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Research paper

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

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ABSTRACT

Despite vast research attention, the knowledge about central mechanisms of appetite regulation in teleost remains inconclusive. A common strategy in studies on appetite regulating mechanisms is to measure the response to feed restriction or – deprivation, but responses vary between fish species and between experiments, and are also likely dependent on the degree of energy perturbation. The anadromous Arctic charr is an interesting model for studying appetite regulation as its feeding cycle comprises months of winter anorexia, and hyperphagia during summer. Here we studied how the gene expression of putative hypothalamic appetite regulators were affected by two days, one week and one month feed deprivation during summer, and subsequent re-feeding and exposure to feed flavour. Short-term feed deprivation caused only a minor reduction in condition factor and had no effect on hypothalamic gene expression. Long-term feed-deprivation caused a marked reduction in weight and condition factor which contrasted the increase in weight and condition factor seen in *ad libitum* fed controls. A marked energy perturbation by feed deprivation was also indicated by a lower hypothalamic expression of the genes encoding insulin-like growth factor 1 (IGF1) and IGF1 binding protein 5 in the feed deprived charr compared to fed controls. Surprisingly, long-term feed deprivation and energy perturbation did not induce changes in hypothalamic appetite regulators. Unexpectedly, re-feeding and exposure to feed flavour caused an increase in the expression of the genes encoding the orexigenic agouti-related peptide and the anorexigenic melanocortin receptor 4 and cocaine- and amphetamine-regulated transcript. Our study gives strong evidence for a role of these in appetite regulation in Arctic charr, but their mechanisms of action remain unknown. We suggest that changes in gene expression are more likely to be registered during transition phases, e.g. from fasting to feeding and upon stimulatory inputs such as feed flavour.

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1. Introduction

Regulation of food intake is pivotal for maintaining energy homeostasis and sustaining metabolism in animals. To date, central appetite regulation is well understood in mammals where it is orchestrated by neuronal circuits located in the arcuate nucleus of the hypothalamus, expressing genes encoding appetite stimulating (orexigenic) neuropeptide Y (NPY) and agouti regulated peptide (AgRP), and appetite inhibiting (anorexigenic) proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) (Schwartz et al., 2000). POMC is cleaved post-translationally resulting in several peptide hormones, including

α-melanocyte stimulating hormone (α-MSH), which binds to the melanocortin receptor 4 (MC4R) at downstream sites such as the paraventricular nucleus and causes a decrease in appetite and an increase in energy expenditure (Cone, 1999). AgRP, on the other hand, is a potent inverse agonist of MC4R and thus counteracts the anorexigenic effect of α-MSH (Cone, 1999). Central appetite regulation is also under the control of peripheral, energy status reflecting cues such as insulin and leptin (Wynne et al., 2005). Eventually, nutrition dependent effects on growth are regulated through signals such as insulin-like growth factor 1 (IGF1) (Baxter, 1988).

The neuroendocrine regulation of appetite in fish has received vast attention during the last two decades, and genes encoding key appetite regulating neuropeptides in mammals are conserved across the vertebrate lineage (Cerda-Reverter and Peter, 2003; Kehoe and Volkoff, 2007; MacDonald and Volkoff, 2009b;

Murashita et al., 2009; Silverstein et al., 1998; Song et al., 2003; Volkoff et al., 2009). However, their responses to treatments such as feed deprivation vary across fish species and even with experimental design within species. Hence, there is still a gap of knowledge on their site of action and role in short-term (meal to meal) and long-term (energy homeostasis) appetite regulation (Hoskins and Volkoff, 2012; Volkoff, 2016). For example, one week feed restriction and 72 h fasting resulted in an upregulation of orexigenic NPY expression in the goldfish (Carassius auratus) brain (Narnaware et al., 2000). In contrast, 6 days feed deprivation lead to a downregulation of both orexigenic AgRP1 and anorexigenic CART expression in brain of Atlantic salmon (Salmo salar), while no change in NPY expression was detected (Murashita et al., 2009). Contrasting results across experiments within the same species and between species may relate to the enormous diversity of teleost species, their adaptations to a variety of environmental conditions, differences in life-history strategies and phenotypic transitions in response to spatiotemporal changing environments.

The appetite of anadromous Arctic charr (Salvelinus alpinus) is seasonally regulated; most of their annual feed intake is obtained during the short, summer feeding residence in the sea while they feed little or nothing during overwintering in fresh water (Johnson, 1980; Jørgensen et al., 1997; Swanson et al., 2011). A similar seasonal feeding cycle is seen in immature offspring of anadromous Arctic charr held in captivity and fed in excess at a constant temperature throughout the year (Tveiten et al., 1996), showing that their temporal changes in appetite is endogenously regulated. However, these strong seasonal changes in food intake were not found to be accompanied by expected changes in the central expression of genes encoding orexigenic and anorexigenic neuropeptides (Striberny et al., 2015). Consequently, the function of these neuropeptides in the Arctic charr is to date unknown.

To identify if the above-described appetite regulators are involved in appetite regulation in Arctic charr we hypothesised that enforced energy perturbation during summer, when they are in a strong anabolic state characterized by hyperphagia, would lead to responses in central appetite signalling. In this study, we investigated the effects of one week and one month feed deprivation on gene expression of known, key actors in appetite regulation in the charr hypothalamus. In the long-term feed deprived charr we also analysed hypothalamic IGF1 and IGFBP5 expression, since energy perturbation generally is accompanied by a reduction in endocrine and paracrine IGF1 signalling in teleosts (Beckman, 2011; Safian et al., 2012). Furthermore, we tested whether 1 and 5 h of re-feeding stimulates responses in central appetite regulation in feed deprived charr. Since it may be a challenge to distinguish whether observed changes in anorexigenic and orexigenic signalling in response to re-feeding reflect an active regulation of food intake or simply are a consequence of it, we also investigated if 1 and 5 h exposure to fish feed flavour induces responses in central appetite regulators in the long-term feed deprived charr.

2. Material and methods

2.1. Experiment 1: Short-term feed deprivation

Experiment 1 was conducted at Tromsø Aquaculture Research Station with hatchery reared offspring of anadromous Arctic charr derived from a broodstock captured in Lake Vårfluesjøen, Svalbard, in 1990. The eggs hatched in winter 2011 and juveniles were held in fresh water at 6 °C under continuous light until July 2011 and thereafter at ambient water temperature and natural light (69 °N; transparent roof) conditions until the start of the experiment. On June 4, 2013, 200 individuals were randomly sorted out and distributed equally among two, 300 L circular tanks supplied

with fresh water at ambient temperature. Temperature was on average 11.2 °C ± 0.2 °C during the experiment. The fish were fed in excess with commercial dry-pellet feed (Skretting, Stavanger, Norway) provided continuously by automatic feeders. From July 2 and until July 9 fish in tank 1 were feed deprived, whereas the fish in tank 2 were fed in excess. Five fish per tank were sampled on July 2 (time zero), July 4 (two days feed deprivation or feeding) and July 9 (one week feed deprivation or feeding). Fish were sampled in the morning and were killed by an overdose of Benzocaine (150 ppm). Fork length and weight were measured and stomach contents removed if present. The brain was dissected out and the hypothalamus was separated from the other brain compartments and stored in 1 ml of RNAlater. Samples were kept at 4 °C for ca. 24 h, and thereafter stored at −20 °C until extraction of total RNA. Stomach contents were placed on aluminum foil and dried overnight in a drying oven at 120 °C and dry weight was measured the day after.

2.2. Experiment 2: Long-term feed deprivation followed by exposure to feed flavour and re-feeding

On June 25, 2014, a total of 264 size-sorted immature individuals of Arctic charr were equally distributed among two 300 L fresh water tanks (132 fish per tank). The charr were 2-years-old offspring of the anadromous Hammerfest strain, originating from wild charr caught in 1984 and since then bred at Tromsø Aquaculture Research Station, where the experiment was carried out. A number of 15 fish in each group were injected with Alcian Blue staining dye with a Pan Jet needleless injector (Wright Dental, Dundee, UK) in order to follow their size and weight development throughout the experiment. Following establishment, the fish in tank 1 were feed deprived and the fish in tank 2 were provided two main meals of commercial dry-pellet feed (Skretting, Stavanger, Norway) daily at 08.00 AM and 3.00 PM and in addition fed by hand to ensure excess provision of feed. Fish were held at simulated natural photoperiod (69 °N) and ambient water temperature, which increased from 5.1 °C on June 25 (beginning of the experiment) to 13 °C on July 24 (end of experiment).

On July 23, 108 feed deprived fish from tank 1 were randomly distributed among three tanks supplied with 300 L fresh water, while 36 fed charr from tank 2 were transferred to a fourth 300 L tank (Fig. 1). On July 24, the charr in the four tanks were exposed to four different treatments: Fish from the fed group were fed ad libitum by hand (control), whereas the fish from the feed deprived group were either re-fed by hand (re-fed), exposed to fish feed flavour (flavour) or continued to be feed deprived in combination with a dummy (dummy). The flavour of fish feed was applied through a fine-meshed nylon bag filled with commercial dry pellet feed that was hung into the tank. In order to test the possibility that the presence of a bag hanging in the water could agitate the fish in the feed flavour group and cause central neuroendocrine responses, we hang a dummy that consisted of a fine-meshed nylon bag filled with small rocks in the un-fed control group, which was a continuation of the feed deprivation treatment (Fig. 1). The experiment started at 9:00 AM and ended at 2:00 PM. A number of 12 fish per treatment were sampled as described for experiment 1 after 1 h (10:00 AM) and 5 h (2:00 PM).

Both experiments were approved by the Norwegian Animal Research Authority ID6491.

2.3. Extraction of mRNA, DNase treatment, reverse transcription and qPCR

Hypothalami were disrupted using TissueLyser II (QIAGEN, Hilden, Germany), and RNA was extracted using the RNeasy Plus Universal Mini Kit (QIAGEN, Hilden, Germany) according to the
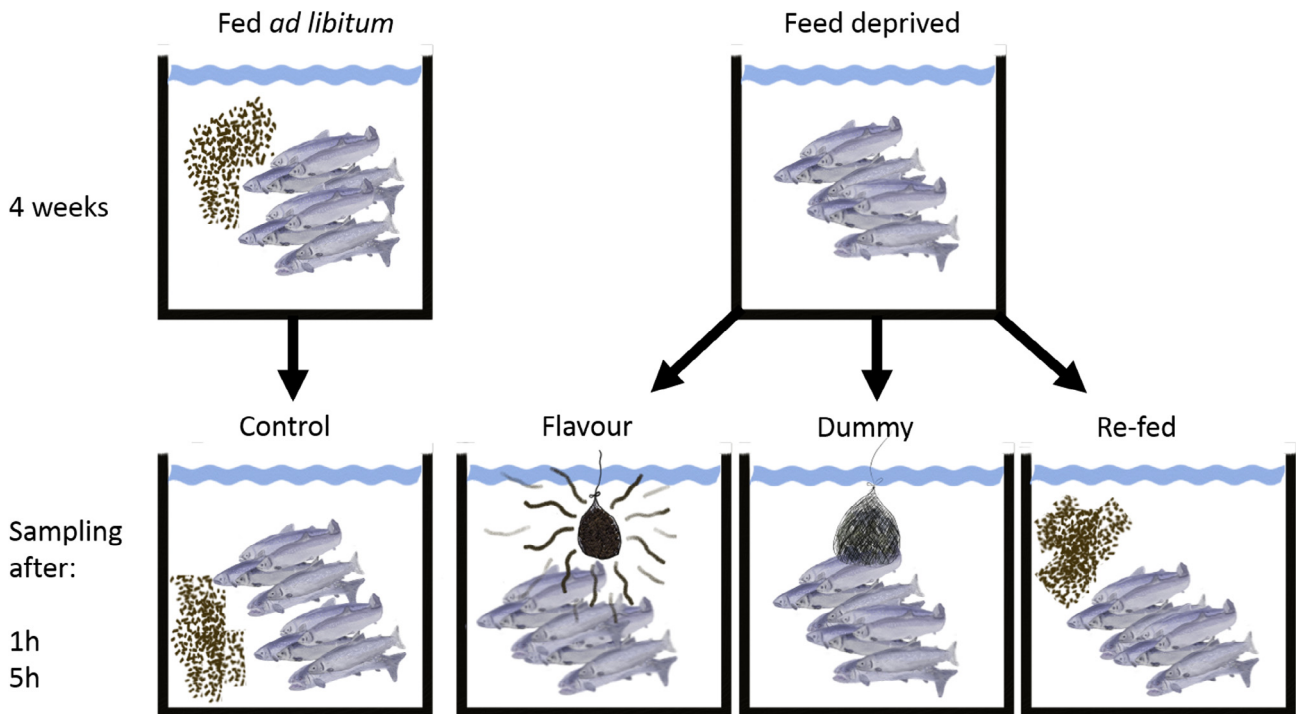
Fig. 1. Design of experiment 2. Two groups of Arctic charr were either fed or feed deprived for one month, after which the feed deprived fish were either re-fed or exposed to feed flavour or a dummy (continued feed deprivation). The previously fed controls were fed in the same manner as the re-fed group.

manufacturer's protocol. Samples were further purified using ethanol precipitation. Concentration and purity of RNA were assessed using NanoDrop ND2000c (Thermo Scientific Inc., MA, USA) with a quality threshold of 1.8 for the 260/280 and 260/230 absorbance ratio. Genomic DNA was removed by treating the RNA with Ambion TURBO DNA-free™ Kit (Life Technologies, CA, USA). A total of 2000 ng (750 ng in experiment 2) RNA were reverse transcribed to cDNA using iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, CA, USA) in experiment 1 and High-Capacity RNA-to-cDNA™ Kit (Thermo Scientific Inc., MA, USA) in experiment 2. No-transcriptase controls were included in the transcription step. Prior to qPCR cDNA was diluted 10-fold.

Gene specific primers were designed by Primerdesign (Southampton, UK) and Sigma Life Science (Sigma-Aldrich, MO, USA). Efficiency was tested using twofold serial dilutions. Primer sequences are presented in Table 1. Both no-RT controls, and one no-template control was included in amplification step for each target gene. The amplification steps were as follows: 50 °C for 10 min, 95 °C for five minutes, [95 °C for 10 s, 60 °C for 30 s, plate read] × 40, 95 °C for 10 s. 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 verify 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).

2.4. Data treatment and statistics

Fulton's condition factor K was calculated according to Ricker (1975): $K = (W \times L^{-3}) \times 100$, where W is body weight in g, and L is fork length in cm. Relative fold change of gene expression was calculated using the $\Delta\Delta Ct$ method (Livak and Schmittgen, 2001). Elongation factor 1 alpha (EF1α), a stable reference gene in the closely related Atlantic salmon (Olsvik et al., 2005), was used to normalize the Ct values of the target genes.

In experiment 1, body weight and condition factor data were shown to be normally distributed using a Shapiro-Wilk test. Possible differences between treatments and sampling time points were tested using a two-way ANOVA. When overall, significant differences were found, post hoc testing was carried out using a Tukey's Honestly Significance test for pairwise comparisons. Gene expression data was not normally distributed and possible differences between treatment groups were assessed using a Kruskal-Wallis test.

Data in experiment 2 were normally distributed (Shapiro-Wilk test). Possible differences in stomach contents between the re-fed and control Arctic charr in experiment 2 were tested using a Two-Sample t-Test. A one-way ANOVA was used to test for possible differences in body weight, condition factor and gene expression between treatments. When overall, significant differences were found, post hoc testing was carried out using a Tukey's Honestly Significance test for pairwise comparisons in body weight and condition factor and a Games-Howell Significance test for pairwise comparisons in gene expression. All statistical testing was done with SYSTAT 13 and figures were drawn using SigmaPlot 13 (both Systat Software, CA, USA). A linear regression in SigmaPlot 13 (Systat Software, CA, USA) was used to test for a correlation between the ΔCt of $IGF1$ and $IGFBP5$ and Fulton's condition factor K. The significance level was set to p < 0.05.

3. Results

3.1. Experiment 1: Short-term feed deprivation

Short-term feed deprivation did not result in a lower body weight of the feed deprived fish than of the *ad libitum* fed fish (Fig. 2A). However, the feed deprived fish had a significant lower Fulton's condition factor after one week compared to the beginning of the experiment and the condition factor tended to be lower

Table 1
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	AGGCATTGACAAGAGAACCATT	119	AF498320.1	99.9
	R	TGATACCACGCTCCCTCTC			
Pro-opiomelanocortin (POMC) A1	F	ACTGTTCAAAAATGTCATCATCAAAG	83	AB462418	106.4
	R	CACCTATCCTCCCTTCCTCTC			
POMC A2	F	GTTGGAGGAAAGAAGAGAGAA	119	AB462420	106.9
	R	CAATAACCACGCAGGACACA			
Cocaine and amphetamine regulated transcript (CART)	F	GTCCATCGTTCTTAGTGCTGAA	115	AB455538	107.1
	R	CAGTTGCTTTTCGTTGGTCAA			
Melanocortin receptor 4 (MC4R)	F	TTCTCACACTGGGGATAGTCA	113	AY534915.1	106.7
	R	CACAGCCAAAGAACAGATGAAT			
Leptin receptor (LEPR)	F	CTTTGCTCGGGAGTCAGGA	129	KC683373.1	105.3
	R	CCTGTGCTTTGAGTGGACTG			
Agouti related peptide (AgRP)	F	TCGCCGAAGACCTGAAGAG	123	CA343080.1	104.0
	R	CGTGGTGCTGTCCCTGAT			
Insulin like growth factor 1 (IGF1)*	F	TGGACACGCTGCAGTTTGTGTGT	109	M95183	107.1
	R	CACTCGTCCACAATACCACGGT			
Insulin like growth factor binding protein 5 (IGFBP5)*	F	GTGTACACCCCACACGACTG	185		104.3
	R	CTGGCAACGCTTCTCCTAAG			
Neuropeptide (NP) Y	F	AGAATTGCTGCTGAAGGAGAG	83	AF203902	107.2
	R	GGGACAGACACTATTACCACAA			

Primers were produced and verified by PrimerDesign Ltd (Southampton, UK). Bp = product length in base pairs. Efficiencies were tested using serial dilutions. *Produced by Sigma Life Sciences.
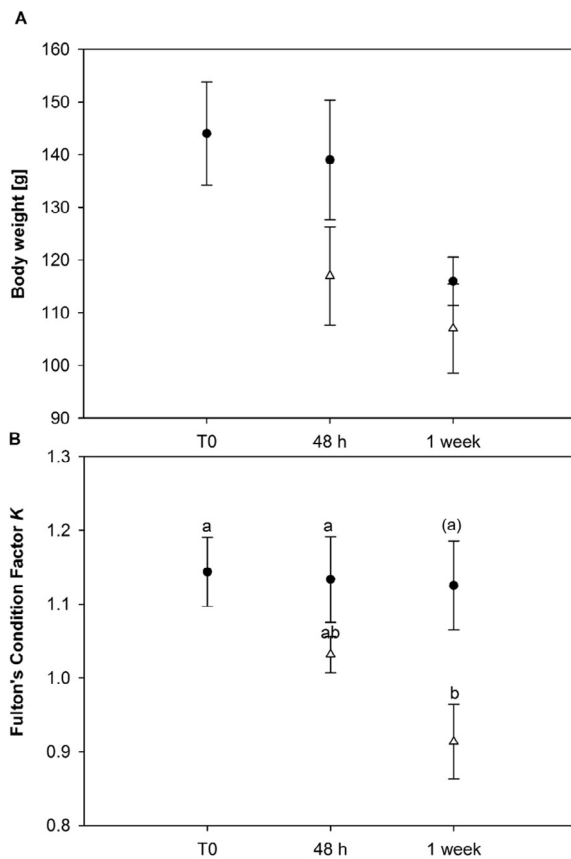
Fig. 2. Mean ± SEM body weight (A) and Fulton's condition factor K (B) of fed and feed deprived Arctic charr sampled for gene expression analysis in experiment 1. Black dots: Control group fed *ad libitum*. Triangles: feed deprived group. Different letters denote groups that were significantly different and those in brackets groups that tend to be different (0.1 > p > 0.05). N = 5.

(p = 0.051) in feed deprived fish than in the control group after 1 week (Fig. 2B).

The gene expression data of hypothalamic appetite regulators was characterized by large individual differences within the sampling groups and no differences in gene expression were found between feed deprived and fed fish at 48 h or 1 week (Fig. 3).

3.2. Experiment 2: Long-term feed deprivation and re-feeding

One month feed deprivation during summer led to a twofold lower body weight, and a markedly lower condition factor in the feed deprived fish than in the fed controls (Fig. 4A,B). Furthermore, the average weight-specific dry stomach content of the re-fed fish sampled after 1 and 5 h (16 of 24 fish had stomach content) was twofold lower than that of the fed controls (23 of 24 had stomach content) (Fig. 5).

At 1 h, the hypothalamic gene expression of IGF1 and IGFBP5 was significantly lower in all groups that had previously been feed deprived compared to the fed controls (Fig. 7A,B). A linear correlation analysis that included all fish sampled in this experiment revealed a positive correlation between Fulton's condition factor and hypothalamic expression of IGF1 and IGFBP5 (Fig. 6A,B).

None of the different treatments (feed flavour, dummy, re-feeding) lead to changes in hypothalamic gene expression of CRF, NPY, LEPR and POMCA1 compared to fed controls (Fig. 7E,F,G,I). The gene expression of AgRP (Fig. 7C) was significantly higher in the flavour group than in the dummy group at 1 h and significantly higher in the re-fed group than in the dummy group at 5 h. There was a trend, albeit not significant (p = 0064), for a higher expression of AgRP in the flavour group than in the fed controls and a higher expression in the re-fed group than in the dummy group at 1 h (p = 0.084). In addition, the gene expression of MC4R (Fig. 7D) was significantly higher in flavour exposed and re-fed fish than in fed controls at 5 h. However, there were no significant differences in MC4R expression between the flavour exposed, re-fed and feed deprived (dummy) fish groups at 5 h.

The gene expression of CART was significantly higher in the re-fed group, and tended to be higher (p = 0.051) in the flavour group, than in the dummy group at 1 h, while no differences between treatments were seen at 5 h (Fig. 7H). The gene expression of POMCA2 did not differ between treatments at 1 h, but was significantly higher at 5 h in the flavour group than in fed controls. There was a trend (p = 0.051) for a higher expression of POMCA2 in the dummy group than in the fed controls at 5 h, while the expression of POMCA2 in re-fed fish at 5 h did not differ from any of the other groups at 5 h (Fig. 7J).
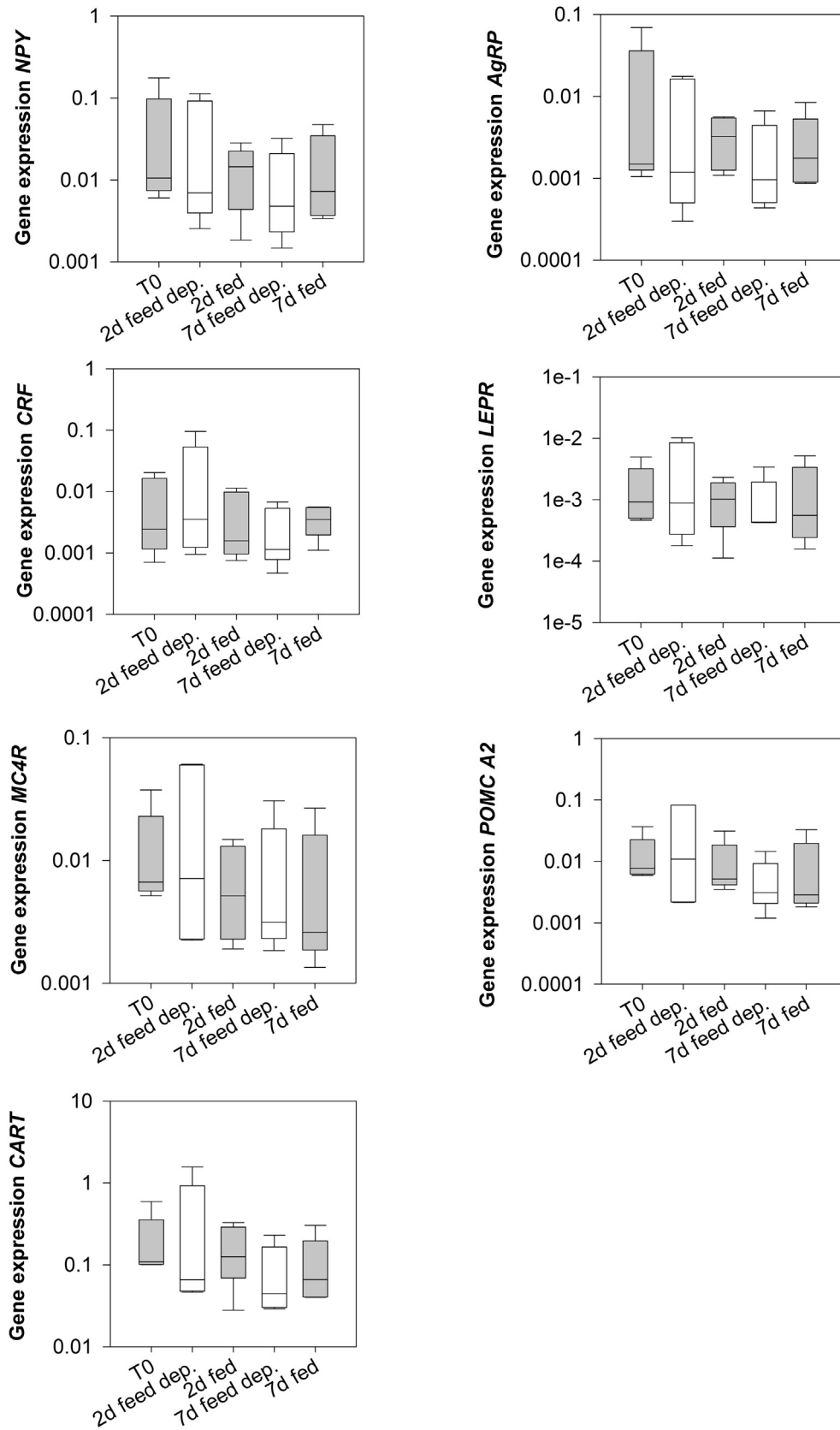
Fig. 3. Normalized gene expression in the hypothalamus of feed deprived (feed dep.) and fed Arctic charr in experiment 1. Box-Whisker plots with median and 25th, 75th, 90th and 10th percentiles. N = 5.
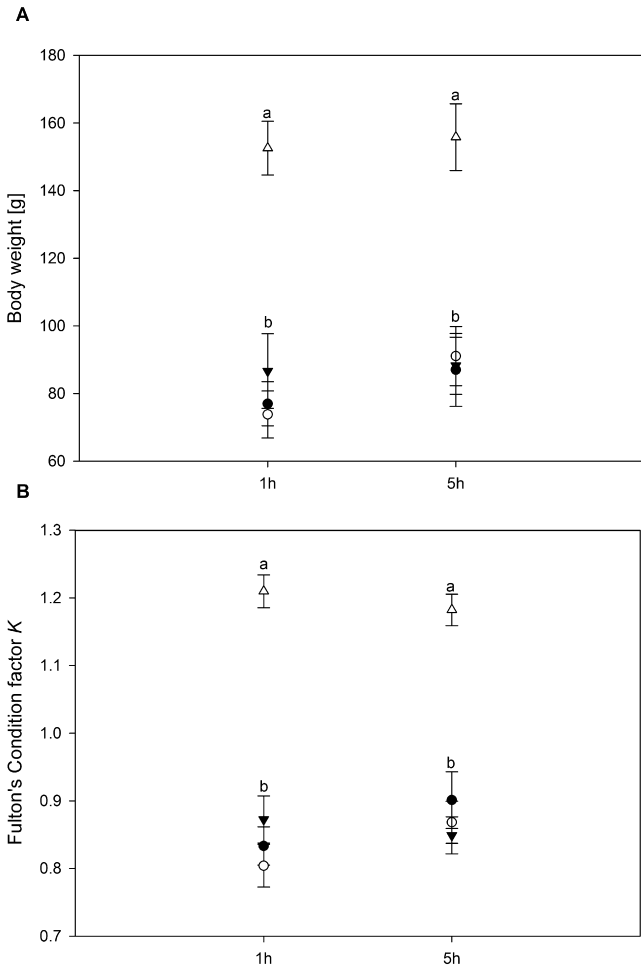
Fig. 4. Mean ± SEM body weight (A) and Fulton's condition factor *K* (B) of previously *ad libitum* fed Arctic charr, fed for 1 and 5 h (white triangles), previously feed deprived Arctic charr exposed to re-feeding (black triangles), fish flavour (white dots) or a dummy (black dots) for 1 and 5 h in experiment 2. *N* = 12. Different letters denote groups that were significantly different.
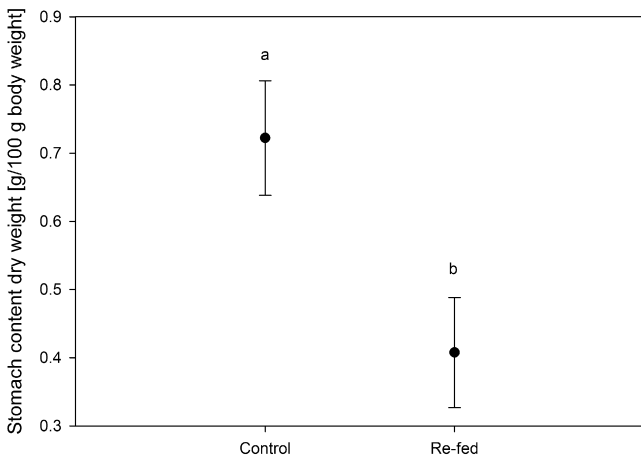
Fig. 5. Mean ± SEM stomach content (dry weight per 100 g of body mass) of fed, control Arctic charr and re-fed charr sampled after 1 and 5 h in experiment 2. Fish with empty stomach were not included in the analysis. Different letters denote groups that were significantly different.

4. Discussion

In this study, we aimed at elaborating the effects of short- and long-term feed deprivation on expression of central key appetite
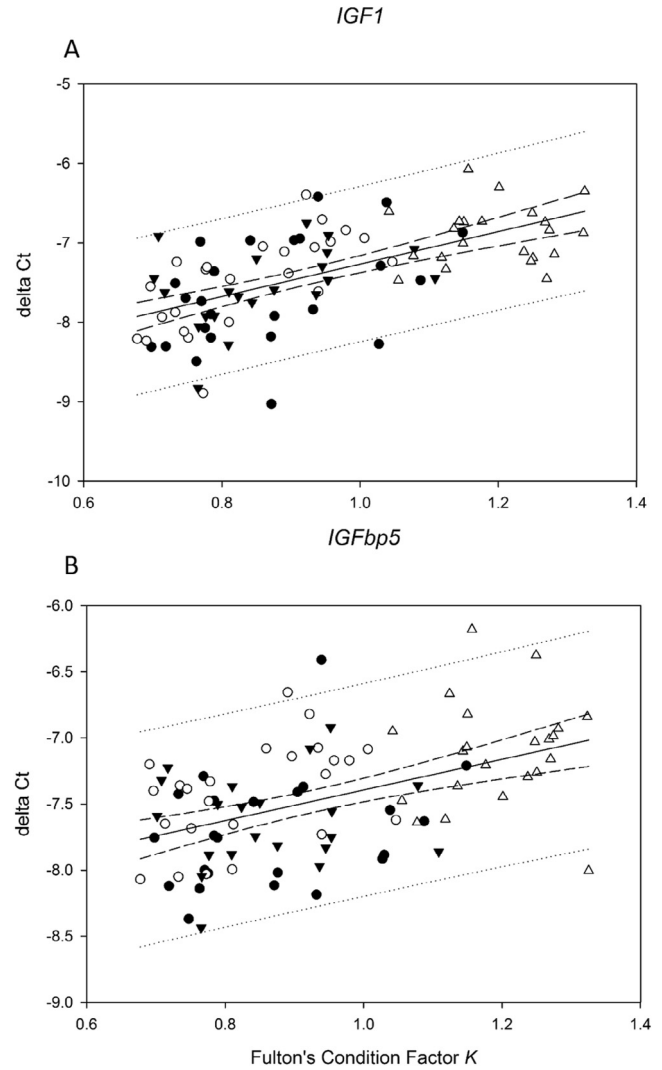

Fig. 6. Correlation between the expression of *IGF1* (A) and *IGFBP5* (B) in hypothalamus and Fulton's condition *K* of all Arctic charr sampled in experiment 2. Gene expression presented as delta Ct values. *IGF1*: R = 0.6073, R^2 = 0.368, p < 0.0001. *IGFBP5*: R = 0.4679, R^2 = 0.2189, p < 0.0001. Black dots: Dummy. White dots: Flavour. Black triangles: re-fed. White triangles: Control.

regulators in the seasonal Arctic charr during summer, a period when they are in a strong hyperphagic state. While there were no responses to long- and short-term feed deprivation, the reappearance of feed, and feed flavour, stimulated the expression of genes encoding both anorexigenic and orexigenic signalling.

4.1. Effects of short-term feed deprivation

One week feed deprivation caused a decrease in condition factor (Fig. 2B), indicating fat mobilization upon feed deprivation. Despite this, there were no responses to feed deprivation in hypothalamic expression of key appetite regulating genes (Fig. 3). Both changes and lack of changes in response to shorter periods of feed deprivation have been reported in previous studies on fish (Volkoff et al., 2009). For example, a decrease in brain expression of the anorexigenic *CART* was seen after one week feed deprivation in Atlantic cod (*Gadus morhua*) (Kehoe and Volkoff, 2007) and Atlantic salmon (Murashita et al., 2009). On the other hand, and in accordance with our data, there was no effect of feed deprivation on *CART* expression in dourado (*Salminus brasiliensis*) (Volkoff et al., 2016). Differences in responses to feed deprivation between fish species are
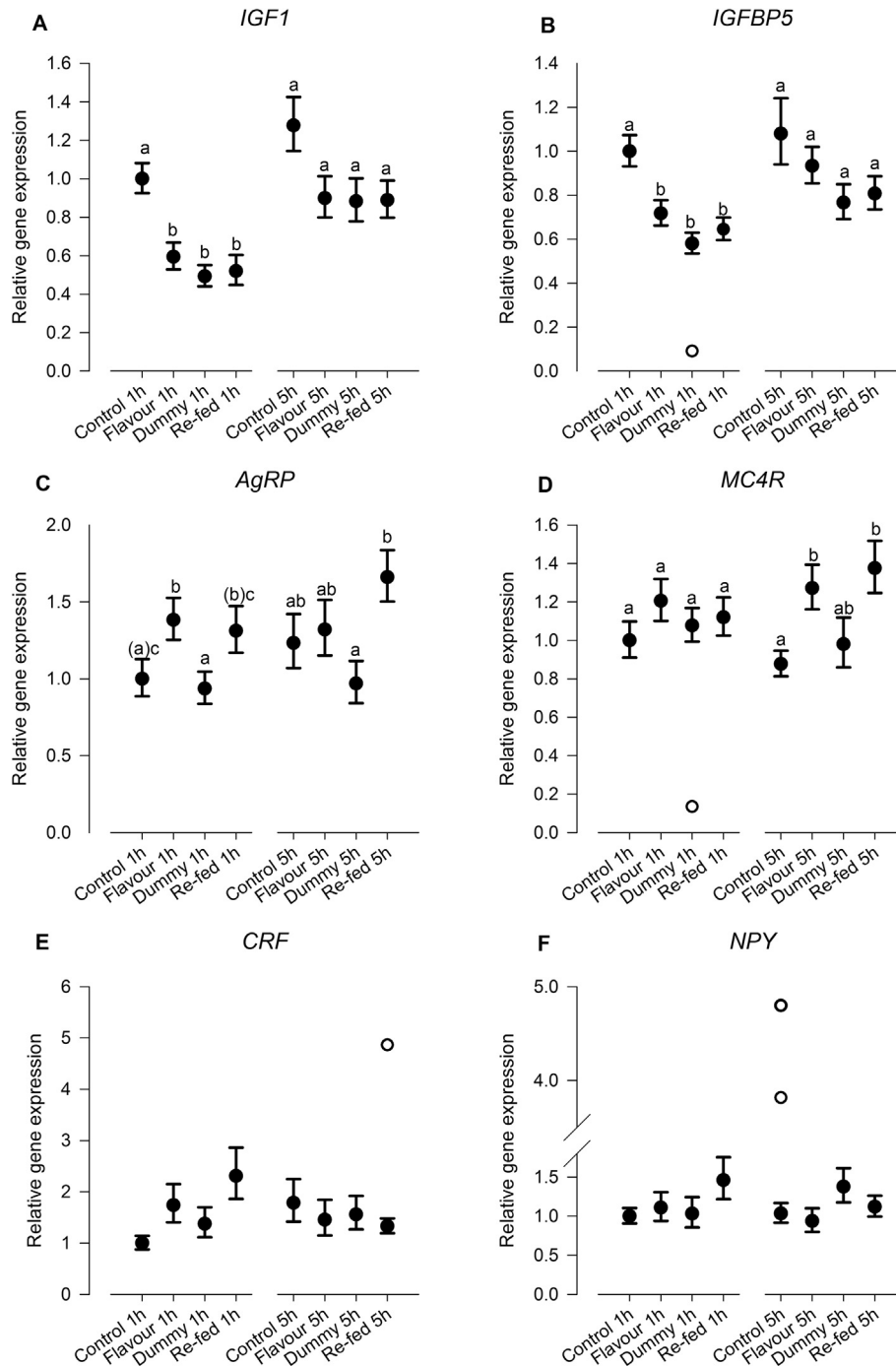
Fig. 7. Mean ± SEM relative expression of *IGF1* (A), *IGFBP5* (B), *AGRP* (C), *MC4R* (D), *CRF* (E), *NPY* (F), *LEPR* (G), *CART* (H), *POMCA1* (I) and *POMCA2* (J) in the hypothalamus of Arctic charr after 1 h and 5 h of treatment in experiment 2. Gene expression of control group after 1 h was set to 1. C: control, F: Flavour, D: Dummy, R: re-fed. *N* = 7–12. Different letters denote groups that were significantly different and those in brackets groups that tend to be different (0.1 > p > 0.05). White dots: Outliers that were excluded from the statistical analyses.

also seen for orexigenic appetite regulators; in Atlantic salmon, the brain gene expression of *AgRP 1* was decreased after 6 days feed deprivation (Murashita et al., 2009), whereas it was up-regulated in the goldfish hypothalamus after 3, 5 and 7 days feed deprivation (Cerda-Reverter and Peter, 2003). Likely, there are multiple underlying causes for the different responses, most of which are currently unknown; for example, cold-water, seasonal species that are adapted to variations in feeding opportunities in their natural environment are expected to respond differently to feed-

deprivation than warm-water species such as the goldfish (Volkoff, 2016). The lack of responses to short-term feed deprivation seen in the Arctic charr in the present study may correspondingly reflect their outstanding adaptation to a nutrient poor and seasonally shifting environment at high latitudes (Jørgensen and Johnsen, 2014).

As illustrated in Fig. 3, there were substantial individual variations in hypothalamic gene expression within sampling groups. This is a common challenge when studying appetite regulation in
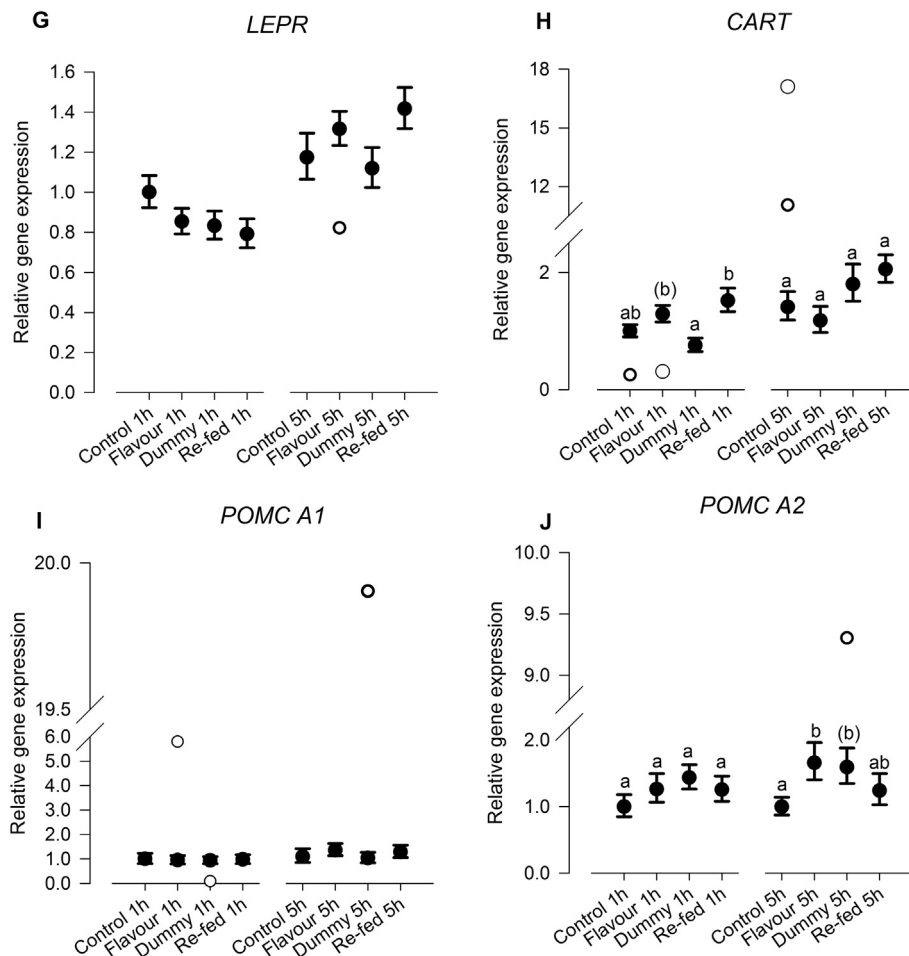
Fig. 7 (*continued*)

fish (Hoskins and Volkoff, 2012) but possibly a particular problem when working with a species such as the Arctic charr, which has a strong tendency to establish social hierarchies (Brännäs and Alanärä, 1993; Jobling and Reinsnes, 1986). Formation of social hierarchies within the groups in the present study may have been one reason for the high individual variation in gene expression. Based on these results, both a higher number of fish sampled, and a stronger energy perturbation were implemented in the second experiment.

4.2. Effects of long-term feed deprivation (fed controls vs. "dummies")

Four weeks feed deprivation during summer caused a strong difference in weight and condition factor between the feed deprived and fed fish (Fig. 4A,B). The marked increase in body weight throughout the experiment in the fed control group confirmed that the charr were in a strong feeding mode when feed deprivation was applied. The finding of a significant, positive relationship between nutritional status (denoted by condition factor) and hypothalamic *IGF1* expression in the fish in the present study (Fig. 6A,B) correspond to the positive relationship between nutritional status and hepatic *IGF1* expression and plasma IGF1 levels seen in numerous fish species (Beckman, 2011) and the reduction in plasma IGF1 levels in feed deprived Arctic charr shown before (Cameron et al., 2007). The lower IGF1 signalling may be explained by a decreased scope for growth and a need for downregulation of anabolic processes in response to long-term feed deprivation (Beckman, 2011). Little is known about extra-hepatic, paracrine

roles of IGF1, but in rainbow trout (*Oncorhynchus mykiss*) it was shown that fasting reduces not only liver *IGF1* expression, but also the expression in adipose tissue and gill (Norbeck et al., 2007). There has been even less focus on paracrine roles of IGF1 in the fish brain, but a positive relationship between nutritional status and *IGF1* mRNA levels in the brain has been documented in GH transgenic coho salmon (*Oncorhynchus kisutch*) (Kim et al., 2015) and in rainbow trout treated with growth-promoting somatotropin (Biga et al., 2004). The function of IGFBP5 in fish is not well understood, but is known to potentiate the function of IGF1 in mammals (Baxter, 2000). The decrease in hypothalamic expression of *IGFBP5* seen with fasting and reduced body nutritional status in the present study corresponds to findings in the Atlantic salmon, where an increase in expression of this binding protein (in a pool of 11 tissues, including whole brain) was registered upon post-fast refeeding (Macqueen et al., 2013). Taken together, our data indicate that central IGF1 signalling responds to nutritional status in a similar manner as in the periphery, as known from other studies in fish. If so, the lower hypothalamic *IGF1* and *IGFBP5* expression in the long-term feed deprived Arctic charr may, in addition to the reduction in Fulton's condition factor *K*, indicate that the charr perceived a negative energy balance after a one month enforced feed deprivation during summer. Consequently, if the appetite regulators here investigated functioned as hunger and satiety signals in the charr, one would expect to see changes in the expression of these in response to long-term feed deprivation. However, this expectation was not met; apart from the discussed differences in *IGF1* and *IGFBP5* expressions, there were no differences in gene expression

of hypothalamic appetite regulators between fed controls and feed deprived (i.e. the dummy group) fish (Fig. 7C–J). In future studies, it would be very interesting to test whether there is a link between hypothalamic IGF1 signalling and long-term appetite regulation in the charr.

Very few reports exist on the effect of long-term feed deprivation on appetite regulating mechanisms in fish and previous studies have not revealed consistent responses. For example, there were no changes in hypothalamic expression of the appetite stimulator NPY and – inhibitor CART in cunner (*Tautogolabrus adspersus*) after 3 weeks feed deprivation (Babichuk and Volkoff, 2013), whereas 4 weeks feed deprivation increased hypothalamic NPY expression in winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009a) and AgRP expression in sea bass (*Dicentrarchus labrax*) (Agulleiro et al., 2014). Surprisingly, in 4 months feed deprived rainbow trout hypothalamic expression of anorexigenic POMCA1 and POMCB was increased while the expression of NPY, AgRP and CART was unaffected (Jørgensen et al., 2016). This tempted the authors to suggest that an upregulation of satiation signals may act as a protective mechanism to avoid searching for food, and thus wasting energy, during periods when feed is absent. Likewise, it is possible that the absence of an upregulation of hypothalamic hunger signals in Arctic charr in the present study is a mechanism to reduce energy expenditure as long as feed is absent.

4.3. Re-feeding and fish feed flavour stimulation

Surprisingly, the Arctic charr that had been feed deprived for one month were not feeding vigorously during the 5 h re-feeding trial. Indeed, their average stomach content (standardized to 100 g of fish body weight) was just above half of the content of those that had been fed throughout the experiment (Fig. 5), and many of the re-fed fish had empty stomachs. This result contrasts to that seen during re-feeding trials with other fish species; 4 months feed deprived rainbow trout showed panicky feeding and a much higher feed intake upon re-feeding than those that had been fed (Jørgensen et al., 2016). Also, in Atlantic salmon, feed consumption was higher than expected on the first day of re-feeding after 40 days of feed deprivation (Krogdahl and Bakke-McKellep, 2005). The reason for the low feed intake in charr during the first hour of re-feeding is not known. However, a stimulation of social competition upon feed appearance may be one underlying factor, since appetite arrest was previously seen during the first days of an experiment with charr held at a low stocking density (Jørgensen et al., 1993). Further, a functional downregulation of the gastrointestinal system during feed deprivation has been reported in other fish species (Zeng et al., 2012) and is likely to happen also in a strongly seasonal fish such as the Arctic charr. Such a process may affect appetite since the gastrointestinal system plays an important role in short-term appetite regulation via the neuroendocrine gut-brain crosstalk (Volkoff et al., 2009).

The increase in orexigenic AgRP gene expression after 1 h in the flavour exposed group and a trend for a higher expression in the re-fed group (Fig. 7C) indicate that the presence of feed or feed flavour is needed to activate appetite signalling after feed deprivation. To the best of our knowledge, the present study is the first to report on responses in appetite signalling mechanisms in fish exposed to feed flavour. The response of the flavour-exposed group indicates that increased AgRP expression functions as a hunger signal, and likely as a signal triggering feeding. The fact that AgRP was higher expressed at 5 h in the re-fed group than in the dummy group, while no differences were detected in the flavour group compared to the dummy group at 5 h may be explained by the continuous presence of feed combined with a low feed intake, and an initial response in the flavour group that may have subsided as the feed did not appear.

Surprisingly, there was also an increase in gene expression of the anorexigenic MC4R in the flavour and re-fed group compared to the fed controls at 5 h, and in expression of CART in the re-fed charr compared to the unfed dummies at 1 h (Fig. 7D,H). It seems paradoxical that both anorexigenic and orexigenic signalling occurs in response to exposure to feed flavour and feed. However, a remarkably similar result was obtained in a previous study with common carp (*Cyprinus carpio*), in which the brain expression of AgRP, CART and MC4R was elevated after 2 h of re-feeding (Wan et al., 2012). Likewise, an increase in both orexigenic (AgRP) and anorexigenic (CART) genes was seen in Atlantic salmon 3 h after feeding a single meal (Valen et al., 2011). Taken together, the abrupt response of both AgRP, CART and MC4R to the appearance of feed and feed flavour in previously long-term feed deprived charr strongly indicates a role of these in the regulation of appetite in Arctic charr. However, the upregulation of both orexigenic and anorexigenic signals together with a putative increase in the sensitivity of the satiety system indicated by an increased MC4R mRNA abundance in re-fed and flavour stimulated charr reveal that we are far from understanding how anorexigenic and orexigenic signalling pathways mutually interact in the regulation of appetite. For example, it was recently shown, but not understood, that mice that detected food responded with an immediate decrease in the activity of hypothalamic AgRP neurons and an increase in the activity of POMC neurons already before feeding (Chen et al., 2015).

Taken together, findings in the present study show that the expression of central appetite regulators in the charr may only be seen during transition phases such as re-feeding after long-term feed deprivation, whereas long-term, stable differences in feeding/nutritional status are not necessarily reflected by differences in the expression of these actors. Finally, the list of actors found to be involved in appetite signalling in fish is expanding at high pace (Volkoff, 2016), and the role of novel peptides in appetite signalling have not yet been investigated in Arctic charr.

5. Conclusion

Neither short- nor long-term feed deprivation resulted in measurable effects on hypothalamic expression of appetite regulators. However, the long-term feed deprivation decreased hypothalamic IGF1 expression, indicating a change in paracrine IGF1 signalling linked to the nutritional constraints imposed by feed deprivation. Our results indicate that feed deprivation does not induce an upregulation of hunger signals or downregulation of key hypothalamic satiation signals after 4 weeks feed deprivation. re-feeding following long-term feed deprivation resulted in an upregulation of AgRP, MC4R, and CART expression in the hypothalamus, providing strong evidence for an involvement of AgRP, MC4R and CART in appetite regulation in the Arctic charr. However, the way these orexigenic and anorexigenic actors interact to exert a net orexigenic or anorexigenic effect remains unknown. The presentation of fish feed flavour to long-term feed deprived charr resulted in responses similar to those seen during re-feeding and proved to be an interesting method to investigate the function of appetite regulators in fish. However, in this study, gene expression was measured in the entire hypothalamus and gene expression patterns may differ at more confined areas. Mapping of appetite regulators using *in situ* hybridization is needed to improve the picture of the spatial distribution of appetite regulators in the charr brain. Finally, there is a need to look at protein levels of the described appetite signals, as an upregulation of these on the mRNA level does not necessarily imply similar changes at the protein level.

Acknowledgments

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