UiT
THE ARCTIC UNIVERSITY OF NORWAY

Department of Arctic and Marine Biology

Feeding and fasting in Arctic charr (*Salvelinus alpinus* (Linnaeus, 1758)): Central regulation of food intake in a highly seasonal fish species

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Anja Striberny

A dissertation for the degree of Philosophiae Doctor – April 2018

UNIVERSITY OF TROMSØ

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Title picture

Wild anadromous Arctic charr captured during the return from their feeding migration to the sea. Photo credit: Even H. Jørgensen

“Life need not be easy, provided only that it is not empty.”

Lise Meitner

Table of contents

I. Acknowledgements

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II. Thesis abstract

Central control mechanisms of food intake vary between different fish species. Few studies have investigated the role of central appetite regulators in fish species that show natural seasonal variations in food intake. Yet, seasonal fishes are particularly interesting because their potential seasonally sliding set-points in appetite and energy homeostasis may reveal new information about the long-term control of food intake in fish. Arctic charr inhabit the oligotrophic freshwater systems of the North. The anadromous (sea-migrating) charr cope with the poor nutritional conditions found in fresh water by performing short feeding migrations to the nutrient-rich sea for a few weeks in summer. These charr obtain their nutrients almost entirely from marine food resources and feed little or nothing while residing in fresh water during the rest of the year. The seasonal feeding rhythm is considered to be under tight endogenous control because it is sustained in captive offspring of anadromous charr despite year-round feed access. The mechanisms underlying the seasonal feeding behaviour of the charr are not understood.

In this work we aimed at shedding light on the role of central appetite regulators in the control of appetite in Arctic charr. We measured the gene expression of putative appetite regulators in captive immature offspring of anadromous Arctic charr held at a simulated natural conditions, as well as in Arctic charr that were subjected to a short and a long period of feed deprivation. In addition we tested the effects of re-feeding and fish feed flavour on the expression of hypothalamic appetite regulators in previously long-term feed deprived charr. Lastly, we compared the brain transcriptome between fed and feed deprived charr over a four weeks period during summer in order to assess effects of feed deprivation on a larger scale.

Our data indicate that the reduction and increase in appetite during winter and summer, respectively, are not regulated by changes in central satiety and hunger signalling in the charr. Furthermore, we could not find differences in central appetite signalling between long-term energy perturbed and fed charr during summer. We conclude from these findings, that the charr may enter a steady-state like regulation of appetite when feed is present or absent for a longer period of time. However, the re-feeding or feed flavour presentation evoked responses in some appetite regulating genes. Consequently, we suggest that changes in the expression of central appetite regulators may only be seen during transition phases, from fasting to feeding, and vice versa, in the Arctic charr. The transcriptomic approach revealed two new interesting candidate genes, nerve growth-factor inducible (VGF) and deiodinase 2 (Dio2), which are previously described to participate in the regulation of energy homeostasis in seasonal mammals. As such this work expands current knowledge about the role of known appetite regulators and depicts new aspects that may be linked to the food intake control of seasonal fishes.

III. List of papers

Paper I

Seasonal Differences in Relative Gene Expression of Putative Central Appetite Regulators in Arctic Charr (*Salvelinus alpinus*) Do Not Reflect Its Annual Feeding Cycle

Anja Striberny[1], Chandra Sekhar Ravuri[1], Malcolm Jobling[1], Even Hjalmar Jørgensen[1], 2015

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PLoS One **10**(9):e0138857

Paper II

Feedback from Arctic charr: Feed flavour stimulation and re-feeding after feed deprivation stimulate genes encoding both orexigenic and anorexigenic neuropeptides

Anja Striberny[1], Even H. Jørgensen[1], 2017

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General and Comparative Endocrinology **246**:71-80.

Paper III

Strong effect of season, but subtle responses to feed deprivation in the brain transcriptome of Arctic charr

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Manuscript

IV. Abbreviations

AgRP	Agouti related peptide
ARC	Arcuate nucleus
BBB	Blood-brain barrier
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CRF	Corticotropin releasing factor
DNA	Deoxy-ribonucleic acid
GABA	Gamma-aminobutyric acid
GH	Growth hormone
Ghr	Ghrelin
GHSR	Growth hormone secretagogue receptor
icv	Intracerebroventricular
IGF	Insulin-like growth factor
Ins	Insulin
Ip	Intraperitoneal
Jak/STAT	Janus kinase/Signal transducer and activator of transcription
K	Fulton’s condition factor
Lep	Leptin
LepR	Leptin receptor
LHA	Lateral hypothalamic area
MC4R	Melanocortin receptor 4
MCH	Melanin concentrating hormone
mRNA	Messenger ribonucleic acid
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
PYY	Peptide YY
RNA-seq	Ribonucleic acid sequencing
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
VGF	Nerve growth factor inducible
YR	Neuropeptide Y receptor
α-MSH	Alpha-melanocyte-stimulating hormone

1. Introduction

1.1 Regulation of food intake and energy homeostasis in mammals

Unlike plants, which are autotrophic, animals as heterotrophs need to obtain the macronutrients they survive on from an external source. Feeding is a time and energy consuming process and a tight regulation of energy intake and expenditure is pivotal for survival.

Due to the global increase in obesity and obesity-related diseases there have been intense research efforts to improve our understanding of the control of food intake and energy homeostasis in mammals. As a consequence, most of the knowledge in this research field is rooted in mammalian model organisms. On that account, the following chapters will first introduce the basic concepts of the endocrine and neuroendocrine control of food intake and energy expenditure in mammals; thereafter, a comparative overview of how these mechanisms apply to fish will be given.

1.1.1 Central integration of endocrine and neuroendocrine anorexigenic and orexigenic signalling

At its simplest, food intake is provoked or inhibited by hunger and satiety, respectively. Hunger and satiety are opposing physiological states that arise based on the integration of central and peripheral appetite stimulating (orexigenic, derived from Greek órexis, “desire”), and inhibiting (anorexigenic) signals in the brain. The control of food intake consists of a complicated network which includes endocrine, neural and neuroendocrine signalling. Short-term signalling pathways regulate food intake on a meal-to-meal basis. In turn, meal size and frequency, and the amount of energy expenditure are determined by the central integration of long-term signals reporting about energy status, with the presumed aim of maintaining energy homeostasis.

In 1950 Kennedy demonstrated that lesions in the ventromedial area of the hypothalamus in rats (*Rattus norvegicus*) generated an obese, hyperphagic phenotype. He concluded that this part of the hypothalamus acts as a “feeding control centre” where peripheral signals reporting about nutritional status are perceived and integrated (Kennedy, 1950). Later it was established that food intake and energy balance are under the control of central orexigenic and anorexigenic signalling circuits with an origin in the arcuate nucleus (ARC) (Fig. 1) (Schwartz et al., 2000). The anatomical location of the ARC, at the base of the hypothalamus, is ideal for receiving information about the peripheral status via endocrine signalling. In most brain areas, the blood-brain barrier (BBB) prevents large, potentially toxic, molecules from entering the brain (Reese and Karnovsky, 1967). However, the median eminence, which is located adjacent to the ARC in the mediobasal hypothalamus, lacks the BBB and hence allows the passage of circulating molecules (Coll and Yeo, 2013).

In the ARC, the peripheral conveyers of energy status are perceived by two populations of “first order” neurons. One population of neurons co-expresses the anorexigenic melanocortin precursor proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) whereas the other population co-expresses the orexigenic neuropeptide Y (NPY) and agouti related peptide (AgRP) (Wynne et al., 2005). In addition to the release of appetite stimulating neuropeptides, AgRP/NPY neurons have been shown to impair POMC/CART signalling by the release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Cowley et al., 2001). Depending on the peripheral input signal, these populations of neurons

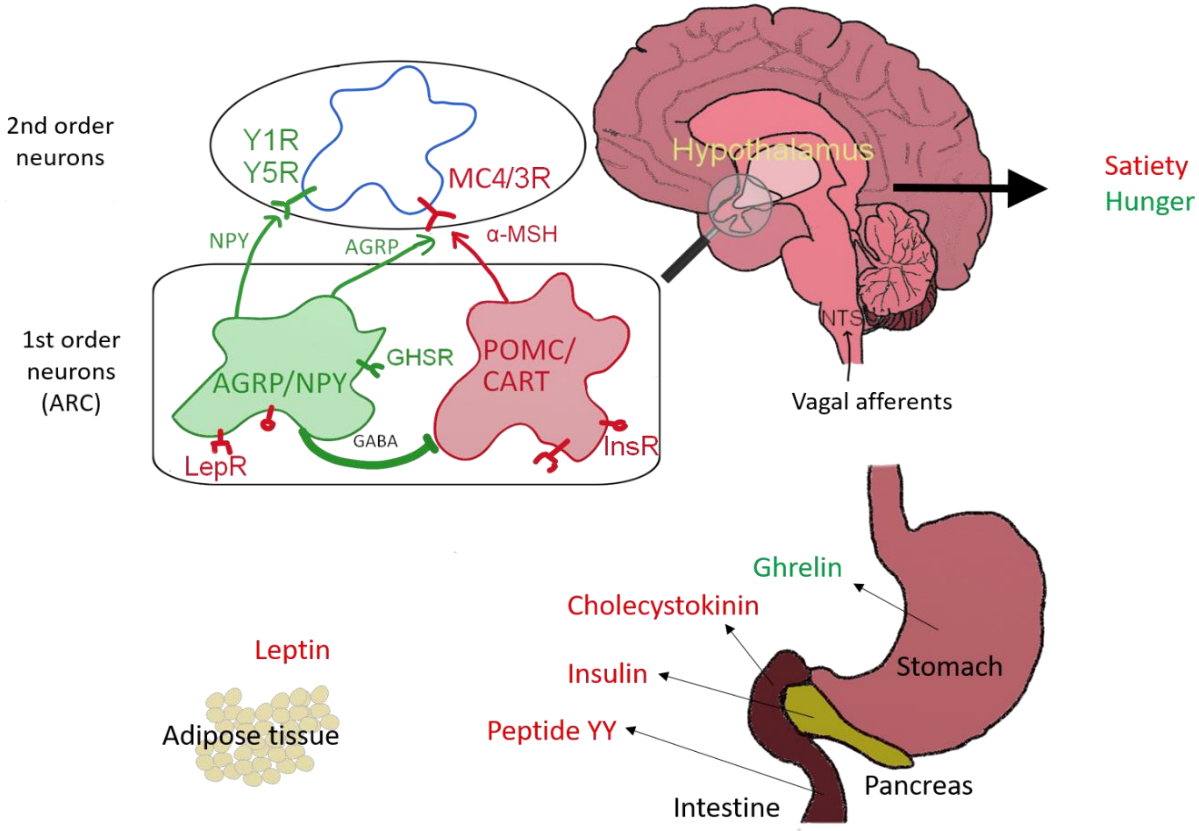

Figure 1 Schematic overview of central and peripheral key players involved in the control of food intake and energy homeostasis in mammals. Peripheral short-term signals, produced by and released from endocrine cells of gastrointestinal tract report about hunger (ghrelin, green) and satiation (cholecystokinin, peptide YY, red) to the brain via the blood stream and vagal afferent nerves. Short-term term signals are integrated by the NTS. The anorexigenic hormone leptin is a conveyer of fat status. Being produced by white adipocytes, leptin levels vary in proportion to the amount of body fat. In the ARC, leptin and insulin inhibit and stimulate AgRP/NPY and POMC/CART neurons, respectively. Modified from: Schwartz et al. 2000

implement orexigenic and anorexigenic signalling cascades through projections to “second order” neurons located in proximal hypothalamic nuclei. “Second order” neurons involved in the control of food intake and energy homeostasis are found in the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial nucleus and ventromedial area of the hypothalamus (Schwartz et al., 2000).

Meal initiation and termination are controlled by neural and hormonal short-term signals that are perceived and integrated in the nucleus tractus solitarius (NTS). The hypothalamic derived anorexigenic and orexigenic signalling cascades are conveyed to and integrated by the NTS, eventually leading to adjustments of meal frequencies and size in dependence of energy status for example by a modulation of CCK sensitivity (Blevins et al., 2009).

1.1.2 Anorexigenic and orexigenic signals in first order neurons of the ARC

In the following paragraphs, a selection of important neuropeptides involved in the regulation of food intake and energy homeostasis is described. However, one needs to bear in mind that many of these neuropeptides are also involved in other physiological processes, which will not be described here.

Melanocortins

POMC is a precursor protein which is cleaved into several smaller peptides in a tissue specific manner (Bicknell, 2008). In the ARC, POMC exerts its anorexigenic effects mainly via the cleavage product alpha-melanocyte stimulating hormone (α-MSH) which has high affinities to the G protein coupled receptors melanocortin receptor 3 and 4 (MCR3/4) (Ellacott and Cone, 2004; Mountjoy et al., 1992). Genetically modified mice (*Mus musculus*) that lack a functional MC4R are obese after onset of maturity, which highlights the important role of MC4R in the control of energy balance (Huszar et al., 1997). Several studies have shown that ARC *POMC* expression is reduced in response to feed deprivation (Mizuno et al., 1998; Schwartz et al., 1997). Conversely, the peripheral satiety signals leptin (Lep) and insulin (Ins) stimulate the expression of *POMC* (Morton et al., 2006).

CART

An anorexigenic role for CART peptides has been postulated in a number of studies. For example, it has been demonstrated in rats, that *CART* mRNA abundance in the ARC is decreased in response to feed deprivation and increased upon peripheral administration of Lep (Kristensen et al., 1998). Moreover, intracerebroventricular (icv) administration of CART peptides has been shown to reduce food intake in rodents (Lambert et al., 1998; Stanley et al., 2001). The exact mechanisms of how CART participates in the integration of appetite signalling remain to be elucidated, but CART icv injections have indicated that the anorexigenic neurons in the PVN are one target of CART (Vrang et al., 1999). A potential CART receptor has yet to be identified, but it is suggested that CART peptides mediate their effects through G-protein coupled receptor(s) (Rogge et al., 2008).

AgRP

AgRP is an orexigenic neuropeptide that is co-expressed with NPY in first order neurons of the ARC (Hahn et al., 1998). AgRP expression is stimulated by feed deprivation and decreased in response to Lep and Ins (Mizuno and Mobbs, 1999). AgRP has been demonstrated to mediate its orexigenic effects by inversely agonising MC4R signalling (Nijenhuis et al., 2001). Both Ins and Lep are negative regulators of AgRP signalling in the ARC (Varela and Horvath, 2012).

NPY

Neuropeptide Y is a 36 amino acid peptide of the NPY-family (Tatemoto et al., 1982). It is considered to be the most potent appetite stimulator in the hypothalamus (Mercer et al., 2011) and chronic administration of NPY is associated with hyperphagia and obesity in rats (Stanley et al., 1986). NPY is abundantly expressed in the brain (Allen et al., 1983) and is highly conserved across the vertebrate lineage (Larhammar et al., 1993). In mammals, the orexigenic effects of NPY are mainly mediated through the NPY receptors Y1 and Y5 (Wynne et al., 2005). In rodents, *NPY* mRNA expression in the ARC increases in response to feed deprivation in (Hahn et al., 1998) and administration of ghrelin in rats (Nakazato et al., 2001), and is reduced following central administration of Ins (Schwartz et al., 1992) and Lep (Schwartz et al., 1996).

1.1.3 Peripheral signals involved in the control of food intake and energy homeostasis

1.1.3.1 Short-term signalling of hunger and satiety

In the short-term, onset and termination of food intake are controlled by communication between the gastrointestinal tract and the brain. Information about the prandial status is directly exchanged through efferent and afferent vagal and spinal nerves that innervate the gastrointestinal tract. For example, pre- and post-prandial changes in stomach distension are sensed by gastric mechanoreceptors, which in turn activate signal transmission through vagal and spinal afferent nerves to the hindbrain where the information is integrated and processed by neurons of the NTS (Konturek et al., 2004). In addition, several hormones that report about nutritional status, are produced in and released by the gastrointestinal tract in a peri-prandial manner. Some important gastrointestinal messengers of hunger and satiation are briefly described below.

Gastric ghrelin

To date, ghrelin (Ghr) is the only known peripheral “hunger” signal identified in mammals (Cummings, 2006). Plasma Ghr concentrations rise and fall pre- and postprandially, respectively (Cummings et al., 2001). The 28 amino acid peptide is predominantly expressed by the mucosa cells of the stomach, and exerts its orexigenic function through binding to growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999). GHSR has been localised in AgRP/NPY neurons of the ARC and has been functionally linked to the orexigenic effect of Grh through icv injections (Nakazato et al., 2001). Ghr also acts in the periphery by increasing hunger signalling through the blockade of vagal afferent nerves (Date et al., 2002).

Intestinal CCK and PYY

A direct role for cholecystokinin (CCK) in satiation signalling was first demonstrated by Gibbs and co-workers (1973) who showed that intraperitoneal injections of this gut hormone suppressed food intake in rats. CCK is a potent satiation signal that is mainly produced and released by the duodenum in response to nutritional stimuli (Berthoud, 2008). It is involved in the short-term control of food intake and is an important signal for meal termination (Cummings and Overduin, 2007). Satiation signalling is mediated through two CCK receptors (CCKA and CCKB) that are located on the vagal afferent nerves in the gastrointestinal tract (Schwartz et al., 2000; Wank, 1998). Peptide YY (PYY), a member of the NPY family of peptides, is another satiation signal that is produced and secreted by the intestine (Batterham et al., 2002; Larhammar, 1996). It is postprandially released in the lower part of the gastrointestinal tract and is implicated in the control of meal termination (Batterham et al., 2002). Like CCK, PYY also stimulates vagal afferent nerves (Koda et al., 2005).

1.1.3.2 Long-term control of energy homeostasis

Leptin and insulin

The lipostat model postulated by Kennedy (1953) implies that energy homeostasis is maintained by adjusting energy intake and expenditure to target a defined set-point indicated by adiposity. Even though it is a matter of debate whether the set-point model applies to the regulation of energy balance in mammals (Speakman, 2018), it is generally accepted that Lep is a peripherally derived satiety signal that gives feedback about the amount of energy stores

to the ARC (Friedman and Halaas, 1998). Lep is a 167 amino acid peptide predominantly produced and secreted by white adipose cells (Zhang et al., 1994). In mammals, plasma Lep levels are directly proportional to the concentration of body fat and hence Lep may be considered the lipostat hormone (Maffei et al., 1995). The receptor of Lep (LepR) is widely expressed in the brain with high abundance in the first order neurons of the ARC (Varela and Horvath, 2012). LepR is a type 1 cytokine receptor that transduces the Lep signal via the Janus kinase/Signal transducer and activator of transcription (Jak/STAT) signalling cascade (Sweeney, 2002). In brief, binding of Lep to LepR causes a phosphorylation of Jak that in turn activates the transcription factor STAT3. STAT3 translocates to the nucleus where it stimulates and inhibits the expression of *POMC* and *AgRP*, respectively (Varela and Horvath, 2012). Furthermore, STAT3 induces the expression of Suppressor of cytokine signalling 3 (*SOCS3*), a negative feedback signal that prevents further Lep signalling by blockade of Jak (Bjorbaek et al., 1999). Lep may activate several other pathways including the phosphatidylinositol 3-kinase signalling pathway, which is also utilized by Ins (Sweeney, 2002). Ins is released by the pancreas and is positively correlated with the proportion of body fat (Polonsky et al., 1988). Similar to Lep, Ins stimulates anorexigenic and catabolic signalling and inhibits orexigenic and anabolic signalling cascades in the ARC (Baskin et al., 1999). In the ARC “first order” neurons, LepR has been found to be co-expressed with Ins receptor (InsR) implying that Lep and Ins exert their central anorexigenic effects in a synergistic manner (Niswender et al., 2004).

1.1.4 Anticipating lean times: the special case of food intake control in seasonal animals

At high latitudes, long winters and short summers lead to dramatic changes in the abundance of food during the year. Arctic and sub-arctic animals have adapted to cope with this (Blix, 2016). Resident Arctic mammals and birds show seasonal changes in appetite, resulting in high feeding activity during summer, and reduced food intake during winter. These seasonal changes in food intake are suggested to be endogenously regulated as they are sustained even under controlled experimental conditions and constant, excess food availability (Mortensen and Blix, 1985; Steinlechner et al., 1983). Considering the underpinning feedback mechanisms involved in the control of long-term energy homeostasis, it has been suggested that seasonal animals are able to adjust their body weight set-point according to season (Mrosovsky and Fisher, 1970).

The means by which the “sliding set-point” is controlled on a seasonal basis is not entirely understood (Ebling, 2015). Studies on Siberian hamster (*Phodopus sungorus*) have indicated that seasonal feeding behaviour is not governed by seasonal changes in the abundance of orexigenic and anorexigenic signals in the hypothalamus (Mercer et al., 1995; Reddy et al., 1999). Paradoxically, the Siberian hamster sustains a high food intake during summer despite high Lep levels and food intake during winter remains low even though Lep levels are decreased (Klingenspor et al., 1996; Klingenspor et al., 2000). The finding that ARC SOCS3 expression is up-regulated by the increase in day length in spring led to the suggestion that a high feeding rate during summer may be maintained by a seasonal decrease in Lep sensitivity (Tups et al., 2006). However, this model does not provide any mechanistic explanation about how changes in Lep sensitivity are implemented downstream in the appetite signalling cascade. Even though the exact mechanisms are not entirely understood, the current model suggests that the neuronal wiring of food intake control in the hypothalamus of seasonal mammals is plastic, and that the modulations of food intake control centres are driven by seasonal differences in the production of triiodothyronine in populations of tanycytes adjacent to the hypothalamus (Ebling, 2015).

The special case of food intake control and energy homeostasis in seasonal mammals illustrates the complexity of long-term regulation of food intake and energy homeostasis, and highlights the need to study non-model organisms in order to get a broader knowledge about the mechanisms underpinning energy homeostasis.

1.2 Control of food intake in fish

Although there are many morphological and physiological differences between mammals and fish, several physiological mechanisms are similar. Food intake and its control are fundamentally important for all animals. So it is no surprise to find many of the above described control mechanisms in fish.

1.2.1 Central control of food intake in fish

Neuropeptides known to be involved in the regulation of food intake in mammals have been studied in several fish species (Lin et al., 2000; Rønnestad et al., 2017; Volkoff et al., 2005). Compared to mammals, the picture of the neuronal organisation of food intake control centres in fish is still incomplete. For example, there remain uncertainties about the location of “first order” and “second order” neurons involved in the control of food intake and energy homeostasis in fish. The hypothalamic nucleus lateral tuberis is considered to be a homologue of the mammalian ARC (Peter et al., 1980), and the preoptic area has been defined as a homologue of the mammalian PVN (Herget et al., 2014). As such, the hypothalamus has received special attention in studies on central control of food intake. However, as the underlying central mechanisms are not entirely understood other parts of the brain have been investigated in connection with fish appetite control.

During the course of evolution, teleosts underwent three rounds, and salmonids and some cyprinids even four rounds of genome duplication. Duplicated genes may either be lost as a consequence of non-functionalisation, or preserved due to sub- or neo-functionalisation. The latter becomes apparent in tissue or stimulus specific expression patterns of paralogous genes (Glasauer and Neuhauss, 2014). Many of the neuropeptides and their receptors described in the previous chapters are expressed as several different paralogues, and the fact that some of these show distinct expression patterns, for example in response to feed deprivation, adds a level of complexity to the elucidation of regulating mechanisms involved in food intake and energy homeostasis.

Several important central signalling systems that control food intake known from mammalian models have also been described in fish. An overview of involved actors is presented in Figure 2 (Rønnestad et al., 2017).

The melanocortin system is highly conserved across vertebrates and presumably arose over 400 million years ago (Cortes et al., 2014; Ringholm et al., 2002). Several melanocortin receptors have been described in fish and a number of studies have provided evidence that melanocortin signalling via MC4R is involved in the control of food intake and energy homeostasis in fish (Cerda-Reverter et al., 2011). Similar to mammals, POMC is post-translationally processed in a tissue specific manner in fish (Dores and Baron, 2011). Icv injections of the POMC cleavage product alpha-MSH reduce food intake in goldfish (*Carassius auratus*), an effect which is reversed by injections of a MC4R antagonist, thereby indicating the role of melanocortin signalling in satiety (Cerdá-Reverter et al., 2003; Cerdá-Reverter et al., 2003).

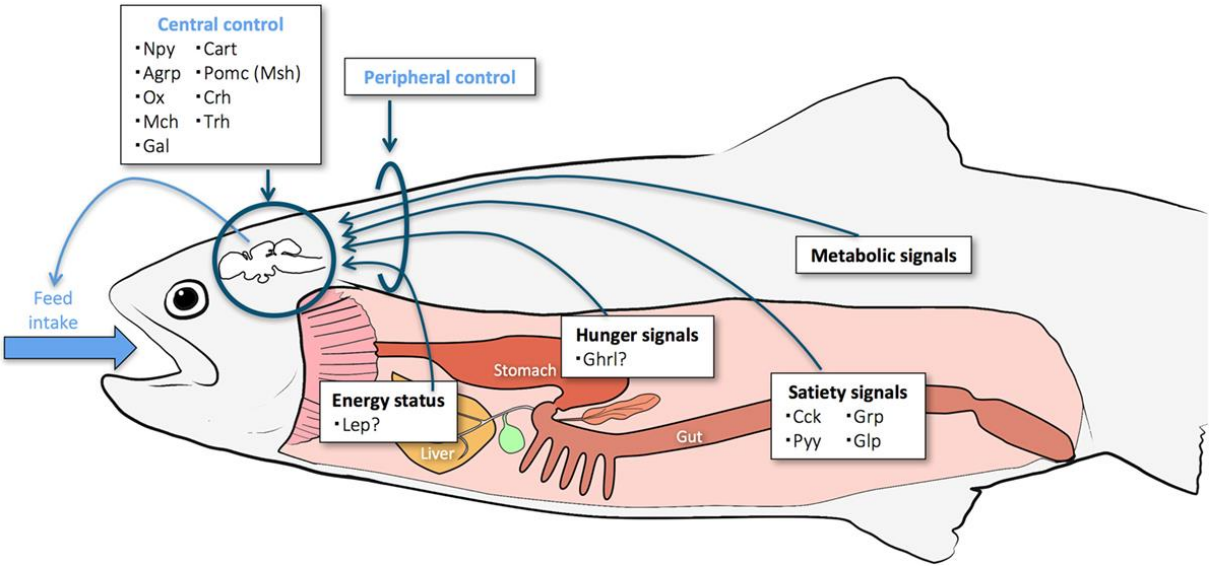

Figure 2 Overview of central neuropeptides and peripheral signals involved in the control of food intake in fish. Reprinted from: Rønnestad et al., 2017 Appetite-controlling endocrine systems in teleosts. Frontiers in Endocrinology.8:73.

Feed deprivation experiments used to investigate effects of nutritional status on the central expression of *POMC*s in fish have provided some inconsistent results when comparing between different fish species and when examining outcomes from studies in fish with feed deprivation experiments in mammals. For example, hypothalamic *POMC* expression was unaltered by 7 days of feed deprivation in goldfish (Cerdá-Reverter et al., 2003), and after 2 and up to 15 days of feed deprivation in zebrafish (*Danio rerio*) (Song et al., 2003), whereas *POMCA1* was reduced in 28 days feed deprived rainbow trout (*Oncorhynchus mykiss*) (Leder and Silverstein, 2006). In a recent study, *POMCA1* was found to be increased after 4 months feed deprivation in rainbow trout (Jørgensen et al., 2016). Furthermore, a postprandial increase in the brain expression of *POMCA1* and *POMCB* has been found in Atlantic salmon (*Salmo salar*), which may indicate a role of central *POMC* in short-term satiation signalling in this fish species (Valen et al., 2011). As such, the melanocortin system seems to be involved in food intake control in fish, but there are species specific differences in the responses to energy perturbations.

As in mammals, AgRP is generally regarded as an orexigenic signal in fish that exerts its orexigenic effect by inhibiting anorexigenic signalling via MC4R (Cerda-Reverter and Peter, 2003). AgRP overexpressing zebrafish show increased feeding activity and are obese, a finding which provides strong support for an orexigenic role of AgRP in fish (Song and Cone, 2007). Furthermore, hypothalamic *AgRP* mRNA abundance increases in response to feed deprivation in both zebrafish and goldfish (Cerda-Reverter and Peter, 2003; Song et al., 2003). On the other hand, the finding that brain *AgRP1*, but not *AgRP2*, expression decreased in response to 6 days feed deprivation in Atlantic salmon highlights the possibility for species specific differences regarding the function of AgRP in the control of food intake (Murashita et al. 2009). This assumption is supported by data from a 7 days feed deprivation study on common carp (*Cyprinus carpio*), which resulted in a reduction in brain *AgRP1* and *AgRP2* expression (Wan et al., 2012) and in a study on rainbow trout, where *AgRP* expression was unchanged in response to 4 months of feed deprivation (Jørgensen et al., 2016). Species-specific differences are also found in studies that investigated potential short-term effects of feeding and feed deprivation on central *AgRP* expression. For example, hypothalamic *AgRP*

expression was increased at 3 and 4 hours post-fasting in the cyprinid *Schizothorax prenanti* (Wei et al., 2013), whereas it was increased at 3 hours post-feeding in Atlantic salmon (Valen et al. 2010).

The mechanisms by which CART mediates its effect are not known, neither in fish nor in mammals. A role of CART in the control of food intake in fish has been suggested as icv injections of CART peptides have been shown to reduce feeding in goldfish (Volkoff and Peter, 2000). The effects of feed deprivation on CART expression differ between experimental designs and fish species investigated. For example *CART* expression decreased in 6 days feed deprived Atlantic cod (*Gadus morhua*) and Atlantic salmon, but was unaltered in 2 weeks feed deprived winter skate (*Raja ocellata*), as well as in 4 months feed deprived rainbow trout (Jørgensen et al., 2016; Kehoe and Volkoff, 2007; MacDonald and Volkoff, 2009b; Murashita et al., 2009).

The NPY family of peptides and their receptors are highly conserved across the vertebrate lineage (Cerdá-Reverter and Larhammar, 2000). However canonical Y1 and Y5 receptors found in mammals are not present in fish, suggesting that appetite related NPY signalling in fish may be mediated via different receptor subtypes (Larhammar and Salaneck, 2004). A recent study provided details about the localisation and mechanisms of NPY and AgRP signalling in the brain of zebrafish larvae. It was demonstrated that *NPY* is, in contrast to mammals, not co-expressed with *AgRP*, but that these neuropeptides are expressed in distinct areas of the hypothalamus. However, similar to mammals, it was found that the *AgRP* and *NPY* expressing neurons are also GABAergic (Jeong et al., 2018). An orexigenic role of NPY in fish has been proposed. Food intake is increased in response to icv injection of NPY in goldfish (López-Patiño et al., 1999). In addition, the NPY induced increase in food intake in goldfish could be attenuated by the administration of a receptor antagonist targeting the Y receptor family (de Pedro et al., 2000). Feed deprivation studies have evoked different responses in *NPY* expression. In goldfish, *NPY* expression was increased after 3 days of feed deprivation, and the increase was reversed by re-feeding (Narnaware and Peter, 2001). Similarly, *NPY* expression was increased in the preoptic area after 2 and 3 weeks of feed deprivation in chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutsch*) (Silverstein et al., 1998). In contrast, brain *NPY* expression was unaffected by 6 days feed deprivation in Atlantic salmon (Murashita et al., 2009).

Although the important central regulatory systems of food intake control are present in fish, the underlying mechanisms by which they act are far from understood. There is a diversity in responses of the various neuropeptides to feed deprivation between fish species. Established laboratory fish models such as zebrafish and goldfish are held in relatively stable environments and are likely to show different responses to changes in food abundance than fish, for example salmonids, that inhabit shifting environments at higher latitudes.

1.2.2 Peripheral short-term signals involved in food intake control

As in mammals, meal initiation and termination in fish are controlled by short-term feedback circuits between the gastrointestinal tract and the brain involving both neural and endocrine signalling.

An important short-term satiation signal in fish, as in mammals, is CCK. Both icv and intraperitoneal injections of CCK reduced food intake in goldfish (Himick and Peter, 1994). The peripheral action of CCK involves signal transduction via vagal afferent fibres (Kang et al., 2010). A role in satiation signalling of CCK has been implied in rainbow trout because food

intake was increased in response to the oral administration of a CCK receptor antagonist (Gelineau and Boujard, 2001).

Based on mammalian research, Ghr is considered orexigenic. Ghr has been identified and described in many fish species. It is predominantly expressed in the gastrointestinal tract, but has also been localised in a number of other peripheral tissues (Unniappan and Peter, 2005). Studies on goldfish have shown that Ghr exerts its orexigenic effects by signal transduction through vagal and splanchnic nerves (Matsuda et al., 2006). In addition, Ghr has a suppressive effect on the gene expression of anorexigenic *CKK* and *PYY* in the intestine of goldfish (Blanco et al., 2017). Whereas results from various studies on goldfish indicate an orexigenic role for Ghr (Miura et al., 2006; Miura et al., 2007; Unniappan et al., 2002), intraperitoneal injections of Ghr did not affect short-term food intake in rainbow trout (Jönsson et al., 2007). Furthermore, the finding that long-term Ghr treatment caused a reduction in food intake of juvenile rainbow trout gave rise to the suggestion that Ghr may be involved in the regulation of long-term energy balance in rainbow trout (Jönsson et al., 2010).

1.2.3 Integration of energetic status in the control of food intake in fish

In contrast to mammals, large knowledge gaps exist about how energy homeostasis is maintained in fish. A distinctive feature of many fishes compared to mammals is that they have indeterminate growth (Mommsen, 2001). Consequently, the question arises as to whether an energy homeostasis is needed.

Fish often respond to periods of energy shortage with an accelerated growth rate and increased food intake, in order to compensate for the previously lost body mass and to return to their original growth curve (Fig. 3) (Ali et al., 2003; Jobling and Johansen, 1999). Such growth compensation may only be partial, and some studies with salmonids have given support for the presence of a lipostat-like mechanism. The higher growth rate and increased food intake during compensatory growth may only be sustained until a replenishment of body lipids has occurred (Jobling and Johansen, 1999). Furthermore, it has been proposed for salmonids, that

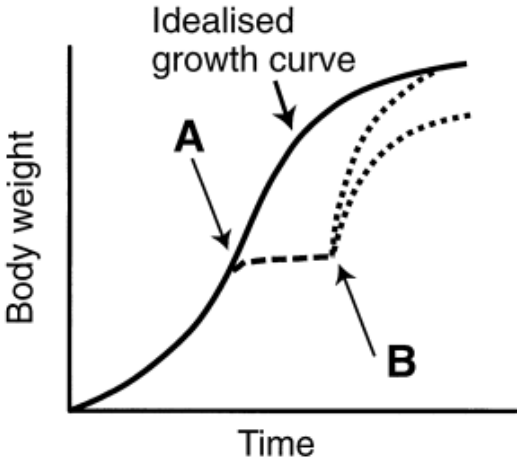

Figure 3 Model of compensatory growth in fish. A feeding restriction is imposed at time point A, and animals are returned to full, or ad libitum, feeding at point B. After the return to full feeding, growth rate is initially rapid, but then slows. The recovery of body weight may be either complete or partial. From: Hypophagia and catch-up growth, by Jobling and Johanson, 1999, Aquaculture Research, 30, 473-478. Reprinted by permission of John Wiley & Sons, Inc.

important gating mechanisms to life-history trajectories such as migration and reproduction depend on lipid status (Thorpe et al., 1998). Consequently, physiological assessment mechanisms of energy stores seem to be important in fish.

The identification of a gene encoding for a homologue to mammalian Lep in pufferfish (*Takifugu rubripes*) by Kurokawa and co-workers (2005) gave hope that this could shed light on the endocrine mechanisms involved in energy homeostasis in fish. Since its discovery, Lep has been identified in many fish species. In fish Lep is highly expressed in the liver, rather than

in adipocytes as observed in mammals (Frøiland et al., 2010; Gorissen et al., 2009; Rønnestad et al., 2010). Intraperitoneal administration of recombinant Lep led to a short-term decrease in food intake in rainbow trout (Murashita et al., 2008), and chronic Lep administration via osmotic pumps was associated with a reduced growth rate and an increased brain *POMCA1* expression in Atlantic salmon (Murashita et al., 2011). However, in contradiction, plasma Lep levels were elevated after 3 weeks of feed deprivation in rainbow trout (Kling et al., 2009). Furthermore, neither plasma Lep levels nor *Lep* expression in the liver were correlated with the amount of body fat in anadromous Arctic charr (Frøiland et al., 2012). Consequently, Lep is not regarded as a conveyer of energy status in fish and the endocrine mechanism involved in the adipostat of fish remain an enigma.

1.3 The seasonal anadromous Arctic charr

The Arctic charr (*Salvelinus alpinus* (Linnaeus, 1758)) belongs to the family Salmonidae. As its name suggests, the Arctic charr has a circumpolar distribution (Fig. 4) that ranges as far North as Ellesmere Island (Jørgensen and Johnsen, 2014).

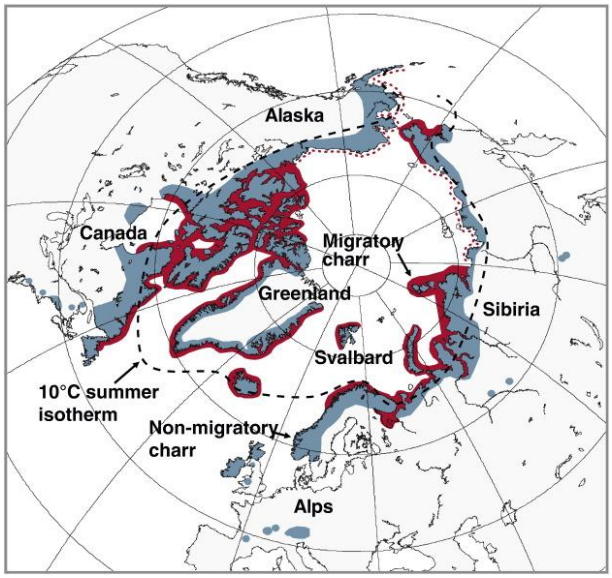

Figure 4 Circumpolar distribution of Arctic charr. Red: distribution of anadromous Arctic charr. From Rhythmic life of the Arctic charr: Adaptations to life at the edge, by Jørgensen and Johnsen, 2014, Marine Genomics, 14, 71-81. Reprinted by permission of Elsevier.

The Arctic charr is the northernmost freshwater fish in the world. Inhabiting oligotrophic and ultra-oligotrophic lakes of the North, the charr has to cope with an uttermost varying environment that includes periods of low food abundance. As a consequence, fish that display different life-history strategies can be found within this species (Klemetsen et al., 2003). For example, within the same population, some individuals live at the bottom of a lake, grow slowly, and reach maturity at a size of 7 cm, whereas their conspecifics in the littoral zone become larger and reach maturity at a size of 20-40 cm (Klemetsen et al., 2003).

Undertaking annual migrations to richer feeding grounds in the sea in order to overcome limited food resources in fresh water is a strategy found in Northern Arctic charr populations (Johnson, 1980). The distribution range of sea-migrating behaviour found in Arctic charr populations is shown in Figure 4.

The seawater migratory behaviour, so-called anadromy, is a common feature found in many salmonid species. However, in contrast to other sea-migrating salmonids that stay in the ocean for several years before returning to fresh water in order to spawn, the seawater

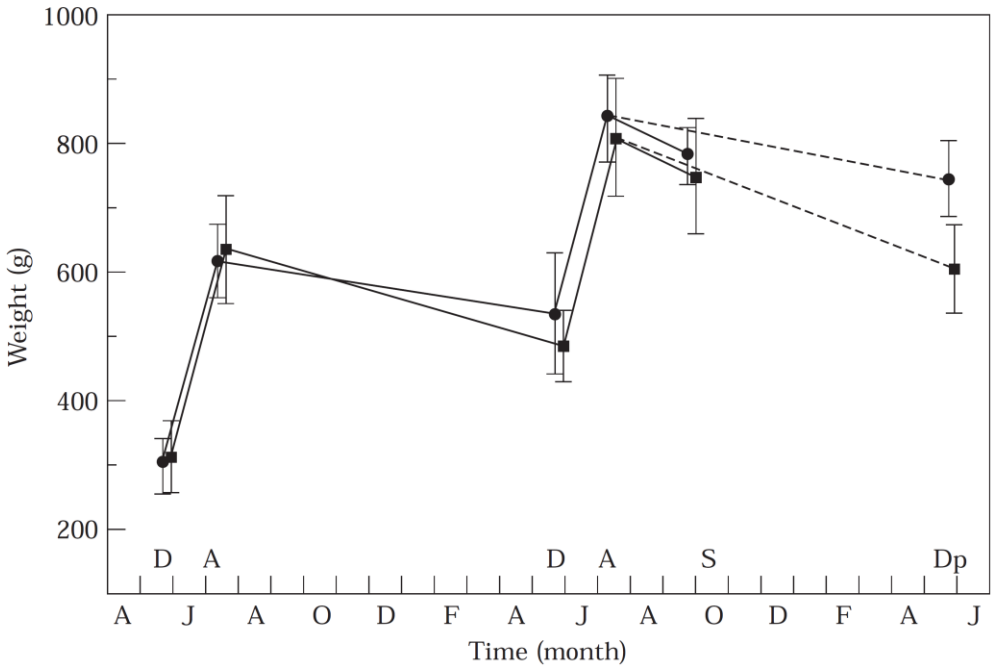

Figure 5 Seasonal variations in body weight of male (circle) and female (square) anadromous Arctic charr during the course of two annual migratory cycles. Data are presented as means ± S.D. for five fish each sex. D, descending; A, ascending; S, Spawning; Dp, descending post-spawners. From Seasonal patterns of growth, lipid deposition, and lipid depletion in anadromous Arctic charr by Jørgensen et al., 1997, Journal of Fish Biology, 51, 312-326. Reprinted by permission of John Wiley & Sons, Inc.

residence of anadromous Arctic charr lasts only for a few weeks during summer and occurs in both immature and mature individuals (Klemetsen et al., 2003). The annual food intake of anadromous charr is almost entirely dependent on feeding on marine prey during the relatively short period of the year that the fish is at sea (Swanson et al., 2011). As a consequence, anadromous Arctic charr undergo extreme seasonal shifts growth rate and body mass (Fig. 5) (Jørgensen et al., 1997). Wild anadromous Arctic charr may double their body mass and show an up to fivefold increase in body lipids during their short stay in seawater during summer (Jørgensen et al. 1997). It has been suggested that anadromous charr are feeding in the sea until replenishment of body lipids has occurred, which would imply the involvement of a lipostat-like control of food intake (Jobling and Johansen, 1999).

The observation that the seasonal feeding behaviour was maintained in captive anadromous Arctic charr which were provided with year-round *ad libitum* access to feed has given rise to the conjecture that the annual changes in feed intake of Arctic charr are endogenously regulated (Sæther et al., 1996). The mechanisms underlying the strong seasonal shifts in food intake and energy metabolism in charr are largely unknown. As such, the anadromous charr represents a fascinating fish species for the study of neuroendocrine control of feeding, both with regards to short-term and long-term control of food intake.

2. Aims of the study

The mechanisms of central control of food intake are fairly well understood in mammalian model organisms. Many of the key players involved in central appetite regulation in mammals have been described in fish and a number of studies, especially those on well-known fish models such as zebrafish and goldfish, have provided evidence for a similar function of these in the central control of food intake in fish. In contrast, several studies on fish species that inhabit shifting environments have provided inconclusive results and as such emphasise a need for studies on additional fish species to get a broader picture of the regulatory appetite mechanisms in fish. A better knowledge of food intake and energy homeostasis control in fish is directly relevant for fish production, and is sought after by the aquaculture industry. In addition, the comparative perspective offers insights into the function and evolution of the control of food intake and energy homeostasis across vertebrates.

In this work we aimed at shedding light on the role of central neuropeptides in the control of food intake in the highly seasonal anadromous Arctic charr. We were particularly interested in the question about how the charr can fast for months without getting hungry.

3. Project proceeding

Firstly, we wanted to assess the seasonal gene expression patterns of known putative appetite regulators in captive anadromous Arctic charr during the course of its circannual feeding rhythm in order to identify potential differences in orexigenic and anorexigenic signalling between voluntarily feeding and fasting charr (Paper I). Secondly, we wanted to reveal the role of central appetite regulators in the control of food intake in the charr by exposing the fish to feed deprivation and re-feeding in an attempt to provoke responses (Paper II, Paper III). Finally, we aimed at unravelling global effects of long-term feed deprivation on the charr brain transcriptome (Paper III). The implementation of high-throughput sequencing in this project should also aid to identify new actors that may be involved in the control of food intake and energy homeostasis in fish.

4. Discussion of main findings

The highly seasonal anadromous Arctic charr exhibits huge differences in feeding activity across season that are maintained in captivity and hence are suggested to be endogenously regulated (Jørgensen and Johnsen, 2014). The seasonal alternations between voluntarily fasting and voraciously feeding make this species a fascinating model for studying appetite mechanisms in general, and long-term regulation of energy homeostasis in particular. The question whether central appetite regulators are involved in the control of the circannual feeding rhythm in the Arctic charr had not been addressed prior to this work.

4.1 Expression patterns of appetite regulators do not mirror the charr's seasonal feeding behaviour

Our first aim was to describe the central expression patterns of anorexigenic and orexigenic neuropeptides and related receptors during the course of a seasonal feeding cycle in the Arctic charr (paper I). We hypothesised to find an up-regulation of orexigenic signalling in feeding

charr during summer, and an up-regulation of anorexigenic signalling in non-feeding charr during spring and winter provided that central orexigenic and anorexigenic neuropeptides were involved in the control of seasonal food intake in the charr. In order to test this hypothesis, we followed two year old, captive, immature Arctic charr during a 10 months period and measured gene expression in telencephalon, mesencephalon and hypothalamus of non-feeding charr sampled in May and January, and feeding charr sampled in July. The charr used in the experiment were all tagged with passive integrated transponders which enabled us to obtain the individual growth history of each fish. Gene expression of orexigenic *AgRP*, *NPY* and anorexigenic *POMC*s, *MC4R*, *CART*, *Lep* and *LepR* were measured by RT-qPCR.

As expected, the charr displayed typical seasonal growth patterns during the experimental period. An initial decrease in body mass and condition factor from April to June was followed by a marked increase from July to end of August and thereupon, body weight and condition factor decreased from September and until end of January, when the experiment ended. However, the gene expression data did not provide an indication for an up-regulation of satiety signalling in voluntarily fasting charr in winter and spring and a hunger signalling in voraciously feeding charr in summer. On the contrary, in the hypothalamus *LepR*, *MC4R* and *LepA1* were lower expressed in fasting charr in winter and spring than in feeding charr in summer. No differences were found for *AgRP*, *POMC*s and *CART*. In the mesencephalon, *AgRP* and *NPY* were higher expressed in non-feeding charr sampled in May and January than in feeding charr sampled in July. Given the anorexigenic role of melanocortin signalling and the orexigenic role of NPY and AgRP in many fishes (Rønnestad et al., 2017), this finding could imply a down-regulation of satiety signalling and an up-regulation of hunger signalling in non-feeding charr. Similar to the Arctic charr, hypothalamic *NPY* was higher and gut *CCK* was lower expressed in voluntarily fasting winter flounder (*Pseudopleuronectes americanus*) in winter than in summer (MacDonald and Volkoff, 2009a). The higher expression of *NPY* and *AgRP* in the mesencephalon and the lower expression of *MC4R*, *LepR* and *LepA1* seen in non-feeding charr in this work may suggest a presence of central orexigenic signalling in non-feeding charr. We did not measure *Ghr* expression in this study, but Ghr has previously been reported to be higher expressed in the stomach of voluntarily fasting charr during spring and autumn than in feeding charr during summer (Frøiland et al., 2010). Ghr has been demonstrated to exert its orexigenic effects by stimulation of brain *NPY* expression in goldfish (Miura et al., 2006). Future studies are needed to test for a functional link between Ghr and central appetite regulators in the Arctic charr. Taken together, the data on expression patterns of appetite regulators obtained in this study do not indicate, at least not on the mRNA level, that the charr's seasonal feeding behaviour is governed by changes in the expression of central appetite regulators. Hence, our results would imply that the seasonal feeding in Arctic charr is controlled by some other, to date unknown, mechanism.

However, the results must be interpreted with caution as the gene expression data of the sampled fish represent only snapshots taken at single time points of the charr's seasonal feeding cycle. One challenge in this, and other experiments that involve terminal sampling, is the choice of the right sampling time point. In this study, the criteria for sampling of voluntarily fasting fish were a preceding stagnation in growth rate and the absence of food in the gastrointestinal tract, whereas the criteria for sampling of the feeding fish in July were a preceding increase in growth rate, and the presence of food in the gastrointestinal tract. As a result, we sampled charr that were either in a steady-state winter-fasting or in a steady-state summer-feeding mode. Therefore, we cannot exclude the possibility that changes in central appetite signalling may have occurred during the transition phases, i.e. during the periods of changing the set-point from voluntarily fasting to feeding and vice versa. Furthermore, mRNA

measurements are only indicators of changes occurring on the transcript level, and due to post-transcriptional and post-translational processing it is uncertain whether the observed changes in gene expression were also present on a functional level.

4.2 Effects of short-term feed deprivation on appetite regulators

A number of studies on different fish species have used inflicted energy perturbation by feed deprivation to provoke changes in appetite signalling (Volkoff, 2016). As we were unable to identify any clear seasonal differences in the expression of putatively orexigenic and anorexigenic actors comparing voluntarily feeding and fasting charr, we subjected summer-feeding charr to 2 and 7 days feed deprivation (paper II). No differences in the expression of hypothalamic appetite regulators were found after 2 and 7 days of feed deprivation. A major problem encountered in the short-term feed deprivation experiment were large within-group variations in gene expression. The Arctic charr tends to form social hierarchies that may cause huge differences in feed intake between dominant and subordinate fish as well as clear signs of stress responses in the latter (Jobling and Wandsvik, 1983; Øverli et al., 1998). Whereas, one might expect that social hierarchies weaken during feed deprivation as the monopolisation target, feed, is absent, the contrary has been shown to be the case in Atlantic salmon (Symons, 1968). Thus, the establishment of social hierarchies may be one explanation for the variations found in both experimental groups. Yet, it may just be so that individual Arctic charr show variable responses to short-term feed deprivation. We addressed the problem of within-group variations by increasing the stocking density in a second feed deprivation experiment (paper II). In addition, we increased the length of feed deprivation to 4 weeks, in order to assure that the feed-deprived charr were in a negative energetic state and we increased the number of fish sampled for analyses.

4.3 Feed flavour and re-feeding stimulate responses of central appetite regulators in long-term feed deprived charr

Based on the gene expression patterns seen during the seasonal feeding cycle, we considered that the feed deprived and fed charr might adjust the central appetite regulation to the respective feeding trial. We assumed that, if a steady-state like regulation of appetite was the case, differences in hypothalamic appetite signalling would not necessarily be observed between the ad libitum fed and feed deprived charr. Consequently, we simulated a transition phase by re-feeding the previously feed deprived charr at the end of the experiment. A major draw-back of re-feeding trials is, however, that it might be difficult to discriminate potential hunger signalling in response to the reappearance of feed from satiation signalling in response to the feed ingested. To overcome this problem, we decided to provoke hunger signalling by exposing the previously feed deprived fish to fish feed flavour. Consequently, we subjected the feed deprived fish at the end of the experiment for 1 and 5 hours to either fish feed flavour (fish feed pellets presented into a nylon bag in the water), a dummy (similar nylon bag but with clean stones), or we re-fed them, whereas the control group was fed as before.

Despite significant differences in body weight and condition between fed and feed-deprived fish at the time of sampling, with lower *K* and *IGF1* expression in brain and liver (data not shown) of feed deprived fish, we did not find any differences in hypothalamic gene expression of neuropeptides, or neuropeptide receptors between fed and feed deprived charr. Re-fed and flavour exposed charr responded to the reappearance of feed with an increase in *AgRP* expression after 1 hour. Furthermore we found an increase in *CART* expression in the

flavour and re-fed group after 1 hour. In addition, expression of *MC4R* was increased in both the flavour and re-fed group after 5 hours. Even though the data provide no basis to discuss the underlying mechanisms of the observed changes in gene expression, these results provide strong evidence for a role of AgRP, MC4R and CART in the regulation of food intake in the Arctic charr. Remarkably, the responses were very similar in re-fed fish and feed flavour exposed fish and it would be interesting to increase the duration of feed flavour exposure and re-feeding after long-term feed deprivation as well as to test the effects of feed flavour in naturally feeding charr at different times of the year.

In summary, we demonstrated in paper II that the expression of central appetite regulators was similar in strongly energy perturbed fish and well-fed fish, while abrupt provision of feed or exposure to feed flavour caused responses. As such, the results from paper II confirm the findings from paper I, in which we found that hypothalamic expression of most neuropeptides did not differ between naturally feeding and non-feeding charr and give support for the suggestion that differences in central appetite signalling may only be seen during phases when appetite either returns or ceases.

4.4 A transcriptomic approach to study regulation of food intake in the charr

A major limitation of gene expression studies using RT-qPCR is the relatively narrow focus on a selected number of genes. For this reason, we decided to employ a transcriptomic approach to evaluate global effects of long-term feed deprivation on the brain of Arctic charr. In order to do so, we compared the brain transcriptomes of fed and feed-deprived charr over a 4 weeks period during the summer feeding season (paper III).

The brain is a heterogeneous tissue and it was discussed whether the transcriptome of an isolated single compartment or of several pooled brain compartments that are known to be involved in food intake control should be sequenced. As we did not know where to expect differences and to what extent they would occur, we decided to pool equal amounts of RNA from the telencephalon, mesencephalon and hypothalamus for RNA sequencing.

High-throughput sequencing and subsequent bioinformatics analyses resulted in a *de novo* transcriptome which consisted of 49829 contigs. Strikingly, despite significant differences in K between fed and feed deprived fish, the global transcriptome expression patterns differed strongly over time (T_1 versus T_0), but only marginally between fed and feed deprived fish at the end (T_1) of the experiment. Differential gene expression analysis identified a total of 4570 differentially expressed genes between the feed deprived fish at T_1 and fed fish sampled from the stock tank at T_0, 2819 differentially expressed genes between the fed fish at T_1 and fed fish sampled from stock at T_0, and 175 differentially expressed genes between feed deprived fish and fed fish at T_1. We further used gene enrichment analysis in order to identify biological processes that differed over time, and between the feed deprived and fed charr at T_1. We found a few indications for metabolic suppression in the group of feed deprived charr compared with the group of fed charr. The expression of haemoglobin subunits was found to be reduced by feed deprivation. A similar result was found in the brain transcriptome of 21 days feed deprived zebrafish (Drew et al., 2008).

In line with the previous findings in this work (paper I, paper II), candidate appetite regulators involved in food intake control differed between T_0 and T_1, albeit not between fed and feed deprived charr at T_1. However, we identified significant differences in *deiodinase 2b* (*Dio2b*) expression. *Dio2b* was lower expressed in feed deprived and fed charr at T_1 compared with fed charr at T_0 and it was significantly lower expressed in feed deprived charr than in fed charr at T_1. Dio2, encoding an enzyme responsible for activating thyroxin by catalysing

deiodination of thyroxin to the active hormone triiodothyronine, seem to be involved in seasonal control of food intake in Siberian hamster (Cubuk et al., 2017). Furthermore, studies on Atlantic salmon have shown that brain expression of *Dio2b* is stimulated in response to long-day photoperiod (Lorgen et al., 2015). Our finding indicates that Dio2b is modulated by both season and feed deprivation in the Arctic charr. In context with the already known functions of Dio2b in seasonal mammals and fish, our finding suggests that Dio2b is a very relevant candidate to be followed up in connection with the seasonal feeding rhythm of the Arctic charr.

Another new potential candidate identified in the transcriptome study that may be involved in the regulation of food intake is VGF (non-acronymic). We found a higher expression of *VGF* in feed deprived than in fed charr at T_1. In mammals, there is evidence for a role of VGF derived peptides in the control of energy homeostasis (Lewis et al., 2015). To the best of our knowledge, VGF has not received any attention in the field of fish physiology. Further investigations are needed to characterise VGF in fish.

In summary, we showed in paper III that strong shifts in the brain transcriptome occur over time, whereas feed deprivation generally only enhanced changes that was seen over time. Similar to the data in paper I and paper II, we did not identify differences in appetite regulators between fed and feed deprived fish at T_1. However, pooling may have masked tissue specific changes in gene expression. Yet, we identified two new candidate genes, *Dio2b* and *VGF*, which may be involved in the control of energy homeostasis and food intake in the charr. These will be further investigated.

4.5 Conclusion

The results of the present work indicate that orexigenic and anorexigenic appetite regulators do not seem to be the underlying cause of the tremendous seasonal changes in food intake in the Arctic charr. The mechanisms allowing the seasonal anadromous charr to remain anorexic through many months of emaciation during winter, without “being hungry”, remain to be described. We have demonstrated by the use of feed deprivation experiments that the Arctic charr does not seem to respond to energy perturbation with an increase in central hunger signalling in the hypothalamus and in the brain. Wild anadromous Arctic charr inhabit the oligotrophic lakes of the North where they feed little to nothing for several months during winter. The absence of central hunger signalling in response to feed deprivation may reflect their strong resilience to restricted food availability.

However, we found that the presentation of fish feed and fish feed flavour to long-term feed deprived charr provokes short-term changes in the hypothalamic gene expression of *AgRP*, *CART* and *MC4R*. As such, changes in appetite signalling in the charr may be seen during transition phases, but not when comparing continuously fed with continuous feed deprived charr. By studying the brain transcriptomes of fed and feed deprived Arctic charr, we could identify that a few metabolic related processes e.g. oxygen transport are affected by long-term energy perturbation during summer. We observed strong shifts in the transcriptome from the start and the end of the experiment that were independent of the feeding trial. Among the few genes that were found differentially expressed between fed and feed deprived charr at T_1 were *Dio2b* (down-regulated) and *VGF* (up-regulated). Both are interesting candidates that need to be further investigated in connection with the control of food intake in the Arctic charr.

5. Future perspectives

The Arctic charr is not only a fascinating fish, but also turned out to be a difficult species for studying the control of food intake. Hence, many questions are left unanswered but a valuable addition to a framework for the understanding of food intake control in Arctic charr and seasonal fishes could be acquired. In order to cover the open questions regarding the control of seasonal feeding in the charr, a tighter sampling schedule during the transition stages is required. Such a strategy should be applied in future studies. The speculation that differences in mesencephalic *NPY* and *AgRP* expression found in the present study, together with the differences in stomach *Ghr* expression found in a previous study, may be an indication for a downstream regulation of appetite. This hypothesis requires further testing as only little is known about a possible link between peripheral factors, such as Ghr and CCK, and central neuropeptides. Co-expression studies of *GHSR* and *NPY* and *AgRP* in the charr brain using in situ hybridisation may be a starting point to shed light on this possible link. Furthermore, functional linking between other peripheral afferent signalling and central neuropeptides may be evaluated by the use of pharmaceutical inhibitors of the vagal afferents, for example capsaicin. In general, a detailed mapping of the central neuropeptides investigated in the present studies by the use of *in situ* hybridisation is highly needed, as many of these exhibit pleiotropic functions. As a consequence, their expression may vary in a tissue specific manner. The use of fish feed flavour in experimental designs has a potential to reveal new aspects of food intake control in fish. This method should be further verified in future experiments by the parallel administration of an olfactory inhibitor in order to confirm the olfactory-hypothalamic link. Such an inhibitor could for example be copper as it has been shown to impair olfactory receptor pathways in salmonids when administered to surface water (Baldwin et al., 2003).

The functional role and localisation of VGF and Dio2b in the seasonal Arctic charr should be further explored. To begin with, their exact localisation in the brain needs to be identified by the use of *in situ* hybridisation studies. Consequently, VGF and Dio2b should be studied in a seasonal context in the charr in order to target their possible involvement in the control of seasonal feeding.

Little is known about the functional role of the different paralogues of neuropeptides in the context of appetite regulation. Most studies that have investigated appetite control mechanisms in fish, including the present work, have only measured gene expression levels, and there is a general need for measurements at the protein level.

Finally, the CRIPR/Cas methodology has been proven a powerful tool to knockout genes in non-model organisms. Consequently, the application of this modern technology in future studies may strongly improve the knowledge about the function of specific actors in the regulation of food intake and energy homeostasis in fish, and may also aid to shed light on the mechanisms underlying the seasonal feeding behaviour in Arctic charr.

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