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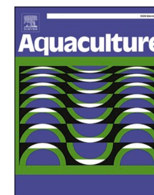

Impact of long-term fasting on the stomach-hypothalamus appetite regulating genes in Atlantic salmon postsmolts

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ABSTRACT

Atlantic salmon will experience periods of fasting during its lifecycle. In nature, prolonged fasting periods occur owing to seasonal fluctuations in available feeds, migration or in relation to reproduction. In a culture setting, salmon is fasted mainly as part of planned operational handling prior to vaccination, delousing, transfer etc., and where fasting may last up to nine days. The mechanisms regulating the appetite during long-term fasting may vary among fish species. Here, we studied the impact of long-term fasting on neuro-endocrine regulation of appetite through the stomach-hypothalamic axis in Atlantic salmon post smolts (1.2 kg, ~46 cm), reared in two experimental conditions (Fed and Fasted; triplicated tanks), and sampled after 4 weeks and 6 weeks of fasting. Fasted fish showed lower condition factor and hepatosomatic index at both sampling points compared to Fed group. In qPCR analysis, hypothalamic relative mRNA expression of agouti-related protein 1 (*agrp1*) was upregulated in fasted group at both sampling points. Among neuropeptide Y (*npy*) paralogs, only *npya1* at 4 weeks was upregulated by fasting. As for cocaine- and amphetamine-regulated transcripts (*cart*), *cart2a* was elevated at 4 weeks, and *cart2b* at both 4 and 6 weeks in fasted group, while *cart3a* and *cart4* showed no response to fasting. The pro-opiomelanocortin (*pomc*) *a1*, *a2* and melanocortin-4 receptor (*mc4r*) *a2* increased only after 6 weeks of fasting, while *mc4rb1* did not respond to fasting. In stomach, 6 weeks of fasting resulted in a decrease of ghrelin1 (*ghrl1*), while expression of *mboat4* was unaffected. The elevated levels of hypothalamic *agrp1* and *npya1* in fasted group support orexigenic roles for these neuropeptides. In addition, upregulation of *cart2a*, *cart2b*, *pomca1* and *pomca2* indicate that these play vital roles in appetite regulation and that fasting may halt and/or counteract hunger signals (*agrp1* and *npya1*) to save energy from foraging search activities during catabolic conditions. Another possibility is that these neuropeptides play a role in fasting-induced stress. Based on the drop in mRNA expression of *ghrl* under catabolic conditions, we hypothesize that Ghrl might return as hunger signal once feed becomes available. We also propose that *agrp1* is a potential appetite biomarker gene under feed deprived conditions.

1. Introduction

In vertebrates, the central control of appetite is regulated by the central nervous system with hypothalamus as the main center. Particularly, two major regions in the hypothalamus named arcuate nucleus (ARC) and paraventricular nucleus (PVN), with distinct neuronal populations and networks, are involved in appetite regulation (Hall, 2011; Timper and Brüning, 2017). Depending on the feeding and nutritional status of the animal, the neurons in the ARC receive peripheral signals and release neuropeptides which act on receptors of second order neurons in the PVN, to induce or inhibit feeding (Nuzzaci et al., 2015). In a fasting (hunger) state, ghrelin (GHRL) acts as a hunger signal predominantly from the stomach and activates neurons expressing the agouti-related protein (AGRP) and neuropeptide Y (NPY) peptides in the ARC of mammalian species. In turn, AGRP and NPY act on the melanocortin-4 receptor (MC4R) receptors of second order neuron in PVN to activate feeding (Rønnestad et al., 2017; Timper and Brüning, 2017). On the other hand, the lipostatic hormone leptin acts as anorexigenic factor, stimulates the pro-opiomelanocortin (POMC)/ cocaine- and amphetamine-regulated transcripts (CART)-expressing

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neurons and inhibits feed intake also through the MC4R receptors. The mechanisms involved in the control of appetite are generally well-conserved among vertebrates, including teleosts (Volkoff, 2016; Rønnestad et al., 2017; Soengas et al., 2018). The hypothalamic nucleus lateralis tuberis pars ventralis (NLTv) has been described as homologous to the mammalian ARC (Rønnestad et al., 2017; Soengas et al., 2018; Soengas, 2021) and *pomc* and *agrp* transcripts were found in the NLT and in rostral hypothalamus of goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003b; Otero-Rodiño et al., 2019), and *npy* and *cart* transcripts were also described in the NLTv of juvenile Atlantic cod (*Gadus morhua*) (Le et al., 2016). In zebrafish (*Danio rerio*), however, the neuropeptide expression and function appear to be conserved between the PVN and the neurosecretory preoptic area (Herget et al., 2014). Further, *mc4r* paralogs were predominantly expressed in the telencephalon and hypothalamus in Atlantic salmon (*Salmo salar*) (Kalananthan et al., 2020a), and in the telencephalon, thalamus, preoptic area, and hypothalamus in goldfish (Cerdá-Reverter et al., 2003a) and spotted sea bass (*Lateolabrax maculatus*) (Zhang et al., 2019). However, probably due to the vast diversity in fish species depending on their ecological niches and aquatic habitats, as well as life history adaptations, transitions between life stages, energy requirement, anatomy and physiology of gastrointestinal system and availability of feed, large variations in regulation of appetite and feeding behavior among species and within species have been reported (Volkoff, 2016; Rønnestad et al., 2017). Thus, the response of these mechanisms to long-term feed deprivation may also vary between teleost species.

Several studies in fish have explored the impact of long-term feed deprivation on the brain/hypothalamic appetite key regulators, ranging from one week of feed deprivation in Atlantic cod (Kehoe and Volkoff, 2007) and in arctic charr (*Salvanius alpinus*) (Striberny and Jørgensen, 2017), 17 days in medaka (*Oryzias latipes*) (Murashita and Kurokawa, 2011), 3 weeks in cunner (*Tautogolabrus adspersus*) (Babichuk and Volkoff, 2013), 29 days in sea bass (*Dicentrarchus labrax*) (Agulleiro et al., 2014), one month in arctic charr (Striberny and Jørgensen, 2017) and in winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009), 8 weeks in spotted sea bass (Zhang et al., 2019), to 4 months feed deprivation in rainbow trout (Jørgensen et al., 2016). In these studies, the duration of feed deprivation was largely different, and the expression response of the appetite regulators differed among the species studied. However, in long-term feed deprivation studies, i.e., duration of more than one week, it is expected that liver gluconeogenesis will have been enhanced (Soengas et al., 1996). Further, Atlantic salmon liver increases its potential for glucose release, through glycogenolysis in a first stage and through gluconeogenesis in a second stage of long-term feed deprivation, and a decrease in the glycolytic capacity of liver due to food deprivation completely reverses by refeeding (Soengas et al., 1996).

To date, studies have reported the effects of long-term feed deprivation on Atlantic salmon brain and liver metabolism (Soengas et al., 1996), in the body composition and shape, filet yield (Lie and Huse, 1992; Einen et al., 1998), growth and sexual maturation (Duston and Saunders, 1999), metabolic rate (Hvas et al., 2020), swimming performance and stress recovery (Hvas et al., 2021) and welfare. Refeeding and compensatory growth (Hvas et al., 2022) have also been studied. Ghrl, produced mainly by the stomach, is the only known peripheral orexigenic hormone in mammals. Ghrelin's response during food deprivation is of high interest to understand the metabolic response to hunger and motivation to feed during this state. Previous studies in Atlantic salmon, has this far explored the control mechanisms for appetite in the stomach *ghrl* during short-term (Murashita et al., 2009; Del Vecchio et al., 2021) and 2 weeks of feed deprivation (Hevrøy et al., 2011). In rainbow trout (*Salmo gairdneri*), a clear inverse relationship between gastric filling and feed intake has been observed (Grove et al., 1978). Indeed, in Atlantic salmon, a statistically significant correlation was found between stomach filling and *agrp1* mRNA expression during short-term fasting (Kalananthan et al., 2020b). However, a link between stomach filling, *ghrl* and hypothalamic neuropeptides expression during long-term feed deprivation has not yet been investigated in Atlantic salmon.

Atlantic salmon belongs to the salmonid family which went through four rounds of whole genome duplications (Lien et al., 2016). The additional rediploidization resulted in an increased gene copy numbers that enable multiple protein isoforms with potentially different physiological functions. While the presence of multiple paralogs is likely linked to facilitate salmon's anadromous life history (Warren et al., 2014; Lien et al., 2016), it also increases the complexity in determining the functional role of the different paralogs.

As a common farming procedure, Atlantic salmon is fasted mainly as part of planned operational procedures, such as, handling prior to vaccination, delousing, transfer etc., normally lasting up to nine days of food deprivation (Kristiansen and Samuelsen, 2006). In nature, long-term feed deprivation in the salmonids is a common phenomenon during the migration back to fresh water for spawning and overwintering (Bower et al., 2009; Rønnestad et al., 2017), while in cultured Atlantic salmon deprivation of feed may occur due to suboptimal environmental conditions, such as seasonal thermal fluctuations and hypoxia (Wade et al., 2019), or due to infectious diseases (McVicar, 1987).

In this study we investigated the effect of 4 and 6 weeks fasting on the mRNA expression of key melanocortin factors involved in the appetite control, such as *npy (a1* and *a2), agrp1, pomc (a1* and *a2)*, *cart* (*2a, 2b, 3a, 4*), and *mc4r* (*a2* and *b1*) in the hypothalamus. Feed deprivation for more than a week was considered as long-term fasting which is equivalent to starvation in the current study. This study contributes to increase our understanding on how Atlantic salmon adjust to cope with different periods of feed deprivation. We also analyzed the mRNA expression of *ghrl* (*1* and *2*) and membrane-bound O-acyltransferase domain-containing 4 (*mboat4*) in the stomach of Atlantic salmon post smolts under the same experimental conditions, as we examined a possible correlation between the gut hormone *ghrl* and the melanocortin neuropeptides expression.

2. Materials and method

2.1. Ethical treatment of animals

The research and sampling were conducted in accordance with the Norwegian Animal Research Authority regulations and was approved by the Norwegian Food Safety Authority under the permit number 21448.

2.2. Experimental setup and sampling

Atlantic salmon post smolts from Aquagen were maintained in six large indoor open flow-through tanks (Ø = 3 m) at 12 °C and 25 ppt under a natural simulated photoperiod at the Institute of Marine Research, Matre Research Station, Norway (60.8754°N). Each tank had constant water supply with a flow rate of 150 $Lmin^{-1}$. Water was aerated, filtered and UVC treated, and oxygen levels were kept >80% saturation. Each tank was allocated with 150 fish (body weight of 1179 ± 11 g and length 45.6 ± 0.1 cm) and fed with commercial pellets (Optiline 4.5, Skretting, Norway) in excess via automated feeders during two daily meals between 09 – 11 and 13–15. The post smolts were acclimatized to the experimental tanks for 6 weeks. Then, three of the tanks were randomly designated as control (Fed) and the other three as treatment (Fasted). Fasting treatment started on 28th January 2020. The last meal for the Fed group of each sampling was from 13:00–15:00 the previous day to sampling. On the 25th of February and 10th of March 2020 (corresponding to 4 and 6 weeks of treatment) a total of 60 fish (5 fish per tank) were sampled from both control and treatment groups. At both samplings, fish were killed with an overdose of MS-222 (1 gL^{-1}) before weight and length was recorded. Immediately after, the fish were opened, and gastrointestinal tract was removed (see **Supplementary**

Fig. 1A - D for dissection procedure). The whole brain of each fish was dissected out (see **Supplementary Fig. 2A - C** for dissection procedure) and transferred in RNA*later* (Thermo Fisher Scientific, MA, USA), kept at 4 °C overnight, and stored at −80 °C until further analysis. The hypothalamus (HYP) was separated prior to analysis. The whole liver was removed, and its weight measured during sampling.

2.3. Biometry

The fitness of the fish was determined at 4 and 6 weeks using the Fulton's condition factor (*K*) equation (**eq. 1**) and hepatosomatic index (HSI) (**eq. 2**):

$$K = 100\frac{W}{L^3} \quad (1)$$

$$\mathrm{HSI} = 100\frac{Lw}{W} \quad (2)$$

where, *W* is the fish weight (g), *L* is the length of the fish (cm), and *Lw* is the liver weight (g) (Froese, 2006; Higgs et al., 2009; Babaei et al., 2016).

2.4. Collection of gastrointestinal tract inner contents and stomach tissue

Each fish was opened carefully, and the gastrointestinal (GI) tract was removed using surgical clamps at both ends. It was then divided into stomach (ST), midgut (MG), and hindgut (HG) as previously described by Kalananthan et al. (2020b), using surgical clamps to avoid loss or transfer of content between the compartments (**Supplementary Fig. 1A - D**). Each segment was emptied of inner content (feed and digesta) by gently stroking the content out into pre-weighed, labeled vials. Thereafter, a distinct piece of stomach at the 'U' region from each individual fish was sampled (**Supplementary Fig. 1D**), rinsed in 1× PBS (phosphate buffered saline), flash frozen, and stored at −80 °C until further analysis. The wet weight of the contents in each segment was measured and dry weight was obtained after incubating in an oven at 110 °C for at least 3 h until it was completely dried.

2.5. Relative mRNA abundance analysis by RT-qPCR

Total RNA was isolated from the hypothalamus (**Supplementary Fig. 1C**) and stomach using TRI reagent (Sigma-Aldrich, MO, United States) according to the manufacturer's instructions. Samples were treated with TURBO DNA-free Kit (Thermo Fisher Scientific, MA, United States) to eliminate possible genomic DNA contamination. The quality of the DNase treated total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). All samples had RNA integrity number (RIN) higher than 7 (scale 1–10). The cDNA was synthesized from 2.0 μg and 2.75 μg of DNase treated total RNA from hypothalamus and stomach, respectively using oligo (dT) primer from SuperScript III First-Strand Synthesis system for RT-PCR kit (Thermo Fisher Scientific).

For qPCR analysis two genes actin beta (*actb*) and ribosomal protein s20 (*s20*) were used as reference genes (Olsvik et al., 2005). For each primer pair, quantification cycle (Cq), primer efficiency and melting peaks were analyzed. All the primer pairs used, except for *mboat4* (GenBank Acc no. XM_045703012.1, Primers 5′-3′ F:GGGTTGGCAAACATTCTGGC, R:ACACTGATAGGAGAAGCCTGG and the amplicon size is 89 bp), are established and validated in previous studies (Kalananthan et al., 2020a, 2021; Del Vecchio et al., 2021; Tolås et al., 2021). The efficiencies ranged between 86 and 109% and R^2 ranged from 0.984 to 0.999.

All qPCR assays were performed using 10 μL of iTaq Universal SYBR Green supermix (Bio-Rad, CA, United States), 0.3 μM of each forward and reverse primers, and cDNA stock dilution template for target genes in hypothalamus 40 and in stomach 60 ng/reaction; for reference genes in hypothalamus 10 and in stomach 12 ng/reaction in 20 μL final reaction volume made with ultra-pure water (Biochrom, Berlin, Germany). All the reactions were performed in duplicate, and a non-template control, no-reverse transcriptase control and a positive between plate control were included in every plate. The qPCR was performed using a CFX96 Real-Time System (Bio-Rad Laboratories, CA, United States) in connection to CFX Manager Software version 3.1 (Bio-Rad, Laboratories, CA, United States) with the following conditions: (1) 95 °C for 30 s, (2) 95 °C for 5 s, (3) 60 °C for 25 s, (4) repeating steps 2–3 for 39 more times. Melting curve analysis over the range of 65–95 °C (increment of 0.5 °C for 2 s) allowed for detection of possible non-specific products and/or primer dimers.

$$Copy\ number = 10^{\left(\frac{Cq-intercept}{slope}\right)} \quad (3)$$

The copy number was normalized using the total ng of RNA used in the reaction for each target and reference gene (**eq. 3**). The ratio of the target gene copy number to the geometric mean copy number of reference genes are used in the statistical analyses and graphs.

2.6. Statistical analysis

The data analysis was carried out using GraphPad version 8 (GraphPad Software, San Diego, CA, USA). Normality of distribution and equal variances of gene expression data were assessed using Shapiro-Wilk normality test and F-test, respectively. The data was log transformed to ensure normal distribution before statistical analysis. A two-way ANOVA followed by the Tukey multiple comparison test was used to reveal possible effects of time and treatment. When either the F-test or the normality test failed, the nonparametric Mann-Whitney test was performed. A $p < 0.05$ was considered significant. *P*-values <0.01, 0.001 and 0.0001 are reported as $p < 0.01$, < 0.001 and $< 0,0001$ respectively in the text. All data are presented as mean ± SEM.

3. Results

3.1. Biometry

The weight and length of fish of the Fed group was significantly higher ($p < 0.001$) than the Fasted group at 6 weeks (Fig. 1 **& Supplementary Table 1**). While the Fed group (1.70 ± 0.082 kg) had an increase in weight of 44%, the Fasted group (1.12 ± 0.052 kg) decreased 5% compared to the mean weight (1.179 ± 0.011 kg) of group at the start of experiment. The *K* factor of the Fasted group significantly ($p <$ 0.001) decreased compared to Fed group at both sampling points (Fig. 1 **& Supplementary Table 1**). After 4 weeks fasting *K* was 1.104 (± 0.017) for the Fasted group and 1.229 (± 0.015) for the Fed group, while at 6 weeks *K* was 1.089 (± 0.012) for the Fasted group and 1.238 (± 0.024) for the Fed. After 4 weeks fasting the HSI for Fed group was 1.017 (± 0.165) and 0.814 (± 0.031) for the Fasted group, whereas at 6 weeks 1.031 (± 0.13) for the Fed group and 0.852 (± 0.084) for the Fasted group respectively (Fig. 1 **& Supplementary Table 1**). The HSI was approx. 20 and 17% lower in Fasted than Fed fish at 4 weeks ($p < 0.001$) and 6 weeks ($p < 0.001$), respectively.

3.2. Gastrointestinal tract compartments filling

In the Fed fish sampled at 4 and 6 weeks, stomach filling varied between 0.147 (± 0.037) and 0.138 (± 0.034) % of BW (Fish wet body weight). As expected, all compartments of the GI tract were empty in the long-term Fasted fish (both 4 weeks and 6 weeks) (**Supplementary Fig. 4 & Supplementary Table 1**). A high correlation between wet and dry content was found in each of the GI tract compartments ($n = 60$): ST ($R^2 = 0.89$, $p < 0.0001$), MG ($R^2 = 0.99$, $p < 0.0001$), and HG ($R^2 = 0.97$, $p < 0.0001$) (**Supplementary Fig. 3 & Supplementary Table 2**).

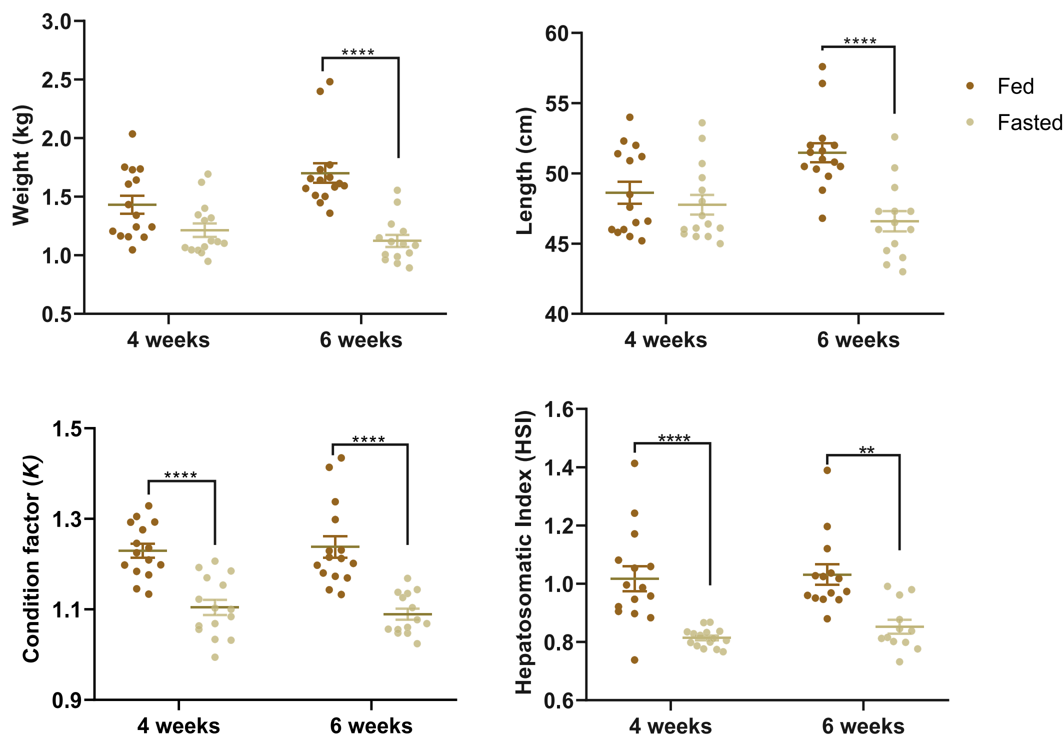

Fig. 1. The weight (kg), length (cm), Fulton's condition factor (K) (4 weeks: Fed $n = 15$; Fasted $n = 15$; 6 weeks: Fed $n = 15$; Fasted $n = 14$) and Hepatosomatic Index (HSI) of fish sampled at 4 weeks (Fed $n = 15$; Fasted $n = 15$) and 6 weeks (Fed $n = 14$; Fasted $n = 12$) of experimental conditions. The data was analyzed using two-way ANOVA followed by Tukey multiple comparison test. Dark brown dots represent Fed group and light brown dots represent the Fasted group. All data are presented as mean ± SEM. Statistical significance is shown as ** when $p < 0.01$ and **** when $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Relative mRNA abundance analysis by RT-qPCR

No significant difference in the mRNA expression of the target genes was found between the Fed group at 4 weeks and 6 weeks (**Supplementary Table 3**). However, the hypothalamic mRNA expression of *agrp1* in the Fasted group was significantly upregulated compared to the Fed group at both 4 weeks ($p < 0.01$) and 6 weeks of fasting ($p < 0.01$) (Fig. 2A, **Supplementary Table 3**). Among the *npy* paralogs, *npya2* was more abundant in hypothalamus, but its mRNA expression level was not affected by fasting treatment, both at 4 and 6 weeks. In contrast, *npya1* mRNA expression in Fasted fish was significantly higher compared to the Fed group ($p < 0.01$) at 4 weeks, but no significant difference was observed at 6 weeks. Among the *cart* genes, *cart2b* was the highest expressed in hypothalamus, and its expression was upregulated following both 4 and 6 weeks of fasting treatment ($p < 0.05$). *cart2a* was upregulated ($p < 0.05$) in fasted group at 4 weeks, while the other two *cart* paralogs, *cart3a* and *cart4,* did not show any significant responses to fasting. The hypothalamic *pomca1* ($p < 0.05$), *a2* ($p < 0.01$) and *mc4ra2* ($p < 0.01$) increased significantly following 6 weeks of fasting compared to the Fed group, while *mc4rb1,* the highest expressed *mc4r* paralog, did not respond to either 4 or 6 weeks of fasting. In the stomach (Fig. 2B, **Supplementary Table 3**), expression of *ghrl1* ($p < 0.05$) was significantly lower at 6 weeks of fasting, while the expression of *ghrl2* and *mboat4* had no differences between the groups at 4 or 6 weeks.

To investigate a possible communication link between the stomach and the hypothalamus, we performed a correlation analysis between the relative mRNA expressions of the hypothalamic melanocortin neuropeptides and receptors with *ghrl1*mRNA expression (Fig. 3A & B **and Supplementary Table 4**). The *pomca2*, *cart4* and *mc4ra2* mRNA expression was negatively correlated to *ghrl1* ($p < 0.05$) while the *agrp1*, *cart2b*, *pomca1* and *mc4rb1* showed negative correlation (not significant but negative coefficient). No other significant correlations were found.

4. Discussion

In the current study, we investigated effects of long-term fasting in Atlantic salmon on known-key players in the appetite control. One of the aims was to explore a putative signaling pathway between the stomach and the hypothalamus (feeding center), in which the neurons from the hypothalamic NLTv receive peripheral signals from the stomach (Rønnestad et al., 2017; Timper and Brüning, 2017) in response to the nutritional status of the animal. Thus, we have selected the melanocortin paralogs to be investigated here based on our previous studies on brain distribution of appetite regulators (Kalananthan et al., 2020a, 2021; Tolås et al., 2021).

4.1. Fish performance under long-term fasting

In the current study, 4 and 6 weeks of fasting under experimental conditions resulted in a reduction of biomass and a drop in the K factor (Fig. 1) from 1.2 (Fed) to 1.1 & 1.0 (Fasted fish groups at 4 and 6 weeks, respectively). This shows that feed deprivation led to changes in metabolism that provided cellular energy through catabolic processes (Bower et al., 2009; Bar, 2014). The HSI decline at 4 weeks of fasting was greater than that at 6 weeks of fasting, which supports the theory of Bar (2014): mobilization of various tissues during prolonged fasting of fish tends to be sequential, with carbohydrates utilized first, followed by fat and lastly protein. Bar (2014) also stated that there are three phases of starvation: the first phase appears to be quite short with a relatively large decrease of body mass, the second phase is generally characterized by relatively long periods of a mild decrease of mass, and the last phase has generally higher rates of mass loss. In a parallel study conducted in the same experiment here presented, continued fasting period up to 8 weeks resulted in a relative high rate of fish weight loss between 6 and 8 weeks of feed deprivation (Hvas et al., 2022), similar to that found after 86 days of feed deprivation (Einen et al., 1998). Einen et al. (1998)

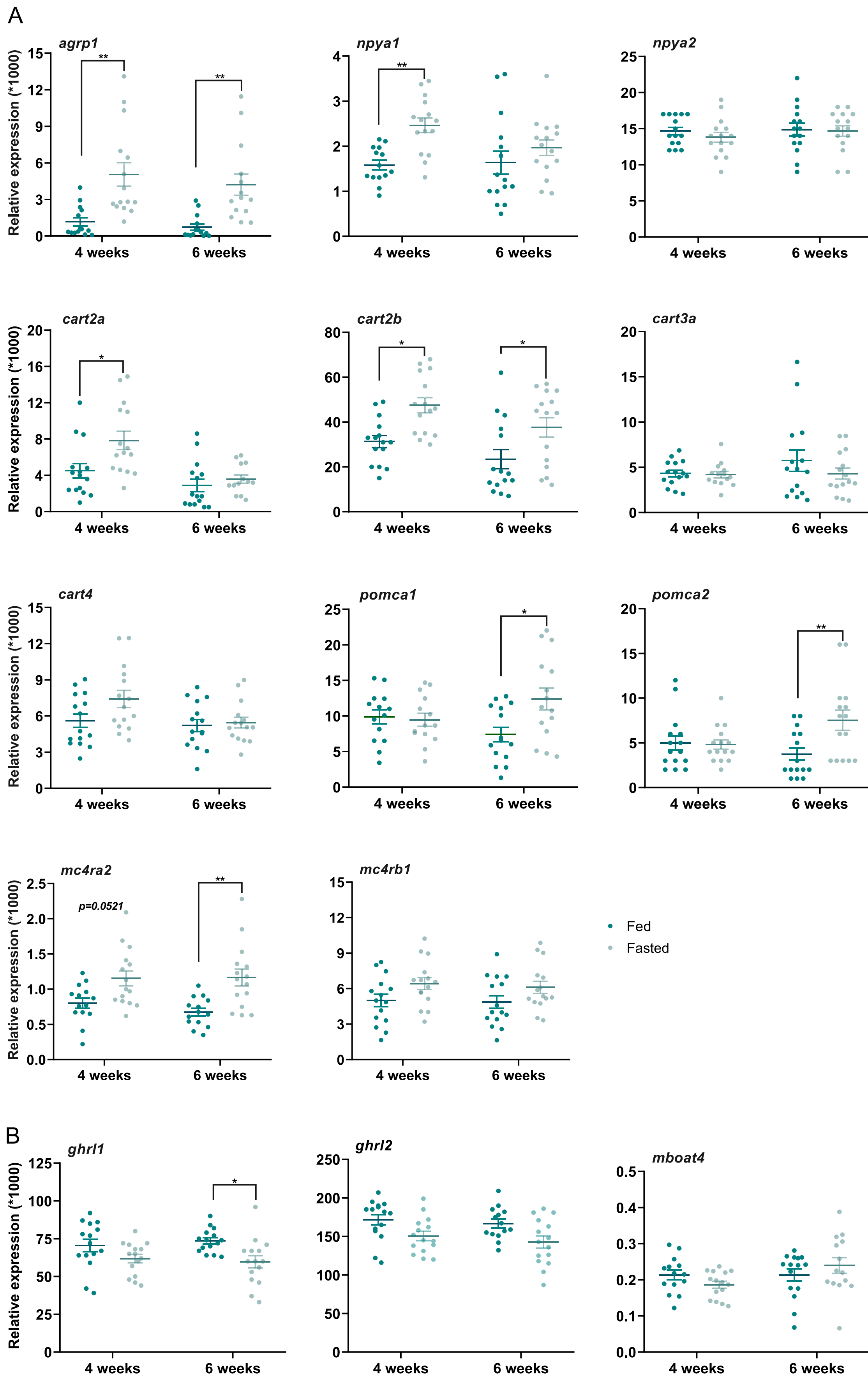

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Fig. 2. Effect of 4 and 6 weeks of fasting on the mRNA expression of **A.** *agrp1*, *npya1*, *npya2*, *cart2a*, *cart2b*, *cart3a*, *cart4*, *pomca1*, *pomca2*, *mc4ra2* and *b1* in hypothalamus and **B.** *ghrl1*, *ghrl2* and *mboat4* in stomach of Atlantic salmon. Dark and light blue dots represent Fed ($n = 14$ or 15) and Fasted ($n = 13$ to 15) groups, respectively. The graphs show the normalized mRNA copy number of each gene to the geometric mean copy number of *actb* and *s20*. A two-way ANOVA followed by the Tukey multiple comparison test was used to analyze effects of time and treatment. The error bars represent mean ± SEM and asterisks show the significant level ($*p < 0.05$, $**p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

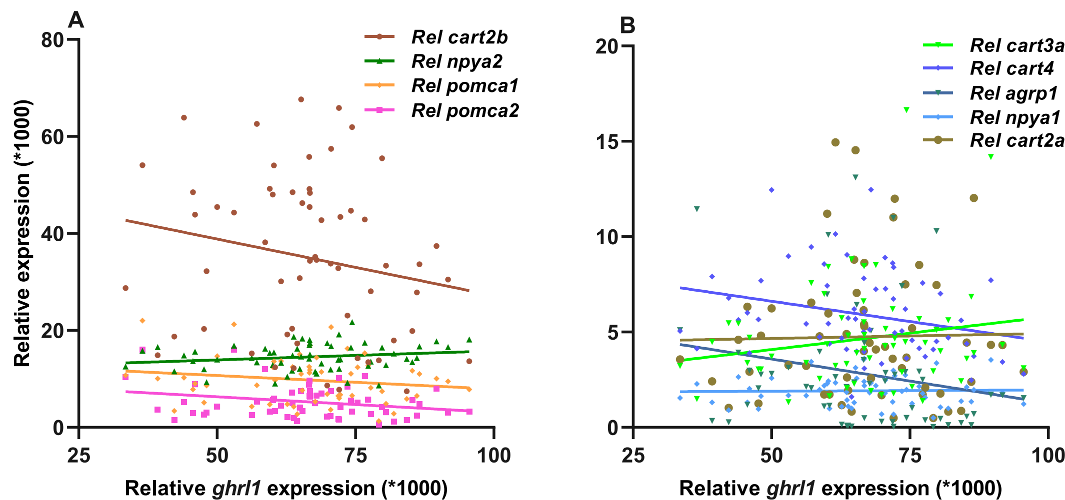

Fig. 3. **A.** Relative mRNA expression of abundant hypothalamic melanocortin neuropeptides *cart2b*, *npya2*, *pomca1* and *pomca2* versus relative mRNA expression of *ghrl1*. **B.** The relative expression of less abundant hypothalamic melanocortin neuropeptides *cart3a, cart4, agrp1, npya1* and *cart2a* mRNA expression versus the relative mRNA expression of *ghrl1*.

further found that fillet fat content was only slightly lowered beyond 58 days of starvation compared to fed fish with decline in fillet yield indicating that fillet fat started to be used as fuel beyond 58 days of starvation following viscera and liver. A significant decline in HSI (related to glycogen reduction) with feed deprivation has been previously reported in Atlantic salmon (Soengas et al., 1996), and other fish species (Sheridan and Mommsen, 1991; Metón et al., 2003; Bayir et al., 2011; Pérez-Jiménez et al., 2012; Ashouri et al., 2013; Babaei et al., 2016). In our study, HSI data suggest that the liver as fuel storage of both glycogen and fat has possibly been utilized during 4 to 6 weeks fasting. Hvas et al. (2022) have refed the fish after the 8 weeks of fasting and the results showed a gradual increase in feed intake over the first month. After three months, the weight difference between the experimental groups (Fed vs. Fasting) was minor, while the *K* factor was highest in the Fasted/refed fish. Full compensatory growth was found after 7 months of refeeding and no effect of fasting was found on welfare parameters (Hvas et al., 2022). This highlights the flexibility of growth trajectories and compensatory mechanisms in Atlantic salmon, similar to reported effects of photoperiod manipulation on seasonal growth rate and *K* factor (Stefansson et al., 1991; Oppedal et al., 1999).

4.2. Stomach regulation of appetite

In long-term fasted fish (4 and 6 weeks), each compartment of GI-tract was, as expected, almost empty (**Supplementary Fig. 4**) with a minor presence of bright yellow fluid (most likely bile) in the gut. The GI-tract likely had reduced functionality after the long-term fasting as previous studies in salmon showed that fasting led to decreased GI mass and enzyme capacities of the GI-tract by 20–50% within two days, and 40–75% after 40 days (Krogdahl and Bakke-McKellep, 2005).

In vertebrates, ghrelin from the GI-tract particularly from stomach is involved in regulating gut motility and stimulate growth hormone release, feed intake and energy homeostasis (Stengel and Taché, 2012; Tine et al., 2016). Ghrelin O-acyltransferase (GOAT), or MBOAT4, is a critical enzyme that modifies (acylates) ghrelin to enable the action on growth hormone secretagogue receptors (GHSR) in ARC (Kojima et al., 1999; Yang et al., 2008; Shlimun and Unniappan, 2011). In Atlantic salmon, *ghrl2* was the most abundant form in the stomach (Fig. 2B) (Del Vecchio et al., 2021) but was not affected by feed deprivation, while *ghrl1* significantly declined after 6 weeks of fasting. This is in line with a study with 2 days of fasting in the same species (Hevrøy et al., 2011). In contrast, studies in seabass (Terova et al., 2008) and in arctic charr (Frøiland et al., 2010) showed that stomach *ghrl* mRNA level was upregulated during starvation, and down-regulated during re-feeding. Additionally, in a previous study in Atlantic salmon, 6 days of starvation led to an increase in stomach *ghrl1* mRNA level (Murashita et al., 2009). However, studies also report no effect of short-term (Del Vecchio et al., 2021), long-term feed deprivation in stomach *ghrl* mRNA level in Atlantic salmon (Hevrøy et al., 2011) and in Atlantic cod (Xu and Volkoff, 2009). The *ghrl* mRNA expression level in response to fasting varies among studies depending on the environmental conditions of the experiment (i.e., temperature, oxygen level, etc.), time of sampling in response to feeding time (Hevrøy et al., 2011), length of the fasting period, subdivision of stomach region (Del Vecchio et al., 2021) and differences in energy metabolism among species (Xu and Volkoff, 2009). The plasma Ghrl level elevated in 24 h post feeding in rainbow trout (Pankhurst et al., 2008), in feed deprivation of 2 days in Atlantic salmon (Hevrøy et al., 2011), 5 days in goldfish (Unniappan et al., 2004), 2 weeks in tilapia (*Oreochromis mossambicus*) (Fox et al., 2009), and 21 days in sea bream (*Sparus aurata*) (Perelló-Amorós et al., 2019) showing its orexigenic action. Whereas a decline in plasma Ghrl level after long-term feed deprivation in burbot (*Lota lota*) (Nieminen et al., 2003) and in rainbow trout showed Ghrl release was suppressed under catabolic conditions (Jönsson et al., 2007). In the current study, there is no data for plasma Ghrl level available to explore more on Atlantic salmon Ghrl in response to long-term fasting. Based on our results, as we hypothesized previously, to save energy from foraging/feed search activity during catabolic conditions, the stomach *ghrl* mRNA level might possibly return as hunger signal only when there is feed available in the vicinity.

The interlink between Ghrl and Mboat4 has been reported in few teleosts (Hatef et al., 2015; Blanco et al., 2016, 2017). Mboat4 has been reported as orexigenic peptide in appetite regulation in zebrafish (Hatef

et al., 2015). In this agastric species, *mboat* mRNA increased in unfed compared to those of fed fish at 3 and 7 days of fasting. This is comparable to findings in mammals where 21 days of fasting led to an increase in *Mboat* mRNA in rat stomach mucosa (González et al., 2008). However, in Atlantic salmon we observed low mRNA level of *mboat4* in stomach and no significant effect on the level of expression (Fig. 2B) in response to long-term fasting. Given that Mboat4 is an essential enzyme for acylation of Ghrl, as we previously hypothesized for expression levels of *ghrl*, the *mboat4* might also turn into a hunger signal when feed is available following a long fasting period in Atlantic salmon.

4.3. Hypothalamic regulation of appetite

The significant increase of hypothalamic *agrp1* mRNA levels (Fig. 2A) to long-term fasting (at 4 and 6 weeks) in Atlantic salmon is in line with the mammalian model, supporting the hypothesis that this neuropeptide provides an important orexigenic drive to stimulate appetite. Our previous study on short-term fasting in the same species also supported the orexigenic role of hypothalamic *agrp1* (Kalananthan et al., 2020b). These observations are also in line with *agrp1* expression response in sea bass (Agulleiro et al., 2014) (8–29 days of fasting) and spotted sea bass (8 weeks of fasting) (Zhang et al., 2019). However, *agrp1* expression was unaffected by starvation in rainbow trout (4 months fasting) (Jørgensen et al., 2016) and in arctic charr (one month) (Striberny and Jørgensen, 2017). In mice, hypothalamic AgRP neuron activity is high during hunger and is rapidly reduced by the sight and smell of food (Su et al., 2017). In our study, the *agrp1* was high during fasting, but due to Covid-19, it was not possible to explore further as no samples were collected after refeeding in the same experiment.

The *npya1* mRNA expression increased significantly following 4 weeks of fasting (Fig. 2A), in line with its proposed role as an orexigenic factor as in winter flounder, where hypothalamic *npy* increased after one month fasting (MacDonald and Volkoff, 2009). However, our results are incongruous with several studies where no effects of fasting on hypothalamic *npy* was observed following one week fasting in Atlantic cod (Kehoe and Volkoff, 2007), 4 weeks in arctic charr (Striberny and Jørgensen, 2017), 8 weeks in spotted sea bass (Zhang et al., 2019) and 4 months in rainbow trout (Jørgensen et al., 2016). There are several possible explanations for the varied observations between these studies, importantly, species specificity (Sheridan and Mommsen, 1991), and experimental conditions such as, variation in duration of fasting since the last meal, and temperature (Kehoe and Volkoff, 2007). Indeed, a previous study in our group focusing on the effects of short-term fasting on *npy* in post smolt Atlantic salmon found no effect on hypothalamic *npy* mRNA expression (Tolås et al., 2021). Moreover, no difference in *npya1* expression was found between the Fed and Fasted groups following 6 weeks of treatment in the current study. Together, this suggests that the role of *npya* in response to fasting is highly dependent on duration of fasting, and further studies into the timing of *npya* fluctuation in response to feed deprivation will likely provide valuable insight (Tolås et al., 2021).

The *cart* genes are described to serve an anorexigenic function according to the mammalian model. In this study, among the *cart* paralogs analyzed, *cart2a* and *2b* increased at 4 weeks of fasting while *cart2b* (the highest expressed in hypothalamus) continued to be higher than the Fed group also at 6 weeks of fasting (Fig. 2A). A similar increase in *cart2b* was also observed in short-term fasting studies in the post smolts of the same species (Kalananthan et al., 2021), and in Siberian sturgeon (*Acipenser baerii*) (Zhang et al., 2018) where *cart* mRNA in whole brain increased after 3–17 days of fasting. However, our finding is contrary to what has been observed in several other species. For example, one week of fasting decreased *cart* mRNA in silver dollar (*Metynnis argenteus*) (Butt et al., 2019), and in yellowtail (*Seriola quinqueradiata*) *cart2a* mRNA expression decreased in the telencephalon and hypothalamus following 8 days of fasting (Fukada et al., 2021), and in medaka *cart2b* (previously named *cart* ch3) expression decreased with 17 days of fasting (Murashita and Kurokawa, 2011). Additionally, *cart* remained unchanged following 3 weeks of fasting in cunner (Babichuk and Volkoff, 2013), one month in arctic charr (Striberny and Jørgensen, 2017), either 2 weeks or 4 weeks fasting in winter flounder (MacDonald and Volkoff, 2009) and 4 months in rainbow trout (Jørgensen et al., 2016). Though the hypothalamic *cart3a* was unaffected by long-term fasting, an upregulation was observed in short-term fasting in Atlantic salmon (Kalananthan et al., 2021). The unaffected mRNA expression level of *cart4* both in the short/long-term fasting in Atlantic salmon suggests a potentially different functionality other than appetite regulation (Kalananthan et al., 2021). Our results further suggest the possibility that the various paralogs might exert their functions at different phases of starvation.

According to the mammalian model, POMC is an anorexigenic/satiety neuropeptide. In the current study in Atlantic salmon, both *pomca1* & *a2* (Fig. 2A) showed an upregulation at 6 weeks of fasting indicating an orexigenic role which is also in line with increasing *pomca1* levels after 4 months of fasting in rainbow trout (Jørgensen et al., 2016). Contrary to these results, *pomca1* was downregulated in 28 days fasted rainbow trout, (Leder and Silverstein, 2006) while no effect was observed in arctic charr (Striberny and Jørgensen, 2017). Our previous 3 days of fasting study resulted in a declining trend in mRNA expression of hypothalamic *pomca2* in Atlantic salmon (Kalananthan et al., 2020b) and 4 days of fasting treatment did not affect either *pomca1* or *a2* expression levels (Kalananthan et al., 2020a). As such, these results suggest that the roles of these paralogs in the hypothalamus differ with fed or fasted condition and the period of fasting (short or long-term) among or even within the species. As mentioned previously, the experimental fish in the fasted group was deprived of feed for >4 weeks and we considered this as equivalent to starvation. Starvation is an ecologically relevant stressor, and it is likely that *pomc* expression was upregulated in the Fasted group in response to starvation induced stress. Pomc is a precursor peptide of other peptides like α-MSH, adrenocorticotropin hormone belonging to melanocortin system functioning in the Hypothalamus-Pituitary-Interrenal axis, and is involved in mobilizing energy during periods of stress (Bernier and Peter, 2001; Lu et al., 2003; Smart et al., 2007).

As previously mentioned, MC4R serves as a receptor in the melanocortin system. Of the *mc4r* paralogs included in our study, only the *mc4ra2* mRNA level (Fig. 2A) significantly increased after 6 weeks in the Fasted group. The *pomc* and *mc4r* upregulation might have been related to a starvation induced stress response. This is supported by data that shows that POMC neurons acts on CRH neurons via MC4R in the hypothalamus (Lu et al., 2003). However, the study of Lu et al. (2003) and several other studies (Wan et al., 2012; Kalananthan et al., 2020a; Zhou et al., 2022) have only investigated their response in short-term feed deprivation (4–7 days) experiments. Consequently, since it is quite probable that the metabolic changes observed in in short-term food deprivation differs from those under long-term food deprivation, as already demonstrated in the same species (Soengas et al., 1996), these studies cannot be directly comparable. However, our results are in line with another study in gibel carp (*Carassius auratus gibelio*) that fasting from 1 to 7 days upregulated the *mc4r* transcript level (Zhou et al., 2022), while, the opposite action for *mc4r* was reported in common Carp (*Cyprinus carpio*) following 7 days of fasting and boosted with refeeding (Wan et al., 2012). Moreover, 4 days of fasting did not affect the paralogs of *mc4r* (*a1*, *a2*, *b1* and *b2*) mRNA expression in Atlantic salmon (Kalananthan et al., 2020a), similar to no response observed in the hypothalamus/brain *mc4r* expression following fasting in arctic charr (Striberny and Jørgensen, 2017) and in barfin flounder (*Verasper moseri*) (Kobayashi et al., 2008). However, Mc4r was activated and regulated by Agrp binding to the receptor rather than gene expression levels of *pomc* or *mc4r* in case of progressive fasting in sea bass (Sánchez et al., 2009). As such, considering *mc4r* expression levels upon feed deprivation may not be the golden standard for proving involvement in appetite regulation. Our investigation demonstrates that both *agrp1* and *mc4ra2* increased after prolonged fasting while *mc4rb1* remained unchanged,

and it is thus uncertain which of the Mc4r paralogs, either a2 or b1, that involve in Agrp1 (as inverse agonist) binding in this context. However, studies in teleost fish report that *mc4r* is expressed in peripheral tissues like the gastrointestinal tract, eye and ovaries in zebrafish (Ringholm et al., 2002), in the eye, ovaries, testis and the liver in barfin flounder (Kobayashi et al., 2008), and in the retina, fat tissue, testis and white muscles in sea bass (Sánchez et al., 2009). This suggests that Mc4r is potentially involved in many physiological functions that are not fully explored. In mice, MC4R selectively in the PVN of hypothalamus (confined to central regions) contributes to glucose homeostasis by regulating glucose reabsorption via circulating adrenaline and renal GLUT2 (De Souza Cordeiro et al., 2021). Taken together, studying the tissue distribution analysis for paralogs of *mc4r* in peripheral tissues of Atlantic salmon could enable identifying the specific paralog that potentially involves in feed intake.

Based on our data, we hypothesize that the upregulation of hypothalamic *cart2a, cart2b, pomca1, pomca2* during long-term fasting in Atlantic salmon indicate that these neuropeptides play a vital role in appetite regulation during fasting by shutting down and/or neutralizing hunger signals by *agrp1* and *npya1* to save energy from foraging/feed search activity during catabolic conditions when no feed is available, or to limit a fasting-induced stress effect. Furthermore, how the interaction between *agrp1* and *npya1,* and between the paralogs of *cart* and *pomc* contributes to the net orexigenic action in long-term fasted Atlantic salmon postsmolts remain unresolved.

4.4. Stomach-hypothalamus interaction in appetite regulation

In the current study, we found a significant negative correlation between relative mRNA expression of *ghrl1* and *ghrl2* in the stomach to *pomca2* and *cart4* in the hypothalamus (Fig. 3A & B**, Supplementary Table 4**). During fasting *ghrl1* and *2* expressions decreased while *pomca2* increased suggesting these have similar functional response to long-term fasting, by reducing hunger during periods when feed is not available. Though *cart3a* showed positive correlation to *ghrl2,* there were no significant differences in the expression in response to long-term fasting. As we previously demonstrated in Kalananthan et al. (2021), the structural difference of salmon Cart4 from mammalian CART might indicate that it serve other roles than controlling feed intake. Previous studies in Atlantic salmon demonstrated that low levels of *ghrl* expressed in the adipose tissue (Murashita et al., 2009) might also suggest that salmon Ghrl is involved in other functions along with feed intake. This is still an area for future research.

The discrepancies among the studies on appetite regulation in fish can possibly be explained due to existence of multiple copies for most appetite regulating genes in some species, differences in the method of analysis (in situ vs. qPCR) or varied methodological approaches (use of whole brain vs. specific brain regions), different developmental stages, the duration of fasting, and the temperature and thus the metabolic rate of the fish. However, it might well be that appetite regulators which respond to feeding are also species-specific according to their feeding strategy and behavior. The mRNA expression of the appetite regulators may not necessarily contribute to the same trend of protein expression of a specific gene and activity at its corresponding receptors (Haider and Pal, 2013). A combined study with mRNA and protein expression could provide a clear understanding of orexigenic and anorexigenic action of appetite regulators (specified paralogs) in response to prolonged feed deprivation.

5. Conclusion

Among the genes we analyzed, *agrp1*, *npya1*, *cart2a* & *2b*, *pomca1* & *a2*, *mc4ra2* and *ghrl1* responded to long-term fasting. Whereas the data supported an orexigenic role for *agrp1* and *npya1* at 4 weeks of fasting, after 6 weeks of fasting *agrp1* alone continued to serve an apparent orexigenic role. The upregulation of *cart2a*, *cart2b*, *pomca1* and *pomca2* could indicate that the neuropeptides play a vital role in appetite regulation by inducing shutting down hunger and/or neutralizing hunger signals (*agrp1* and *npya1*) to save energy from foraging search activity during catabolic conditions. Alternatively, these neuropeptides could play a fasting-induced stress response to mobilize energy. We also postulate that the drop in *ghrl* mRNA expression under catabolic conditions in Atlantic salmon might return as a hunger signal when feed is available in the vicinity. We also propose *agrp1* as a potential appetite biomarker gene under feed deprived conditions in this species. However, the dynamic response related to the duration of feed deprivation needs further investigation.

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Authors' contributions

OF and IR conceived and designed the study. OF conducted the experiment. TK, OF, FL, IT and VG contributed to sampling. FL and ASG did the primers design and cloning of *mboat4*. TK and VG did preparatory lab work. TK performed qPCR and statistical analysis and drafted the manuscript. All authors contributed to the interpretation of the data, writing of the manuscript, read and approved the final version.

Declaration of Competing Interest

The authors declare no competing or financial interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738917.

References

Agulleiro, M.J., Cortés, R., Leal, E., Ríos, D., Sánchez, E., Cerdá-Reverter, J.M., 2014. Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 205, 251–259. https://doi.org/10.1016/j.ygcen.2014.02.009.

Ashouri, G., Yavari, V., Bahmani, M., Yazdani, M.A., Kazemi, R., Morshedi, V., et al., 2013. The effect of short-term starvation on some physiological and morphological parameters in juvenile Siberian sturgeon, *Acipenser baerii* (Actinopterygii: Acipenseriformes: Acipenseridae). Acta Ichthyol. Piscat. 43, 145–150. https://doi.org/10.3750/AIP2013.43.2.07.

Babaei, S., Abedian Kenari, A., Hedayati, M., Yazdani Sadati, M.A., Metón, I., 2016. Effect of diet composition on growth performance, hepatic metabolism and antioxidant activities in Siberian sturgeon (*Acipenser baerii*, Brandt, 1869) submitted to starvation and refeeding. Fish Physiol. Biochem. 42, 1509–1520. https://doi.org/10.1007/s10695-016-0236-0.

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

Bar, N., 2014. Physiological and hormonal changes during prolonged starvation in fish. Can. J. Fish. Aquat. Sci. 71, 1447–1458. https://doi.org/10.1139/cjfas-2013-0175.

Bayir, A., Sirkecioglu, A.N., Bayir, M., Haliloglu, H.I., Kocaman, E.M., Aras, N.M., 2011. Metabolic responses to prolonged starvation, food restriction, and refeeding in the brown trout, *Salmo trutta*: oxidative stress and antioxidant defenses. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 159, 191–196. https://doi.org/10.1016/j.cbpb.2011.04.008.

Bernier, N.J., Peter, R.E., 2001. The hypothalamic-pituitary-interrenal axis and the control of food intake in teleost fish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 639–644. https://doi.org/10.1016/S1096-4959(01)00360-8.

Blanco, A.M., Sánchez-Bretaño, A., Delgado, M.J., Valenciano, A.I., 2016. Brain mapping of ghrelin O-acyltransferase in goldfish (*Carassius Auratus*): novel roles for the ghrelinergic system in fish? Anat. Rec. 299, 748–758. https://doi.org/10.1002/ar.23346.

Blanco, A.M., Gómez-Boronat, M., Alonso-Gómez, Á.L., Yufa, R., Unniappan, S., Delgado, M.J., et al., 2017. Characterization of Ghrelin O-Acyltransferase (GOAT) in goldfish (*Carassius auratus*). PLoS One 12, 1–28. https://doi.org/10.1371/journal.pone.0171874.

Bower, N.I., Taylor, R.G., Johnston, I.A., 2009. Phasing of muscle gene expression with fasting-induced recovery growth in Atlantic salmon. Front. Zool. 6, 1–13. https://doi.org/10.1186/1742-9994-6-18.

Butt, Z.D., O'Brien, E., Volkoff, H., 2019. Effects of fasting on the gene expression of appetite regulators in three Characiformes with different feeding habits (*Gymnocorymbus ternetzi*, *Metynnis argenteus* and *Exodon paradoxus*). Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 227, 105–115. https://doi.org/10.1016/j.cbpa.2018.10.002.

Cerdá-Reverter, J.M., Peter, R.E., 2003. Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. Endocrinology 144, 4552–4561. https://doi.org/10.1210/en.2003-0453.

Cerdá-Reverter, J.M., Ringholm, A., Schiöth, H.B., Peter, R.E., 2003a. Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. Endocrinology 144, 2336–2349. https://doi.org/10.1210/en.2002-0213.

Cerdá-Reverter, J.M., Schiöth, H.B., Peter, R.E., 2003b. The central melanocortin system regulates food intake in goldfish. Regul. Pept. 115, 101–113. https://doi.org/10.1016/S0167-0115(03)00144-7.

De Souza Cordeiro, L.M., Elsheikh, A., Devisetty, N., Morgan, D.A., Ebert, S.N., Rahmouni, K., et al., 2021. Hypothalamic MC4R regulates glucose homeostasis through adrenaline-mediated control of glucose reabsorption via renal GLUT2 in mice. Diabetologia 64, 181–194. https://doi.org/10.1007/s00125-020-05289-z.

Del Vecchio, G., Lai, F., Gomes, A.S., Verri, T., Kalananthan, T., Barca, A., et al., 2021. Effects of short-term fasting on mRNA expression of ghrelin and the peptide transporters PepT1 and 2 in Atlantic Salmon (*Salmo salar*). Front. Physiol. 12, 1–12. https://doi.org/10.3389/fphys.2021.666670.

Duston, J., Saunders, R.L., 1999. Effect of winter food deprivation on growth and sexual maturity of Atlantic salmon (*Salmo salar*) in seawater. Can. J. Fish. Aquat. Sci. 56, 201–207. https://doi.org/10.1139/f98-165.

Einen, O., Waagan, B., Thomassen, M.S., 1998. Starvation prior to slaughter in Atlantic salmon (*Salmo salar*). Aquaculture 166, 85–104. https://doi.org/10.1016/s0044-8486(98)00279-8.

Fox, B.K., Breves, J.P., Hirano, T., Grau, E.G., 2009. Effects of short- and long-term fasting on plasma and stomach ghrelin, and the growth hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis mossambicus*. Domest. Anim. Endocrinol. 37, 1–11. https://doi.org/10.1016/j.domaniend.2009.01.001.

Froese, B.R., 2006. Cube law, condition factor and weight – length relationships: history, meta-analysis and recommendations. J. Appl. Ichthyol. 22, 241–253. https://doi.org/10.1111/j.1439-0426.2006.00805.x.

Frøiland, E., Murashita, K., Jørgensen, E.H., Kurokawa, T., 2010. Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. Gen. Comp. Endocrinol. 165, 136–143. https://doi.org/10.1016/j.ygcen.2009.06.010.

Fukada, H., Murashita, K., Senzui, A., Masumoto, T., 2021. Multiple cocaine- and amphetamine-regulated transcript genes in yellowtail *Seriola quinqueradiata*: cloning, tissue distribution in the brain, and response to fasting and fish meal soluble fraction. Fish. Sci. 87, 55–64. https://doi.org/10.1007/s12562-020-01475-5.

González, C.R., Vázquez, M.J., López, M., Diéguez, C., 2008. Influence of chronic undernutrition and leptin on GOAT mRNA levels in rat stomach mucosa. J. Mol. Endocrinol. 41, 415–421. https://doi.org/10.1677/JME-08-0102.

Grove, D.J., Loizides, L.G., Nott, J., 1978. Satiation amount, frequency of feeding and gastric emptying rate in *Salmo gairdneri*. J. Fish Biol. 12, 507–516. https://doi.org/10.1111/j.1095-8649.1978.tb04195.x.

Haider, S., Pal, R., 2013. Integrated analysis of transcriptomic and proteomic data. Curr. Genomics 14, 91–110. https://doi.org/10.2174/1389202911314020003.

Hall, J.E., 2011. Metabolism and Temperature Regulation: Dietary balances; regulation of feeding; obesity and starvation; vitamins and minerals. In: Guyton and Hall Textbook of Medical Physiology, pp. 843–855 ed. Saunders (Philadelphia: Elsevier Inc).

Hatef, A., Yufa, R., Unniappan, S., 2015. Ghrelin O-acyl transferase in zebrafish is an evolutionarily conserved peptide upregulated during calorie restriction. Zebrafish 12, 327–338. https://doi.org/10.1089/zeb.2014.1062.

Herget, U., Wolf, A., Wullimann, M.F., Ryu, S., 2014. Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. J. Comp. Neurol. 522, 1542–1564. https://doi.org/10.1002/cne.23480.

Hevrøy, E.M., Azpeleta, C., Shimizu, M., Lanzén, A., Kaiya, H., Espe, M., et al., 2011. Effects of short-term starvation on ghrelin, GH-IGF system, and IGF-binding proteins in Atlantic salmon. Fish Physiol. Biochem. 37, 217–232. https://doi.org/10.1007/s10695-010-9434-3.

Higgs, D.A., Sutton, J.N., Kim, H., Oakes, J.D., Smith, J., Biagi, C., et al., 2009. Influence of dietary concentrations of protein, lipid and carbohydrate on growth, protein and energy utilization, body composition, and plasma titres of growth hormone and insulin-like growth factor-1 in non-transgenic and growth hormone transgenic coho s. Aquaculture 286, 127–137. https://doi.org/10.1016/j.aquaculture.2008.08.036.

Hvas, M., Stien, L.H., Oppedal, F., 2020. The metabolic rate response to feed withdrawal in Atlantic salmon post-smolts. Aquaculture 529, 735690. https://doi.org/10.1016/j.aquaculture.2020.735690.

Hvas, M., Stien, L.H., Oppedal, F., 2021. The effect of fasting period on swimming performance, blood parameters and stress recovery in Atlantic salmon post smolts. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 255 https://doi.org/10.1016/j.cbpa.2021.110913.

Hvas, M., Nilsson, J., Vågseth, T., Nola, V., Fjelldal, P.G., Hansen, T.J., et al., 2022. Full compensatory growth before harvest and no impact on fish welfare in Atlantic salmon after an 8-week fasting period. Aquaculture 546, 737415. https://doi.org/10.1016/j.aquaculture.2021.737415.

Jönsson, E., Forsman, A., Einarsdottir, I.E., Kaiya, H., Ruohonen, K., Björnsson, B.T., 2007. Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 147, 1116–1124. https://doi.org/10.1016/j.cbpa.2007.03.024.

Jørgensen, E.H., Bernier, N.J., Maule, A.G., Vijayan, M.M., 2016. Effect of long-term fasting and a subsequent meal on mRNA abundances of hypothalamic appetite regulators, central and peripheral leptin expression and plasma leptin levels in rainbow trout. Peptides 86, 162–170. https://doi.org/10.1016/j.peptides.2015.08.010.

Kalananthan, T., Lai, F., Gomes, A.S., Murashita, K., Handeland, S., Rønnestad, I., 2020a. The melanocortin system in Atlantic salmon (*Salmo salar* L.) and its role in appetite control. Front. Neuroanat. 14, 48. https://doi.org/10.3389/fnana.2020.00048.

Kalananthan, T., Murashita, K., Rønnestad, I., Ishigaki, M., Takahashi, K., Silva, M.S., et al., 2020b. Hypothalamic *agrp* and *pomc* mRNA responses to gastrointestinal fullness and fasting in Atlantic salmon (*Salmo salar*, L.). Front. Physiol. 11, 61. https://doi.org/10.3389/fphys.2020.00061.

Kalananthan, T., Gomes, A.S., Lai, F., Tolås, I., Jordal, A.-E.O., Norland, S., et al., 2021. Brain distribution of 10 cart transcripts and their response to 4 days of fasting in Atlantic Salmon (*Salmo salar* L.). Front. Mar. Sci. 8, 763766 https://doi.org/10.3389/fmars.2021.763766.

Kehoe, A.S., Volkoff, H., 2007. Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 146, 451–461. https://doi.org/10.1016/j.cbpa.2006.12.026.

Kobayashi, Y., Tsuchiya, K., Yamanome, T., Schiöth, H.B., Kawauchi, H., Takahashi, A., 2008. Food deprivation increases the expression of melanocortin-4 receptor in the liver of barfin flounder, *Verasper moseri*. Gen. Comp. Endocrinol. 155, 280–287. https://doi.org/10.1016/j.ygcen.2007.05.010.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402, 656–660.

Kristiansen, T.S., Samuelsen, O.B., 2006. Utredning for Mattilsynet: Fiskevelferd ved bruk av slaktemerd for oppdrettsfisk. Havforskningsinstituttet, Bergen, Norway, pp. 1–20.

Krogdahl, Å., Bakke-McKellep, A.M., 2005. Fasting and refeeding cause rapid changes in intestinal tissue mass and digestive enzyme capacities of Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 141, 450–460. https://doi.org/10.1016/j.cbpb.2005.06.002.

Le, H.T.M.D., Angotzi, A.R., Ebbesson, L.O.E., Karlsen, Ø., Rønnestad, I., 2016. The ontogeny and brain distribution dynamics of the appetite regulators NPY, CART and pOX in larval Atlantic cod (*Gadus morhua* L.). PLoS One 11 (4), e0153743. https://doi.org/10.1371/journal.pone.0153743.

Leder, E.H., Silverstein, J.T., 2006. The pro-opiomelanocortin genes in rainbow trout (*Oncorhynchus mykiss*): duplications, splice variants, and differential expression. J. Endocrinol. 188, 355–363. https://doi.org/10.1677/joe.1.06283.

Lie, Ø., Huse, J., 1992. The effect of starvation on the composition of Atlantic salmon (*Salmo salar*). Fisk. Dir. Skr. Ernæring 5, 11–16.

Lien, S., Koop, B.F., Sandve, S.R., Miller, J.R., Kent, M.P., Nome, T., et al., 2016. The Atlantic salmon genome provides insights into rediploidization. Nature 533, 200–205. https://doi.org/10.1038/nature17164.

Lu, X.Y., Barsh, G.S., Akil, H., Watson, S.J., 2003. Interaction between α-melanocyte-stimulating hormone and corticotropin-releasing hormone in the regulation of feeding and hypothalamo-pituitary-adrenal responses. J. Neurosci. 23, 7863–7872. https://doi.org/10.1523/jneurosci.23-21-07863.2003.

MacDonald, E., Volkoff, H., 2009. 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. 56, 58–65. https://doi.org/10.1016/j.yhbeh.2009.03.002.

McVicar, A.H., 1987. Pancreas disease of farmed Atlantic salmon, *Salmo salar*, in Scotland: epidemiology and early pathology. Aquaculture 67, 71–78. https://doi.org/10.1016/0044-8486(87)90009-3.

Metón, I., Fernández, F., Baanante, I.V., 2003. Short- and long-term effects of refeeding on key enzyme activities in glycolysis-gluconeogenesis in the liver of gilthead seabream (*Sparus aurata*). Aquaculture 225, 99–107. https://doi.org/10.1016/S0044-8486(03)00281-3.

Murashita, K., Kurokawa, T., 2011. Multiple cocaine- and amphetamine-regulated transcript (CART) genes in medaka, *Oryzias latipes*: cloning, tissue distribution and effect of starvation. Gen. Comp. Endocrinol. 170, 494–500. https://doi.org/10.1016/j.ygcen.2010.11.005.

Murashita, K., Kurokawa, T., Nilsen, T.O., Rønnestad, I., 2009. Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (Salmo salar): molecular cloning and tissue expression. Gen. Comp. Endocrinol. 160, 223–235. https://doi.org/10.1016/j.ygcen.2008.11.024.

Nieminen, P., Mustonen, A.M., Hyvärinen, H., 2003. Fasting reduces plasma leptin- and ghrelin-immunoreactive peptide concentrations of the Burbot (*Lota Iota*) at 2°C but not at 10°C. Zool. Sci. 20, 1109–1115. https://doi.org/10.2108/zsj.20.1109.

Nuzzaci, D., Laderrière, A., Lemoine, A., Nédélec, E., Pénicaud, L., Rigault, C., et al., 2015. Plasticity of the melanocortin system: determinants and possible consequences on food intake. Front. Endocrinol. Lausanne). 6 https://doi.org/10.3389/fendo.2015.00143.

Olsvik, P.A., Lie, K.K., Jordal, A.E.O., Nilsen, T.O., Hordvik, I., 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol. Biol. 6, 21. https://doi.org/10.1186/1471-2199-6-21.

Oppedal, F., Taranger, G.L., Juell, J.-E., Hansen, T., 1999. Growth, osmoregulation and sexual maturation of underyearling Atlantic salmon smolt *Salmo salar* L. exposed to different intensities of continuous light in sea cages. Aquac. Res. 30, 491–499.

Otero-Rodiño, C., Rocha, A., Sánchez, E., Álvarez-Otero, R., Soengas, J.L., Cerdá-Reverter, J.M., 2019. Sensing glucose in the central melanocortin circuits of rainbow trout: a morphological study. Front. Endocrinol. (Lausanne). 10, 1–7. https://doi.org/10.3389/fendo.2019.00254.

Pankhurst, N.W., King, H.R., Ludke, S.L., 2008. Relationship between stress, feeding and plasma ghrelin levels in rainbow trout, *Oncorhynchus mykiss*. Mar. Freshw. Behav. Physiol. 41, 53–64. https://doi.org/10.1080/10236240701661156.

Perelló-Amorós, M., Vélez, E.J., Vela-Albesa, J., Sánchez-Moya, A., Riera-Heredia, N., Hedén, I., et al., 2019. Ghrelin and its receptors in gilthead sea bream: nutritional regulation. Front. Endocrinol. (Lausanne). 9, 1–14. https://doi.org/10.3389/fendo.2018.00399.

Pérez-Jiménez, A., Cardenete, G., Hidalgo, M.C., García-Alcázar, A., Abellán, E., Morales, A.E., 2012. Metabolic adjustments of *Dentex dentex* to prolonged starvation and refeeding. Fish Physiol. Biochem. 38, 1145–1157. https://doi.org/10.1007/s10695-011-9600-2.

Ringholm, A., Fredriksson, R., Poliakova, N., Yan, Y.L., Postlethwait, J.H., Larhammar, D., et al., 2002. One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. J. Neurochem. 82, 6–18. https://doi.org/10.1046/j.1471-4159.2002.00934.x.

Rønnestad, I., Gomes, A.S., Murashita, K., Angotzi, R., Jönsson, E., Volkoff, H., 2017. Appetite-controlling endocrine systems in teleosts. Front. Endocrinol. (Lausanne). 8, 73. https://doi.org/10.3389/fendo.2017.00073.

Sánchez, E., Rubio, V.C., Thompson, D., Metz, J., Flik, G., Millhauser, G.L., et al., 2009. Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (*Dicentrarchus labrax*). Am. J. Phys. Regul. Integr. Comp. Phys. 296, 1293–1306. https://doi.org/10.1152/ajpregu.90948.2008.

Sheridan, M.A., Mommsen, T.P., 1991. Effects of nutritional state on in vivo lipid and carbohydrate metabolism of coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 81, 473–483. https://doi.org/10.1016/0016-6480(91)90175-6.

Shlimun, A., Unniappan, S., 2011. Ghrelin O-acyl transferase: bridging ghrelin and energy homeostasis. Int. J. Pept. 2011 https://doi.org/10.1155/2011/217957.

Smart, J.L., Tolle, V., Otero-Corchon, V., Low, M.J., 2007. Central dysregulation of the hypothalamic-pituitary-adrenal axis in neuron-specific proopiomelanocortin-deficient mice. Endocrinology 148, 647–659. https://doi.org/10.1210/en.2006-0990.

Soengas, J.L., 2021. Integration of nutrient sensing in fish hypothalamus. Front. Neurosci. 15, 1–8. https://doi.org/10.3389/fnins.2021.653928.

Soengas, J.L., Strong, E.F., Fuentes, J., Veira, J.A.R., Andres, M.D., 1996. Food deprivation and refeeding in Atlantic salmon, *Salmo salar*: effects on brain and liver carbohydrate and ketone bodies metabolism. Fish Physiol. Biochem. 15, 491–511. https://doi.org/10.1007/BF01874923.

Soengas, J.L., Cerdá-Reverter, J.M., Delgado, M.J., 2018. Central regulation of food intake in fish: an evolutionary perspective. J. Mol. Endocrinol. 60, R171–R199. https://doi.org/10.1530/JME-17-0320.

Stefansson, S.O., Bjornsson, B.T., Hansen, T., Haux, C., Taranger, G.L., Saunders, R.L., 1991. Growth, parr– smolt transformation and changes in growth hormone of Atlantic salmon (*Salmo salar*) reared under different photoperiods. Can. J. Fish. Aquat. Sci. 48, 2100–2108.

Stengel, A., Taché, Y., 2012. Ghrelin-a pleiotropic hormone secreted from endocrine X/A-like cells of the stomach. Front. Neurosci. 6, 1–16. https://doi.org/10.3389/fnins.2012.00024.

Striberny, A., Jørgensen, E.H., 2017. Feedback from Arctic charr: feed flavour stimulation and re-feeding after feed deprivation stimulate genes encoding both orexigenic and anorexigenic neuropeptides. Gen. Comp. Endocrinol. 246, 71–80. https://doi.org/10.1016/j.ygcen.2017.03.012.

Su, Z., Alhadeff, A.L., Betley, J.N., 2017. Nutritive, post-ingestive signals are the primary regulators of AgRP neuron activity. Cell Rep. 21, 2724–2736. https://doi.org/10.1016/j.celrep.2017.11.036.

Terova, G., Rimoldi, S., Bernardini, G., Gornati, R., Saroglia, M., 2008. Sea bass ghrelin: molecular cloning and mRNA quantification during fasting and refeeding. Gen. Comp. Endocrinol. 155, 341–351. https://doi.org/10.1016/j.ygcen.2007.05.028.

Timper, K., Brüning, J.C., 2017. Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. DMM Dis. Model. Mech. 10, 679–689. https://doi.org/10.1242/dmm.026609.

Tine, M., Kuhl, H., Teske, P.R., Tschöp, M.H., Jastroch, M., 2016. Diversification and coevolution of the ghrelin/growth hormone secretagogue receptor system in vertebrates. Ecol. Evol. 6, 2516–2535. https://doi.org/10.1002/ece3.2057.

Tolås, I., Kalananthan, T., Gomes, A.S., Lai, F., Norland, S., Murashita, K., et al., 2021. Regional expression of npy mRNA paralogs in the brain of atlantic Salmon (*Salmo salar* L.) and response to fasting. Front. Physiol. 12, 720639 https://doi.org/10.3389/fphys.2021.720639.

Unniappan, S., Canosa, L.F., Peter, R.E., 2004. Orexigenic actions of ghrelin in goldfish: feeding-induced changes in brain and gut mRNA expression and serum levels, and responses to central and peripheral injections. Neuroendocrinology 79, 100–108. https://doi.org/10.1159/000076634.

Volkoff, H., 2016. The neuroendocrine regulation of food intake in fish: a review of current knowledge. Front. Neurosci. 10, 540. https://doi.org/10.3389/fnins.2016.00540.

Wade, N.M., Clark, T.D., Maynard, B.T., Atherton, S., Wilkinson, R.J., Smullen, R.P., et al., 2019. Effects of an unprecedented summer heatwave on the growth performance, flesh colour and plasma biochemistry of marine cage-farmed Atlantic salmon (*Salmo salar*). J. Therm. Biol. 80, 64–74. https://doi.org/10.1016/j.jtherbio.2018.12.021.

Wan, Y., Zhang, Y., Ji, P., Li, Y., Xu, P., Sun, X., 2012. Molecular characterization of CART, AgRP, and MC4R genes and their expression with fasting and re-feeding in common carp (*Cyprinus carpio*). Mol. Biol. Rep. 39, 2215–2223. https://doi.org/10.1007/s11033-011-0970-4.

Warren, I.A., Ciborowski, K.L., Casadei, E., Hazlerigg, D.G., Martin, S., Jordan, W.C., et al., 2014. Extensive local gene duplication and functional divergence among paralogs in Atlantic Salmon. Genome Biol. Evol. 6, 1790–1805. https://doi.org/10.1093/gbe/evu131.

Xu, M., Volkoff, H., 2009. Molecular characterization of ghrelin and gastrin-releasing peptide in Atlantic cod (*Gadus morhua*): cloning, localization, developmental profile and role in food intake regulation. Gen. Comp. Endocrinol. 160, 250–258. https://doi.org/10.1016/j.ygcen.2008.12.004.

Yang, J., Brown, M.S., Liang, G., Grishin, N.V., Goldstein, J.L., 2008. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132, 387–396. https://doi.org/10.1016/j.cell.2008.01.017.

Zhang, X., Gao, Y., Tang, N., Qi, J., Wu, Y., Hao, J., et al., 2018. One evidence of cocaine- and amphetamine-regulated transcript (CART) has the bidirectional effects on appetite in Siberian sturgeon (*Acipenser baerii*). Fish Physiol. Biochem. 44, 411–422. https://doi.org/10.1007/s10695-017-0444-2.

Zhang, K.Q., Hou, Z.S., Wen, H.S., Li, Y., Qi, X., Li, W.J., et al., 2019. Melanocortin-4 receptor in spotted sea bass, *Lateolabrax maculatus*: cloning, tissue distribution, physiology, and pharmacology. Front. Endocrinol. (Lausanne). 10, 1–13. https://doi.org/10.3389/fendo.2019.00705.

Zhou, Y., Li, Y., Lei, L., Deng, X., Duan, Y., Fu, S., 2022. The melanocortin-4 receptor (MC4R) gene in the gibel carp *Carassius auratus gibelio*: cloning, tissue distribution, and fasting effects. Aquac. Int. https://doi.org/10.1007/s10499-022-00911-w.