which normalizer was most stable. qPCRs were run on the Eppendorf 2S Realplex system (Eppendorf, Hamburg, Germany) using the Kapa Fast Sybr Green System [D-Mark Biosciences, Toronto, Ontario, Canada; final concentration: 1X Kapa Sybr Green Master Mix in a 10 µL reaction, 0.2 µM of each sense and antisense

Table 3.2. Atlantic cod (*Gadus morhua*) specific primers and associated transcripts.

Transcript	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon length
uba52	GGC CGC AAA GAT GCA GAT	CTG GGC TCG ACC TCA AGA GT	69 bp
pmch	GAC CGA GGG CTG TCC TAC TAC	CTC ATG GTG TCC CGC TTG	87 bp
hcrt	CTG AGG AAT CGA GAA AAA CAC C	GCA CTA ACA CGA GGA CTT GGA T	113 bp
npy	GAC AAA GGT ACG GGA AGA GG	CAA TGA CGG GTC ATA TCT GC	112 bp
cart	CCA ACG TGT GAT ATC GGA GA	CTA CAT TCG AAC CAC GTT CC	140 bp

primer, 6.25 ng of input total RNA in 1 µL of diluted cDNA (diluted with nuclease-free water)] using the cycling parameters as follows: 95 °C for 3 minutes and the following profile repeated for 40 cycles: 95 °C for 30 s, 58 °C for 15 s, 72 °C for 20 s. A normalizer gene was chosen based on the criteria that its cycle threshold (C_T) value range was no greater than 2 cycles when comparing all treatment groups (diets) and amongst samples (individual fish) within each tissue to ensure that growth and diet did not affect normalizer regulation. Standard curves were generated using a six point two-fold dilution series where samples were run in triplicate to determine primer efficiencies and R^2 values for genes of interest (GOI) as well as the normalizer gene in each brain region (Pfaffl 2001). Ranges were 0.89 - 1.08 for amplification efficiencies and 0.994 - 0.999 for R^2 values (Table 3.3). Melting curves were run to ensure a single product (without primer-dimers) was being amplified using the following thermal cycler program: 95°C for 15 s, followed by a temperature ramp for 20 min ranging from 55 - 95°C for 15 s rising at 0.1°C s^{-1}. A "no cDNA template control" was run on all plates to ensure no contamination was being amplified, while "no reverse transcriptase controls" using individual samples were run to ensure genomic DNA was not being amplified. These quality control (QC) tests were also completed for the telencephalon/preoptic area and the hypothalamus to ensure consistency across tissues. Since *uba52* had the smallest C_T range (< 2 C_Ts) of the candidate normalizer genes and was consistently expressed between brain regions and treatments, it was used as the normalizer in this study.

Once the primers passed our quality control assessment, qPCRs of the Atlantic cod samples for each brain region (telencephalon/preoptic area, optic tectum/thalamus

Table 3.3. Primer efficiencies (E) and R^2 using a 6-point standard curve for each brain region. A * indicates a 5-point standard curve.

Transcript	Efficiencies and R^2					
	Telencephalon/ preoptic area		Optic tectum/ thalamus		Hypothalamus	
	E	R^2	E	R^2	E	R^2
uba52	1.02	0.995	1.04	0.997	0.96	0.995
pmch	1.08	0.996	0.98	0.997	0.97	0.998
hcrt	1.03	0.998	0.99	0.999	1.00	0.996
npy	1.00	0.999	0.91	0.994	0.97	0.994*
cart	0.95	0.998	0.89	0.997	0.94	0.994

and hypothalamus) were completed using the Kapa Sybr Green System for the treatments (15% CM and 30% CM) against our control group and samples were run in duplicate. For *pmch* and *hcrt*, a 1:2 cDNA dilution was used (25 ng of input total RNA in 1 µL cDNA) for telencephalon/preoptic area and optic tectum thalamus, as these genes have low transcript expression in these regions, and a 1:4 cDNA dilution (12.5 ng of input total RNA in 1 µL cDNA) was used for the hypothalamus, a region in which *pmch* and *hcrt* have higher mRNA expression. *npy* and *cart* have relatively high mRNA expression levels throughout the brain, therefore a 1:4 cDNA dilution (12.5 ng of input total RNA) was used for the optic tectum/thalamus and hypothalamus and a 1:8 cDNA dilution (6.25 ng of input total RNA) for the telencephalon. If any samples were outside of the normalizer's 2 C_T range, the most extreme sample (furthest sample from the normalizer C_T mean) was removed from the analyses; no more than 2 individuals per treatment were removed from analyses. An inter-run calibrator (IRC) cDNA was randomly chosen at the beginning of the experiment and used on all plates in the study for both the normalizer and the GOI to ensure inter-plate reproducibility. The IRC mean C_T was compared between plates within a given tissue, and the C_T range was within 0.5 C_Ts. All thresholds were set automatically by the Realplex program and a baseline drift correction was used to ensure any drifting baselines, especially with the transcripts with lower expression, were taken into consideration for C_T values.

Relative quantities (RQ; $2^{-\Delta\Delta CT}$) of gene expression were calculated manually using the comparative C_T method (Livak and Schmittgen, 2001; Pfaffl, 2001). Since primer efficiencies of the GOIs did not equal the normalizer efficiency the Pfaffl equation

(Pfaffl, 2001) was used:

$$RQ = [(E_{control})^{CT\ sample} / (E_{target})^{CT\ sample}] / [(E_{control})^{CT\ calibrator} / (E_{target})^{CT\ calibrator}]$$

The mean C_T for each individual as well as calculated primer amplification efficiencies (Table 3.3) were used in the above equation to determine RQ values. The lowest expressing individual in each study was used as the calibrator.

3.2.7. Statistical analyses

Statistical analyses and figures were generated using GraphPad prism v6.0 (GraphPad Software Inc., San Diego, California, USA).

3.2.7.1. Growth and feed consumption analyses

A Shapiro-Wilk normality test was used to determine if the datasets were normally distributed. If they were normally distributed, a one-way ANOVA followed by Tukey's post-hoc test were used to compare growth and feeding metrics among groups. If the data were not normally distributed, a non-parametric Kruskal-Wallis *H*-test followed by Dunn's multiple comparison test were used to compare groups.

3.2.7.2. qPCR analysis

RQs were $\log_2$ transformed in Excel (Microsoft Co., Seattle, Washington, USA). Normality for each group was assessed by the Shapiro-Wilk test. One-way ANOVAs or Kruskal-Wallis *H*-tests were run for each transcript (*pmch, hcrt, npy*, and *cart*) in each brain region (telencephalon/preoptic area, optic tectum/thalamus, and hypothalamus). If the data were normal, a one-way ANOVA was used with Tukey's multiple comparison correction following the overall test. For non-normal data, the non-parametric Kruskal-Wallis *H*-test was used with Dunn's multiple comparisons to determine differences among diets. Tukey's multiple comparisons were completed following the ANOVA if an overall significant effect ($p < 0.10$) was observed.

A priori effective sample sizes were calculated with the G*Power software (Faul *et al*. 2007; Faul *et al*. 2009) for qPCR data to determine if an $\alpha = 0.10$ (as opposed to $\alpha = 0.05$), could be used in this study based on the small sample sizes. Calculated means, average standard deviations and effect size (a measure describing the magnitude of difference between experimental groups), as well as values of $\alpha = 0.05$ or $\alpha = 0.10$ and $\beta = 0.20$ (power = $1 - \beta = 0.80$) were used in the algorithm based on Cohen's *d* guidelines (Cohen, 1992). Once the effective sample sizes were determined, post-hoc achieved power tests were completed using total population size ($N = 20 - 27$), number of groups ($n = 3$), $\alpha = 0.05$ and 0.10 and calculated effect size as parameters.

Overall fold-change values were calculated as 2^{A-B} as in Hori *et al*. (2012), where A is the average $\log_2$ transformed RQ from an experimental group (30% CM being

considered experimental relative to 15% CM) and B is the average $\log_2$ transformed RQ from the diet-matched control diet group.

3.3. Results

3.3.1. Effects of camelina meal supplemented diets on growth in Atlantic cod

No significant differences between initial mass or initial length were detected between tanks and diets (ANOVA, $p > 0.10$, Table 3.4).

Overall, an effect of diet was seen for mass specific growth rate (SGR_M: ANOVA, $p < 0.001$, Table 3.4). A significant difference between the control and 30% CM diet was observed for SGR_M, where 30% CM diet fed fish had lower growth rates compared to the group fed the control diet. No differences in fish growth were seen between the control and 15% CM or between 15% and 30% CM diets for SGR_M.

CM-supplemented diets affected final mass and length of fish after 13 weeks (ANOVA, $p < 0.0001$; Table 3.4). Control fish were significantly heavier and longer at the end of the feeding trial and had significantly greater weight gain than fish fed either CM inclusion diet, while 15% CM fish had significantly increased mass and length, as well as higher weight gain compared to the 30% CM fish. Condition factor was also affected by diet (ANOVA, $p < 0.0001$; Table 3.4) and differences were observed among all three diets. Control fish had the highest CF, 30% CM fish had the lowest CF, and 15% CM fish had intermediate CF.

Table 3.4. The effect of *Camelina sativa* meal-supplemented diets (15% and 30%) on growth parameters in Atlantic cod (*Gadus morhua*) for week 13 sampling point. All means are given with standard deviation. Significance is indicated by a lowercase letter at a *p*-value < 0.10. All data for the control fish diet are recorded in Hixson and Parrish (2014), since both studies are based on the same feeding trial and use the same control diet (but different experimental diets).

	Control fish oil/fish meal	**15% camelina meal**	**30% camelina meal**
Initial mass (g)	14.3 ± 1.2	14.1 ± 1.4	13.7 ± 1.4
Final mass (g)	50.8 ± 10.3^{a}	43.4 ± 9.0^{b}	33.0 ± 8.5^{c}
Mass gain (g)	36.6 ± 1.4^{a}	28.2 ± 4.5^{b}	19.4 ± 1.4^{c}
Initial length (cm)	11.3 ± 0.3	11.3 ± 0.5	11.3 ± 0.6
Final length (cm)	17.0 ± 1.1^{a}	16.4 ± 1.1^{b}	15.2 ± 1.2^{c}
Specific growth rate (mass) ($g \bullet day^{-1}$)	1.3 ± 0.03^{a}	1.1 ± 0.1^{ab}	0.9 ± 0.1^{b}
Condition factor	1.01 ± 0.1^{a}	0.97 ± 0.1^{b}	0.91 ± 0.1^{c}

3.3.2. Effects of camelina meal supplemented diets on feed intake in Atlantic cod

The overall total feed consumed over the 13-week period was significantly different among diets (ANOVA, $p = 0.0002$; Figure 3.1A). The control fish ate the most feed (1661 ± 13 g), while both the 15% (1464 ± 41 g) and 30% (1158 ± 45 g) CM diet fish ate significantly less than the controls (Tukey's multiple comparisons, $p = 0.0194$ and $p = 0.0002$, respectively). Fish fed the 15% CM diet consumed significantly more feed than fish fed the 30% CM diet (Tukey's multiple comparisons, $p = 0.0024$).

Feed consumption per fish was significantly lower in the CM diets compared with the fish oil/fish meal control diet (Figure 3.1B, ANOVA, $p = 0.0059$). During week 13 (*i.e.* the final week of the feeding trial), individual fish fed the control diet ate an average of 3.262 ± 0.288 g of feed; this was significantly greater than both the CM diets (15% CM: 2.345 ± 0.045 g, $p = 0.0221$; 30% CM: 2.037 ± 0.066 g, $p = 0.0058$), although no differences between fish fed the CM diets were observed. Feed conversion ratio (FCR) was significantly different between diets (ANOVA, $p = 0.0530$; Figure 3.1C). The FCR of control and 15% CM fed groups did not differ (0.9014 ± 0.0151 and 1.016 ± 0.0822, respectively), while the FCR was greater in the 30% CM fish group compared with the control (30% CM: 1.131 ± 0.0305; Tukey's multiple comparisons, $p = 0.0448$).

error. Significance is indicated by a lowercase letter at a p-value < 0.10.

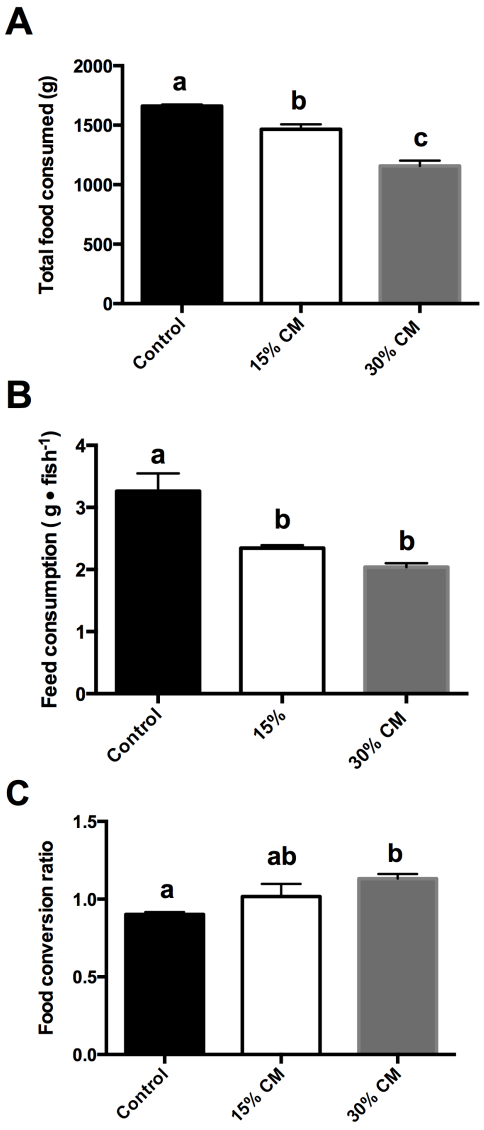

Figure 3.1. The effect of camelina meal-supplemented diets (15% and 30%) on food intake parameters in Atlantic cod. A) Total food consumed over 13 week experiment (grams), B) Relative food consumption for week 13 (gram food ingested • gram wet mass fish $^{-1}$), and C) Food conversion ratio for week 13. All means are given with standard error. Significance is indicated by a lowercase letter at a *p*-value < 0.10

3.3.3. Effects of camelina meal supplemented diets on appetite-related transcript expression in Atlantic cod

Effective population and group sizes for each transcript and brain region are presented in Table 3.5 for both $\alpha = 0.05$ and $\alpha = 0.10$. In this study, using $\alpha = 0.05$ might have biased the interpretation of the results given the small sample sizes (N = 6-9 for each treatment). Based on the power analyses, population sizes of N = 27-39 (group size = 3) with an $\alpha = 0.10$ would be required for sufficient statistical power. Statistical power ranged from 0.0782 to 0.8212 for $\alpha = 0.05$ and 0.1432 to 0.9005 for $\alpha = 0.10$. A p-value = 0.10 was used as our level of significance henceforth, since it provides a higher level of power with lower effective population sizes (comparable to ones used in this study).

3.3.3.1. *pmch*

No significant differences in *pmch* transcript expression were detected between fish fed the control and/or CM diets in the telencephalon/preoptic area or in the hypothalamus (Kruskal-Wallis H-test, $p > 0.10$; Figure 3.2A,C).

CM diet fed fish had significant effects on *pmch* mRNA expression in the optic tectum/thalamus (ANOVA, $p = 0.0315$; Figure 3.2B). No differences were seen in *pmch* mRNA expression between the control fish oil/fish meal diet and both the 15% and 30% CM diets. *pmch* mRNA expression was 13.00-fold higher in the 30% CM fish compared with 15% CM diet (Tukey's multiple comparisons, p = 0.0318, Figure 3.2B).

Table 3.5. Effective population sizes based on calculated means, average standard deviations and effect size for each brain region and transcript using $\alpha = 0.05$ and 0.10, $\beta = 0.20$ (where power = $1 - \beta = 0.80$) and number of groups = 3. Achieved power is based upon calculated effective population size (as described above) for each $\alpha = 0.05$ and 0.10, actual population size (N = 20-27) and group size (n = 3).

Brain region	Transcript	Effect size	Achieved power (1 - β)		Effective population size		Group size	
			α = 0.05	α = 0.10	α = 0.05	α = 0.10	α = 0.05	α = 0.10
Telencephalon/ preoptic area	*pmch*	0.1768	0.0970	0.1715	312	249	104	83
	hcrt	0.2501	0.1374	0.2288	159	126	53	42
	npy	0.1868	0.1119	0.1923	279	225	93	75
	cart	0.1788	0.1064	0.1845	306	246	102	82
Optic tectum/ thalamus	*pmch*	0.5828	0.681	0.7963	33	27	11	9
	hcrt	0.1914	0.1152	0.1970	267	213	89	71
	npy	0.3423	0.3017	0.4306	87	69	29	23
	cart	0.1229	0.0782	0.1432	642	513	214	171
Hypothalamus	*pmch*	0.3196	0.2377	0.3567	99	78	33	26
	hcrt	0.4786	0.5030	0.6404	48	39	16	13
	npy	0.6830	0.8212	0.9005	24	21	8	7
	cart	0.1804	0.1075	0.1861	300	240	100	80

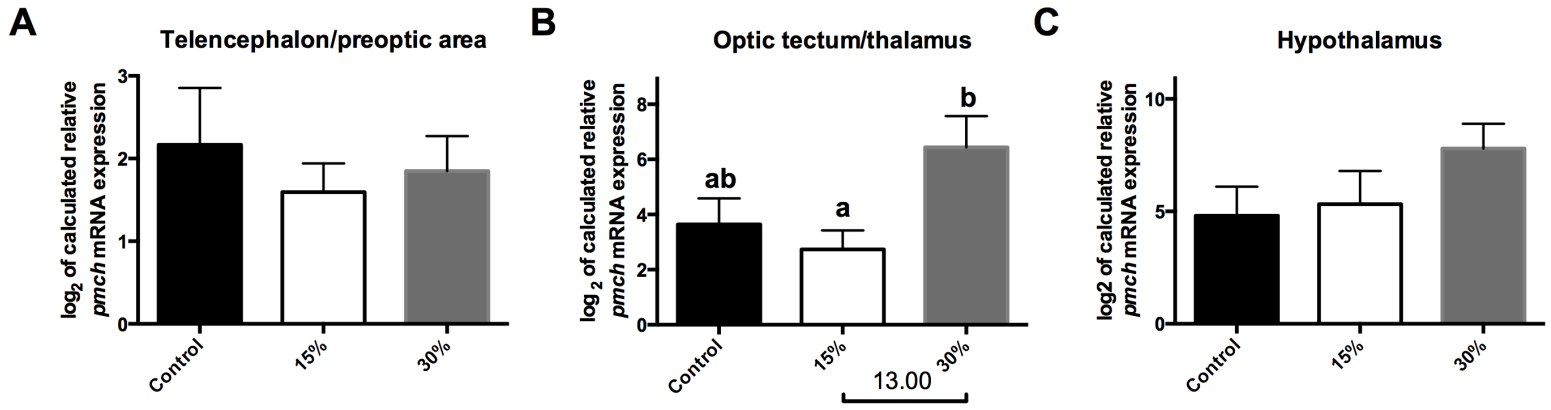

Figure 3.2. The effects of camelina meal-supplemented diets (15% and 30%) on *pmch* mRNA expression (log_2 calculated relative quantification values) in Atlantic cod. A) Telencephalon/preoptic area, B) Optic tectum/thalamus, and C) Hypothalamus. All means are given with standard error. Fold-changes between diets with significant differences are shown at the bottom of figure. Significance is indicated by a lowercase letter at a p-value < 0.10.

3.3.3.2. *hcrt*

No significant differences in *hcrt* transcript expression were observed in either the telencephalon/preoptic area or optic tectum/thalamus from fish in the different treatment groups (telencephalon/preoptic area: ANOVA, $p > 0.10$; optic-tectum/thalamus: Kruskal-Wallis, $p > 0.10$; Figure 3.3A,B).

In the cod hypothalamus, CM diets showed a statistically significant effect on *hcrt* transcript expression (ANOVA, $p = 0.0793$, Figure 3.3C). The fish fed the 30% CM diet had 14.95-fold significantly higher *hcrt* transcript expression than the control diet fish (Tukey's multiple comparisons, $p = 0.0654$).

3.3.3.3. *npy*

No significant differences were seen in *npy* mRNA expression between fish fed the three diets in either the telencephalon/preoptic area or optic tectum/thalamus (Kruskal-Wallis *H*-test, $p > 0.10$; Figure 3.4A,B).

Overall, CM diets affected cod hypothalamic *npy* transcript expression (Kruskal-Wallis *H*-test, $p = 0.0134$; Figure 3.4C). *npy* mRNA expression was 7.71-fold higher in 15% CM fish compared with the control fish (Dunn's multiple comparisons, $p = 0.0123$). No significant differences in *npy* transcript expression were observed between fish fed the 30% CM and control diets ($p > 0.10$).

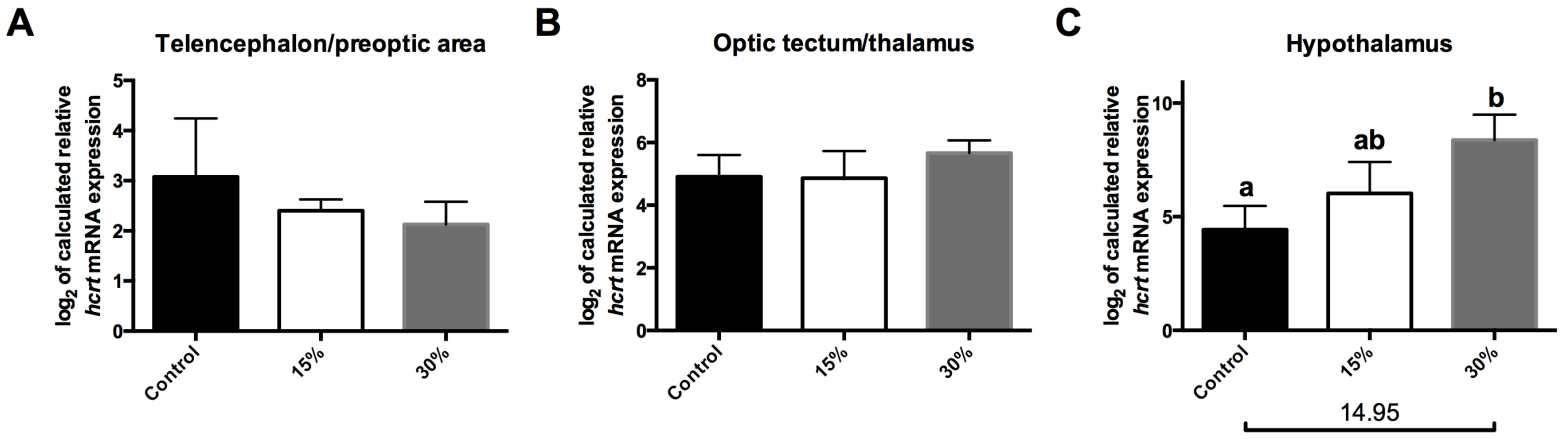

Figure 3.3. The effects of camelina meal-supplemented diets (15% and 30%) on *hcrt* mRNA expression (log_2 calculated relative quantification values) in Atlantic cod. A) Telencephalon/preoptic area, B) Optic tectum/thalamus, and C) Hypothalamus. All means are given with standard error. Fold-changes between diets with significant differences are shown at the bottom of figure. Significance level of p-value < 0.10 is indicated by lower case letters.

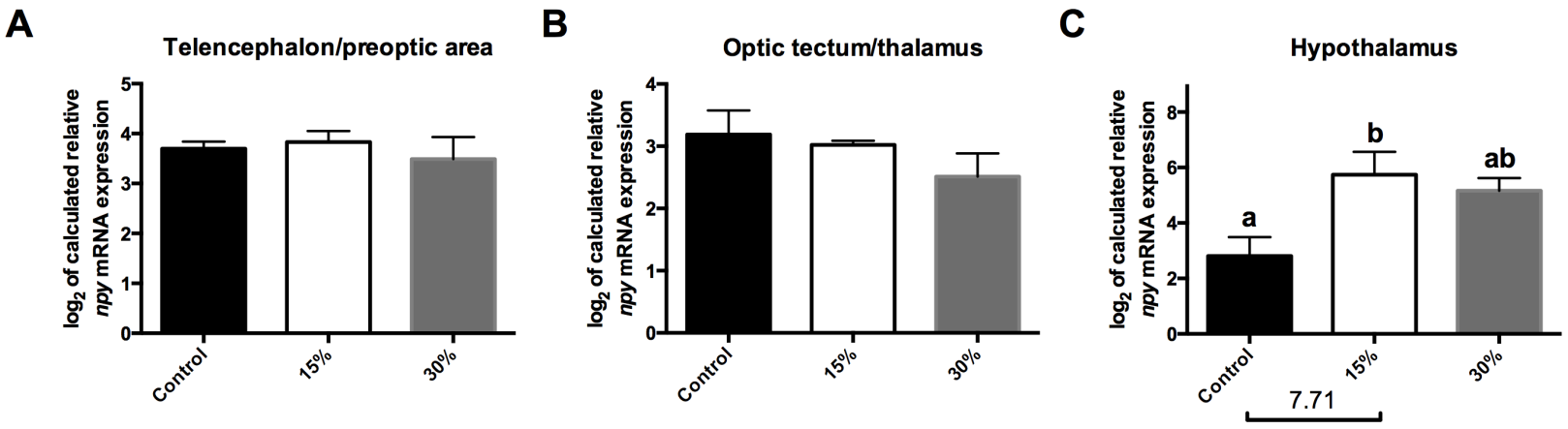

Figure 3.4. The effects of camelina meal-supplemented diets (15% and 30%) on *npy* mRNA expression (log_2 calculated relative quantification values) in Atlantic cod. A) Telencephalon/preoptic area, B) Optic tectum/thalamus, and C) Hypothalamus. All means are given with standard error. Fold-changes between diets with significant differences are shown at the bottom of figure. Significance is indicated by a lowercase letter at a p-value < 0.10.

3.3.3.4. *cart*

No significant differences in *cart* transcript expression were observed between fish fed the control and CM diets in the telencephalon/preoptic area, optic tectum/thalamus or hypothalamus (telencephalon/preoptic area: ANOVA, $p > 0.10$; optic tectum/thalamus, hypothalamus: Kruskal-Wallis *H*-test, Figure 3.5A-C).

3.4. Discussion

3.4.1. Effects of camelina meal supplemented diets on growth in Atlantic cod

High (30%), but not moderate (15%), levels of CM were associated with a decrease in SGR_M compared with the fish meal diet. Atlantic cod fed soybean-supplemented (SB; *Glycine max*) diets are able to tolerate partial replacements between 20-25% SB meal, whereas decreases in final mass and SGR_M are seen with the 30% SB meal diet (von der Decken and Lied 1993; Refstie *et al*. 2006). In aquaculture, high SGRs are beneficial, as they reduce production time and maintain high protein turnover rates.

Although cod fed 15% CM diets maintain SGR_M similar to fish fed the control fish meal diet, all size parameters (*i.e.* final mass, condition factor) need to be taken into consideration. Thus, even lower inclusions of CM (less than 15%) may be necessary to ensure all growth parameters are not negatively influenced by the feed.

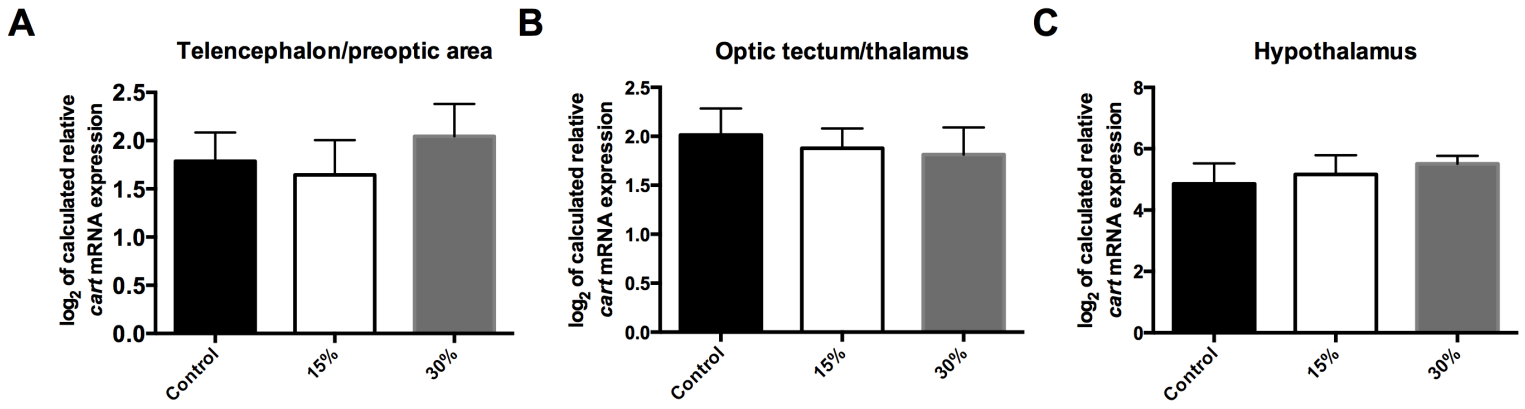

Figure 3.5. The effects of camelina meal-supplemented diets (15% and 30%) on *cart* mRNA expression (log_2 calculated relative quantification values) in Atlantic cod. A) Telencephalon/preoptic area, B) Optic tectum/thalamus, and C) Hypothalamus. All means are given with standard error. No significant differences were observed in between fish fed the control and CM diets.

Comparable growth-related deficits in other fish species fed high concentrations of plant-supplemented diets have previously been shown. For example, in Nile tilapia (*Oreochromis niloticus*) fed 25-50% faba bean (FB; *Vicia faba*) meal diets, growth is not affected, however higher FB meal supplemented diets (75 and 100%) cause decreases in SGRs (Gaber 2006). Rainbow trout (*Oncorhynchus mykiss*) fed faba bean/soybean mixed (FBSB) inclusion diets display a similar trend where fish fed 15% FBSB meal diets have SGRs similar to individuals fed the fish meal diet, while higher levels of FBSB inclusion in diets (30 and 45% FBSB) cause declines in growth (Ouraji *et al*. 2013). Together, these results suggest that fish can tolerate an "ideal" concentration of plant meal supplement in their feed before growth deficits are observed and that the responses to the degree of supplementation are species-specific.

Atlantic cod fed either CM diet had lower final mass and condition factor compared with the control fish. Unlike CM, SB meal inclusion did not affect cod condition factor and only affected final mass at high concentrations (30% SB) (Refstie *et al*. 2006). The condition of fish is a measure of how much energy the fish accumulates through feeding. In our study, the 15% CM fish appeared to be growing at the same rate as the control diet fish, but were not retaining mass from the food stuffs (as seen by the reduced final mass and condition factor), suggesting that cod fed moderate concentrations of CM feed are possibly using some internal energy stores (fat) to maintain normal growth.

The reduced growth (and food intake, see below) in cod fed CM diets seen in this study might have been due to the detrimental effects of ANFs. Phytates and glucosinolates are the primary ANFs in camelina and both have been shown to have

detrimental effects on fish feeding and growth (Francis *et al*. 2001; Matthaus and Angelini 2005). It is possible that in cod, phytates might form bonds with proteins reducing protein abundance or trigger intestinal epithelial inflammation as seen in carp fed diets high in phytates (Hossain and Jauncey 1990). Both processes would result in lowered nutrient and amino acid availability and uptake causing decreased growth rates (Spinelli *et al*. 1983; Hossain and Jauncey 1991). Another ANF, saponin, is known to cause reduced food intake in Chinook salmon (*Oncorhynchus tsawytscha*) fed plant-based diets (Bureau *et al*. 1998), as well as damage to the intestinal epithelium and depressed mucosal enzyme activity, which would lead to reduced nutrient uptake and suppressed growth.

3.4.2. Effects of camelina meal supplemented diets on feed intake in Atlantic cod

CM significantly decreased total feed consumption in Atlantic cod, with the 30% diet showing the most extreme reductions. Total feed consumption started to decline within three weeks for both the CM diets (data not shown), indicating that palatability and/or digestibility may be factors contributing to low total feed intake. Since plant material has been shown to decrease palatability in fish feed (Mackie and Mitchell 1985), it is possible that cod were less attracted to their food.

Feed consumption per individual fish was lower in camelina diets compared to the control fish oil/fish meal diet. Feeding Atlantic cod with low percentage SB-supplemented feed does not affect food consumption, although a decrease is observed in the higher 30% SB inclusion diet (von der Decken and Lied 1993). In this study, it seems

that Atlantic cod fed the CM diets did not compensate for a possible decrease in digestibility by eating more feed, as seen with fish fed soybean meal diets (Refstie *et al*. 2006). However, fish in each tank as a whole ate less, growing more slowly and becoming leaner.

Fish fed the 30% CM-supplemented diet had higher feed conversion ratios (FCR) than fish fed the control fish oil/fish meal diet. Low SGRs and high feed conversion ratios are indicators of underfed fish (Cho 1992). Similarly, in both rainbow trout and Nile tilapia, high levels of FBSB meal and FB inclusion in diets cause increases in FCR and decreases in food intake, indicative of a decrease in digestibility, conversion of food into body reserves and palatability of feed (Gaber 2006; Ouraji *et al*. 2013). It has been suggested that fish might be able to build muscle with different amounts of plant meals in their diets, but are unable to put on fat, as indicated by lower fat stores seen in fish fed higher FBSB meal diets (Gaber 2006; Ouraji *et al*. 2013). Furthermore, since feed intake tends to be lower in the higher inclusion diets, fish are likely using their endogenous fat reserves instead of exogenous feedstuffs to maintain basal energy requirements.

Lack of plant material digestibility could be another factor causing inhibition of growth and decrease in gross feed intake in fish fed high plant meal diets. Atlantic cod fed SB meal (SBM)-supplemented diets have reduced dietary amino acid, lipid and starch digestibility, indicating that the gastrointestinal tract of the carnivorous cod may not be able to adapt to high plant meal-based diets (Refstie *et al*. 2006). Similarly, Nile tilapia fed high levels of FB meal-supplemented diets have lower apparent digestibility coefficients (ADC) for dry matter, protein and energy compared with control fish fed with fish meal diets, indicating that a high plant content in the diet does impair feed

digestibility, lowering nutrient availability and leading to decreased growth rates (Gaber 2006).

3.4.3. Effects of camelina meal-supplemented diets on appetite-related transcript expression in Atlantic cod brain regions

CM supplemented diets caused concomitant increases in expression of transcripts encoding all orexigenic peptides in this study. Few studies have examined the effects of plant-based diets on food intake related hormones and/or their transcripts.

The high CM inclusion diet (30%) triggered an increase in *pmch* transcript compared to the 15% CM, but not control, diet expression in the cod optic tectum/thalamus. Previous studies have shown that food deprivation causes increases in *pmch* mRNA expression in brain appetite regulating centres in Atlantic cod (Tuziak and Volkoff 2013 – Chapter 2), winter flounder (Tuziak and Volkoff 2012), starry flounder (*Platichthys stellatus*) (Kang and Kim 2013), barfin flounder (Takahashi *et al*. 2004), and zebrafish (Berman *et al*. 2009). Although cod receiving the 30% CM diet were not starved, food intake of these fish was significantly lower compared to both the control and 15% CM diets, suggesting that fish fed the 30% CM diet were still "hungry". Although fish were fed to satiation, the 30% CM feed may not be providing adequate nutrients and energy to maintain satiety (feeling "full") until the time of sampling. Previous studies in fish have shown that changes in *pmch* mRNA expression with respect to feeding usually occur in the hypothalamus (Berman *et al*. 2009; Tuziak and Volkoff 2012; Tuziak and Volkoff 2013 – Chapter 2). Interestingly, in our study,

only optic tectum/thalamus *pmch* transcript levels changed significantly between fish fed the each of the CM diets, but not between the CM diets and the control, which might suggest that distinct regions of the brain react differently to various levels of fasting. The optic tectum is known to regulate food-searching behaviours in fish and, in goldfish, a high number of MCH-ir fibres are detected in this region (Huesa *et al*. 2005). Therefore, elevated *pmch* mRNA expression in the cod optic tectum could also be partially linked to an increase in food searching behaviour - as indicated by reduced food consumption – as fish continue to search for more favourable/palatable food stuffs.

Atlantic cod hypothalamic *hcrt* transcript expression was 14.95-fold higher in fish fed the 30% CM diet compared with fish fed the control fish meal diet. Previous studies show that Atlantic cod fed low food rations (*i.e.* food-restricted fish) display an increase in *hcrt* levels compared with fish fed high food rations (*i.e.* fish fed to satiation) suggesting that *hcrt* mRNA expression is affected by different feeding regimens (Xu and Volkoff 2007). Similar increases in *hcrt* expression following food deprivations have been demonstrated in other fish species (MacDonald and Volkoff 2010; Amiya *et al*. 2012; Wall and Volkoff 2013). High *hcrt* mRNA levels and decreased food intake in fish fed the 30% CM diet suggest that these fish were not satiated. In both fish and mammals, high *hcrt* transcript expression levels in the hypothalamus are correlated with high activity (*i.e.* increases in locomotion), indicating that HCRT may be involved in locomotion (Volkoff *et al*. 1999; Martins *et al*. 2004). As previously mentioned, high *pmch* transcript levels in the brain could imply heightened food seeking behaviours and thus increased swimming activity would be expected. Concomitant up-regulation of *pmch* and *hcrt* mRNA expression could indicate that fish are searching for more palatable food

and not eating the provided CM pellets.

A 7.71-fold increase in hypothalamic *npy* mRNA expression was seen in fish fed the 15% CM diet compared with the cod fed the fish meal control feed. Several reasons could account for the high *npy* transcript levels:

First, as for *pmch* and *hcrt*, the fish might not have been fed to satiation and still be "hungry", as gross food intake declined in both the 15% CM and 30% CM diets compared to the control fish meal diet. This is consistent with the high brain *npy* mRNA expression observed in many fish species following food deprivation (Narnaware and Peter 2001; Kehoe and Volkoff 2007; MacDonald and Volkoff 2009b; MacDonald and Volkoff 2009a).

Secondly, the increase in *npy* mRNA expression in fish fed the 15% CM diet might be due to the role of *npy* in the modulation of fatty acid metabolism. Recent studies show that Atlantic cod fed camelina oil supplemented diets convert most ALA into long chain fatty acids in peripheral tissues with some oxidation of extra ALA (Morais *et al*. 2012; Hixson and Parrish 2014). Camelina contains high levels of medium chain fatty acids, including ALA, and *npy* may be up-regulated in fish fed CM to convert ALA into longer chain fatty acids (*i.e.* 20:3ω3). In orange-spotted grouper, hypothalamic *npy* mRNA expression has been shown to stimulate carnitine palmitoyltransferase 1a (*cpt1a*) transcript expression (Tang *et al*. 2013). In mammals, *cpt1a* is an enzyme responsible for moving fatty acids, such as malonyl-CoA, into mitochondria for β-oxidation. Malonyl-CoA is thought to be a key satiety factor that inhibits *cpt1a* and is involved in fatty acid elongation (Lane *et al*. 2008; Lopaschuk *et al*. 2010). The conversion of malonyl-CoA

into longer chain fatty acids, such as ALA, by *cpt1a* and other downstream enzymes, as well as the β-oxidation of fatty acids could have *npy* regulatory inputs and both mechanisms (elongation and oxidation) are possibly occurring in CM fed fish.

Lastly, increased *npy* mRNA expression in fish hypothalami fed the 15% CM diet may be due to nutritional imbalances of essential amino acids, in particular lysine and arginine, which are known to affect food intake in fish. Lysine is important for β-oxidation of long-chain fatty acids and has been shown to be an appetite stimulant (Walton *et al*. 1984; Berge *et al*. 2002; Harpaz 2005), while arginine is involved in endocrine signalling pathways (Jobgen *et al*. 2006; Yao *et al*. 2008) and can be a feeding deterrent in fish feeds (Adron and Mackie 1978; Papatryphon and Soares 2000). In cobia (*Rachycentron canadum*), fish fed low lysine to arginine diets (ratio: 0.8) have reduced food intake and growth after one week of feeding compared to fish fed commercial pellet diets (lysine to arginine ratio: 1.5) (Nguyen *et al*. 2013). Changes in amino acid composition in diets also affect transcription of *npy*: post-prandial decreases in *npy* mRNA expression are seen in control (ratio of lysine to arginine: 1.0) and high lysine to arginine (ratio: 1.8) fish but not low lysine to arginine fish, suggesting that the amino acid composition of diets might affect how fish sense satiation and the expression of appetite regulators, such as *npy* (Nguyen *et al*. 2013). Since camelina has a low lysine to arginine ratio (lysine % = 4.95. arginine % = 8.15, ratio = 0.61) (Zubr 1997), the reduction in food intake seen in fish fed diets rich in camelina meal might be due to a feeding deterrent effect of high arginine levels or the ability of the fish to sense a poor nutritional feed. Lysine supplementation might be necessary in camelina diets to stimulate feeding in cod

and decrease amino acid imbalances.

No differences in *cart* mRNA expression were seen between control and camelina diets fed Atlantic cod. Since appetite-stimulating factors are up-regulated in fish fed CM diets - possibly an indication of hunger - there is perhaps no need for *cart* to be produced in higher quantities in fish fed these diets. In cobia, neither food intake nor periprandial cholecystokinin (*cck*) - another potent anorexigenic peptide - mRNA expression are influenced by plant diets compared to fish meal food, suggesting that the mRNA expression of anorexigenic peptides is being "shut-down" or a response may only be detected at the translational or post-translational level (Nguyen *et al*. 2013). Although fish were fed to satiation, no changes in *cart* transcript expression further suggest that cod were still "hungry" at the time of sampling following CM-supplemented meals.

3.5. Conclusions

Atlantic cod do not appear to fully tolerate CM-supplemented diets at the levels used in the current study. Growth and feed intake are markedly reduced in cod fed 15% and 30% CM diets compared to the fish fed a control meal, which is potentially caused by reduced feed palatability and/or anti-nutritional factors. Orexigenic peptide mRNA expression levels are affected by CM diets. *pmch* and *hcrt* transcript expressions are increased in the optic tectum/thalamus and hypothalamus, respectively, in cod fed 30% CM compared with the 15% CM diet, but not the control for *pmch* and both diets for *hcrt*, suggesting that these peptides play an integral role as hunger signals and possibly in food seeking behaviours. Our data suggest that *npy* plays a role in food intake regulation in

Atlantic cod, however other functions may include modulation of long chain fatty acid synthesis and sensing of feed with poor nutritional value (*i.e.* amino acid imbalances). *cart* mRNA expression is not affected by CM-supplemented diets in Atlantic cod suggesting that CART is not affected by plant-supplemented diets or modifications are occurring solely at the protein level. The increase in expression of orexigenic factors and no differences in *cart* mRNA may indicate that cod are still "hungry". CM-supplemented diets may be useful in the development of a sustainable cod aquaculture industry, however further research is needed to determine the maximal CM-inclusion percentage - 15% CM appears to be too high based on the results in this study - and which factors in the CM diet (*i.e.* anti-nutritional factors, amino acids, palatability, digestibility) cause detrimental changes in growth and reductions in food consumption as well as changes in the brain expression levels of transcripts encoding appetite-related hormones.

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Chapter 4: Effects of background colour on feeding, locomotion and the expression of appetite-related hormones in Atlantic cod (*Gadus morhua*)

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Abstract

This study examines how feeding, responses to fasting and locomotion, as well as the brain mRNA expression of some important hormones involved in food intake [melanin-concentrating hormone (MCH; gene: *mch*), orexin (OX; gene: *ox*), gonadotrophin-releasing hormones (GnRH; genes: *gnrh2* and *gnrh3*)], are affected by background colour in juvenile Atlantic cod (*Gadus morhua*). Fish were held in pairs under constant light in 40 L glass aquaria with either black or white backgrounds for 10 days with half of the tanks fed and half fasted. Background colour did not affect either locomotor or feeding behaviour, or the forebrain transcript expression of any of the hormones examined. Fasting induced reductions in locomotion in both backgrounds and in skin colouration (*i.e.* fasted fish were paler than fed fish) in white backgrounds. The brain mRNA expression levels of both *mch* and *ox* were lower and those of *gnrh*3 were higher in fasted fish compared to fed fish held in both backgrounds, whereas *gnrh*2 transcript expression was not affected by fasting. Our results suggest that background colour has little effect on feeding and locomotion and the response to fasting in juvenile Atlantic cod, whereas fasting has more pronounced effects, as it reduces activity, induces skin paleness and affects transcript expression levels of *mch*, *ox* and *gnrh*.

4.1. Introduction

Feeding in fish, as in all vertebrates, is regulated by a number of hormones, which include melanin-concentrating hormone (MCH; gene: *mch*), orexin (OX; gene: *ox*) and gonadotrophin-releasing hormones (GnRHs; gene: *gnrh2* and *gnrh3*). MCH, first identified in fish as a key factor involved in skin colour adaptation through aggregation of melanosomes (Fujii and Oshima 1986; Kawauchi *et al*. 1983), has been shown to act as an orexigenic (appetite-stimulating) hormone in mammals (Qu *et al*. 1996), but its role in regulating feeding in fish appears to be variable and species-specific (Matsuda *et al*. 2009; Shimakura *et al*. 2008; Tuziak and Volkoff 2012). In both mammals (Tsujino and Sakurai 2013) and fish (Appelbaum *et al*. 2009; Volkoff *et al*. 1999; Yokobori *et al*. 2011; Yokogawa *et al*. 2007), OX acts as an orexigenic peptide and is involved in the regulation of locomotor activity and sleep-wake cycles. Although, in both mammals (Millar 2005) and fish (Zohar *et al*. 2010), the primary function of the GnRH family of peptides is the control of reproduction, these peptides have also been shown to regulate feeding. To date, fourteen different variants of the GnRH peptide have been identified in vertebrates, eight of which are found in teleost fish (van der Kraak 2009). Diploid fish genomes generally include three *gnrh* forms (*gnrh1*, *gnrh2* and *gnrh3*). Among the GnRH forms, GnRH2 appears to be the form linking reproduction with energy status (Temple *et al*. 2003). For example, in female shrews, food deprivation reduces both GnRH2 protein levels and reproductive activity (Kauffman *et al*. 2006). Similarly, central administration of GnRH2 inhibits feeding in both goldfish (*Carassius auratus)* (Hoskins *et al*. 2008;

Matsuda *et al*. 2008) and zebrafish (*Danio rerio*) (Nishiguchi *et al*. 2012), and fasting decreases *gnrh*2 brain expression in zebrafish (Nishiguchi *et al*. 2012), winter flounder (*Pseudopleuronectes americanus*) (Tuziak and Volkoff 2013a) and Ya fish (*Schizothorax prenanti*) (Wang *et al*. 2014).

Feeding is a crucial aspect of aquaculture, as feeds are expensive and food wastage increases production costs. However, the mechanisms regulating feeding behaviour and appetite in most farmed fish are still largely unknown and optimization of feeding and growth in fish is often achieved by empirical choices of feeding parameters [*e.g.* feeding times and frequencies (Charles *et al*. 1984; Cui *et al*. 1997; Lee *et al*. 2000; Riche *et al*. 2004; Zhou *et al*. 2003)] and/or environmental rearing conditions [*e.g.* tank colour (Jentoft *et al*. 2006; Kang and Kim 2013b; Martinez-Cardenas and Purser 2007; Yamanome *et al*. 2005) and photoperiod (Taranger *et al*. 2006; Veras *et al*. 2013)]. Background colour can play a key role in fish feeding behaviour. However, to date, few fish species have been examined with regards to the effects of background colour on feeding, growth and appetite-related hormones. Exposure to light backgrounds increases food intake and growth in barfin flounder (*Verasper moseri*) (Sunuma *et al*. 2009), Eurasian perch (*Perca fluviatilis*) (Strand *et al*. 2007) and rainbow trout (*Oncorhynchus mykiss*) (Papoutsoglou *et al*. 2005) and induces increases and decreases in hypothalamic *mch* and *gnrh2* mRNA abundances, respectively, in barfin flounder, suggesting an effect of background on feeding and appetite endocrine regulators (Amiya *et al*. 2008)

Although species-specific differences exist, in fish, food deprivation is usually accompanied by increases in the mRNA expressions of orexigenic hormones such as *mch*

and *ox* [*e.g.* winter flounder (Buckley *et al*. 2010; Tuziak and Volkoff 2012), barfin flounder (Amiya *et al*. 2012), Atlantic cod (*Gadus morhua*) (Tuziak and Volkoff 2013b – Chapter 2), Mexican blind cavefish (*Astyanax fasciatus mexicanus*) (Wall and Volkoff 2013), goldfish (Nakamachi *et al*. 2006) and zebrafish (Berman *et al*. 2009)], and a down-regulation of anorexigenic (appetite-inhibiting) hormones such as *gnrh* [*e.g.* in winter flounder (Tuziak and Volkoff 2013a – Chapter 2) and the African cichlid *Astatotilapia burtoni* (Grone *et al*. 2012)].

Atlantic cod are cold water, demersal fish of commercial interest for both fisheries and aquaculture. In order to determine if background colour affects feeding in cod, the response to fasting, locomotion, as well as the expression transcripts of *mch*, *ox* and the active *gnrh* forms present in cod [*gnrh2* and *gnrh3* (Hildahl *et al*. 2011)], fed and fasted juvenile fish were exposed to either white or black backgrounds for 10 days. The effects of background colour on skin colour were also assessed.

In the wild, cod are a demersal fish that are known to feed in both light and dark habitats. Although cod live in "dark" environments with less light penetration, they also exhibit periodical vertical migrations following pelagic prey items, moving up in the water column into "lighter" environments (Le Bris et al., 2013; Strand and Huse, 2007). In aquaculture settings, cod are typically reared in dark (usually green) tanks, and it could be expected that cultured fish used in this study might feed better in " familiar" darker backgrounds than in lighter environments, which might be novel and stressful to the animals.

It might also be expected that food deprived cod in both backgrounds will

decrease locomotion compared to fed cod in order to preserve energy, since, in general, fasted fish reduce energy expenditure to balance the lack of energy intake (Ware, 1975).

Finally, previous studies in Atlantic cod indicate that both *mch* and *ox* are appetite stimulators (Xu and Volkoff, 2007; Tuziak and Volkoff, 2013b – Chapter 2), while *gnrh* might not be directly involved in food intake regulation (Tuziak and Volkoff, 2013b – Chapter 2). Therefore, it could be expected that both *mch* and *ox* transcripts will be up-regulated in fasted fish compared with fed fish, while no differences in *gnrh* mRNAs will be observed. Furthermore, since light backgrounds have previously been shown to up regulate *mch* mRNA expression in flounder (Amiya et al., 2008), the fasting-induced increase in *mch* expression might be more pronounced in fasted fish exposed to light backgrounds. Similarly, dark backgrounds down regulate *gnrh* mRNA expression in barfin flounder (Amiya et al., 2008), suggesting that a decrease in *gnrh* transcript expression might be observed in cod exposed to dark background compared to light backgrounds with a possibly more pronounced down-regulation in fed fish.

We also assessed the effects of background colour on skin palor, which is important to determine whether changes in *mch*, *ox* and *gnrh* transcripts are directly correlated to skin colour change. *mch* is well known for its regulation of skin colour, in particular through the concentration of melanosomes, leading to the lightening of skin (Kawauchi et al., 1983), which is most apparent during exposure to light backgrounds (Suzuki et al., 1995). It could thus be expected that cod will have lighter skin colour and higher *mch* transcript expression during light background exposure compared to dark backgrounds, while *gnrh* mRNA expression will be reduced in light backgrounds, as seen

in barfin flounder where *gnrh* protein expression demonstrates the opposite of *mch*. Presently, the effects of background colour on *ox* transcript expression have not been examined in fish.

4.2. Methods

4.2.1. Animals

Juvenile Atlantic cod were cultured at the St. Andrews Biological Station (SABS, St. Andrews, New Brunswick, Canada). Stock fish were maintained in 450 L flow-through seawater tanks at a stocking density of 500 cod per tank with an average temperature of 12.0°C (range: 10 - 15 °C), dissolved oxygen concentration of 103% and a 16h light: 8h dark constant photoperiod. Fish were fed 2 mm commercial Europa cod pellets (Skretting, Vancouver, BC, Canada) containing 51% protein, 21% fat and 9% crude carbohydrate twice daily (08:30 and 19:30) to satiation. These times were chosen so that the fish were fed in the morning and in the evening with sufficient time left between feedings to ensure that fish were hungry at the times of feeding. Animals were maintained and sacrificed in accordance with animal care protocols approved by the Memorial University of Newfoundland Animal Care Committee following the guidelines of the Canadian Council on Animal Care.

For the experiment, 56 cod [average standard length (SL) = 9.38 ± 0.82 cm; initial

mass = 6.92 ± 1.83 g] were randomly selected from the stock tanks. Sex ratios could not be determined since gonads are not visible in juvenile fish. Pairs of fish from the stock tank were transferred into a total of 28 flow-through (rate: 800 mL min^{-1}) 40 L glass aquaria. Each aquarium had either a black (*N*= 28 fish, 14 pairs in 14 tanks) or white (*N*= 28 fish, 14 pairs in 14 tanks) background covering each vertical side, leaving the bottom uncovered to mimic the natural environment (brown painted shelf). Two fish were used per tank as Atlantic cod are social feeders and fish alone in a tank might become stressed (*i.e.* no food consumption, hiding in a corner of the tank, sporadic movements; S.M.T., H.V., personal observations). Since juvenile cod were too small for external tagging, slightly different sizes or body forms or pigmentations were used to distinguish between fish in a single tank. Water temperature was 15.0 ± 0.20 °C. A 24 h light cycle (average light intensity was 195.7 ± 9.7 lux using daylight-spectrum fluorescent lights) was used based on previous studies showing that Atlantic cod grow quicker and, possibly feed more under these photoperiod conditions (Puvanendran and Brown 2002; Taranger *et al*. 2006). Furthermore, as *gnrh* [*e.g.* grass puffer (*Takifugu niphobles)* (Ando *et al*. 2014); orange-spotted grouper (*Epinephelus coioides*) (Chai *et al*. 2013)], *mch* [*e.g.* rainbow trout (Lyon and Baker 1993)] and *ox* [cod (Hoskins and Volkoff 2012a); goldfish (Hoskins and Volkoff 2012b; Nisembaum *et al*. 2014)] have been shown to display diel patterns in expression linked to photoperiod, the use of constant light ensured that sampling was not performed at a trough or a peak of expression/synthesis.

All fish were acclimated for 10 days under these conditions. During the 10-day acclimation, fish were fed twice daily (08:30 and 19:30). Satiation was determined when

fish stopped consuming pellets and started mouthing and spitting out pellets 8-10 times consecutively. Feeding until satiation lasted on average 291 ± 6 s.

Following the acclimation period, fish in black or white backgrounds were randomly either fasted (*N*= 28 fish, 14 groups of 2 fish per tank, 7 fasted with white backgrounds, 7 fasted with black backgrounds) or fed twice a day (between 08:30 and 9:30 and between 19:30 and 20:30, as feeding all the tanks took about 1 h) to satiation (*N*= 28 fish, 14 groups of 2 fish per tank, 7 fed with white backgrounds, 7 fed with black backgrounds), for 10 days.

Every day, for each feeding time, the number of pellets consumed per fish was recorded for fed fish, as well as the time spent feeding. Feed intake (g food per g of fish) was calculated by multiplying the number of pellets eaten by the mass of a pellet (8 mg) and dividing this number by the mass of the fish [food intake (g food/g fish) = number of pellets eaten * 0.008/ mass of fish].

Swimming activity was measured for two minutes in both fed and fasted fish twice daily, 1 h prior to the scheduled feeding times, by counting the number of times a fish swam across a line dividing tanks in half width-wise using tape adhered to the bottom. Preliminary analyses (S.M.T., personal observation) showed that fish within a tank displayed a random swimming behaviour (*i.e.* in all directions, with no preference for either the width-wise or length-wise axis), suggesting that a single width-wise division of the tank accurately depicts activity levels. Swimming activity assessments were performed before feeding to avoid a possible bias induced by variations in activity between fed and fasted fish, as feeding often induces hyperactivity (or food anticipatory

activity, FAA) (Davis and Bardach 1965). All recordings were done manually, without a video recorder.

The final recordings were completed the morning of the 11th day. Fish were killed with an overdose of MS-222 (TMS, tricaine methanesulfonate, Syndel Laboratories, Vancouver, BC, Canada) 1 h post-feeding to allow some digestion to occur, as some appetite-related peptides are stimulated or inhibited by digestive processes (Sobrino Crespo *et al*. 2014). Lengths and masses were recorded and brains were dissected and stored in RNAlater at -20°C until further use.

4.2.2. Effects of background colour and feeding status on skin colour

In order to estimate skin colour changes, each sampled fish was photographed with a Pentax S20 digital camera (Ricoh Imaging Company, Tokyo, Japan) affixed to a stationary holder. Images were converted to grey scale using Adobe Photoshop 4 software (Adobe, San Jose, CA, USA) and mean "greyness" was determined using the "Analyze → Measure" function in ImageJ software (Schneider *et al*. 2012), which determines the mean pixel grey value of a selected area: high values correspond to a "darker" colouration (*i.e.* 100), whereas lower numbers represent a "lighter" fish (*i.e.* 0).

A polygon using specific anatomical landmarks (eyes, fins, tail) was used to ensure that measurements were consistent among fish [Figure 4.1(a-d)]: lines were drawn between several points located on the dorsal surface of the fish just behind the eye, the ventral surface of the fish just behind the eyes, the most anterior point of the pelvic fin,

the most posterior point of the anal fin, the anterior portion of the tail, around the tail to the most posterior area of the second dorsal fin, and the most anterior point of the first dorsal fin. The anterior of the head, including the eyes, and the fins, were excluded to avoid any bias from the eye, shadows on the head and translucency of the fins.

4.2.3. RNA extraction and cDNA synthesis

Brains were dissected into forebrain (telencephalon/preoptic area, optic tectum/thalamus and hypothalamus) and hindbrain (cerebellum, medulla oblongata and spinal cord) regions, as described previously (Tuziak and Volkoff 2013b – Chapter 2). Whole forebrains were used in the transcript expression analyses as brains were small and using distinct brain regions was not expected to provide enough RNA for analyses. In addition, appetite-related hormones have previously been shown to be mostly expressed in cod forebrain (Kehoe and Volkoff 2007; Tuziak *et al*. 2014 – Chapter 3; Tuziak and Volkoff 2013b – Chapter 2; Volkoff *et al*. 2009; Xu and Volkoff 2007). Total RNA was extracted using the TRI reagent/chloroform (BioShop, Burlington, ON, Canada) method as per the manufacturer's instructions. Final RNA concentrations, 260/280 (> 1.8) and 260/230 (> 1.8) ratios were obtained using a Nanodrop spectrophotometer (Thermo-

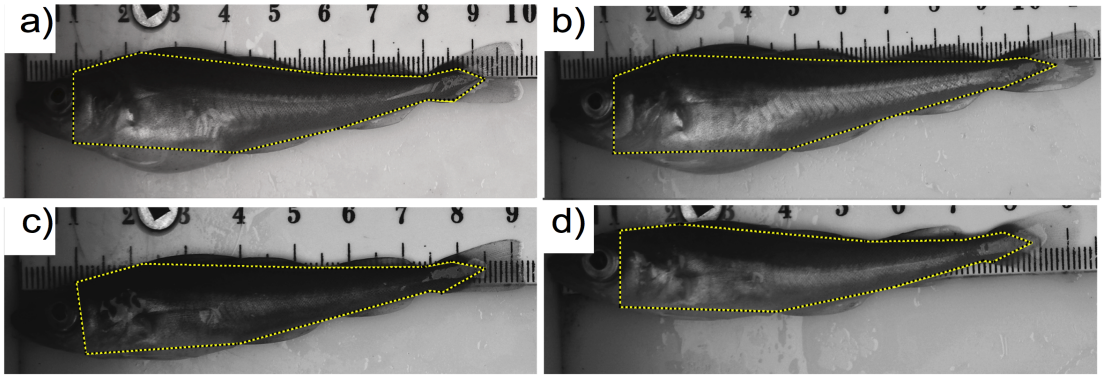


Figure 4.1. Examples of photos used to determine skin colour (mean greyness) differences between cod with different feeding status (**a** and **b**: fed ; **c** and **d:** fasted) and background colour adaptation (**b** and **d**: white**, a** and **c:** black). Polygons represent area used to determine skin colour for each fish.

Fisher Scientific, Waltham, MA, USA). RNAs were also run on 1.15% agarose gel electrophoresis to assess their quality. Samples were then DNase-treated (Sigma, St. Louis, MI, USA) and column purified using the GeneJET RNA clean up and concentration micro kit (Thermo-Fisher Scientific) as per the manufacturer's protocol. Sample quality was then re-analyzed with the Nanodrop and run on gel electrophoresis, as previously described, to ensure these procedures did not cause any RNA degradation.

cDNA synthesis was completed with the Superscript II cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) using 1 µg total purified RNA and 250 ng random primers (Promega, Fitchburg, WI, USA) as per the manufacturer's protocol, with the exception that the RNaseOUT step was removed since > 50 ng μl^{-1} of RNA was used.

4.2.4. Quantitative real-time RT-PCR (qPCR)

qPCR was used to quantify the forebrain transcript expressions of *mch*, *ox*, *gnrh2* and *gnrh3*. Ubiquitin A-52 residue ribosomal protein fusion product 1 (*uba52*) was used as a housekeeping gene (HKG) as its expression has previously been shown to be stable in the brain of cod submitted to different feeding statuses or diets (Tuziak *et al*. 2014 – Chapter 3, Tuziak and Volkoff 2013b – Chapter 2). Primers for *mch* (Ensembl: **ENSGMOT00000010147**), *gnrh2* (GenBank accession no. **GU332294**) and *gnrh3* (GenBank accession no. **GU332295**) were designed in two separate exons spanning an intron to avoid genomic DNA amplification, using previously annotated cod sequences. Primers for *uba52* were obtained from Olsvik *et al*. (2008) (Table 4.1). All

Table 4.1. Atlantic cod (*Gadus morhua)* primer sequences and associated transcripts used for quantitative real-time polymerase chain reaction (qPCR).

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Ubiquitin (*uba52*)	GGC CGC AAA GAT GCA GAT	CTG GGC TCG ACC TCA AGA GT
Melanin-concentrating hormone (*mch)*	GAC CGA GGG CTG TCC TAC TAC	CTC ATG GTG TCC CGC TTG
Orexin (*ox*)	CAC AGT GTC CCA AAC ACC TG	CTG TGA GCG TCG CAG AGC
Gonadotrophin-releasing hormone 2 (*gnrh2*)	GTC TCA TGG CTG GTA CCC TGG	GCC TCA GGT AGC TAC ACT CC
Gonadotrophin-releasing hormone 3 (*gnrh3*)	CCA TAC AGT CTG GTC AAC GACG	CAA TCG ACC AAT GTG GGT GTT CAC

primers were obtained from Eurofins MWG Operon (Huntsville, AL, USA). Primer optimization was conducted with 1:2 cDNA dilution 6-point standard curves using a 10 µl reaction containing 2X Kapa SYBR Fast master mix (Kapa Biosystems, Inc., Woburn, MA, USA), either 2.5 or 1.25 ng cDNA (*ox* and *gnrh3*: 1:2 dilution, *mch* and *gnrh2*: 1:4 dilution) and 10 µM of each forward and reverse primers on an Eppendorf Mastercycler ep *realplex* 2s (Eppendorf, Hamburg, Germany). The qPCR cycling parameters used for primer optimization were: 95°C for 3 min, 95°C for 30 s, 58°C for 15 s, 72°c for 20 s repeated for 40 cycles, followed by a melting curve: 95°C for 15 s, then a temperature ramp for 20 min ranging from 55 - 95°C for 15 s increasing at 0.1°C s^{-1}. All samples were run in triplicate. Primer efficiencies (*uba52*: 0.95, *mch*: 0.92, *ox*: 0.96, *gnrh2*: 0.92, *gnrh3*: 0.95), R^2 values ($0.993 \leq R^2 \leq 0.999$) and single peaks in the melting curve were determined. Once primers passed the quality control measures, qPCRs for each target were run under the same conditions for the experimental samples using either 2.5 ng (*ox* and *gnrh3*) or 1.25 ng (*mch* and *gnrh2*) of cDNA, depending on the standard curve analyses, using *uba52* as the HKG. Since multiple plates were used for a given study (1 HKG + 2 target transcripts), a linker individual sample was used to ensure inter-assay reproducibility was maintained. All samples in a single plate were run in duplicates.

Raw data were $\log_2$ transformed and relative mRNA expression for each individual was analyzed using the Pfaffl (2001) method. The mean C_T and primer efficiencies were calculated using the Realplex software (Eppendorf) and inputted into the following equation to determine relative quantification (RQ) values. The individual fish with the lowest expression was used as the calibrator.

$$\mathrm{RQ} = \frac{\left(E_{\mathrm{ctrl}}\right)^{\mathrm{CTsample}}}{\left(E_{\mathrm{target}}\right)^{\mathrm{CTsample}}} \div \frac{\left(E_{\mathrm{ctrl}}\right)^{\mathrm{CTcalibrator}}}{\left(E_{\mathrm{target}}\right)^{\mathrm{CTcalibrator}}}$$

4.2.5. Statistical analysis

Statistical analyses and figures were completed using GraphPad Prism v6.0c (GraphPad Inc., San Diego, CA, USA). Shapiro-Wilk normality tests were conducted for all data sets.

The effects of background colour on food intake and time spent feeding were assessed for fed fish using Student's unpaired *t*-tests assuming equal standard deviations for both populations. For all fish, two-factor ANOVAs were used to determine the effects of background colour and feeding status on swimming activity, relative transcript expression (*mch*, *ox*, *gnrh2* and *gnrh*3) and skin colour. Post-hoc Tukey HSD tests were run to determine significant differences between treatments (*i.e.* colour:feeding status). Linear regressions were used to identify relationships between swimming activity and skin colour with appetite-related peptide mRNA expression. For all tests, significance was at a *p*-value of 0.05.

4.3. Results

4.3.1. Effects of background colour on feeding and locomotion

The average daily feed consumption (both meals combined) was not affected by background colour (Student's *t*-test, $p > 0.05$, Figure 4.2A). Background colour did not affect time spent feeding (Student's *t*-test, $p > 0.05$, Figure 4.2A).

There were no significant differences in locomotor behaviour between black and white background adapted fish in either fed or fasted animals (two-factor ANOVA, $p > 0.05$, Figure 4.2B). Background affected fasting-induced changes in locomotor behaviour as fasted fish showed a significant reduction in swimming activity compared with fed fish in black backgrounds, but not in white backgrounds (two-factor ANOVA, $p = 0.003$, Tukey HSD: white fed and white fasted $p > 0.05$, white fed and black fed $p > 0.05$, white fasted and black fasted $p > 0.05$, black fed and black fasted $p = 0.03$, Figure 4.2B).

4.3.2. Effects of background colour and feeding status on mRNA expression

Background colour did not affect the transcript expression of any of the appetite regulators examined (two-factor ANOVA, $p > 0.05$, Figures 4.3 A-D). In both white and black background-adapted fish, fasting induced significant decreases in both *mch* and *ox* mRNA expressions (two-factor ANOVA, $p = 0.0243$ and $p = 0.0273$, respectively, Figures 4.3 A,B) and an increase in *gnrh3* mRNA expression (two-factor ANOVA, $p = 0.0203$, Figure 4.3D). No significant differences were seen for *gnrh2* mRNA expression between fed and fasted cod in either background (two-factor ANOVA, $p > 0.05$, Figure 4.3C).

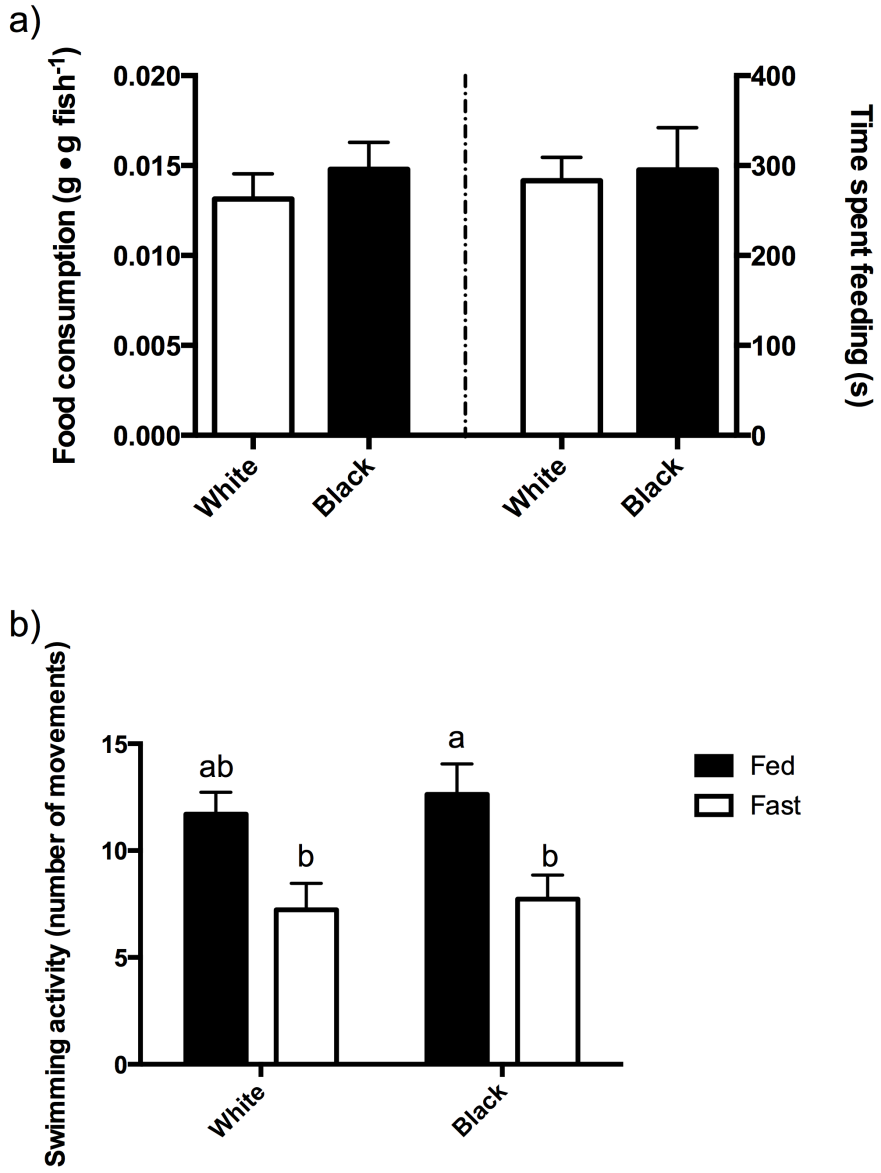


Figure 4.2. Effects of background colour on feed consumption (g • g fish $^{-1}$) and time spent feeding (s) (A), and swimming activity (B) in juvenile Atlantic cod. Significant differences between groups are indicated by an * (Students unpaired *t*-test assuming equal standard deviations, $p < 0.05$) or by different lowercase letters (Tukey HSD, $p < 0.05$). Data are presented as means ± SEM.

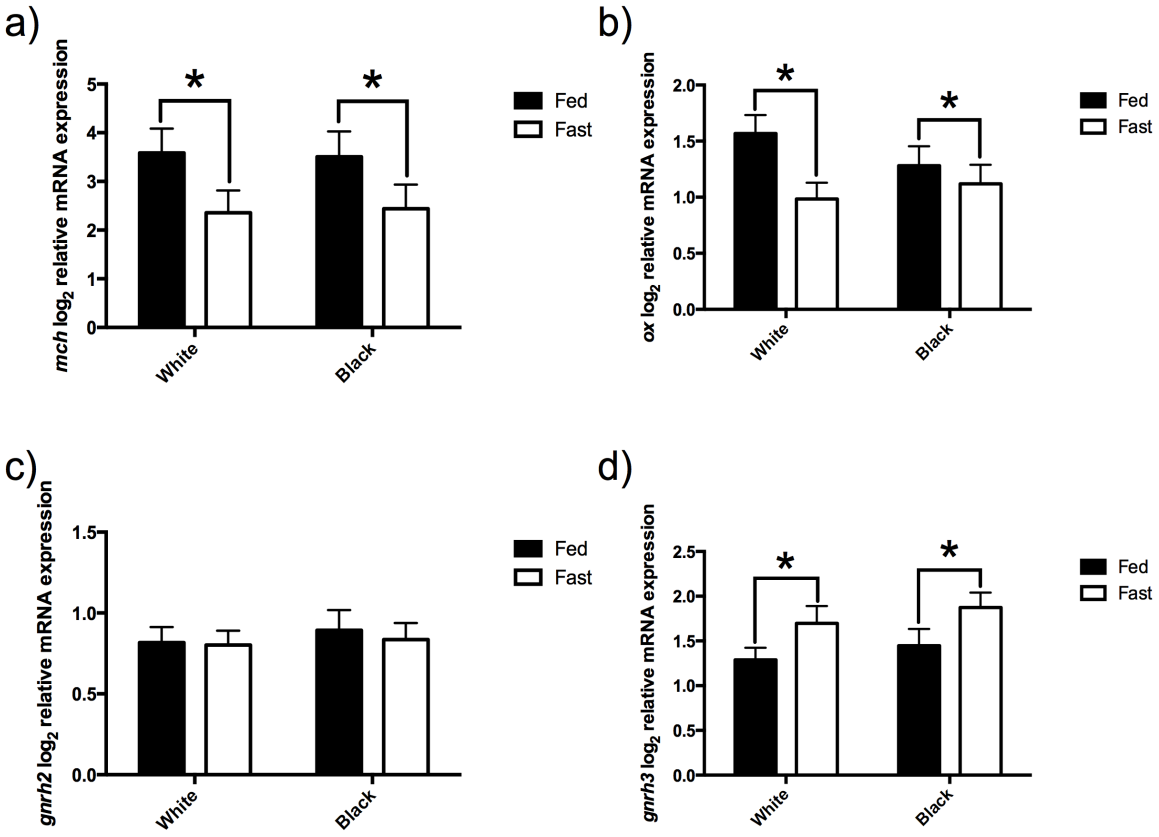


Figure 4.3. Effects of background colour and feeding status on appetite-related transcript expression. All profiles are expressed in $\log_2$ relative to the housekeeping gene (*ubi52*). (A) Melanin-concentrating hormone (*mch*), (B) Orexin (*ox*), (C) Gonadotrophin-releasing hormone 2 (*gnrh2*) and D) *gnrh3*. Significant differences between groups are indicated by an * (Two-factor ANOVA, Tukey HSD post-hoc test, $p < 0.05$). Data are presented as means ± SEM

4.3.3. Effects of background and feeding status on skin colour

Overall, no significant differences in skin colour were detected between white and black background-adapted fish when both fed and fasted treatments were combined (two-factor ANOVA, $p > 0.05$, Figure 4.4E). However, when considering only fed fish, white background-adapted fish were darker than black background-adapted fish (Tukey HSD: white fed and black fed $p = 0.0053$ Figure 4.4E). When considering only fasted fish, there were no significant differences between white background- and black background-adapted fish (Figure 4.4E).

Fasting induced colour changes in white background-adapted fish, as fasted fish were lighter than fed fish, but no differences in skin colour were detected in black background-adapted fish (two-factor ANOVA, $p = 0.0095$, Tukey HSD: white fed and white fasted $p = 0.0004$, white fed and black fed $p = 0.0053$, white fasted and black fasted $p > 0.05$, black fed and black fasted $p > 0.05$, Figure 4.4E).

4.4. Discussion

4.4.1. Effects of background colour on feeding and locomotion in juvenile Atlantic cod

In our study, background colour did not affect food intake or time spent feeding in Atlantic cod. Similar to our results, in juvenile seahorses (*Hippocampus abdominalis)*, tank colour background has no effects on feeding rates (Martinez-Cardenas and Purser

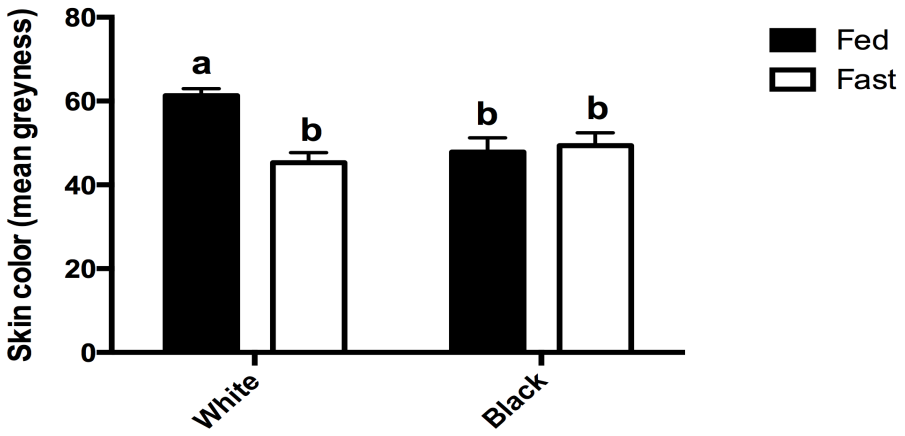


Figure 4.4. Effects of background colour and feeding status on skin colouration in juvenile Atlantic cod. Significant differences between groups are indicated by different lowercase letters (Two-factor ANOVA, Tukey HSD post hoc test, $p < 0.05$). Data are presented as means ± SEM.

2007). However, in juvenile Caspian Kutum (*Rtilus frisii Kutum),* feeding rates of fish kept in black backgrounds are higher than those of fish in clear (yellow) backgrounds (Imanpoor and Abdollahi 2011) and in barfin flounder (Mizusawa *et al*. 2011, Sunuma *et al*. 2009; Takahashi *et al*. 2004) and perch (*Perca fluviatilis)* (Jentoft *et al*. 2006; Strand *et al*. 2007), higher feeding rates are seen under white than black backgrounds. It has been suggested that feeding rates might depend on the ability of fish to see and capture their prey/food and are thus influenced by parameters such as prey density, orientation and intensity of lights, contrast of prey against the background, tank and prey colour (Browman and Marcotte 1987; Jentoft *et al*. 2006; Martinez-Cardenas and Purser 2007; Strand *et al*. 2007). It is possible that the variations in response seen in different studies might be due to species-specific differences in feeding habits and sensory detection of food, or to different experimental protocols (*e.g.* different colour distributions of the background, different light intensities).

Fasting, but not background colour, affected cod locomotor activity, as fasted fish displayed a reduced swimming activity compared with fed fish. Consistent with our results, barfin flounder (Sunuma *et al*. 2009) and Nile tilapia (*Oreochromis niloticus)* (Merighe *et al*. 2004) show no differences in locomotor activity when exposed to different backgrounds. A reduction in swimming activity during fasting is not surprising as fish conserve energy during food depletion (Ware 1975). Our results are consistent with other studies showing that fasting cod display reductions in both swimming speed and metabolism (Bjornsson 1993) and fasting roach (*Rutilus rutilus*) decrease their daily activity levels by 50% (van Dijk *et al*. 2002).

4.4.2. Effects of background colour and feeding status on the expression of appetite-regulators

No differences in transcript expression were detected between fish held in different background colours for any of the appetite regulators examined.

To our knowledge, there are no published reports on the effects of background colour on *ox* mRNA expression. Our results suggest that the orexin system is not affected by background colour.

The lack of effect of background colour on *gnrh* expression in our study contrasts with previous studies showing that in barfin flounder, higher brain *gnrh2* levels are seen in fish held in black tanks than those kept in white tanks (Amiya *et al*. 2008). Also in contrast to our results, previous studies in fish have shown that the MCH system can be affected by environmental colour. For example, white backgrounds significantly increase MCH/*mch* protein plasma levels and brain mRNA expression in barfin flounder (Amiya *et al*. 2005; Amiya *et al*. 2008) and the number of MCH-immunoreactive cells in the brains of barfin flounder (Amiya *et al*. 2005; Amiya *et al*. 2008), tilapia (Groneveld *et al*. 1995) and rainbow trout (Suzuki *et al*. 1995). In addition, goldfish brain *mch* mRNA levels are significantly higher in fish exposed to green or purple lights than in fish exposed to white or red lights (Shin and Choi 2014). It is possible that the lack of effect of background colour on forebrain *mch* mRNA expression in our study might be due to the presence of several *mch* forms in cod. Indeed, some fish [*e.g.* zebrafish (Berman *et al*. 2009) and flatfish (Kang and Kim, 2013a; Tuziak and Volkoff 2012)] display two forms

of *mch* with different expression profiles and putative physiological functions. For example, in zebrafish, hypothalamic *mch1* (equivalent to the cod *mch* examined here) mRNA expression increases in white-adapted compared to black-adapted fish, whereas *mch2* mRNA expression is not affected by background colour (Berman *et al*. 2009).

Another possible reason that no differences in mRNA expression were observed for any of the peptides examined between white and black background-adapted cod is that the variation in background colour may not have been perceived by the fish, as the artificial background was attached to the sides of the tanks and fish were placed in a relatively bright environment. In addition, the exposure time used in the present study might not have been long enough to induce changes in expression. Furthermore, changes in expression might have occurred in specific brain regions, so that the use of whole forebrain in our analyses might have masked local, small changes in expression. Future studies in Atlantic cod using different backgrounds, different light settings and exposure/sampling times, as well as specific brain regions instead of the whole forebrain might reveal changes in expression of feeding-related peptides.

Background had no effect on the fasting-induced response in mRNA expression. In both backgrounds, fasting induced a decrease in both *mch* and *ox* mRNA expressions, and an increase in *gnrh3* mRNA expression, and did not affect *gnrh2* mRNA expression.

The decrease in *mch* mRNA expression levels in fasted fish compared with fed fish contrasts with previous studies showing a fasting-induced increase in *mch* brain expression in Atlantic cod (Tuziak and Volkoff 2013b – Chapter 2) and other fish [zebrafish (Berman *et al*. 2009); winter flounder (Tuziak and Volkoff 2012); starry

flounder (*Platichthys stellatus*) (Kang and Kim 2013a)]. Similarly, a decrease in *ox* mRNA expression in fasted fish contrasts with results in other fish, including the Mexican blind cavefish (Wall and Volkoff 2013), winter flounder (Buckley *et al*. 2010), barfin flounder (Amiya *et al*. 2012) and winter skate (*Leucoraja ocellata*) (MacDonald and Volkoff 2010) for which fasting up-regulates in *ox* transcript expression.

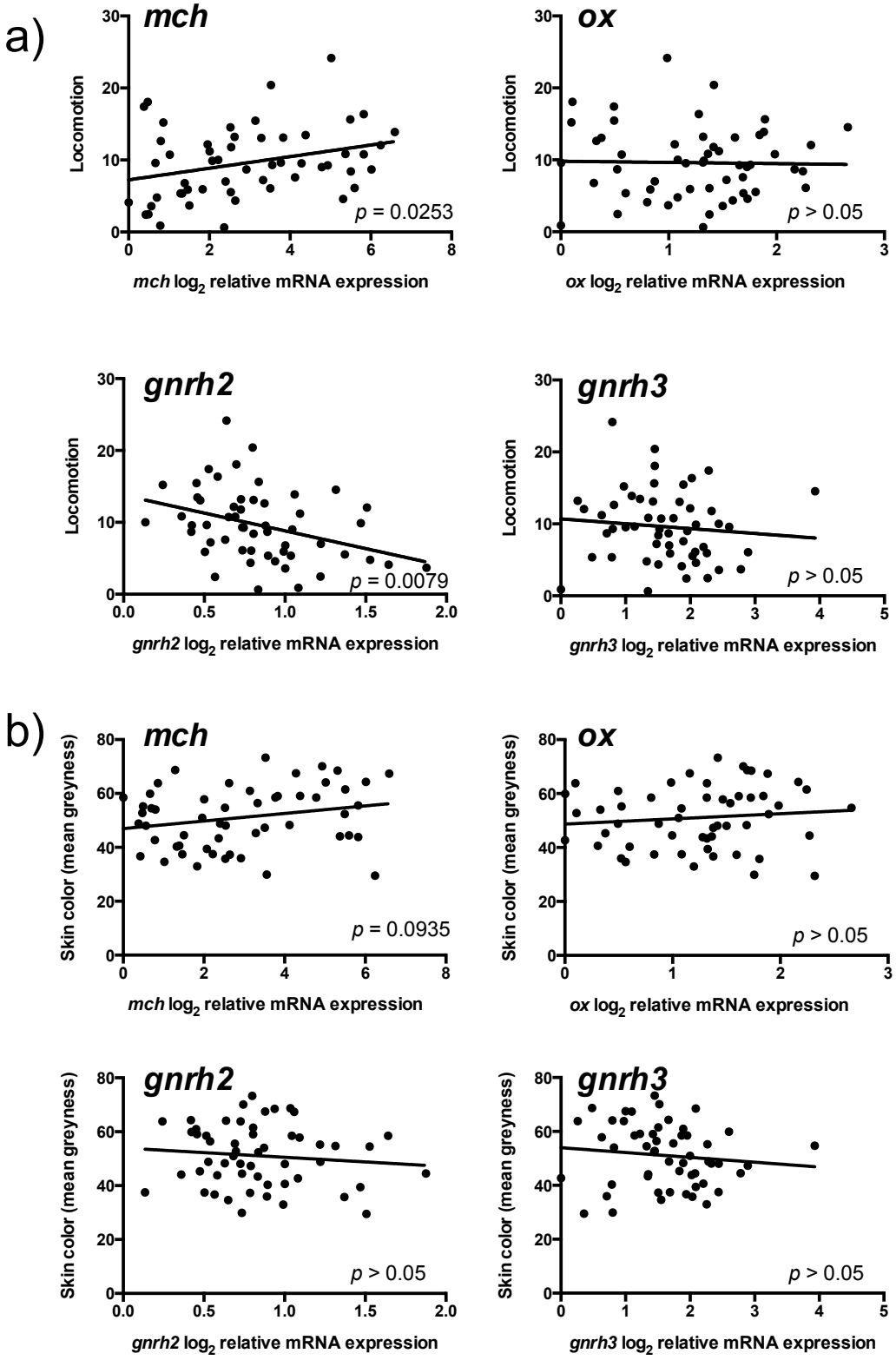
The variation in results among studies might indicate that, in cod, as opposed to most fish, MCH and OX act as anorexigenic factors or, most probably, that feeding regulation might not be the major function of either peptide. This hypothesis is consistent with the fact that in our study, no significant relationships were detected between the amount of food consumed and the expression of either *mch* or *ox* (data not shown). The fact that *mch* mRNA expression is correlated with activity levels (Figure 4.4A) and that fasted fish, with reduced *ox* and *mch* levels, also display low swimming activity levels, suggest that these two peptides might have important roles in the regulation of locomotor activity. Previous studies in mammals (Bonnavion and de Lecea 2010) and fish [*e.g.* zebrafish (Prober *et al*. 2006); goldfish (Volkoff *et al*. 1999; Nakamachi *et al*. 2006); ornate wrasse (*Thalassoma pavo)* (Facciolo *et al*. 2010)] have shown that OX promotes hyperactivity and wakefulness. Reports on the effects of MCH on activity levels are inconsistent among studies: in both rats (Sanchez *et al*. 1997) and goldfish (Matsuda *et al*. 2006), central administration of mch do not appear to affect locomotion, but *mch* deficient (*mch*-/-) mice are hyperactive, albeit only when fasted (Willie *et al*. 2008), and injecting MCH potentiates cocaine-induced hyperactivity in mice (Chung *et al*. 2009).

gnrh3, but not *gnrh2*, mRNA was influenced by feeding status, as higher forebrain

gnrh3 expression levels were seen in fasted compared with fed fish, which contrasts with previous studies in Atlantic cod showing that hypothalamic mRNA expressions of neither *gnrh*s are affected by fasting (Tuziak and Volkoff 2013b – Chapter 2). Also in contrast to our results, *gnrh2* brain expression is affected by fasting in winter flounder (Tuziak and Volkoff 2013a), goldfish (Hoskins *et al*. 2008) and zebrafish (Nishiguchi *et al*. 2012) and in winter flounder, fasting reduces telencephalic *gnrh3* mRNA expression (Tuziak and Volkoff 2013a). Interestingly. *gnrh2*, but not *gnrh3*, expression levels were correlated to locomotor activity (Figure 4.5A), suggesting that GnRHs might influence swimming activity in fish, as seen in salmon, for which treatment with a GnRH significantly enhances swimming activity and jumping motivation (Plate *et al*. 2003).

Overall, the discrepancies in the fasting-induced changes in mRNA expression studies might be due to species-specific responses to fasting, or to different fasting periods used among experiments and time-dependent changes in the response to fasting. For example, in the case of Atlantic cod, the present study used a fasting period of 10 days whereas in Tuziak and Volkoff 2013b (Chapter 2), fish were fasted for two weeks. Furthermore, in Tuziak and Volkoff 2013b (Chapter 2) fish were exposed to 11°C water temperatures, whereas in this study the average water temperature was 15°C. In addition, the use of forebrain for expression analyses in the present study might have masked more punctual changes in expression.

Figure 4.5. A) Overall correlations between transcripts [melanin-concentrating hormone (*mch*), orexin (*ox*), gonadotrophin-releasing hormone 2 (*gnrh2*) and *gnrh3*] and swimming activity in juvenile Atlantic cod. A p-value < 0.05 is used to detect a slope significantly different from zero using a linear regression. B) Relationships between melanin-concentrating hormone (*mch*), orexin (*ox*), gonadotrophin-releasing hormone 2 (*gnrh2*) and *gnrh3* with skin colour in Atlantic cod. A p-value < 0.05 is used to detect a slope significantly different from zero using a linear regression


a)
mch
Locomotion
p = 0.0253
mch log2 relative mRNA expression
ox
Locomotion
p > 0.05
ox log2 relative mRNA expression
gnrh2
Locomotion
p = 0.0079
gnrh2 log2 relative mRNA expression
gnrh3
Locomotion
p > 0.05
gnrh3 log2 relative mRNA expression
b)
mch
Skin color (mean greyness)
p = 0.0935
mch log2 relative mRNA expression
ox
Skin color (mean greyness)
p > 0.05
ox log2 relative mRNA expression
gnrh2
Skin color (mean greyness)
p > 0.05
gnrh2 log2 relative mRNA expression
gnrh3
Skin color (mean greyness)
p > 0.05
gnrh3 log2 relative mRNA expression

4.4.3. Effects of background colour and feeding status on skin colour

Although when *all* fish (fed and fasted) were taken into consideration, no overall differences were observed in skin colour between white and black background-adapted fish, background colour did affect skin colour when fed fish were analyzed separately, as fed fish exposed to a white background were significantly darker than those adapted to black backgrounds. Our results contrast with previous studies in red porgy (*Pagrus pagrus*) (Rotllant *et al*. 2003), guppy (*Poecilia reticulata*) (Rodgers *et al*. 2013) and barfin flounder (Amiya *et al*. 2008), for which fish exposed to white backgrounds become paler than black background-adapted fish. As mentioned previously, it is possible that variations in background colour were not well perceived by the fish, or that exposure times were not long enough to induce changes in skin colour. It is also possible that colouration was affected by a 24h light exposure. Indeed, photoperiod has previously been shown to influence skin colour in animals, including amphibians [e.g. *Bufo ictericus* (Filadelfi *et al*. 2005)] and fish [zebrafish (Shiraki *et al*. 2010)]. Interestingly, in our study, fasted fish were paler than fed fish in white background-adapted fish only. This phenomenon has previously been reported in gilthead sea bream (*Sparus aurata*) for which a discolouration of fish skin is observed after one week of fasting (Grigorakis and Alexis 2005). The mechanisms regulating this fasting-induced colour change are not known.

There were no significant correlations between appetite-related transcript expressions and skin colour (mean greyness; Figure 4.4). In other fish it has been shown

that MCH plays a role in the regulation of colour, as shown in winter flounder (Amiya *et al*. 2005; Mizusawa *et al*. 2011), starry flounder (Kang and Kim 2013a), zebrafish (Berman *et al*. 2009), and rainbow trout (Baker *et al*. 1986) demonstrated through differences in *mch* transcript expression with varied background colour (high *mch* transcript expression in white/light backgrounds compared to low expression in black/dark backgrounds). However, in cod, *mch* mRNA expression was higher in fed/darker fish compared with fasted/pale fish, which is unexpected, given the known function of MCH in skin colour adaptation (*i.e.* concentration of melanin granules resulting in pale skin). The lack of correlation between appetite-related transcripts and skin colour could be a result of high individual variation and other confounding factors (*i.e.* perceived stress and stress-related hormone production interacting with appetite-related hormones).

Conclusions

In conclusion, Atlantic cod feeding and locomotion do not appear to be influenced by background colour overall. Locomotor activity was reduced in fasted fish compared with fed fish perhaps to reduce wastage of energy by routine swimming. *mch* and *gnrh2* mRNAs were related to locomotion, which could indicate that these two peptides regulate activity or vice versa. During fasting, decreases in *mch* and *ox* mRNA expression levels might be linked to decreased swimming activity and high *gnrh3* transcript expression could be linked to external reproductive signals (*i.e.* photoperiod) rather than food intake

regulation. Finally, fasted fish were paler than fed fish which suggests that, although background colour does not directly influence behaviour and appetite-related transcripts when cod are well fed, it may play a greater role in behavioural changes during fasting conditions.

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Chapter 5: General discussion and conclusions

This thesis focused on the molecular and behavioural regulation of food intake in a marine, aquacultured fish, the Atlantic cod. The underlying molecular mechanisms that integrate internal physiological (*i.e.* energy, developmental) and environmental (*i.e.* photoperiod, habitat and prey characteristics) cues with food intake regulation are not yet known in cod and are important for understanding feeding and growth physiology in aquaculture practices.

The overall objective of my thesis was to characterize appetite-related hormones (*i.e.* MCH, orexin, NPY, GnRH and CART) in Atlantic cod and understand how internal and environmental factors influence food intake-related hormone transcript regulation and behaviour in Atlantic cod.

More specifically, I aimed to identify in which Atlantic cod central and peripheral tissues MCH and GnRH are expressed and their pattern of transcript expressions during cod early development. Furthermore, I intended to demonstrate whether background colour, feed type and internal factors (*i.e.* feeding status, life stage) played a role in regulating Atlantic cod food intake behaviour and related hormone transcript expressions.

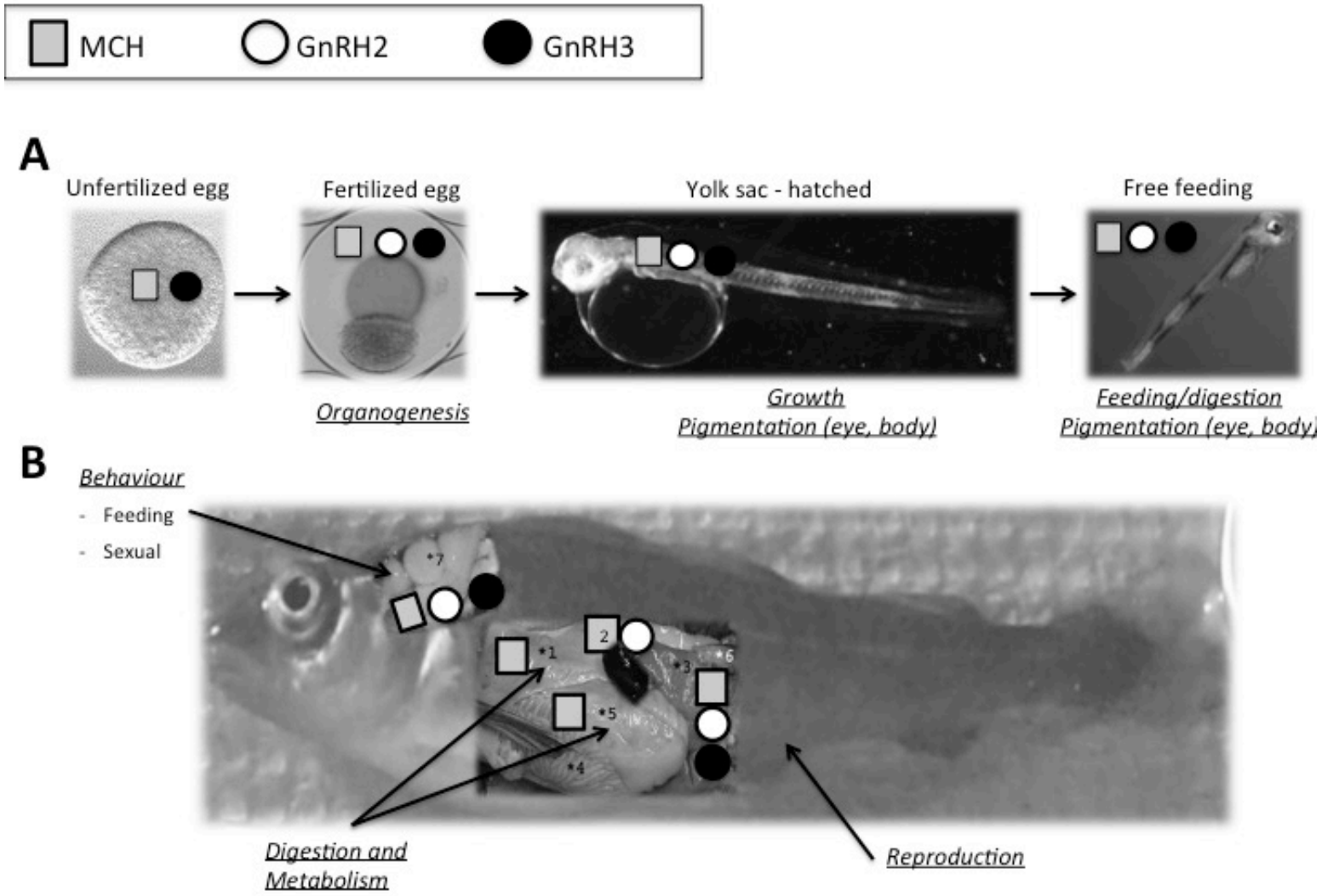
To this end, I examined the mRNA expression levels of various appetite regulators, including melanin-concentrating hormone (MCH), orexin, neuropeptide Y (NPY), gonadotrophin-releasing hormone (GnRH) and cocaine- and amphetamine-regulated transcript (CART) in Atlantic cod (*Gadus morhua*) under the influence of food deprivation (Chapters two and four), plant meal-supplemented diets (*Camelina sativa*

meal; Chapter three) and differing background colours (black versus white; Chapter four). Behavioural indices, such as food intake and swimming activity (Chapters three and four), were assessed as well as growth rates (Chapter three) and skin colour (Chapter four). I also determined when some appetite regulators (MCH and GnRH) "turn-on" during early development and where transcripts are expressed in central and peripheral tissues of adult fish (Chapter two).

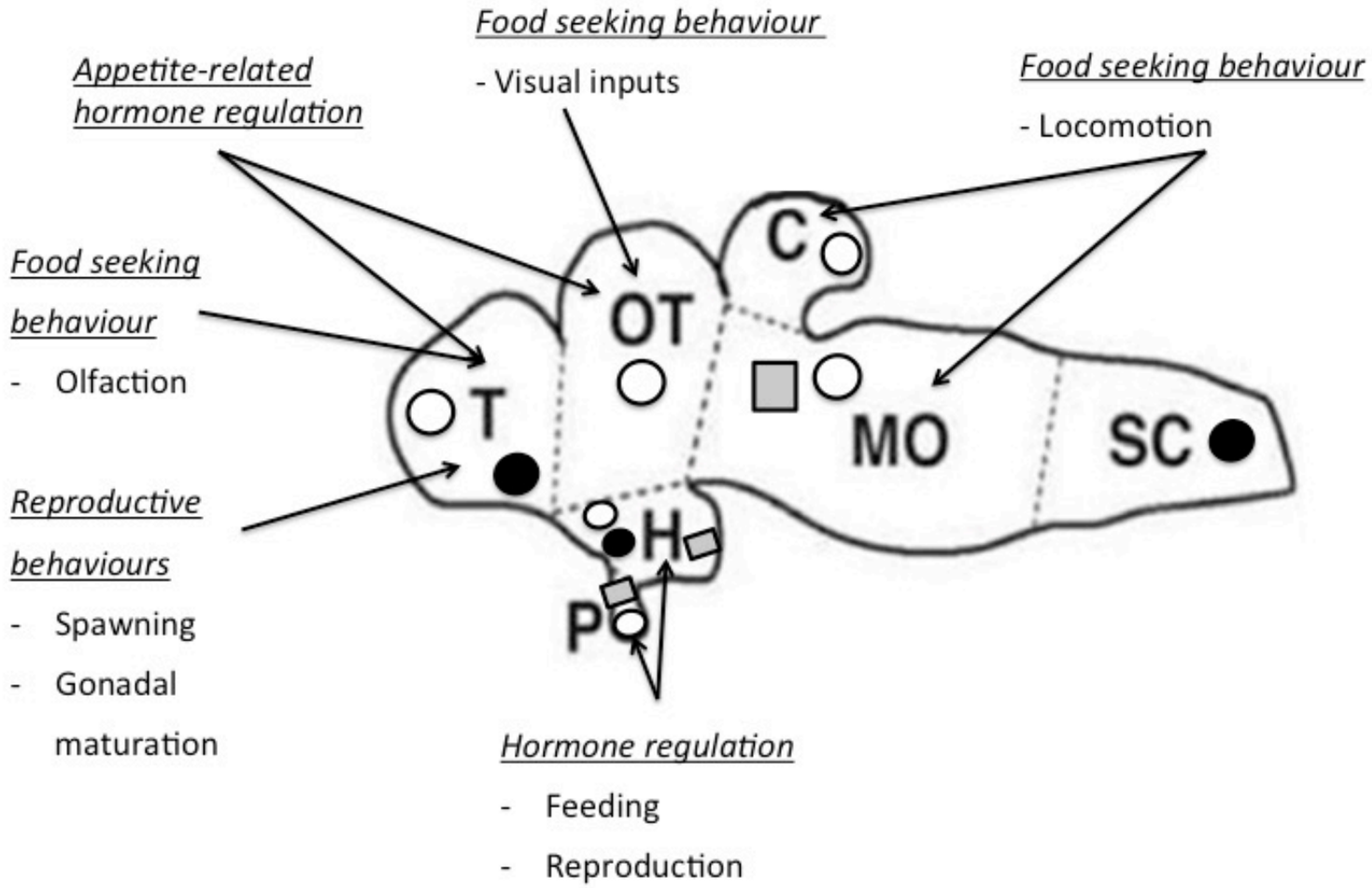
5.1. MCH and GnRH play a role in development and have a widespread distribution in adult fish

Results in this thesis and literature support the notion that MCH and GnRHs play roles in various processes, including, but not limited to, food intake regulation, skin colour adaptation, reproduction and neuromodulation of other hormone systems. In cod, MCH and GnRH3 mRNAs are maternally deposited into eggs, indicative of a neuromodulatory role during early development (Figure 5.1A; Chapter 2). MCH has variable expression throughout early development, while GnRH2 and GnRH3 mRNAs "turn-on" during mid-segmentation during brain development. Although the exact role of GnRH2 is unknown in cod, it likely plays a role as an appetite inhibitor during early development. For both MCH and GnRHs, highest expression appears during the exogenous feeding stages, perhaps supporting their functions as appetite regulators during early larval development.

Figure 5.1. Summary diagrams showing melanin-concentrating hormone (MCH) and gonadotrophin-releasing hormone (GnRH) transcript expressions and possible functions (underlined text) in Atlantic cod (*Gadus morhua*). A) transcript expressions throughout early development, B) tissue distributions (1 – liver, 2 – spleen, 3 – ovary, 4 – pyloric caeca, 5 – stomach, 6 – intestine, 7 – brain), and C) central tissue distributions (T – telencephalon/preoptic area, OT – optic tectum/thalamus, H – hypothalamus, P – pituitary gland, C – cerebellum, MO – medulla oblongata, and SC – spinal cord).


MCH
GnRH2
GnRH3
A
Unfertilized egg
Fertilized egg
Yolk sac - hatched
Free feeding
Organogenesis
Growth
Pigmentation (eye, body)
Feeding/digestion
Pigmentation (eye, body)
B
Behaviour
- Feeding
- Sexual
*7
*1
2
*3
*6
*5
*4
Digestion and
Metabolism
Reproduction

C


Food seeking behaviour
- Visual inputs
Appetite-related hormone regulation
Food seeking behaviour
- Locomotion
Food seeking behaviour
- Olfaction
Reproductive behaviours
- Spawning
- Gonadal maturation
C
OT
T
MO
SC
H
P
Hormone regulation
- Feeding
- Reproduction

MCH and GnRH transcripts are present throughout cod central (*i.e.* central nervous system) and peripheral tissues (Figure 5.1B,C; Chapter 2). MCH and GnRH mRNAs are found in brain regions related to appetite regulation (telencephalon/preoptic area, optic tectum/thalamus, hypothalamus), as well as the pituitary gland and the stomach, suggesting a peripheral function in food intake regulation.

In fish, both MCH and GnRH play integral roles in many endocrine-related processes (Kawauchi and Baker 2004; Chen and Fernald 2008), thus it is not surprising that variable transcript expressions are observed in cod early ontogeny as MCH and GnRHs are likely involved in the development (*i.e.* neural connections, cellular organization) of these systems. MCH, GnRH2 and GnRH3 transcript expressions in central and peripheral tissues related to food intake regulation are indicative of their roles in appetite regulation, whether it is through direct innervation of appetite pathways in the brain or other digestive processes in the stomach and GI tract.

These findings are novel in Atlantic cod and could prove to be of value to aquaculture practices. For example, the use of female cod with high MCH or GnRH3 mRNA expressions could be important, as these mRNAs are maternally transferred to eggs and potentially future generations. In cod, MCH is an important appetite stimulator (Tuziak and Volkoff 2013 – Chapter 2), while GnRH3 is known for its role in regulating reproductive processes (*i.e.* sperm and egg development, spawning behaviour) (Hildahl *et al.* 2011). High expressions of both MCH and GnRH3 could improve cod fitness (via increased reproduction) and growth (via increased appetite). GnRH2 regulation of appetite may be developmental stage-specific, thus might be more important during larval

development compared with adults. Detailed studies are needed to determine the relationship between GnRH2 expression, peptide levels, food intake and growth rates in larval cod. Breeding of individuals that have lower expression of GnRH2 mRNA during exogenous feeding could increase growth rates and reduce time spent in tanks.

5.2. Fasting affects the expression of MCH, GnRH, and orexin providing evidence of the role of these peptides in regulating feeding, metabolism and food seeking behaviour

In fish, MCH appears to be an appetite stimulator, while GnRHs might play a role in inhibiting food intake (Shahjahan *et al.* 2014). MCH mRNA levels in Newfoundland-reared juvenile Atlantic cod are up-regulated after a 2-week fasting (larger tanks with >50 fish per tank), while GnRH2 and GnRH3 transcripts do not appear to be influenced by food deprivation (Figure 5.2; Chapter 2). However, in New Brunswick-reared cod, a 10-day fasting event (small tanks with 2 fish) caused brain mRNA expression levels of MCH to be lower and those of GnRH3 higher in fasted fish compared to fed fish, whereas GnRH2 expression was not affected by fasting (Chapter 4). Orexin expression was also lower in fasted fish (Chapter 4).

Differences observed between these two studies might be due to durations of fasting. The length of fasting could affect MCH, orexin and GnRH transcript expression levels, especially in juveniles, as the amount of energy present in a fish dictates its hunger status. Juveniles are still growing thus require more easily obtainable energy and do not tend to store it, therefore an extra four days of fasting may have caused the energy supply

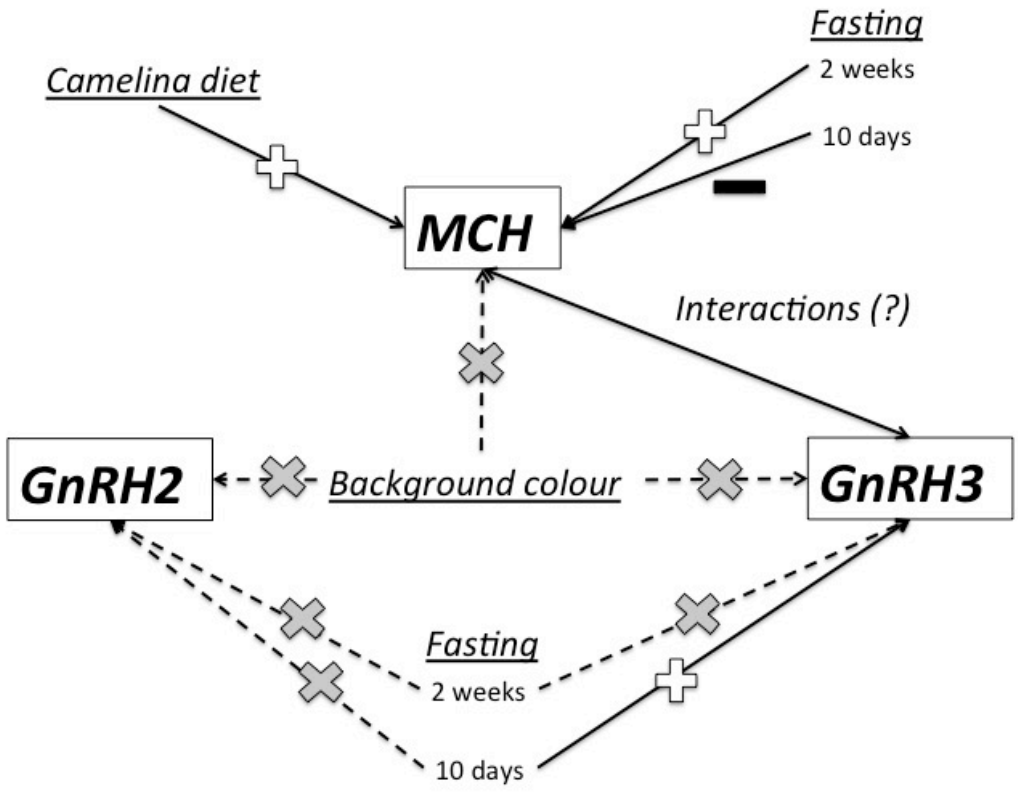


Figure 5.2. Summary model of melanin-concentrating hormone (MCH) and gonadotropin-releasing hormone (GnRH) transcript expression when environmental (*i.e.* background colour and diet) and internal (*i.e.* energy stores) cues are varied in Atlantic cod (*Gadus morhua*). Dashed line and "X" indicates no effect on hormone transcript expressions, solid line indicates an effect (positive with a "+" and negative with a " –") on hormone transcript expressions.

needed to maintain daily processes to cross below the required energy threshold and activate "hunger"-related systems, such as MCH, orexin and GnRH. Prior to full activation of food intake-related hormone systems, fish may sense the lack of nutrients and energy and reduce foraging behaviours, which explains fasting-induced reductions in locomotion in cod (Chapter 4). Whether these changes in locomotion are related to expression changes in MCH and GnRH needs further investigation.

Additionally, water temperature may also play a role in the differential effects observed between appetite-related transcripts in this thesis. For both Chapter 2 and Chapter 4, ambient water temperatures were used (11°C and 15°C, respectively) due to the nature of the experimental set-up (*i.e.* flow through tanks with water being supplied directly from the ocean). Juvenile Atlantic cod within the size range used in the present studies (mean initial mass: 5-15 g; mean final mass: 7-60g) prefer a water temperature range of 12.4-14.7°C (Imsland *et al.* 2005). Furthermore, Atlantic cod feed conversion efficiency has also been shown to significantly increase with increasing temperature (FCR: 1.11 to 1.17; Imsland *et al.* 2005). Water temperatures in Chapter 4 fall within this "preferred" range, whereas temperatures in Chapter 2 are outside of the spectrum, suggesting that temperature may be a factor in the behavioural and molecular differences we observed between these studies.

High stocking densities can induce a stress response in captive fish (Portz *et al.* 2006). Induced stress (*i.e.* density/holding condition) in fish may also contribute to differences in MCH and GnRH expression patterns (Kang and Kim 2013). Atlantic cod prefer to reside in larger groups – but not overcrowded – and changes in group size may

induce stress in fish (Caipang *et al.* 2008), thereby activating the production and release of stress-related hormones, such as cortisol, which in turn may influence MCH (Kang and Kim 2013) and GnRH (Consten *et al.* 2001) regulation. Although fish in smaller groups and tanks did not appear to be stressed (*i.e.* did not show reduced feeding or locomotion, or increased "hiding"), it is possible that they acclimated to the initial stressor of being in a small group, but maintained higher than normal (*i.e.* large group size fish) stress hormone levels, which might have affected MCH, orexin and GnRH transcription.

Furthermore, different genetic backgrounds (New Brunswick *vs.* Newfoundland strains) could have resulted in distinctive regulations of MCH and GnRH, as fish may have been more or less resilient to the perceived fasting and group size stresses. The Newfoundland fish may be more resilient to stress or not perceive an inflicted stress of fasting so that fasted fish may have lower stress hormone levels thus higher MCH transcript expression levels than the New Brunswick strain.

Examining the effects of fasting in juvenile cod had never been carried out before. These results are important for aquaculture practices as they can help discern what factors (*i.e.* fasting, group/tank size, genetic background) affect feeding behaviour and appetite-related hormones, such as MCH and GnRH, in Atlantic cod. Minimal stress is required for maximal growth and, therefore, determining the most cost-effective strategies (*i.e.* increased number of fish in rearing facilities, fish with a genetic background resilient to stressors) is essential for a sustainable aquaculture industry.

5.3. Plant-based diets affect feeding, growth and the expression of peptides

With the decrease in some baitfish populations (Department of Fisheries and Oceans 2013), aquaculturists need to rely on other protein sources, such as plants, to supplement fish oil and meals in feeds. However, whether carnivores can adapt to a plant-supplemented diet is yet to be determined, since their digestive system physiology and anatomy is distinct from omnivores and herbivores (German and Horn 2006; Geevarghese 1983). *Camelina sativa* is a novel plant-based diet, which is being tested in farmed fish, such as Atlantic cod. Camelina meal inclusion diets caused decreases in both growth and food intake in Atlantic cod (Figure 5.2; Chapter 3), suggesting that this compound might not be a viable components for cod diets.

Further evidence that cod are not able to tolerate camelina meal-supplemented diets is provided by changes in appetite-related hormone expression profiles, in particular the up-regulations of "hunger" peptides, suggesting the fish are not obtaining enough assimilable nutrients. Cod optic tectum MCH transcript expression levels were significantly higher in fish fed the 30% camelina meal diet compared to fish fed the 15% camelina meal diet. The study also shows that other appetite regulators might be affected. In the hypothalamus, compared to fish fed the control diet, orexin expression was significantly higher in fish fed the 30% camelina meal diet, while NPY transcript expression was significantly higher in fish fed the 15% camelina meal diet. CART mRNA expression was not affected by diet in any brain region. Additional studies on the effects

of plant-based diets on carnivorous fish feeding behaviour and regulation, specifically Atlantic cod, need to be completed.

Since cod are carnivorous and consume plant matter (*i.e.* algae) mixed with zooplankton at early stages of development (Kane 1984), it is not surprising that they are unable to tolerate plant meal-supplemented diets. This study is important since determining the degree of supplementation (*i.e.* 0-30%) and, in particular, which plant-based by-products (*i.e.* oil *vs.* meal) are tolerated in farmed Atlantic cod is crucial in my understanding of whether cod can tolerate plant-supplemented diets. If cod cannot tolerate (*i.e.* as seen by reduced appetite, growth rates, and increased levels of "hunger" hormones) high levels of plant supplementation, then other sources of protein, aside from baitfish, need to be identified for feed.

5.4. Background colour

In chapter four, I determined how the combination of fasting and varying background colours affect the forebrain transcript expression levels of food intake regulators, as well as feeding and swimming behaviours and skin pallor in juvenile Atlantic cod.

Background colour did not affect either locomotor or feeding behaviour, or the forebrain expression of any of the hormones examined (MCH, orexin and GnRH) (Figure 5.2.). Fasting induced reductions in locomotion in both backgrounds and in skin colouration (*i.e.* fasted fish were paler than fed fish) in white backgrounds.

These results suggest that background colour has little effect on feeding and locomotion and the response to fasting in juvenile Atlantic cod, whereas fasting has more pronounced effects, as it reduces activity, induces skin paleness and affects expression levels of MCH, orexin and GnRH.

The results from this study were surprising, since other fish studies have shown that background colours significantly affect food intake, skin pallor and food intake-related hormone expression (Amiya *et al*. 2005; Amiya *et al.* 2008; Browman and Marcotte 1987; Kang and Kim 2013). As previously mentioned, it is possible that fish perceived the small group size (2 fish per tank) and fasting as greater stressors than background colour. The effects of background colour on cod feeding behaviour and hormone regulation is important to the aquaculture community as it demonstrates that cod may not be affected by background colour if other stressors (*i.e.* tank density, food availability) co-exist. However, whether cod changes in behaviour and expression levels might occur if only changes in background colour (and no other stressors) are present requires further examination.

5.5. Future directions for research

Future directions for this study include:

(1) Examining exact locations of MCH and GnRH neurons in the brain of Atlantic cod through *in situ* hybridization, which would help identify neural connections between these peptide families to discern whether MCH plays a modulatory role

on GnRH regulation. Furthermore, *in vitro* MCH peptide exposure using cell or tissue cultures could help to determine if it affects GnRH transcripts and protein regulation (*i.e.* true antagonists or just opposite effects) and vice versa.

(2) Determining the direct *in vivo* effects of MCH and GnRH on food intake in Atlantic cod through intracerebroventricular (ICV) or intraperitoneal (IP) injections to examine whether these peptides play a role in food intake and associated locomotory behaviours. Through ICV injections we can determine whether the observed effect on appetite is regulated centrally, while IP injections can reveal peripheral food intake behaviour regulation.

(3) Assessing the transcript expression of appetite regulators, such as MCH, orexin, NPY, GnRH and CART, and transcripts involved in fatty acid, carbohydrate and protein metabolism to determine their correlations with appetite-regulators during camelina meal or other plant-supplemented feeding trials, including plants/algae native to Newfoundland, to resolve the interactions between appetite- and metabolic-related systems. *In vitro* and *in vivo* studies using cod hypothalami exposed to various nutrients, such as glucose, fatty acids, or amino acids, as well as fish exposed to diets of varying nutrient compositions could help to determine if appetite-related hormone transcript and protein expressions correlate with known quantities of essential nutrients and decipher if appetite-related hormone cells can "sense" nutrient imbalances. These studies are important because plant-based diets could lack essential nutrients needed for cod and *in vivo* studies would show direct correlations between hormone and nutrient quantities.

(4) Assessing how a variety of background colours, light spectra and photoperiods affect food intake behaviours and related peptide mRNA and protein expression in Atlantic cod would be important for aquaculture practices. If these external cues positively influence food intake behaviour and related hormone production then the industry would be able to construct more efficient procedures for rearing Atlantic cod.

(5) Examine links between feeding and reproduction for MCH and GnRH in mature adults to determine if sex-specific variations are present in Atlantic cod. By determining whether sex- or developmental-specific differences in feeding and MCH and GnRH exist in cod, it would help to design efficient feeding protocols for cod throughout development. For example, if there is a reproductive stage at which cod do not eat much, food can be reduced. If at this stage, feeding is reduced in females, but not males, keeping sexes in separate tanks may also help to eliminate unnecessary feed costs. Furthermore, by identifying whether MCH and GnRH transcript expressions vary during these stages, we can genetically select fish that have better reproductive fitness because of MCH and GnRH genetic variations.

5.6. Conclusions

My study sheds some light on complex interactions between internal and environmental factors, how fish perceive these cues and adjust to them physiologically

with respect to appetite regulation. By examining how food intake is controlled in multiple fish species, a general model can be formulated for fish and other vertebrates to devise a complete picture of vertebrate appetite regulation. Further examination of interactions between food intake regulation and other physiological systems, such as reproduction and growth, would also help to develop an overall representation of appetite regulation in fish.

Finally, the results from this study are also of importance to Atlantic cod aquaculture. By understanding how plant meal-supplemented diets and background colour affects growth and food intake in a carnivorous fish, the industry can decide whether they are valuable alternatives during intensive rearing of juvenile cod. Alternative feed is necessary with the decline of some baitfish stocks, however if the feed reduces growth and market desirability then it is not a plausible option. The same holds true for background colours of tanks, if a specific colour is perceived as "stress"-inducing then it will impact growth. Knowing which factors positively and negatively influence food intake is important for successful aquaculture practices.

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