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, Chandra Sekhar Ravuri, Malcolm Jobling, Even Hjalmar Jørgensen*

Department of Arctic and Marine Biology, UiT—The Arctic University of Norway, Tromsø, Norway

* even.jorgensen@uit.no

Abstract

The highly seasonal anadromous Arctic charr (*Salvelinus alpinus*) was used to investigate the possible involvement of altered gene expression of brain neuropeptides in seasonal appetite regulation. Pro-opiomelanocortin (*POMCA1, POMCA2*), Cocaine and amphetamine regulated transcript (*CART*), Agouti related Peptide (*AgRP*), Neuropeptide Y (*NPY*) and Melanocortin Receptor 4 (*MC4-R*) genes were examined. The function of centrally expressed Leptin (Lep) in fish remains unclear, so Lep (*LepA1, LepA2*) and Leptin Receptor (*LepR*) genes were included in the investigation. In a ten months study gene expression was analysed in hypothalamus, mesencephalon and telencephalon of immature charr held under natural photoperiod (69°38′ N) and ambient temperature and given excess feed. From April to the beginning of June the charr did not feed and lost weight, during July and August they were feeding and had a marked increase in weight and condition factor, and from November until the end of the study the charr lost appetite and decreased in weight and condition factor. Brain compartments were sampled from non-feeding charr (May), feeding charr (July), and non-feeding charr (January). Reverse transcription real-time quantitative PCR revealed temporal patterns of gene expression that differed across brain compartments. The non-feeding charr (May, January) had a lower expression of the anorexigenic *LepA1, MC4-R* and *LepR* in hypothalamus and a higher expression of the orexigenic *NPY* and *AgRP* in mesencephalon, than the feeding charr (July). In the telencephalon, *LepR* was more highly expressed in January and May than in July. These results do not indicate that changes in central gene expression of the neuropeptides investigated here directly induce seasonal changes in feeding in Arctic charr.

Introduction

In mammals, appetite is regulated by feedback mechanisms involving peripheral energy status, metabolic signals and centrally produced appetite regulators. Key to central regulation are two
sets of neurons located in the arcuate nucleus (ARC) of the hypothalamus; appetite stimulating (orexigenic) neurons expressing Neuropeptide Y (NPY) and Agouti related peptide (AgRP), and appetite inhibiting (anorexigenic) neurons expressing Pro-opiomelanocortin (POMC) and Cocaine and amphetamine regulated transcript (CART). These exert their effects via second-order neurons expressing members of the NPY and Melanocortin (MC) receptor families [1, 2]. A major signalling molecule is the 16 kDa peptide Leptin (Lep) [3], which circulates proportionally to the amount of body fat in mammals and enters the ARC [4]. The Lep signal received by the Leptin receptor (LepR) is transduced via the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signalling pathway [5]. This leads to an activation of POMC and CART expression and an inhibition of AgRP and NPY expression [2].

Genes encoding many of the signalling molecules and their receptors found in mammals have been identified in fish [6] but evidence for appetite regulating roles in fish is inconclusive. For example, treatment of fish with NPY has consistently resulted in increased food intake [7–14], but feed deprivation studies have resulted in both an increase [13, 15–18] and no change [19–21] in brain NPY mRNA expression. There is support for the notion that Lep has an anorexigenic function in fish [22, 23], but there are currently no data that have revealed a lipostatic role of Lep in long-term energy regulation in fish [24–29]. In contrast to mammals, fish Lep is expressed in a wide range of tissues, including the brain [28, 30–33]. A possible role of centrally produced Lep in the regulation of appetite and energy homeostasis in fish has, however, received little attention. Most studies on central expression of appetite-regulating genes in fish have focused on either the hypothalamus or the whole brain, whereas the telencephalon and mesencephalon have been studied to a lesser degree [6].

Arctic charr (Salvelinus alpinus) occur in oligotrophic waters of the northern hemisphere [34] and in the northernmost part of their distribution range they can be anadromous and migrate to the sea each summer to feed [34, 35]. Marine prey can account for up to 90% of the annual food intake of the anadromous charr [36] and feeding is reduced or absent during overwintering in fresh water [37–40]. Furthermore, offspring of anadromous charr held in captivity at constant temperature fast voluntarily for several months during winter even though feed is available, implying that their seasonality is endogenously regulated [41]. As such, the anadromous charr can serve as a model for investigating mechanisms associated with long-term regulation of appetite and energy homeostasis in teleosts. A previous study with anadromous Arctic charr revealed no link between plasma Lep and changes in appetite and body adiposity across seasons [25]. To date it has not been investigated whether central Lep and LepR play a role in seasonal appetite regulation in the charr.

We hypothesised that the seasonal feeding cycle of anadromous Arctic charr is orchestrated by changes in gene expression of appetite regulators located in the brain and that differences in central expression of Lep and its receptor may play a role in long-term appetite regulation in Arctic charr. To this end, we investigated the gene expression of putative appetite regulators, including Lep and its signal transducing receptor LepR, in the hypothalamus, mesencephalon, and telencephalon of anadromous Arctic charr sampled during their natural seasonal feeding cycle in May (non-feeding), July (feeding) and January (non-feeding).

**Material and Methods**

**Experimental set-up**

The experiment used offspring of wild anadromous Arctic charr supplied by a government-run restocking program in the Hals watercourse (70°N), Finnmark, northern Norway. In the hatchery, the eggs were incubated in darkness at 6–8°C, and after hatching in April 2010 the fish were held at elevated temperature (~10°C) and exposed to continuous light until July.
From then on they were kept at ambient water temperature and fed in excess on commercial dry-pellet feed (Skretting, Stavanger, Norway) under continuous light until September 9, after which they were held on a 10:14-h light:dark cycle and at ambient water temperature until the start of the experiment. All feeding was by automatic feeders continuously for 24 h.

On March 6, 2012, a total of 420 individually tagged (passive integrated transponders) charr were transported from the rearing facility in Finnmark to the Aquaculture Research Station in Tromsø (ARST; 69°N). At ARST they were held in a 7 m² circular tank supplied with fresh water at ambient temperature under natural light conditions (room with transparent roof) and fed in excess with commercial dry-pellet feed (Skretting, Stavanger, Norway) until April 24, when they were anaesthetized (benzocaine, 60 ppm) and measured for body mass and fork length to the nearest milligram and millimetre, respectively. Thereafter, 152 size-sorted fish with an average weight and length of 154.6 ± 1.6 g and 26.3 ± 0.1 cm, respectively, were distributed among two, 500 L circular tanks supplied with fresh water (76 fish per tank). The fish were then held under simulated natural light conditions (69°N) and fed in excess with commercial dry-pellet feed until the end of the experiment in January 2013.

Every two to four weeks from April 2012 to January 2013, all fish in both tanks were anaesthetized in 60 ppm benzocaine, identified by tag reading and fork length and body mass measured. Periods of negative and positive weight change were considered to reflect non-feeding and feeding states, respectively. Sampling of fish for Reverse Transcription real-time quantitative PCR (RT-qPCR) was carried out between 10.00 am and 2.00 pm on three occasions: on May 15, July 27 (2012) and January 18 (2013). Five fish from each tank (total 10 fish per sampling time point) were killed by an overdose of benzocaine (150 ppm), brains were dissected out and the hypothalamus, mesencephalon, and telencephalon were immediately put separately in 1.5 ml Eppendorf tubes (Sigma-Aldrich Co. LLC, Switzerland) containing 1 ml RNA-later. Samples were kept at 4°C for ca. 24 h, and they were then stored at -20°C until extraction of total RNA. The sex of each fish was noted, the gonad removed and weighed, and when present, stomach contents were removed and weighed. As expected from the temporal change in growth rate of the fish groups, all fish sampled in May and January had empty stomachs, whereas those sampled in July had food in their stomach at the time of sampling (Table 1).

Body condition of the fish sampled for gene expression studies was assessed using Fulton’s condition factor $K$ calculated according to Ricker [42]: $K = (\frac{W \times L^{-3}}{100}$, where $W$ is body mass in g, and $L$ is fork length in cm. Gonadosomatic index (GSI) was calculated as $\frac{GW \times W^{-1}}{100}$, where $GW$ is the weight (g) of the gonad. All data are presented as mean ± standard error of mean (s.e.m.).

The experiment was approved by the Norwegian Committee of Ethics in Animal Experimentation, Id no. 4187.

**Extraction of mRNA and RT-qPCR analyses**

Tissues were disrupted using TissueLyser II (QIAGEN, Hilden, Germany), and RNA was extracted using the RNeasy Plus Universal Mini Kit (QIAGEN, Hilden, Germany) according to

| Sampling Date | Stomach content [%BM ± s.e.m.] | GSI [%BM ± s.e.m.] |
|---------------|---------------------------------|-------------------|
| 15.05.2012    | 0.0 ± 0.0                       | 0.2 ± 0.1         |
| 27.07.2012    | 3.5 ± 0.4                       | 0.1 ± 0.1         |
| 18.01.2013    | 0.0 ± 0.0                       | 0.6 ± 0.1         |

Table 1. Stomach content in % of body mass (BM), and gonadosomatic index (GSI) in % of BM of anadromous Arctic charr ($n = 10$) sampled during study.

doi:10.1371/journal.pone.0138857.t001
the manufacturer’s protocol. Concentration and purity of RNA were measured using NanoDrop ND2000c (Thermo Scientific, MA USA) and when the 260/280 or 260/230 absorbance ratio was below the quality threshold (1.7), samples were further purified using ethanol precipitation. Genomic DNA was removed by treating the RNA with Ambion TURBO DNA-free™ Kit (Life Technologies, CA, USA). A total of 2000 ng RNA was then reverse transcribed to cDNA using iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, CA, USA). Reverse transcription was conducted according to the manufacturer’s protocol in a total reaction volume of 20 μl. No-reverse transcriptase (no-RT) controls were included in the reverse transcription step. The cDNA was diluted ten-fold. Amplification using qPCR was performed in duplicate using Hard-Shell™ Low-Profile Thin-Wall 96-Well Skirted PCR Plates (Bio-Rad, CA, USA), 10 μl 2x SsoAdvanced™ Universal SYBR™ Green Supermix (Bio-Rad, CA, USA), 1 μl primer mix (final concentration 250 nM) and 6 μl cDNA in a total reaction volume of 20 μl. Gene specific primers were designed by PrimerDesign (Southampton, UK) and verified for efficiency by performing RT-qPCR on serial dilutions (Table 2). Both no-RT controls, and one no-template control were included in the amplification step for each target gene. The amplification steps were as follows: 50°C for 10 minutes, 95°C for five minutes, [95°C for 10 seconds, 60°C for 30 seconds, plate read] x 40, 95°C for 10 seconds. The PCR product was subjected to a melt curve analysis with a temperature range of 65°C to 95°C, an increment of 0.5°C, and one plate read after each increment, to ensure product specificity. All qPCR analyses were run with CFX96 Real-Time PCR Detection System (Bio-Rad, CA, USA) and the software CFX Manager 3.0 (Bio-Rad, CA, USA).

Table 2. Forward (F) and reverse (R) primer sequences used for cDNA amplification by qPCR.

| Gene                                | Primer Sequence (5’-3’) | bp | Accession number | Efficiency [%] |
|-------------------------------------|--------------------------|----|------------------|----------------|
| Elongation factor 1 alpha (EF 1α)   | F AGGCATTGACAAAGAGAACCATT 119 | AF498320.1 | 99.9            |
|                                     | R TGATACACGCCTCCCTCTC     |    |                  |                |
| Pro-opiomelanocortin (POMC) A1      | F ACGTTCAAAAATGTCAATCAAAGGAGAAGAGAG 83  | AB462418 | 106.4           |
|                                     | R CACCTATCCTCCCTCCCTCTC   | 119 | AB462420         | 106.9          |
| Pro-opiomelanocortin (POMC) A2      | F GGGAAAGAAAGAGAGAGAGAAG 119 | AB462420 | 106.9          |
|                                     | R CAAATACCCACCCCATCAACA   | 119 | AB462420         | 106.9          |
| Cocaine and amphetamine regulated transcript (CART) | F GTCCATCGTTCTTAGCTGCAAG 115 | AB455538 | 107.1          |
|                                     | R CAGTTCTTTGTGTCTCAAGA    |     |                  |                |
| Melanocortin receptor 4 (MC4-R)     | F TTCTCACACCTGGGATAGTCA   | 113 | AY354915.1       | 106.7          |
|                                     | RCACAGCAGGAGAACAGGATGAAT  |    |                  |                |
| Leptin (Lep) A1                     | F TCCTAGACTGAGGCAGACCT    | 92  | JQ615967.2       | 108.6          |
|                                     | R GCCTGGGAGAGGCTGATAT     |    |                  |                |
| LepA2                               | F TGGCACTAAACAGAGCTCAAGG  | 102 | AB490667.1       | 108.3          |
|                                     | R CTCAGTGTAGATCTCAAGTCA   | 113 | AY354915.1       | 106.7          |
| Leptin receptor (LepR)              | F CTTGCTCGGGGAGAGGAGGA    | 129 | KC683373.1       | 105.3          |
|                                     | R CCAATACCCACCCCATCAACA   |    |                  |                |
| Agouti related peptide (AgRP)       | F TGGCCGAAGACCTGAAGAAG    | 123 | CA34080.1        | 104            |
|                                     | R CGTGGTGCTGCTCTCTGAT     |    |                  |                |
| Neuropeptide (NP) Y                 | F AGAATTGCTGCTGAGAGGAGAGA 83 | AF203902 | 107.2          |
|                                     | R GGGAGAAGGAGGAGGAGGAGAAG |    |                  |                |

All primers were produced and verified by PrimerDesign Ltd (Southampton, UK). Bp = product length in base pairs. Efficiencies were tested using serial dilutions.

doi:10.1371/journal.pone.0138857.t002
Data treatment and statistics

Relative fold change of gene expression was calculated using the $\Delta \Delta Ct$ method [43]. Elongation factor 1 alpha (EF1α), a stable reference gene in Atlantic salmon (*Salmo salar*) [44], was used to normalise the Ct values of the target genes. Normalized qPCR data were LOG transformed prior to statistical testing [45].

A one-way ANOVA was used to test for differences in gene expression across the sampling dates.

The significance level was set to $p < 0.05$. When differences were found, post-hoc testing was carried out using Tukey’s Honestly Significant test. All statistical testing was done with SYSTAT 13 and figures were drawn using SigmaPlot 12.5 (both Systat Software, CA, USA).

Results

Seasonal changes in body mass and condition factor

The body mass of the fish sampled on May 15 had decreased between March 28 and the sampling date, indicating that the fish were in a prolonged non-feeding state (Fig 1A). For fish sampled on July 27 there had been a marked increase in body weight from mid-June onwards, indicating that the fish were feeding and growing. Fish sampled on January 18 had gradually decreased in body mass since October, indicating an extended period without feeding. The condition factor $K$ (Fig 1B) of the fish sampled in May and January had decreased in the period prior to the samplings, whereas there had been a marked increase in condition factor prior to the sampling of fish in July; this provides confirmation of the feeding status of the sampled fish. All sampled charr were immature and had a GSI < 1% (Table 1).

Relative quantification of gene expression in the three brain compartments

In the hypothalamus there were no significant differences in gene expression of either the anorexigenic POMCs, CART and LepA2, or orexigenic AgRP between sampling dates (Fig 2A, Table 3). The expression of NPY was very low (Ct > cycle 35) on all sampling dates and the data were not analysed statistically. In May and January gene expressions of LepA1, MC4-R and LepR were two-fold lower than in July, whereas no significant differences in the expression of these genes were found between the fish sampled in May and January (Fig 2A, Table 3).

In the mesencephalon, relative gene expression of POMCA1 and POMCA2 did not differ between the sampling dates (Fig 2B, Table 3). Gene expression of NPY was twelve-fold higher in January than in July, and about three-fold higher in May than in July. Expression of AgRP was seven-fold higher in May and January than in July (Fig 2B, Table 3). Gene expression of CART was 2.5-fold higher in January than in July. No significant differences were found when comparing the expression of CART in May and July, and January and May (Fig 2B, Table 3). LepR expression was four-fold higher in January than in both May and July, but there was no significant difference between May and July (Fig 2B, Table 3). The expression of LepA1 was three-fold higher in January than in July and seven-fold higher in January than in May, but no significant differences for LepA1 expression were detected between the May and July samples. The expression of both MC4-R and LepA2 was three-fold higher in January than in July and 2.5-fold higher in May than in July (Fig 2B, Table 3).

In the telencephalon, there were no significant differences in gene expression between sampling times for the orexigenic neuropeptides AgRP and NPY, or for the anorexigenic neuropeptides CART and LepA2 (Fig 2C, Table 3). The expression of LepR was two-fold higher in both May and January than in July. Expression of MC4-R was slightly higher in January than in
May, but no significant differences in expression were found between January and July, and May and July (Fig 2C, Table 3).

**Discussion**

We aimed to use the seasonal feeding cycle of anadromous Arctic charr to reveal central mechanisms involved in long-term regulation of appetite and energy homeostasis in fish. The temporal changes in body weight and condition factor (Fig 1A and 1B) correspond with those seen in previous studies [24, 41, 46, 47] and confirm the existence of a pronounced seasonality in appetite and growth of Arctic charr when given continuous access to food. The fact that the charr sampled in May and January had lost weight in the period before sampling and had empty stomachs support the existence of a winter non-feeding state, and that they represented individuals from the large fraction of the anadromous charr population that do not feed during the winter [41].
Although the summer feeding period partly coincided with increased water temperature, the most pronounced increases in appetite and growth of the Arctic charr occurred several weeks after water temperature had started to increase, and growth persisted during the period of decreasing water temperature in late summer and autumn (Fig 1). Even though temperature
has marked effects on food intake in fish and other ectothermic animals [48], data from the present, and previous studies [41, 49], provide evidence that water temperature is not the determinant of feeding and non-feeding state in charr. Further, changes in photoperiod probably did not trigger the switch from the non-feeding to the feeding state, because photoperiod was increasing during the early part of the experiment, and there were 24 hours of daylight by mid-May when fish were still decreasing in weight (Fig 1). Similarly, previous work has provided evidence that charr display seasonality in food intake independent of changes in photoperiod [50].

It can therefore be concluded that the transitions between non-feeding and feeding states in Arctic charr are physiologically regulated on a seasonal basis and, as such, the Arctic charr could represent an excellent model for investigating mechanisms regulating long-term appetite in fish.

Differences in relative mRNA abundance of appetite regulators in the brain do not appear to reflect feeding status

If the brain neuropeptides investigated in the present study were regulating long-term appetite, one would expect to find genes encoding for orexigenic neuropeptides to be more highly expressed in feeding charr, sampled in summer, than in non-feeding charr, sampled in spring and winter, and genes encoding for anorexigenic neuropeptides to have lower expression in feeding charr than in non-feeding charr. These expectations were not met.

In the hypothalamus, the expression of neither the anorexigenic POMC and CART nor the orexigenic AgRP genes differed between sampling dates (Fig 2A, Table 3) whereas the gene expression of NPY was undetectable with RT-qPCR. These findings suggest that seasonal changes in appetite in anadromous charr are independent of gene expression occurring within the hypothalamic POMC/CART and NPY/AgRP system. Furthermore, in the mesencephalon expression of orexigenic NPY and AgRP was higher in non-feeding charr sampled in January and May than in feeding charr sampled in July (Fig 2B, Table 3). In the telencephalon, the only temporal difference in gene expression was seen for MC4-R (Fig 2C, Table 3), but data were not consistent with a direct involvement in seasonal regulation of appetite. Consequently, our findings do not provide support for a cause-and-effect role of these neuropeptides in the seasonal shifts of feeding in the charr.

Table 3. Testing for differences across sampling time points (p-values for ANOVA and Tukey’s Honestly-Significant-Test).

| Gene | Hypothalamus | Mesencephalon | Telencephalon |
|------|--------------|---------------|--------------|
|      | ANOVA        | Ma—Ju | Ju—Ja | Ma—Ja | ANOVA | Ma—Ju | Ju—Ja | Ma—Ja | ANOVA | Ma—Ju | Ju—Ja | Ma—Ja |
| POMCA1 | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.d. | n.d. | n.d. | n.d. |
| POMCA2 | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.d. | n.d. | n.d. | n.d. |
| CART | n.s. | n.s. | n.s. | n.s. | 0.001 | n.s. | 0.001 | n.s. | n.s. | n.s. | n.s. | n.s. |
| MC4-R | <0.001 | <0.001 | <0.001 | <0.001 | 0.017 | n.s. | 0.008 | n.s. |
| LepA1 | <0.001 | <0.001 | <0.004 | <0.001 | n.s. | n.s. | n.s. | n.s. |
| LepA2 | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| LepR | 0.004 | 0.015 | 0.006 | n.s. | <0.001 | n.s. | <0.001 | n.s. |
| AgRP | n.s. | n.s. | n.s. | n.s. | 0.004 | 0.005 | 0.022 | n.s. |
| NPY | n.d. | n.d. | n.d. | n.d. | <0.001 | 0.010 | <0.001 | <0.001 |

Ma—Ju = May tested against July, Ju—Ja = July tested against January, Ma—Ja = May tested against January. n.d.: no data (due to low expression level and/or poor melt curve). n.s.: not significant

doi:10.1371/journal.pone.0138857.t003
Few studies have reported how the gene expression of central appetite regulators varies with seasonal feeding cycles in fish. Cunner (Tautogolabrus adspersus) had lower expressions of hypothalamic and telencephalonic NPY and CART during winter torpor, when they voluntarily fast, than during their summer feeding season [51]. In the winter flounder (Pseudopleuronectes americanus), on the other hand, hypothalamic gene expression of NPY was higher in winter, when food intake was low, than in summer, when food intake was high, whereas the expression of CART did not differ across seasons [52]. Thus, available data do not provide a clear picture about how central appetite regulators participate in the regulation of seasonal feeding cycles in fish, or if they are involved at all. In seasonal mammals, e.g. Siberian hamster (Phodopus sungorus), seasonal changes in appetite and body weight may not be accompanied by seasonal changes in the expression of central appetite regulators, suggesting that the central appetite regulating system predominantly regulates short-term food intake by timing of meals and reactive responses to changes in food availability [53]. This assumption is in accordance with observations that, in contrast to animals that are subjected to feed deprivation, the mechanisms that normally defend body mass and/or energy status are shut down during periods of body weight change in seasonal mammals [54]. Such a mechanism, rooted in models which propose a seasonal sliding set-point in adiposity [55], would imply that a decrease in body mass and adiposity during winter would not induce a re-commencement of feeding. This might explain the observation made during the present (Fig 1, Table 1) and previous [41] studies on Arctic charr.

Seasonal differences in central gene expression of Lep and its receptor point towards involvement in seasonally regulated processes

Lep plays an important role in maintaining energy homeostasis in mammals by signalling adiposity to the brain, but, hitherto, no relationship has been found between plasma Lep levels and adiposity in fish [24, 27, 29, 56]. In anadromous Arctic charr plasma Lep concentrations did not change significantly during the annual feeding cycle, despite there being marked differences in body fat that ranged from 4.5% in early summer to 17% in autumn [24]. In fish, Lep and LepR are expressed in several tissues including the brain [28, 32]. Hence, our study aimed to investigate whether centrally expressed Lep and LepR could be linked to long-term appetite regulation in charr.

To the best of our knowledge, this is the first study in which central expression of Lep and LepR has been analysed through a seasonal feeding cycle in fish, including voluntarily non-feeding periods. Our results do not indicate that centrally expressed Lep acts as an anorexigenic agent in a long-term perspective, because the hypothalamic gene expression of LepA1 and LepR was higher in feeding charr than in non-feeding charr, and the expression of LepA2 did not differ between feeding and non-feeding charr (Fig 2A).

Nevertheless, a role for central LepR in appetite regulation is possible, because recombinant Lep treatment reduced feeding in both rainbow trout (Oncorhynchus mykiss) [23] and Atlantic salmon [57]. In both studies, the anorexigenic effect of Lep was associated with an upregulation of the hypothalamic [23] and brain [57] POMC system, which in mammals promotes Lep-mediated anorexigenic effects through the MCR pathway [1, 2]. In charr, there was not a consistent seasonal pattern in the expression of LepR and POMC A1 and A2, but there was a covariation of expression of LepR and MC4-R in the hypothalamus (Fig 2A).

In the mesencephalon of the Arctic charr LepA2 expression was, in contrast to the hypothalamus, higher in non-feeding fish sampled in January and May than in feeding fish sampled in July, while LepA1 expression was higher in fish sampled in January than in fish sampled in July (Fig 2B). Whether Lep produced in the mesencephalon acts as an anorexigenic agent in non-feeding charr during winter and spring requires further investigation.
Also there is a need to confirm if differing expression patterns of Lep and LepR across brain compartments are indicative of autocrine and paracrine signalling, as has been suggested previously [47, 58].

It is possible that changes in hypothalamic LepR expression (Fig 2A) could be related to seasonal shifts between anabolic and catabolic states and/or to body adiposity. In support of this conjecture, LepR was more highly expressed in the brain of juvenile Atlantic salmon fed to satiety than in conspecifics subjected to feed restriction [56]. On the other hand, central Lep expression was negatively correlated with body fat in juvenile Atlantic salmon sampled during the growth season [59]. These discrepancies may be related to the fact that both studies monitored LepR transcripts in the whole brain. The different seasonal patterns in Lep and LepR expression in different brain compartments in the present study (Fig 2) emphasise the need for conducting analyses in separate brain regions or even at the level of specific nuclei.

Overall, the seasonal patterns of central expression of Lep and LepR and the differences between the three brain compartments support the view that Lep exerts pleiotropic effects in fish [60]. Seasonal differences in central expression of Leps and LepR may be linked to one or more of the physiological events that characterize the seasonal life of anadromous Arctic charr. These encompass substantial changes in feeding, substrate utilization and energy expenditure [37], a vernal smoltification preceding seaward migration [61] and seasonal reproduction [62].

Conclusions

This study did not reveal a link between expression of anorexigenic (CART, POMCs and MC4-R) and orexigenic (NPY and AgRP) neuropeptides in different brain compartments and seasonal changes in appetite, growth and condition in Arctic charr. Nor did we find support for an anorexigenic role of centrally expressed Lep in the long-term regulation of appetite in charr. Some words of caution must, however, be given because changes or lack of changes in mRNA abundance do not necessarily reflect changes in their encoded peptides, as post-transcriptional regulation may occur [29].

Some temporal variations in gene expression were observed, and there were differences across brain compartments. Consequently, our results indicate the need for additional studies to unravel the roles of these neuropeptides in governing physiological functions in fish.

Acknowledgments

We thank the staff at Tromsø Aquaculture Station for taking care of the fish. We also thank Kristin Elisa Ruud Hansen for helping with measuring and sampling of the fish.

Author Contributions

Conceived and designed the experiments: EHJ MJ. Performed the experiments: EHJ CSR AS. Analyzed the data: EHJ AS MJ. Contributed reagents/materials/analysis tools: CSR AS. Wrote the paper: AS EHJ MJ CSR.

References

1. Schwartz MW, Woods SC, Porte D Jr., Seeley RJ, Baskin DG. Central nervous system control of food intake. Nature. 2000; 404 (6778): 661–671. PMID: 10766253
2. Wynne K, Stanley S, McGowan B, Bloom S. Appetite control. J Endocrinol. 2005; 184(2): 291–318. PMID: 15684339
3. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994; 372(6505): 425–432. PMID: 7984236
4. Blum WF, Englaro P, Hanitsch S, Juul A, Hertel NT, Müller J, et al. Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. J Clin Endocrinol Metab. 1997; 82(9): 2904–2910. PMID: 9284717

5. Hegyi K, Fülöp K, Kovács K, Tóth S, Falus A. Leptin-induced signal transduction pathways. Cell Biol Int. 2004; 28(3): 159–169. PMID: 14984741

6. Volkoff H, Unniappan S, Kelly SP. The endocrine regulation of food intake. In: Bernier NJ, Van der Kraak G, Farrell A, Brauner C, editors. Fish Physiology: Fish Neuroendocrinology. Amsterdam: Academic Press; 2009. p. 421–465.

7. Aldegunde M, Mancebo M. Effects of neuropeptide Y on food intake and brain biogenic amines in the rainbow trout (Oncorhyncus mykiss). Peptides. 2006; 27(4): 719–727. PMID: 16253390

8. Carpio Y, Acosta J, Morales A, Herrera F, González LJJ, Estrada MP. Cloning, expression and growth promoting action of Red tilapia (Oreochromis sp.) neuropeptide Y. Peptides. 2006; 27(4): 710–718. PMID: 16202477

9. Carpio Y, León K, Acosta J, Morales R, Estrada MP. Recombinant tilapia Neuropeptide Y promotes growth and antioxidant defenses in African catfish (Clarias gariepinus) fry. Aquaculture. 2007; 272: 649–655.

10. López-Patiño MA, Guijarro AI, Isorna E, Delgado MJ, Alonso-Bedate M, De Pedro N. Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (Carassius auratus). Eur J Pharmacol. 1999; 377(2–3): 147–153. PMID: 10456424

11. Silverstein J, Plisetskaya E. The Effects of NPY and insulin on food intake regulation in fish. Am Zool. 2000; 40(2): 296–308.

12. Volkoff H, Peter RE. Interactions between orexin A, NPY and galanin in the control of food intake of the goldfish, Carassius auratus. Regul Pept. 2001; 101 (1–3): 59–72. PMID: 11495680

13. Yokobori E, Azuma M, Nishiguchi R, Kang KS, Kamijo M, Uchiyama M, et al. Neuropeptide Y stimulates food intake in the zebrafish, Danio rerio. J Neuroendocrinol. 2012; 24(5): 766–773. doi: 10.1111/j.1365-2826.2012.02281.x PMID: 22250860

14. Zhou Y, Liang XF, Yuan X, Li J, He Y, Fang L, et al. Neuropeptide Y stimulates food intake and regulates metabolism in grass carp, Ctenopharyngodon idellus. Aquaculture. 2013; 380-383(383): 52–61.

15. Hosomi N, Furutani T, Takahashi N, Masumoto T, Fukada H. Yellowtail neuropeptide Y: molecular cloning, tissue distribution, and response to fasting. Fish Sci. 2014; 80(3): 483–492.

16. Namaware YK, Peyon PP, Lin X, Peter RE. Regulation of food intake by neuropeptide Y in goldfish. Am J Physiol Regul Integr Comp Physiol. 2000; 279(3): 1025–1034.

17. Silverstein JT, Breininger J, Baskin DG, Plisetskaya EM. Neuropeptide-Y like gene expression in the salmon brain increases with fasting. Gen Comp Endocrinol. 1998; 110(2): 157–165. PMID: 9570936

18. Tang Z, Sun C, Yan A, Wu S, Qin C, Zhang Y, et al. Genes involved in fatty acid metabolism: molecular characterization and hypothalamic mRNA expression responses to fasting. Gen Comp Endocrinol. 2009; 161(2): 252–261. doi: 10.1016/j.ygcen.2009.03.015 PMID: 19332071

19. MacDonald E, Volkoff H. Neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and cholecystokinin (CCK) in winter skate (Raja ocellata); cDNA cloning, tissue distribution and mRNA expression responses to fasting. Gen Comp Endocrinol. 2009; 166(1): 117–127. doi: 10.1016/j.ygcen.2009.03.015 PMID: 19857495

20. Bernier NJ, Gorissen M, Filik G. Differential effects of chronic hypoxia and feed restriction on the expression of leptin and its receptor, food intake regulation and the endocrine stress response in common carp. J Exp Biol. 2012; 215( Pt 13): 2273–2282. doi: 10.1242/jeb.066183 PMID: 22675188

21. Murashita K, Kurokawa T, Ebbessen LOE, Stefansson SO, Rennestad I. Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (Salmo salar). Gen Comp Endocrinol. 2009; 162(2): 160–171. doi: 10.1016/j.ygcen.2009.03.015 PMID: 19332070

22. Li GG, Liang XF, Xie Q, Li G, Yu Y, Lai K. Gene structure, recombinant expression and functional characterization of grass carp leptin. Gen Comp Endocrinol. 2010; 166(1): 117–127. doi: 10.1016/j.ygcen.2009.10.009 PMID: 19857495

23. Murashita K, Uji S, Yamamoto T, Rennestad I, Kurokawa T. Production of recombinant leptin and its effects on food intake in rainbow trout (Oncorhyncus mykiss). Comp Biochem Physiol B: Biochem Mol Biol. 2008; 150(4): 377–384.

24. Freiland E, Jobling M, Björnsson BT, Kling P, Ravuri CS, Jørgensen EH. Seasonal appetite regulation in the anadromous Arctic charr: evidence for a role of adiposity in the regulation of appetite but not for leptin in signalling adiposity. Gen Comp Endocrinol. 2012; 178(2): 330–337. doi: 10.1016/j.ygcen.2012.06.017 PMID: 22732082
25. Freiland E, Murashita K, Jørgensen EH, Kurokawa T. Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. Gen Comp Endocrinol. 2010; 165 (1): 136–143. doi: 10.1016/j.ygcen.2009.06.010 PMID: 19539626

26. Fuentes EN, Kling P, Einarsdottir IE, Alvarez M, Valdes JA, Molina A, et al. Plasma leptin and growth hormone levels in the fine flounder (Paralichthys adspersus) increase gradually during fasting and decline rapidly after refeding. Gen Comp Endocrinol. 2012; 177(1): 120–127. doi: 10.1016/j.ygcen.2012.02.019 PMID: 22429729

27. Kling P, Rennestad I, Stefansson SO, Murashita K, Kurokawa T, Björnsson BT. A homologous salmonid leptin radioimmunoassay indicates elevated plasma leptin levels during fasting of rainbow trout. Gen Comp Endocrinol. 2009; 162(3): 307–312. doi: 10.1016/j.ygcen.2009.04.003 PMID: 19362558

28. Rennestad I, Nilsen TO, Murashita K, Angotzi AR, Gamst Moen AG, Stefansson SO, et al. Leptin and leptin receptor genes in Atlantic salmon: Cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status. Gen Comp Endocrinol. 2010; 168(1): 55–70. doi: 10.1016/j.ygcen.2010.04.010 PMID: 20403358

29. Salmerón C, Johansson M, Angotzi AR, Rennestad I, Jönsson E, Björnsson BT, et al. Effects of nutritional status on plasma leptin levels and in vitro regulation of adipocyte expression and secretion in rainbow trout. Gen Comp Endocrinol. 2015; 210: 114–123. doi: 10.1016/j.ygcen.2014.10.016 PMID: 26448269

30. Kurokawa T, Murashita K. Genomic characterization of multiple leptin genes and a leptin receptor gene in the Japanese medaka, Oryzias latipes. Gen Comp Endocrinol. 2009; 161(2): 229–237. doi: 10.1016/j.ygcen.2009.01.008 PMID: 19523937

31. Tinoco AB, Nisembaum LG, de Pedro N, Delgado MJ, Isorna E. Leptin expression is rhythmic in brain and liver of goldfish (Carassius auratus). Role of feeding time. Gen Comp Endocrinol. 2014; 204: 239–247. doi: 10.1016/j.ygcen.2014.06.006 PMID: 24932715

32. Tinoco AB, Nisembaum LG, Isorna E, de Pedro N. Leptins and leptin receptor expression in the goldfish (Carassius auratus). Regulation by food intake and fasting/overfeeding conditions. Peptides. 2012; 34(2): 329–335. doi: 10.1016/j.peptides.2012.02.001 PMID: 22342497

33. Zhang H, Chen H, Zhang Y, Li S, Lu D, Zhang H, et al. Molecular cloning, characterization and expression profiles of multiple leptin genes and a leptin receptor gene in orange-spotted grouper (Epinephelus coioides). Gen Comp Endocrinol. 2013; 181: 295–305. doi: 10.1016/j.ygcen.2012.09.008 PMID: 23022580

34. Johnson L. The Arctic charr (Salvelinus alpinus). In: Balon EK, editor. Charrs, Salmonid Fishes of the genus Salvelinus. The Hague: Dr. W. Junk b.v. Publishers; 1980. pp. 15–98.

35. Klemetsen A, Knudsen R, Staldvik FJ, Amundsen P-A. Habitat, diet and food assimilation of Arctic charr under the winter ice in two subarctic lakes. J Fish Biol. 2003; 62(1): 195–202.

36. Swanson HK, Kidd KA, Reist JD. Quantifying importance of marine prey in the diets of two partially anadromous fishes. J Fish Aquat Sci. 2005; 20(1): 195–204.

37. Asa-Hansen Ø, Vijayan MM, Johnsen HK, Cameron C, Jørgensen EH. Resmoltification in wild, anadromous Arctic char (Salvelinus alpinus): a survey of osmoregulatory, metabolic, and endocrine changes preceding annual seawater migration. Can J Fish Aquat Sci. 2005; 62(1): 195–204.

38. Bovin TG, Power G. Winter condition and proximate composition of anadromous arctic char (Salvelinus alpinus) in eastern Ungava Bay, Quebec. Can J Zool/Rev Can Zool. 1990; 68: 2284–2289.

39. Jørgensen EH, Johansen SJ, Jobling M. Seasonal patterns of growth, lipid deposition and lipid depletion in anadromous Arctic char. J Fish Biol. 1997; 51(2): 312–326.

40. Mathisen OA, Berg M. Growth rates of the charr (Salvelinus alpinus, L.) in the Vardnes River, Tromsø, northern Norway. Institute of Freshwater Research, Drotthningholm Reports. 1968: 47–178.

41. Tveiten H, Johnsen HK, Jobling M. Influence of the maturity status on the annual cycles of feeding and growth in Arctic char reared at constant temperature. J Fish Biol. 1996; 48(5): 910–924.

42. Ricker WE. Computation and interpretation of biological statistics of fish populations. Bulletin of the Fisheries Research Board of Canada 1975; 191: 1–382.

43. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Method. 2001; 25(4): 402–408. PMID: 11846609

44. Olsvik PA, Lie KK, Jordal AEO, Nilsen TO, Hordvik I. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol. 2005

45. Rieu I, Powers SJ. Real-time quantitative RT-PCR: design, calculations, and statistics. Plant Cell. 2009; 21(4):1031–1033. doi: 10.1105/tpc.109.066001 PMID: 19395682

46. Aarseth JJ, Freiland E, Jørgensen EH. Melatonin implantation during spring and summer does not affect the seasonal rhythm of feeding in anadromous Arctic charr (Salvelinus alpinus). Polar Biol. 2010; 33(3): 379–388.
47. Jørgensen EH, Martinsen M, Strøm V, Hansen KE, Ravuri CS, Gong N, et al. Long-term fasting in the anadromous Arctic charr is associated with down-regulation of metabolic enzyme activity and up-regulation of leptin A1 and SOCS expression in the liver. J Exp Biol. 2013; 216 (Pt 17): 3222–3230.

48. Jobling M. Fish bioenergetics. London: Chapman & Hall; 1994.

49. Bottengård L, Jørgensen EH. Elevated spring temperature stimulates growth, but not smolt development, in anadromous Arctic charr. Comp Biochem Physiol, A: Mol Integr Physiol. 2008; 151(4): 596–601.

50. Sæther BS, Johnsen HK, Jobling M. Seasonal changes in food consumption and growth of Arctic charr exposed to either simulated natural or a 12:12 LD photoperiod at constant water temperature. J Fish Biol. 1996; 48(6): 1113–1122.

51. Babichuk NA, Volkoff H. Changes in expression of appetite-regulating hormones in the cunner (Tautogolabrus adspersus) during short-term fasting and winter torpor. Physiol Behav. 2013; 120: 54–63. doi:10.1016/j.physbeh.2013.06.022 PMID: 23831740

52. MacDonald E, Volkoff H. Cloning, distribution and effects of season and nutritional status on the expression of neuropeptide Y (NPY), cocaine and amphetamine regulated transcript (CART) and cholecystokinin (CCK) in winter flounder (Pseudopleuronectes americanus). Horm Behav. 2009; 56(1): 58–65. doi:10.1016/j.yhbeh.2009.03.002 PMID: 19303880

53. Ebling FJP. Hypothalamic control of seasonal changes in food intake and body weight. Front Neuroendocrinol. 2015; 37: 97–107. doi: 10.1016/j.yfrne.2014.10.003 PMID: 25449796

54. Adam CL, Mercer JG. Appetite regulation and seasonality: implications for obesity. Proc Nutr Soc. 2004; 63(3): 413–419. PMID: 15373951

55. Reddy AB, Cronin AS, Ford H, Ebling FJ. Seasonal regulation of food intake and body weight in the male siberian hamster: studies of hypothalamic orexin ( hypocretin), neuropeptide Y (NPY) and pro-opiomelanocortin (POMC). Eur J Neurosci. 1999; 11(9): 3255–3264. PMID: 10510189

56. Trombley S, Maugars G, Kling P, Björnsson BT, Schmitz M. Effects of long-term restricted feeding on plasma leptin, hepatic leptin expression and leptin receptor expression in juvenile Atlantic salmon (Salmo salar L.). Gen Comp Endocrinol. 2012; 175(1): 92–99. doi: 10.1016/j.ygcen.2011.10.001 PMID: 22019478

57. Murashita K, Jordan AEO, Nilsson TO, Stafasson SO, Kurokawa T, Björnsson BT, et al. Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway. Comp Biochem Physiol, A: Mol Integ Physiol. 2011; 158(1): 79–86.

58. Baltzegar DA, Reading BJ, Douros JD, Borski RJ. Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. J Endocrinol. 2014; 220(1): 61–72. doi: 10.1530/JOE-13-0292 PMID: 24194509

59. Trombley S, Mustafa A, Schmitz M. Regulation of the seasonal leptin and leptin receptor expression profile during early sexual maturation and feed restriction in male Atlantic salmon, Salmo salar L., parr. Gen Comp Endocrinol. 2014; 204: 60–70. doi: 10.1016/j.ygcen.2014.04.033 PMID: 24818969

60. Londraville RL, Macotela Y, Duft RJ, Easterling MR, Liu Q, Crespi EJ. Comparative endocrinology of leptin: assessing function in a phylogenetic context. Gen Comp Endocrinol. 2014; 203: 146–157. doi: 10.1016/j.ygcen.2014.02.002 PMID: 24525452

61. Jørgensen EH, Aas-Hansen Ø, Moriyama S, Iwata M, Tau Strand JE. The parr–smolt transformation of Arctic charr is comparable to that of Atlantic salmon. Aquaculture. 2007; 273(2–3): 227–234.

62. Frantzen M, Damsgård B, Tveiten H, Moriyama S, Iwata M, Johnsen HK. Effects of fasting on temporal changes in plasma concentrations of sex steroids, growth hormone and insulin-like growth factor 1, and reproductive investment in Arctic charr. J Fish Biol. 2004; 65(6): 1526–1542.