Basic mechanisms for control of appetite and feed intake in Atlantic salmon (*Salmo salar*)

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Thesis for the degree of Philosophiae Doctor (PhD)
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To my beloved mother
என்னுயிர் அம்மாவுக்கு o o o

Scientific environment

The work in this PhD study was performed from May 2018 to July 2022 at the Marine Developmental Biology (MDB) research group at the Department of Biological Sciences (BIO), University of Bergen (UoB). Experiment for Paper V was conducted in collaboration with Researcher Dr. Ole Folkedal at the Institute of Marine Research (IMR), Matre. The PhD study was accomplished under the supervision of Professor Ivar Rønnestad (UoB), Researcher Dr. Floriana Lai (UoB) and Professor Sigurd O. Handeland (UoB/NORCE).

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“நன்றி மறப்பது நன்றன்று நன்றல்லது அன்றே மறப்பது நன்று”- குறள் எண்: 108
“எந்நன்றி கொன்றார்க்கும் உய்வுண்டாம் உய்வில்லை செய்நன்றி கொன்ற மகற்கு”- குறள் எண்: 110

Kural No.108: It is not right to forget the help rendered by someone; it is virtuous to forget any harm, the moment it is done.
Kural No.110: There is salvation for faltering on any virtue, but not for ingratitude.

Abstract

Atlantic salmon (*Salmo salar* L.) is a key species in sea food exports of Norway and represents ca. 70 - 80 billion NOK worth annually. The extensive expansion of this fish industry combined with frequent overfeeding has contributed to potentially negative impacts on the ecosystem. As such, an efficient utilization of fish feed, with low feed conversion is vital to ensure sustainable production with regards to the environment, fish welfare, and production costs (>50% of production cost goes to feed). Thus, a clear understanding of the biological mechanisms that underly hunger and satiety, and thereby control of feed intake, would enable optimization of feeding protocols with a considerable positive impact to the salmon industry. The lack of understanding includes the contributions of key neuroendocrine players that modulate feeding and the identification of reliable biomarkers that can be used to assess appetite and potentially growth in fish.

In vertebrates, food intake is controlled by the synergic actions of central and peripheral signals by afferent neurons and circulation in combination with olfactory and visual sensation, which stimulate ingestion in relation to the nutritional status of the animal. As a central control hub, the hypothalamus plays a pivotal role in regulating appetite and feeding. In mammals, hypothalamic neuronal network comprises major distinct cell populations that express the orexigenic neuropeptides (agouti-related protein (AgRP) and neuropeptide Y (NPY)) and the anorexigenic peptides (proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART)). These melanocortin neuropeptides act on the melanocortin-4 receptor (MC4R) in higher order neurons that control both food intake and energy expenditure. The melanocortin system seems to be relatively well-conserved among vertebrates, including teleost species. However, in Atlantic salmon, the salmonid-specific fourth round whole-genome duplication (Ss 4WGD) led to the presence of several paralog genes which may have resulted in divergent functions for each of them.

Therefore, this PhD study focused mainly on 1. Updating the key appetite genes repertoire information. 2. Spatial distribution analysis of key appetite gene paralogs in

the brain regions bulbus olfactorius/olfactory bulb (OB), telencephalon (TEL), midbrain (MB), cerebellum (CE), hypothalamus (HYP), saccus vasculosus (SV), pituitary (PT) and brainstem (BS), including a first assessment of the appetite gene paralogs' role in controlling appetite in short-term fasting (3 or 4 days) in Atlantic salmon. 3. Based on their spatial distribution and quantitative expression in different brain regions, the last focus was to explore the impact of long-term fasting (4 and 6 weeks) on appetite regulating genes in the stomach-hypothalamic axis in Atlantic salmon postsmolts.

Initially, *in silico* analyses were performed to retrieve the sequences of the key appetite genes with paralogs and isoforms from GenBank and Ensembl and phylogenetic trees were predicted. Next, a standard protocol for dissecting fish brain into 8 regions was developed for the brain region distribution study of the key appetite genes. For quantification of mRNA expression of the appetite genes, assays for the retrieved sequences for reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) were developed and confirmed the identity of the novel sequences by cloning the qPCR product.

In silico analyses have confirmed the presence of 2 *agrp* (*agrp1* and *2* (also known as *asip2*)), 3 *npy* (*npya1*, *a*2 and *b*), 3 *pomc* (*pomca1*, *a2* and *b*), 10 *cart* (*cart1a*, *1b1*, *1b2*, *2a*, *2b1*, *2b2*, *3a1*, *3a2*, *3b* and *4*), 4 *mc4r* (*mc4ra1*, *a2*, *b1* and *b2*) and 2 *ghrl* (*ghrl1* and *2*) gene paralogs in the Atlantic salmon genome database. This study reports the first identification and characterization of gene paralogs for *mc4r*, *npy*, *cart* and membrane-bound O-acyltransferase domain containing 4 (*mboat4)*.

Brain regional distribution analyses showed a wide distribution pattern with varying range for all genes analyzed. Among 8 brain regions HYP, TEL, OB, MB, PT and BS showed higher mRNA expressions. The *agrp1*, *pomca1* and *pomca2* were highly expressed in HYP while *agrp2* was high in TEL and *pomcb* in PT. The *npya1* (most abundant among *npy*) and *b* were highly expressed in TEL and *npya2* in HYP. The *cart2b* was the most abundant *cart* paralog, followed by *cart3b*, *3a*, *1b*, *2a*, *4* and *cart1a*. The *cart2b* was abundant in OB and TEL, *cart3b* in BS and MB, *cart3a* in

TEL, MB, and BS and *cart2a* was in MB and HYP whilst *cart4* in HYP and TEL. The *cart1a* and *1b* were with higher expression in MB. The *agrp1*, *pomca1*, *pomca2*, *cart2a, 2b, 3a, 3b, 4* and *npya2* showed considerable expression in HYP in contrast to the other neuropeptides. The *mc4rb1* was the most abundant *mc4r* paralog, and *mc4ra1*, *a2* and *b1* showed higher expression in HYP and TEL whilst *mc4rb2* was highly expressed in HYP.

In the short-term (3 days) fasting experiment a significant upregulation of hypothalamic *agrp1* transcripts levels in the Fasted group was observed. Moreover, the mRNA abundance of *agrp1* was significantly negatively correlated with the stomach dry weight content. The correlation between stomach fullness and *agrp1* mRNA expression suggests a possible link between the stomach filling and satiety signals. The findings reported in this study indicates that hypothalamic *agrp1* acts as an orexigenic signal in Atlantic salmon. Whereas 4 days of fasting resulted in a significant decrease in mRNA expression of *npya1* in the olfactory bulb, increase of *npya2* in the midbrain and a trend of increase of *npya2* in the hypothalamus. Among *cart* paralogs only *cart2b* was upregulated after 4 days of fasting in OB, MB, and HYP compared to Fed group.

In the long-term fasting experiment, the condition (*K*) factor of fish significantly decreased at both samplings for Fasted group compared to Fed group. In qPCR analysis, the hypothalamic relative mRNA expression of *agrp1* showed highly significant upregulation at both 4 and 6 weeks of fasting. Among *npy* paralogs *npya1* and *a2,* only *npya1* level was significantly upregulated at 4 weeks of fasting. Whereas among *cart* paralogs *cart2a* mRNA expression level was significantly high only at 4 weeks fasting, *cart2b* level increased statistically in both 4 and 6 weeks of fasting, while *cart3a* and *cart4* did not respond to fasting. The *pomca1*, *a2* and *mc4ra2* increased significantly at 6 weeks of fasting. Whereas in stomach, at 6 weeks of fasting ghrelin1 (*ghrl1)* declined.

Conclusively, among the appetite regulating genes that was analyzed both in short-term and long-term fasting *agrp1*, *npya1*, *cart2a* & *2b*, *pomca1* & *a2*, *mc4ra2* and *ghrl1* responded to fasting. At short-term fasting hypothalamic *agrp1* acted in an orexigenic

role and *pomca2* showed a trend of an anorexigenic role. Whereas in long-term fasting, the *agrp1* and *npya1* responded as orexigenic in action while *cart2a*, *cart2b*, *pomca1* and *pomca2* upregulation demonstrates that the neuropeptides might play either a vital role in appetite regulation i.e., fasting may be inducing shutting down of hunger and/or counteracting on hunger signals (*agrp1* and *npya1*) to save energy from foraging search activity in catabolic conditions or play a fasting induced stress response. It is postulated that the decline in *ghrl1* mRNA expression under catabolic conditions might return as hunger signal once the food is available in the vicinity. Taken together the data reported in this study suggest a*grp1* as a potential appetite biomarker gene though the temporal dynamics of the expression in relation to a meal is complicated and need to be investigated further.

Sammendrag

Atlantisk laks (*Salmo salar* L.) er en nøkkelart i norsk sjømateksport, og representerer 70 – 80 Mrd NOK i verdi. Den store økningen i produksjon kombinert med overfôring har bidratt til negative effekter på det marine økosystemet. Av den grunn er effektiv utnyttelse av fiskefôret avgjørende for å sikre en bærekraftig produksjon med hensyn til miljø, fiskevelferd og produksjonskostnader (>50 % av produksjonskostnadene er til fôr). En økt forståelse av de grunnleggende biologiske mekanismene som styrer sult, metthetsfølelse, og kontroll av fôropptaket, vil gi mulighet til å forbedre dagens fôringsprotokoller og derav sikre økt fôrutnyttelse. Dette inkluderer kunnskap omkring de enkelte nevroendokrine faktorer som modulerer appetitt og fôringsatferd, samt å identifisere gode biomarkører som kan brukes til å vurdere appetitt og mulig vekst hos fisk.

Hos virveldyr styres matinntaket av et samspill mellom sentrale og perifere signaler som inkluderer afferente nevroner og sirkulerende faktorer som hormoner i kombinasjon med signaler fra lukt- og syn og som bidrar til å stimulere fôrinntak i forhold til dyrets ernæringsstatus. Hypothalamus har en sentral rolle i å regulere appetitt og fôringsatferd. Hos pattedyr er det beskrevet et hypotalamisk nevronalt nettverk av store, distinkte cellepopulasjoner som uttrykker de oreksigene nevropeptidene (agouti-relatert protein (AgRP), nevropeptid Y (NPY)) og de anorexigene peptidene (proopiomelanocortin (POMC), kokain- og amfetaminregulert transkripsjon (CART)). Oreksigene peptider stimulerer appetitt og sult, mens anorexigene peptider stimulerer metthet, og inhiberer sultfølelse. Disse melanocortin-nevropeptidene virker på melanokortin-4-reseptoren (MC4R) i høyere ordens nevroner og som kontrollerer både matinntak og energiforbruk. Melanocortin systemet ser ut til å være relativt godt bevart blant virveldyr, inkludert teleoster. Hos atlantisk laks som har vært gjennom en laksefisk-spesifikk fjerde runde med hel genom duplikasjon (Ss 4WGD) vil de fleste av disse genene ha flere paraloge gener som kan ha ulike funksjoner for hver genvariant.

Med dette som utgangspunkt har denne PhD-studien fokusert på følgende problemstillinger;
1. Å oppdatere dagens kunnskap bilde omkring tilstedeværelse av ulike sentrale appetittgener, 2. Å gjennomføre en distribusjonsanalyse av sentrale appetittgen paraloger i hjerneregionene bulbus olfactorius (OB), telencephalon (TEL), midthjernen (MB), cerebellum (CE), hypothalamus (HYP), saccus vasculosus (SV), hypofyse (PT) og hjernestamme (BS). Dette inkluderte også en vurdering av de ulike variantenes rolle i å kontrollere appetitten ved korttidsfaste (3 eller 4 dager) hos laks. 3. Å kartlegge hvordan appetittregulerende gener i mage-hypothalamus aksen påvirkes av langtidsfaste (4 og 6 uker) hos postsmolt av atlantisk laks med utgangspunkt i fordeling og uttrykk av de ulike paraloger i ulike avsnitt i hjernen hos laks.

Innledningsvis ble det utført et '*in silico'* søk for å hente sentrale appetittgener med paralogene og isoformer fra GenBank og Ensembl. I tillegg ble et fylogenetiske trær etablert. Deretter ble en standard protokoll for å dissekere fiskehjerne i 8 regioner utviklet for å studere fordelingen av de ulike genparaloger i fiskehjernen. Analyser for revers transkriptase- kvantitativ polymerasekjedereaksjon (RT-qPCR) ble utviklet m.h.p. sekvenssammenligningene og kvantifisering av mRNA-ekspresjon av appetittgenene. Parallelt ble identiteten til de nylig identifiserte sekvensene bekreftet ved å klone qPCR produkter.

'In silico' analyser bekreftet tilstedeværelsen av 2 *agrp* (*agrp1* og *2* (også kjent som *asip2*)), 3 *npy* (*npya1, a2* og *b*), 3 *pomc* (*pomca1, a2* og *b*), 10 *cart* (*cart1a, 1b1, 1b2, 2a, 2b1, 2b2, 3a1, 3a2, 3b* og *4*), 4 *mc4r* (*mc4ra1, a2, b1 og b2*) og 2 *ghrl* (*ghrl1* og *2*) genparaloger i genomdatabasen for atlantisk laks. Studien rapporterer den første identifiseringen og karakteriseringen av genparaloger for *mc4r*, *npy*, *cart* og 'membrane-bound O-acyltransferase domain containing 4' (*mboat4*) (Eng).

Analyser av fordelingen av de analyserte genene i ulike regioner i hjernen viste et bredt distribusjonsmønster med varierende områder for alle de analyserte gener. HYP, TEL, OB, MB, PT og BS som viste et høyere mRNA-uttrykk blant de 8 hjerneregionene. *agrp1, pomca1* og *pomca2* var sterkt uttrykt i HYP, mens uttrykk av *agrp2* var høyt i TEL og *pomcb* i PT. *npya1* (mest rikelig blant *npy*) og *npyb* ble sterkt uttrykt i TEL og

npya2 i HYP. Den mest uttrykte *cart* paralogen var *cart2b*, etterfulgt av *cart3b, 3a, 1b, 2a, 4* og *cart1a*. *cart2b* var høyt uttrykt i OB og TEL, *cart3b* i BS og MB, *cart3a* i TEL, MB og BS, *cart2a* ble hovedsakelig uttrykt i MB og HYP, mens *cart4* ble uttrykt i HYP og TEL. *cart1a* og *1b* var hadde størst uttrykk i MB. *agrp1, pomca1, pomca2, cart2a*, *2b, 3a, 3b, 4* og *npya2* viste et betydelig uttrykk i HYP i motsetning til de andre nevropeptidene. *mc4rb1* var den høyest uttrykte *mc4r*-paralogen, og *mc4ra1, a2* og *b1* viste høyere uttrykk i HYP og TEL, mens *mc4rb2* var høyt uttrykt i HYP.

I forsøket med kortvarig (3 dagers) faste ble det registrert en signifikant oppregulering av transkripsjonsnivået i hypothalamus *agrp1* i den fastede gruppen. Dessuten var mRNA uttrykket av *agrp1* signifikant negativt korrelert med tørrvektsinnholdet i magen. Korrelasjonen mellom magefylling og *agrp1* mRNA-uttrykket indikerer en sammenheng mellom magefylling og metthetssignaler. Dataene indikerer videre at hypothalamus *agrp1* fungerer som et oreksigent signal hos laks. Etter 4 dagers Faste viste resultatene en signifikant reduksjon av *npya1* mRNA uttrykket i OB, økt *npya2* mRNA ekspresjon i midthjernen, og det ble også observert en trend med økt *npya2* mRNA uttrykk i hypothalamus. Blant *cart*-paralogene, ble kun *cart2b* oppregulert etter fire dagers faste i OB, MB og HYP sammenlignet med gruppen som ble fôret.

I forsøket hvor postsmolt laks ble fastet over en lengre periode (opp til 6 uker) viste resultatene at *K* faktoren avtok i Faste gruppen sammenlignet med Fôret gruppe. qPCR-analyse viste at mRNA uttrykket av *agrp1* i hypothalamus ble signifikant oppregulert etter 4 og 6 ukers faste. Blant *npy*-paralogene *npya1* og *a2* var bare *npya1*-nivået signifikant oppregulert etter 4 ukers faste. Mens det blant *cart* paralogene ble registrert ett *cart2a* mRNA ekspresjonsnivå som var signifikant høyt etter 4 ukers faste. I tillegg økte *cart2b*-nivået statistisk i etter 4 og 6 ukers faste, mens *cart3a* og *cart4* ikke responderte på faste. Paralogene p*omca1, a2* og *mc4ra2* økte signifikant etter 6 ukers faste og i magevev (etter 6 uker med faste) var det en tendens til at ghrelin1 (*ghrl1*) nivå ble redusert.

Oppsummert m.h.p. de appetittregulerende genene som ble analysert så responderte *agrp1, npya1, cart2a* og *2b, pomca1* og *a2, mc4ra2* og *ghrl1* på matmangel både etter kortvarig og langvarig sult. Ved kortvarig faste spilte *agrp1* i hypothalamus en

tilsynelatende oreksigen rolle, og *pomca2* viste en trend som tilsier en anoreksigen rolle. Under langtidsfaste, reagerte *agrp1* og *npya1* etter et oreksigent mønster, mens *cart2a, cart2b, pomca1* og *pomca2* ble oppregulert. Dette tyder på at disse nevropeptidene deltar aktivt i appetittreguleringen, dvs. faste kan indusere en nedstengning av sult og/eller motvirke sultsignaler (*agrp1* og *npya1*) for å spare energi ved å redusere søkeatferd, eks. svømmeaktivitet under katabolske forhold der mat ikke er tilgjengelig. Alternativt kan disse nevropeptidene være en del av en sultindusert stressrespons. Basert på dataene kan en spekulere i at nedgangen i *ghrl1* mRNA-uttrykk i magesekken under katabolske forhold forårsaket av matmangel, kan komme tilbake som sultsignal når mat igjen blir tilgjengelig. Samlet antyder dataene at *agrp1* er en potensiell appetittbiomarkør, selv om den tidsmessige dynamikken til uttrykket i forhold til et måltid er komplisert og må undersøkes nærmere.

Abbreviations

Abbreviation	Description
AA	amino acid
actb	Actin beta/beta actin
AgRP	agouti-related protein
Amg	anterior midgut
ARC	arcuate nucleus
Asip2	agouti signaling peptide 2
BS	brainstem
BW	brackish water
CART	cocaine and amphetamine-regulated transcript
CCK	Cholecystokinin
cDNA	complement deoxyribonucleic acid
CE	cerebellum
c-fos	cellular oncogene fos
CNS	central nervous system
Cq	quantitation cycle
Crh/Crf	cortocotropin releasing hormone/ cortocotropin releasing factor
Cys	cysteine
DEG	Differentially Expressed Gene analysis
DNA	deoxyribonucleic acid
E	efficiency
ECL	extracellular loop
FW	fresh water
Gb	gallbladder
Ghrl	ghrelin
GHSR	growth hormone secretagogue receptors
GI	gastrointestinal
GLP-1	Glucagon-like-peptide-1
GOAT	Ghrelin O-acyltransferase
GPCRs	G protein coupled receptors
Hg	hindgut
HPI	hypothalamus-pituitary-interrenal
HSI	hepatosomatic index
HYP	hypothalamus
ICL	intracellular loop
ILAB	industrial lab
IMR	Institute of marine research.
ISH	*In situ* hybridization
JTT	Jones-Taylor-Thornton
K	condition factor
LDN (SNP)	simulated natural photoperiod
LL	continuous light

LEP	leptin
Li	liver
MB	midbrain
Mboat4	Membrane Bound O-Acyltransferase Domain Containing 4
MC4R	melanocortin-4 receptor
Mg	midgut
ML	Maximum Likelihood
mRNA	messenger Ribonucleic acid
MS222	Tricaine Methanesulfonate
NPY	neuropeptide Y
OB	olfactory bulb
Ob	obese gene
Pc	pyloric caeca
POA	preoptic area
POMC	proopiomelanocortin
PT	pituitary
PVN	paraventricular nucleus
PYY	Peptide-YY
qPCR	quantitative polymerase chain reaction
RAS	recirculating aquaculture system
Ss 4RWGD	salmonid-specific fourth round whole-genome duplication
s20	ribosomal protein S20
St	stomach
SV	saccus vasculosus
TEL	telencephalon
TMH	transmembrane domains/helices
3R WGD	third round of whole genome duplication
UoB	university of Bergen
α-Msh	alfa-melanocyte stimulating hormone

List of Publications

Paper I

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

Paper II

Kalananthan, T., Murashita, K., Rønnestad, I., Ishigaki, M., Takahashi, K., Silva, M. S., Wakabayashi, Y., Lai, F., Shimizu, M., Nilsen, T. O., Pino Martinez, E. and Gomes, A. S. (2020). Hypothalamic *agrp* and *pomc* mRNA responses to gastrointestinal fullness and fasting in Atlantic salmon (*Salmo salar* L). *Front. Physiol.* 11:61. doi: 10.3389/fphys.2020.00061

Paper III

Kalananthan, T., Gomes, A. S., Lai, F., Tolås, I., Jordal, A.-E. O., Norland, S., Murashita, K. and Rønnestad, I. (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, 1–13. doi:10.3389/fmars.2021.763766.

Paper IV

Tolås, I., **Kalananthan, T.**, Gomes, A. S., Lai, F., Norland, S., Murashita, K. and Rønnestad, I. (2021). Regional Expression of *npy* mRNA Paralogs in the Brain of Atlantic Salmon (*Salmo salar* L .) and Response to Fasting. *Front. Physiol*. 12, 1–11. doi:10.3389/fphys.2021.720639.

Paper V

Kalananthan, T., Folkedal, O., Gomes, A. S., Lai, F., Handeland, S. O., Tolås, I., Gelebart, V. and Rønnestad, I. Impact of long-term fasting on the stomach-hypothalamic appetite regulating genes in Atlantic salmon postsmolts (Manuscript, submitted to Aquaculture).

Contents

1 INTRODUCTION

1.1 *General introduction*

The global demand for seafood is continuously increasing and Norway has been one of the leading countries producing and exporting sea food with Atlantic salmon (*Salmo salar*) as a key commercial species. Atlantic salmon represented an export value of ca. 70 and 80 billion NOK in 2020 and 2021 respectively (Norwegian Seafood Council., 2020, 2021). Norway's long coastline provides ideal conditions for the success of salmon aquaculture. However, the extensive expansion of this industry combined with frequent overfeeding has contributed to potentially negative effects on the ecosystem (Strain and Hargrave, 2005). As such, the efficient utilization of fish feed is vital to ensure sustainable farming with regards to the environment, fish welfare, and production costs. Feed is one of the main expenses in salmon industry (more than 50%, goes to fish feed) (Asche and Oglend, 2016) and there is thus both environmental and economic reasons to optimize feeding schedules to reduce feed conversion ratio and optimize growth. Thus, a clear understanding of the biological mechanisms that underly hunger and satiety, and thereby, control of feed intake can be of great benefit to the salmon industry. This includes a better understanding of the neuro-endocrine players that modulate feeding and the identification of reliable biomarkers that can be used to assess appetite and potentially growth in fish trials.

In vertebrates, the central control of appetite is regulated by the central nervous system and hypothalamus is the feeding center. Particularly, two major regions in the hypothalamus named arcuate nucleus (ARC) and paraventricular nucleus (PVN), with distinct neuronal populations and network involve in appetite regulation (Barsh and Schwartz, 2002; Hall, 2011; Timper and Brüning, 2017) (**Figure 1**). According to the nutritional status of the animal, the neurons from the arcuate nucleus receive the peripheral signals and release the neuropeptides which act on the receptors of second order neurons (PVN) either to induce or inhibit feeding (Nuzzaci et al., 2015). In mammals, at Fasting or hunger state ghrelin (GHRL) act as a hunger signal predominantly from stomach and activate the AgRP/NPY neurons in the ARC which

release AgRP/ NPY that act on the Melanocortin 4 receptors (MC4R) of second order neuron in PVN to activate feeding and act as an orexigenic pathway (Hall, 2011; Rønnestad et al., 2017; Timper and Brüning, 2017) (**Figure 1**). On the other hand, as basal activity at Fed or satiety state, the peripheral signals like insulin, leptin (Lep), peptide-YY (PYY), glucagon-like-peptide-1 (GLP-1) and cholecystokinin (CCK) inhibit AgRP/NPY neurons and activate the POMC/CART neurons in the ARC which in turn generate POMC. POMC is post-translationally cleaved into α-melanocyte stimulating hormone (α-MSH) and released to activate the MC4R in the PVN. This pathway, which reduces or inhibits the food intake and enhance the energy expenditure represents an anorexigenic pathway. This system seems to be relatively well-conserved among vertebrates, including teleost species. There are large variations in regulation of appetite and feeding among species and within the species due to vast diversity in fish species depending on their ecological niches and aquatic habitat as well as life history adaptations, transitions between life stages, energy requirement, feeding behaviors, anatomy and physiology of gastrointestinal system (reviewed in Volkoff, 2016; Rønnestad et al., 2017). Therefore, the central mechanism regulating appetite also vary among fish species in response to feed deprivation.

In Atlantic salmon the salmonid-specific fourth round whole-genome duplication (Ss 4R WGD) led to the presence of several paralog genes which may have resulted in divergent functions of the duplicated genes. Most of the previous studies on appetite controlling systems in the species have analyzed gene expression on whole brain (Rønnestad et al., 2017). It is virtually unknown which areas of the Atlantic salmon brain are involved in the regulation of feed intake and appetite. Also, the receptor systems and interactions are unknown. This is also important information for applying the correct sampling and dissection strategies when exploring temporal differences in the genes involved in appetite control.

In nature, long-term food deprivation in salmonids is a common phenomenon during the anadromous migration to fresh water for spawning and over wintering (Bower et al., 2009; Rønnestad et al., 2017) while in cultured Atlantic salmon cessation of feeding (loss of appetite) occur due to suboptimal environmental conditions like infectious

diseases (McVicar, 1987), seasonal thermal fluctuations, and hypoxia (Wade et al., 2019). Short-term feed restrictions are also common prior to aquaculture practices like handling for vaccination, transportation, and harvest (Waagbø et al., 2017). In Atlantic salmon the response of appetite regulators to short-term and long-term fasting in the gut-brain axis is not well explored.

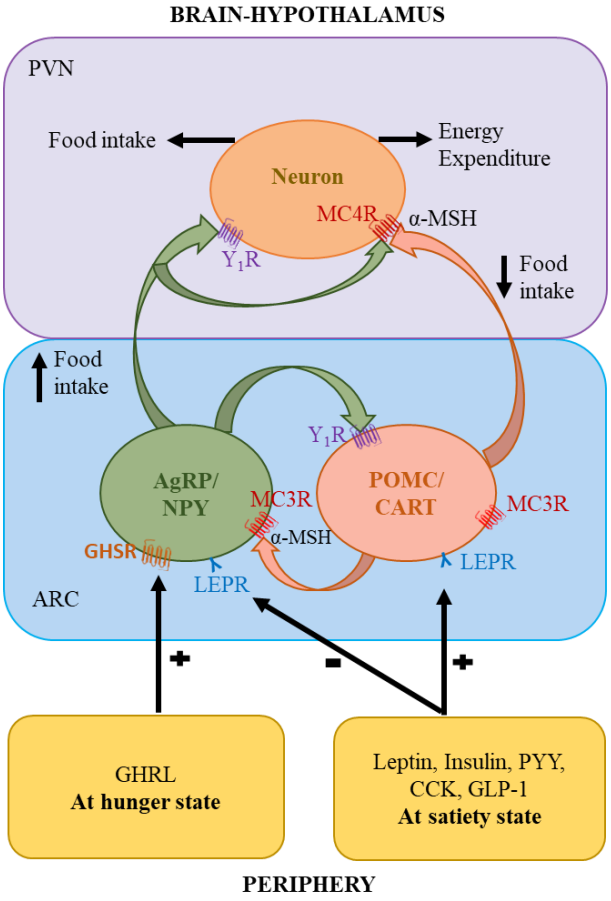

Figure 1. A simplified neuronal network showing interaction between hypothalamus and periphery in appetite regulation in mammals where ARC - arcuate nucleus, PVN - paraventricular nucleus, AgRP - agouti-related protein, NPY - neuropeptide Y, POMC - proopiomelanocortin, α-MSH- melanocyte stimulating hormone and CART - cocaine and amphetamine-regulated transcript. **(+)** activation and **(-)** inhibition and different transmembrane receptors shown in different colors: MC4R & MC3R - melanocortin receptors 4 and 3, LEPR - leptin receptor, Y_1R - neuropeptide Y_1 receptor and GHSR - growth hormone secretagogue receptor. PYY - peptide-YY, GLP-1 - glucagon-like-peptide-1 and CCK - cholecystokinin. The illustration is modified from (Barsh and Schwartz, 2002; Hall, 2011).

1.2 *Key appetite regulators in fish*

In vertebrates, food intake is controlled by the synergic actions of central and peripheral signals by afferent neurons and circulation in combination with olfactory and visual sensation, which stimulate ingestion in relation to the nutritional status of the animal. Previous studies in most model species including mammals and most fish have investigated *agrp*, *npy*, *cart*, *pomc* and *mc4r* as central and *ghrl, glp-1*, *cck*, *pyy*, *leptin and insulin* as peripheral key appetite regulators. The presence of multiple paralogs in Atlantic salmon indicating broad functionality which likely facilitates migration and allows the salmon to maintain its anadromous life history (Warren et al., 2014; Lien et al., 2016), it also increases the complexity in sorting out the roles of the different paralogs for each gene. Unraveling the knowledge on the key appetite regulator genes with their paralogs signaling in the periphery-central axis particularly from gastrointestinal (GI) system to brain enable to understand the basic mechanism of appetite and feed intake in Atlantic salmon.

1.2.1 Key appetite regulators in brain

1.2.1.1 Agouti-related protein

In teleosts the Agrp-mediated action on food intake seems to be conserved as seen in mammals. Two agouti-related protein (Agrp) paralogous genes (*agrp1* and *agrp2*) have been identified in common carp (*Cyprinus carpio*) (Wan et al., 2012), sea bass (*Dicentrarchus labrax*) (Agulleiro et al., 2014), zebrafish (*Danio rerio*) (Shainer et al., 2017), torafugu (*Takifugu rubripes*) (Kurokawa et al., 2006), including Atlantic salmon (Murashita et al., 2009a). The orexigenic role of hypothalamic AgRP appears to be conserved in some of the teleost species studied, such as goldfish (*Carassius auratus*) (Cerdá-Reverter and Peter, 2003), zebrafish (Song et al., 2003; Song and Cone, 2007; Shainer et al., 2019), coho salmon (*Oncorhynchus kisutch*) (Kim et al., 2015), common carp (Zhong et al., 2013) and seabream (*Sparus aurata*) (Koch et al., 2019). However, in Atlantic salmon, previous results indicated that *agrp1* may have an anorexigenic effect based on analyses of the whole brain mRNA expression after 6 days of fasting, while *agrp2* had no effect on the control of appetite (Murashita et al., 2009a). The Agrp2 has been demonstrated as non-appetite regulator depending on the spatial

expression in zebrafish. The pre-optic Agrp2 is a neuroendocrine regulator in stress axis that reduces cortisol secretion in zebrafish (Shainer et al., 2019), whereas *agrp2* from pineal gland showed a possible neuroendocrine role in the regulation of background adaptation (Shainer et al., 2017).

1.2.1.2 Neuropeptide Y

NPY has been reported to be the most potent orexigenic factor in mammals (Mercer et al., 2011) and NPY's functional role as a regulator of energy homeostasis and appetite control is conserved across vertebrates, including some observations in teleosts (Volkoff et al., 2005; Rønnestad et al., 2017). Diversification in the anatomical, physiological and environmental adaptations caused the species-specific appetite regulation mechanism. Studies report the orexigenic role of Npy in several species such as goldfish (Narnaware et al., 2000), grass carp (*Ctenopharyngodon idellus*) (Zhou et al., 2013), zebrafish (Yokobori et al., 2012), rainbow trout (*Oncorhynchus mykiss*) (Aldegunde and Mancebo, 2006), chinook (*Oncorhynchus tshawytscha*) and coho salmon (Silverstein et al., 1998). In Nile tilapia (*Oreochromis niloticus*), evidence of duplicated *npy* gene paralogs *a* and *b* have also been reported (Yan et al., 2017). Previous studies in teleost identified high levels of *npy* in different regions of brain, suggesting that other parts of the brain also involve in appetite regulation or Npy have other functions (Le et al., 2016; Kaniganti et al., 2021).

1.2.1.3 Cocaine and amphetamine-regulated transcript

The name CART derived from a transcript that was found to be released in response to the psychomotor stimulants cocaine and amphetamine in rat brain (Douglass et al., 1995). The mammalian CART is known to be an anorectic peptide controlling feed intake in accordance with energy balance (Kristensen et al., 1998; Yang et al., 2005; Rogge et al., 2008). CART is also involved in other functions as stress response, reward, and as previously mentioned it also responds to drug addiction/abuse (Koylu et al., 2006). Several studies in teleost investigated the *cart* gene's involvement in appetite regulation, and multiple *cart* genes have been identified in species such as goldfish (Volkoff and Peter, 2001), Ya-fish (*Schizothorax prenanti*) (Yuan et al., 2015a), zebrafish (Nishio et al., 2012; Akash et al., 2014; Ahi et al., 2019), yellowtail

(*Seriola quinqueradiata*) (Fukada et al., 2021), medaka (*Oryzias latipes*) (Murashita and Kurokawa, 2011), and Senegalese sole (*Solea senegalensis*) (Bonacic et al., 2015). The differential expression patterns of *cart* genes in brain regions have also been described in these species, with species specific role in appetite regulation and other physiological functions.

1.2.1.4 Proopiomelanocortin

POMC is a precursor peptide which is at post-transcriptional stage cleaved into melanocyte-stimulating hormones (α-, β- and γ-MSH) and adrenocorticotropic hormone (ACTH) (Castro and Morrison, 1997; Ghamari-Langroudi et al., 2011), and it is believed to be involved in a wide range of physiological functions including energy homeostasis in the anorexigenic pathway in mammals (Cone, 1999). Three *pomc* paralogous genes (*pomca1*, *pomca2*, and *pomcb*) and one splice variant (*pomca2s*) have been previously identified and characterized in Atlantic salmon, (Murashita et al., 2011) and in rainbow trout (Leder and Silverstein, 2006). Though MSHs serve a role in appetite control in mammals (Millington, 2007), their function in appetite control in teleost remains ambiguous. For instance, fasting did not change *pomc* expression in goldfish (Cerdá-Reverter and Peter, 2003), zebrafish (Song et al., 2003) while intracerebroventricular administration of α-MSH showed an anorexigenic effect in goldfish. Inconsistent response to fasting in salmonids are also reported with respect to duration of fasting. In rainbow trout a decrease in *pomca1* (but not *pomca2* or *pomcb*) with 28 days of fasting (Leder and Silverstein, 2006) and increase in *pomca1* and *b* after 4 months fasting in the hypothalamus (Jørgensen et al., 2016), as well as no change after 7 days of fasting in Arctic charr (*Salvelinus alpinus*) (Striberny and Jørgensen, 2017) were reported.

1.2.1.5 Melanocortin-4 receptor

MC4R belongs to G protein coupled receptors (GPCRs) family A with seven transmembrane loops connected by alternating extracellular and intracellular loops (Tao, 2010). In mammals, MC4R plays important roles in regulating multiple physiological processes including energy homeostasis, reproduction and sexual function (Zhang et al., 2019a). In energy homeostasis, α- MSH inhibits feeding

primarily by activating MC4R (Cone, 1999) and structural conservation of α-MSH during vertebrate evolution suggests preserved functions throughout the evolutionary process (Cerdá-Reverter et al., 2003). Mc4r is involved in the control of energy balance in a few fish species in the same manner as in mammals (Schjolden et al., 2009). In teleost, the role of Mc4r receptor in relation to energy balance was explored in salmonids rainbow trout (Schjolden et al., 2009; Jørgensen et al., 2016) and Arctic charr (Striberny and Jørgensen, 2017), common carp (Wan et al., 2012), spotted sea bass (*Lateolabrax maculatus*) (Zhang et al., 2019a), goldfish (Cerdá-Reverter et al., 2003), Mexican cave fish (*Astyanax mexicanus*) (Aspirasa et al., 2015), barfin flounder (*Verasper moseri*) (Kobayashi et al., 2008). The functional role of Mc4r, based on expression studies (mRNA expression by qPCR or *in situ*, and central administration of agonist and antagonists of the Mc4r) in energy balance seems to vary among these species depending on their environment and life history.

1.2.2 Key appetite regulators in the periphery

1.2.2.1 Ghrelin

GHRL produced and released from the GI tract is involved in metabolic functions like stimulation of growth hormone release, gut motility, food intake and energy homeostasis (Stengel and Taché, 2012; Tine et al., 2016). Murashita et al. (2009b) have identified and characterized two *ghrl* paralogs *ghrl1* and *ghlr2* in Atlantic salmon. In the previous studies in the same species, high mRNA level of *ghrl* was observed particularly in stomach among GI tract tissues (Murashita et al., 2009b; Del Vecchio et al., 2021). Ghrl has been reported to have an orexigenic action in mammals (Date et al., 2001) which has mainly been consistent with findings in several fish species, including goldfish (Matsuda et al., 2006), tilapia (*Oreochromis mossambicus*) (Riley et al., 2005), brown trout (*Salmo trutta*) (Tinoco et al., 2014), gilthead sea bream (Perelló-Amorós et al., 2019) and grass carp (Yuan et al., 2015b). Salmerón et al. (2015) demonstrated in rainbow trout that Ghrl appears to stimulate synthesis of triglycerides as well as their mobilization and act as enhancer of lipid turn-over. However, discrepancies in the mRNA level between *ghrl* paralogs in response to feed

availability (at varying time frame) have been reported in salmonids (Murashita et al., 2009b; Frøiland et al., 2010; Hevrøy et al., 2011).

1.2.2.2 Membrane-bound O-acyltransferase domain-containing 4

Ghrelin O-acyltransferase (GOAT) or membrane-bound O-acyltransferase domain-containing 4 (MBOAT4) is a critical enzyme that modifies (*n-acyl* modification=acylates) GHRL to enable the action on growth hormone secretagogue receptors (GHSR) in ARC (Kojima et al., 1999; Yang et al., 2008; Shlimun and Unniappan, 2011). *Goat* mRNA expression was detected primarily in mouse stomach and intestine, while other enzymes of MBOAT family were found in several other tissues in addition to the gastrointestinal tract (Shlimun and Unniappan, 2011). Studies in mammals reported that chronic food deprivation resulted increase in *GHRL* mRNA expression in the gastric mucosa and a corresponding increase in *GOAT* mRNA in the same tissue (González et al., 2008). Only few studies in teleost, investigated and characterized *goat* including goldfish (2 variants) (Blanco et al., 2017) and zebrafish (Hatef et al., 2015).

1.2.2.3 Glucagon-like-peptide-1

GLP-1, an anorexigenic intestinal peptide, belongs to the family of glucagon-like peptides encoded by preproglucagon gene (Bertucci et al., 2019; Xie et al., 2022). In mammals the presence of food in the intestines stimulate enteroendocrine cells to secrete GLP-1, and this hormone considered as one of the GI peptides that regulate the quantity of food intake through the short-term regulation (Hall, 2011). The physiological function of Glp-1 in teleosts such as goldfish (Yeung et al., 2002), rainbow trout (Polakof et al., 2011), coho salmon (White et al., 2016) and grass carp (Xie et al., 2022) has been investigated. In grass carp, Xie et al. (2022) demonstrated that GLP-1 administration upregulated *cart* and downregulated *npy* and *agrp* expressions in hypothalamus 2h after injection pointing toward anorexigenic role in appetite regulation. Yeung et al. (2002) suggested that apart from reducing food intake, Glp-1 was involved in gastric emptying and played an important role in regulating liver glycogenolysis and gluconeogenesis in goldfish.

1.2.2.4 Cholecystokinin

CCK a member of the 'gut–brain' family of peptide hormones is secreted by the proximal intestine and characterized by evolutionary conserved biologically active C-terminal octapeptide (CCK-8) among vertebrates (Chandra and Liddle, 2007). The role of CCK in pancreatic secretion and gallbladder contraction while presence of digesta in the gut was already well established where it was also demonstrated that CCK reduced meal size in a dose-dependent manner in rats (reviewed in Murphy and Bloom, 2004). In mammals, CCK is one of the GI hormone that regulate the quantity of food intake through a short-term regulatory effect (Hall, 2011). In teleost, Cck exerts its biological actions by binding to two main types of specific GPCRs Cck-a receptor (Cck-1r) and Cck-b receptor (Cck-2r), which are primarily localized in the GI tract and in the brain (Kurokawa et al., 2003; Rathore et al., 2013). An anorexigenic role of Cck in teleost has been demonstrated in a wide range of species with an increase in postprandial circulating Cck levels in rainbow trout (Jönsson et al., 2006), yellowtail (pyloric caeca) (Murashita et al., 2007), and in goldfish acute administration of CCK resulted in the suppression of food intake (Himick and Peter, 1994). Two types of *cck* (-*l* and -*n*) were identified previously in Atlantic salmon and no significant differences in intestinal expression levels were observed in response to 6 days of fasting, however *cck-l/-n* increased in brain (Murashita et al., 2009b).

1.2.2.5 Peptide-YY

PYY is a peptide hormone belonging to the neuropeptide Y (NPY) family that consists of 36 amino acids and two isoforms of gene *pyya* and *b* (Sundström et al., 2008). In mammals, PYY is secreted in the distal intestine, and a meal particularly with high calories and fat stimulates maximal release of it (Hall, 2011). The mammalian model indicates that PYY suppresses appetite through the inhibition of NPY and subsequent activation of POMC neurons (Hall, 2011; Rønnestad et al., 2017). In the same model, food intake stimulates release of PYY, with blood concentrations rising to peak levels 1 to 2 hours after ingesting a meal as a short-term appetite regulation (Hall, 2011). PYY is expressed in both endocrine cells and enteric neurons (Ekblad and Sundler, 2002). In teleost, being predominantly expressed in the brain and GI-tract, *pyy* gene expression patterns are similar among the fish species, such as Japanese Flounder (*Paralichthys*

olivaceus) (Kurokawa and Suzuki, 2002) and yellowtail (Murashita et al., 2007). Flounder Pyy peptide showed a high identity to Pyy of sea bass and zebrafish (Kurokawa and Suzuki, 2002). Murashita et al. (2009b) have demonstrated spatial distribution in GI tract, where the highest level of mRNA expression of *pyy* was observed in the pyloric caeca and midgut and the level declined toward posterior gut in Atlantic salmon. Observations in teleost species red-bellied piranha (*Pygocentrus nattereri*) (Volkoff, 2014), grass carp (Chen et al., 2014) and yellowtail (Murashita et al., 2007) suggest that *pyy* response to fasting/feeding might be species-specific.

1.2.2.6 Leptin

LEP protein is a product of obese (*Ob*) gene. This gene was first identified in double mutant (*Ob*/*Ob*) mice associated with impaired metabolic functions (Zhang Y et al., 1994; Timper and Brüning, 2017). In mammals, the peptide hormone LEP is mainly released from adipose tissue as an adiposity signal to brain. LEP suppresses the activity of AgRP/NPY neurons and activate the POMC/CART neuron to inhibit feeding (Hall, 2011). LEP has been extensively investigated in both humans and murine models. In humans leptin has been one of the available biomarkers for satiety (De Graaf et al., 2004). Kurokawa et al. (2005) identified the first fish Lep in pufferfish (*Takifugu rubripes*) in 2005. In teleost, *lep* orthologs and several duplicated paralogs, originating from the 3R WGD events (reviewed in Rønnestad et al., 2017) as two *lep* hormone genes, *lepa* and *lepb* have been reported in species including zebrafish (Ahi et al., 2019), medaka (Kurokawa and Murashita, 2009) and mandarin fish (*Siniperca chuatsi* Basilewsky) (Yuan et al., 2020). Previous studies in few salmonids reported a single *lep* gene in response to fasting/feeding such as in rainbow trout (Kling et al., 2009), arctic charr (Frøiland et al., 2010) and in Atlantic salmon (Murashita et al., 2011). Lately, in the latter species 2 isoforms of *lepa1* and *a2* and a receptor *lepr* (Rønnestad et al., 2010), and two *lepb* genes *b1* and *b2* were identified (Angotzi et al., 2013). In Atlantic salmon, the tissue distribution varied widely among the *lep* paralogs. For *lepa1*, higher levels were found in brain, white muscle, liver and ovary, whereas *lepa2* generally had a lower level of mRNA expression than *lepa1,* except for the GI tract (stomach and mid-gut) and kidney (Rønnestad et al., 2010). On the other hand, expression of *lepb* genes was mainly found in gill and brain at all stages (Angotzi et

al., 2013). Further, Atlantic salmon reared on a rationed feeding regime (60% of satiation) for 10 months grew less than control (100%) and had lower *lepa1* mRNA expression in the fat-depositing visceral adipose tissue, while the expression in liver with a higher *lepa2* mRNA in the feed-rationed group (Rønnestad et al., 2010). In Atlantic salmon, the *lepa1* paralog provides the best correlation between energy status and mRNA expression in key tissues, supporting its role as an adiposity signal (Rønnestad et al., 2010).

1.2.2.7 Insulin

Insulin plays a vital role in energy and glucose homeostasis in mammals (Timper and Brüning, 2017) and purified insulin actually excites POMC neurons (Qiu et al., 2014). Disruption of insulin signaling from POMC neurons alone did not affect energy or glucose homeostasis (Könner et al., 2007), but deletion of both insulin and leptin receptors from POMC neurons deteriorates glucose homeostasis in mice (Hill et al., 2010). Any specific roles of insulin in regulation of food intake in fish is largely unknown, however, in goby complete iseletectomy resulted in hyperphagia (*Gillichthys mirabilis*) (Kelley and Grant, 1993) and in rainbow trout, intraperitoneal injections of insulin decreased food intake (Librán-Pérez et al., 2015) which suggests an anorexigenic role for insulin.

1.3 *Fasting/starvation in fish*

For commercial aquaculture industry, it is vital to develop a better feeding strategy with optimal feeding models that is economically sustainable, causing less environmental impact (by minimal use of fish feed and minimal waste) and less total operational costs with consideration to feed intake and fish growth, (Strain and Hargrave, 2005; Adaklı and Taşbozan, 2015). In recent years feed deprivation in connection to compensatory growth have been investigated in different studies on various species. To understand metabolic adaptations during fasted or starved conditions, studies have been performed in various fish species such as European sea bass (Pérez-Jiménez et al., 2007), common dentex (*Dentex dentex*) (Pérez-Jiménez et al., 2012), gilthead seabream (Metón et al., 2003), Siberian sturgeon (*Acipenser baerii*) (Ashouri et al., 2013), European Sea Bass

(Adaklı and Taşbozan, 2015), Arctic charr (Striberny and Jørgensen, 2017), rainbow trout (Jørgensen et al., 2016), including Atlantic salmon (Lie and Huse, 1992; Einen et al., 1998; Krogdahl and Bakke-McKellep, 2005; Waagbø et al., 2017). Fasting dependent metabolic adjustments differ from species to species in connection to various factors, such as fish age, reserves availability, past nutritional history (Pérez-Jiménez et al., 2007). Short or long-term fasting stimulates the genes encoding both orexigenic and anorexigenic neuropeptides and related receptors (Striberny and Jørgensen, 2017) in the central and peripheral system of fish while major energy stores like liver, adipose tissue, white muscle undergo mobilization corresponding to energy homeostasis (De Pedro et al., 2003; Pérez-Jiménez et al., 2012). Bertucci and co-workers (2019) reviewed in detail and summarized in a table how the teleost response to fasting in terms of appetite regulator genes. However, the regular update in genome data bases with changes in number of gene paralogs for the appetite regulators in teleost makes it difficult to compare the corresponding gene/homolog among species.

In the aquaculture production of Atlantic salmon, the frequent use of high energy dry feeds resulted with large deposits of visceral fat as well as high fat content in the fillet (Lie and Huse, 1992). This is an undesired feature from the consumers point of view and introduction of fasting not only stimulate the mobilization and catabolism of lipids before slaughter (Lie and Huse, 1992) but also improve the quality of meat hygienically through complete evacuation of gut (Waagbø et al., 2017). Feed withdrawal has also been implemented prior to other aquaculture practices like handling, transportation and crowding and handling during vaccination. In nature, salmonids go through a long food deprivation period during the anadromous migration to fresh water for spawning and over wintering (Bower et al., 2009; Rønnestad et al., 2017) while in cultured Atlantic salmon cessation of feeding occur due to suboptimal environmental conditions like infectious diseases (McVicar, 1987), seasonal thermal fluctuations, and hypoxia (Wade et al., 2019).

To date, there are some studies that have reported effects of long-term food deprivation on Atlantic salmon body composition and shape, fillet 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), welfare, refeeding and compensatory growth (Hvas et al., 2022). However, not much is known on how the control mechanisms for appetite and the key players in the gut-brain axis are modulated in this species, and how the key players respond to short- and long-term feed restriction.

2 AIMS OF THE PHD STUDY

The overall objective of this study was to update the key appetite genes repertoire information, to study spatial distribution of key appetite gene paralogs in the brain regions including a first assessment of their role in controlling appetite in short-term fasting (3 or 4 days) in Atlantic salmon. Based on their spatial distribution and quantitative expression in different brain regions, the last focus was to explore the impact of long-term fasting on appetite regulating genes in the stomach-hypothalamic axis in Atlantic salmon postsmolts.

The major aims of this PhD study are subdivided into the followings:

- To identify and characterize known and novel appetite regulating neuropetides and the key receptor in melanocortin system in brain of Atlantic salmon postsmolts via *in silico* analyses.

- To establish and validate assays for real-time reverse transcriptase polymerase chain reaction (RT-PCR)/quantitative PCR (qPCR) analysis of key neuropeptides involved in appetite control.

- To develop and standardize a protocol for dissection of Atlantic salmon brain into 8 regions, olfactory bulb (OB), telencephalon (TEL), midbrain (MB), cerebellum (CE), hypothalamus (HYP), saccus vasculosus (SV), pituitary (PT) and brainstem (BS).

- To investigate the spatial distribution of appetite genes in the 8 brain regions.

- To assess the role of the key central appetite regulators in controlling appetite in short-term fasting (3 or 4 days) in Atlantic salmon.

- To evaluate the impact of long-term fasting (4 and 6 weeks) on stomach-brain axis appetite regulating (selected) genes in Atlantic salmon postsmolts.

3 METHODOLOGICAL APPROACH

3.1 *Experimental conditions and fish*

In experiment 1, Atlantic salmon post smolts were obtained from Engesund fish farm (Fitjar, Norway) and randomly distributed into two brackish water 2,000 L tanks (48 fish per tank) at 10°C and continuous light at the Industrial Lab (ILAB) in Bergen (Norway) (Table 1). Fish were fed continuously with commercial dry feed pellets (Biomar intro 75 HH 50 mg Q) using an automatic feeder. After 3 weeks of acclimatization, tanks were assigned to Fed and Fasted groups while continued feeding only to the Fed group. After Fasted group was fasted for 4 days, 26 to 27 fish from each group were sampled.

In experiment 2, Atlantic salmon postsmolts from Bremnes Seashore's RAS facility (Trovåg, Norway) were randomly assigned to tanks (5 fish/tank) into six freshwater indoor tanks (150 L) with water temperature at 8.5°C and continuous day light was used to mimic the standard commercial procedures and to stimulate optimal growth (**Table 1**). All the tanks were fed daily ad libitum from 9:00 to 16:00 with commercial dry fish pellets (Biomar 3 mm) using automatic fish feeders. After 18 days of acclimatization, fish were divided into two groups that were either Fed (sampled 2 h after feeding) or Fasted for 3 and 4 days. In total, seven fish/group were sampled (2 to 3 fish/tank).

In experiment 3, Atlantic salmon post smolts from Aquagen were maintained in six large indoor, open brackish water, flow-through tanks (⌀ = 3 m) at 12°C and under a natural simulated photoperiod at Institute of Marine Research, Matre Research Station, Norway. Each tank was supplied with 150 fish (**Table 1**). The post smolts were allowed to acclimatize to the conditions for one and a half months fed with commercial pellets (Skretting, Norway) in excess via automated feeders twice a day (9:00 to11:00 and 13:00 to 15:00). Then, the post smolts from three tanks were designated as control (Fed) and the other three as treatment (Fasted). At the 4 and 6 weeks of treatment total of 60 fish (5 fish/tank) were sampled from both control and treatment groups. The last

meal for the Fed group of each sampling was from 13:00 to 15:00 the previous day to sampling.

In all experiments, oxygen of water in tanks was maintained above 80%.

Table 1. Experimental conditions and Atlantic salmon postsmolts used in all experiments.

Paper	Experiment	Weight (g)	Length (cm)	Water temperature (^{o}C)	Water	Photoperiod	Fish from	Place
I	1	269.0 ± 65.6	28.5 ± 2.2	10.0	BW	LL	Engesund	ILAB
II	2	214.7 ± 61.7	26.8 ± 2.4	8.5	FW	LL	Bremnes	UoB
III	2	183.8 ± 35.9	25.4 ± 1.7	8.5	FW	LL	Bremnes	UoB
IV	2	183.8 ± 35.9	25.4 ± 1.7	8.5	FW	LL	Bremnes	UoB
V	3	1179.0 ± 11.0	45.6 ± 0.1	12.0	BW	LDN (SNP)	IMR	IMR, Matre

FW - fresh water, BW – brackish water, LL – continuous light, LDN (SNP)- simulated natural photoperiod of 60.9°N, UoB-University of Bergen, ILAB-industrial lab (Bergen), IMR-Institute of Marine Research.

3.2 *General methodological approach*

The general workflow of the methodological approach used for all papers is given in **Figure 2.** To gain knowledge key appetite regulators in Atlantic salmon, the *in silico* analysis for the presence of multiple paralogs and/or isoforms of appetite regulating genes was performed using the GenBank (https://www.ncbi.nlm.nih.gov/) and Ensembl (https://www.ensembl.org/index.html) (publicly available from 2018 to 2020) in parallel to carrying out experiments and samplings. To update the knowledge on the key appetite regulators in Atlantic salmon based on the *in silico* analysis, protein structure and phylogenetic trees were made to the novel genes. Primers for each key genes were designed to differentiate the paralogs/isoforms (whenever possible). For the quantification of mRNA expressions of the genes the qPCR amplicons were cloned into plasmid standards to be used in qPCR analysis (**for paper I** purified qPCR products were used to make standards). Involvement of different brain regions of Atlantic salmon smolt in controlling appetite and feed intake under Fed and Fasted

experimental conditions was investigated parallelly, using qPCR analysis through dissecting fish brain into 8 regions. For this study the protocols for RNA extraction, cDNA synthesis, and qPCR analyses were followed. The protocols and assays were validated according to the brain and stomach tissues of the fish.

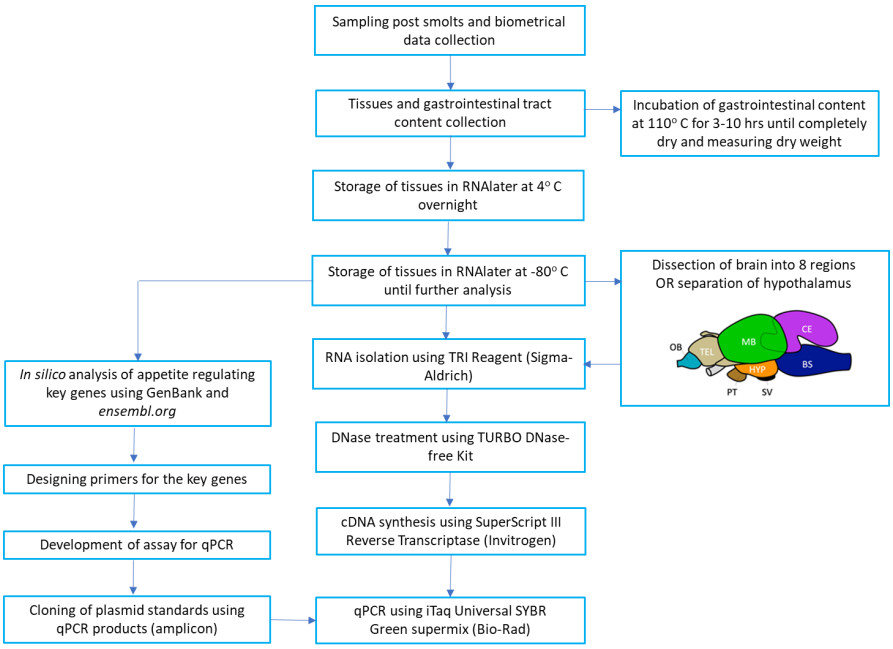

Figure 2. Flowchart showing the steps in the methodological approach used in general for all papers. Inserted illustration is lateral view of Atlantic salmon postsmolt brain for dissection of regions OB - olfactory bulb, TEL - telencephalon, MB - midbrain, CE - cerebellum, HYP - hypothalamus, SV - saccus vasculosus, PT - pituitary, BS - brainstem (Illustration of brain by Prof. Ivar Rønnestad).

3.2.1 Sampling the GI system

During sampling, fish from the Fed and the Fasted group were collected and euthanized using an overdose of 200 mg/L of MS222 (Tricaine methanesulfonate, Scan-Vacc, Hvam, Norway). Length and weight of each fish was recorded for morphometric analyses like condition (*K*) factor (**Paper I - V**). The GI tract was dissected and carefully divided into three compartments (**Figure 3A-D**): stomach (ST), midgut (MG), and hindgut (HG), using surgical clamps to avoid loss or transfer of content

between compartments. Then, each segment was emptied of food and digesta by gently stroking the content out onto pre-weighed pieces of aluminium foil/vials. The weight of contents in each segment was first measured on a wet weight basis, thereafter, the dry weight was obtained by incubating in an oven at 110°C for at least 3 h, until it was completely dried. After removing the digesta from the stomach, the tissue sample for RNA was collected from the triangle ('U' region of stomach), rinsed in PBS (phosphate buffered saline) and transferred immediately to liquid nitrogen (**Figure 3D**). Liver weight was taken for hepatosomatic index (HSI) (**Paper V**).

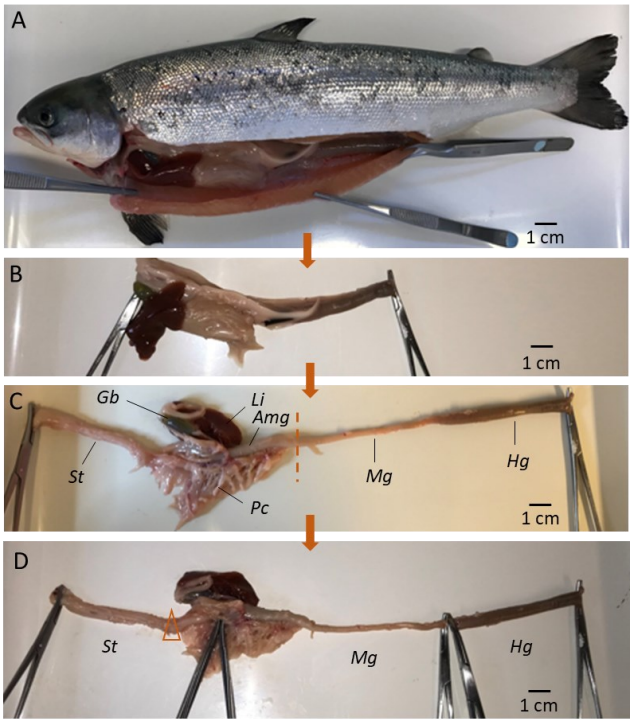

Figure 3. Protocol for the collection of gut content and stomach tissue for gene expression analyses. A. Initial cut opening of Atlantic salmon postsmolt exposing the GI tract. **B**. Removal of GI tract using surgical clamps at both ends of the tract. **C**. Identification of the different organs/tissues of Atlantic salmon digestive system. Dashed line indicate separation of *Amg* and *Mg*. **D**. Preparation to collect digesta/gut contents from each GI tract compartments using surgical clamps. The triangle shows the stomach ('U' region) tissue sampled for gene expression analysis. *St* - stomach, *Gb* - gallbladder, *Li* - liver, *Amg* - anterior midgut, *Pc* - pyloric cecae, *Mg* - midgut and *Hg* - hindgut.

3.2.2 Sampling of brain

The brain samples were removed from the fish skull carefully as shown in the **Figure 4A & 4B** and placed in RNA*Later* immediately, samples were left at 4 °C overnight and stored at -80 °C until further analysis.

3.2.3 Updating structural and phylogenetic analysis of appetite regulator proteins

Putative Atlantic salmon appetite genes were retrieved from Ensembl and GenBank genome databases (from 2018 to 2020) (ICSASG_v2) and compared using ClustalX 2.1 (Larkin et al., 2007). **Paper I** (*mc4r* and *pomc*), **Paper III** (*cart*) and **IV** (*npy*) (Tolås et al., 2021) were updated with novel genes whichever available. The deduced amino acid (AA) sequences of Atlantic salmon Mc4r, Pomc, Cart and Npy and the homolog sequences from human (*Homo sapiens*) and other fish species, including the Callorhinchidae, elephant shark (*Callorhinchus milii*); Latimeriidae and Lepisosteidae, spotted gar (*Lepisosteus oculatus*) as species before the teleost specific whole genome duplication event (Ts WGD); two Cyprinidae, goldfish as species that went through a very recent 4R WGD and zebrafish which did not; Characidae, Mexican tetra/ blind cave fish; Clupeidae, Atlantic herring (*Clupea harengus*) as old teleost; Salmonidae species, rainbow trout; the Esocidae northern pike (*Esox lucius*) as a sister group of salmonids that diverged before the Ss 4R WGD; six Neoteleostei Gadidae Atlantic cod, Adrianichthyidae medaka, Gasterosteidae stickleback (*Gasterosteus aculeatus*), Cichlidae Nile tilapia (*Oreochromis niloticus*) and Scophthalmidae turbot (*Scophthalmus maximus*), Senegalese sole, and coelacanth (*Latimeria chalumnae*) were retrieved from Ensembl and GenBank genome databases. Based on this fish species were selected for the phylogenetic tree for **Paper I**, **III** and **IV.**

Paper I - Phylogenetic tree was constructed based on the predicted full-length Mc4r and Pomc peptide sequences of 17 fish species representatives and the human.

Paper III - Phylogenetic tree was constructed based on the predicted mature peptides of Cart sequences of 15 fish species representatives and the human.

Paper IV - Phylogenetic tree was constructed using the deduced amino acid sequences of the full-length Npy from 13 teleost species and the human (Tolås et al., 2021).

The peptide sequences were aligned using MUSCLE with the default parameters (UPGMA clustering method, Gap opening penalty −2.90, Gap extension 0.0) from MEGAX (Hall, 2013). The alignments were displayed in GeneDoc 2.7 (Nicholas et al., 1997) and percentages of sequence identity calculated. Putative signal peptides were predicted using PrediSi and proteolytic cleavage sites predicted using Neuropred. Phylogenetic analysis was performed using the predicted mature peptide sequences of Cart. Based on best-fit substitution model analysis in MEGAX, phylogenetic tree was constructed using Maximum Likelihood (ML) with a Jones-Taylor-Thornton (JTT) model (Jones et al., 1992; Kumar et al., 2018) with fixed Gamma distribution (+G) parameter with five rate categories and 500 bootstrap replicates. The tree was rooted to corresponding peptide for human appetite gene in each phylogenetic tree.

Tertiary protein structures of Atlantic salmon Mc4r were predicted using the IntFOLD5 (Mcguffin et al., 2019) and human MC4R structure with AgRP (PDB entry 2IQV) was retrieved from UniProt. Mc4r transmembrane domains/helices (TMHI-TMHVII), extracellular loops (ECL1-ECL3), and intracellular loops (ICL1- ICL3) were retrieved from UniProt3 database. Potential cleavage sites of Pomc precursor were acquired using UniProt and ProP 1.04 by using the full-length AA sequences. The Mc4r images were edited, and disulfide bonds were predicted by PyMOL Molecular Graphics System v 2.3.

Searches for Agrp in Atlantic salmon genomic database did not identify any novel paralogs in addition to the ones previously published by Murashita et al. (2009).

3.2.4 Dissection of Atlantic salmon brain

To ensure high RNA yield and quality, the brain was placed on a glass slide on an ice block during dissection under a zoom stereomicroscope (Olympus SZ51) and cleaned from blood vessels and membrane. The pineal gland was removed and not used in any analysis. The PT, OB, TEL, BS, CE, SV, HYP, and MB were separated in this order (**Figure 2**). The HYP region is encircled in blue (**Figure 4C**). The **Figures 4B** and **4C**

show how the brain regions are distinguished in original brain sample. For **paper I**, **III** and **IV** all 8 brain regions were used for analysis and for **paper II** and **V** only hypothalamus region was used in the analysis.

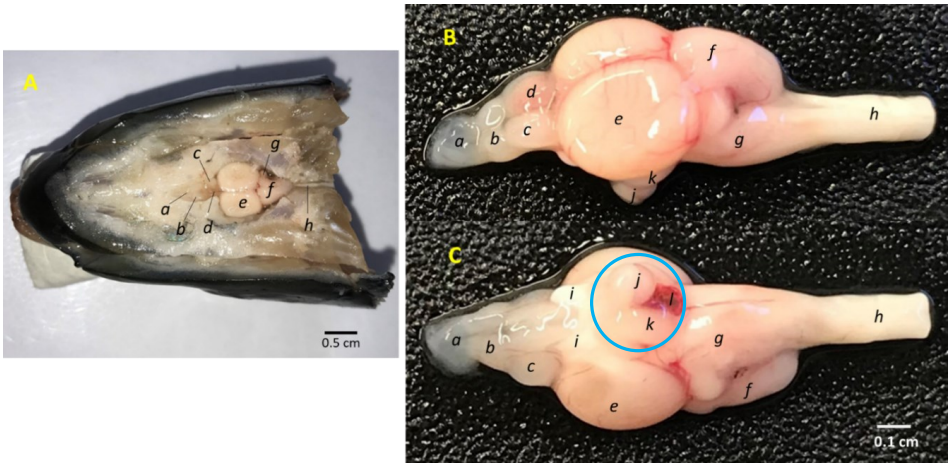

Figure 4 A. Upper view of Atlantic salmon postsmolt brain within the fish skull **B**. Upper view **C**. Bottom view of Atlantic salmon postsmolt brain. ***a*** - olfactory nerves, ***b*** - olfactory bulb (OB), ***c*** - telencephalon (TEL), ***d*** - pineal gland, ***e*** - optic lobe /part of midbrain (MB), ***f*** - cerebellum (CE), ***g*** - medulla oblongata / brainstem (BS), ***h*** - spinal cord, ***i*** - optic nerves, ***j*** - pituitary (PT), ***k*** - hypothalamus (HYP), ***l*** - saccus vasculosus (SV). The hypothalamus region is encircled in blue if saccus vasculosus and pituitary is removed.

3.2.5 RNA isolation and cDNA synthesis

Total RNA was isolated from each section of the brains using TRI Reagent (Sigma-Aldrich, MO, United States) following the manufacturer's protocol. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, United States) and a 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, CA, United States) were used to assess the quantity and the quality of the extracted total RNA, respectively. To avoid any remnants of genomic DNA, 5 to 10 µg of total RNA was treated with TURBO DNase-free Kit (Ambion Applied Biosystems, CA, United States) with 1 µl of DNase (2 Units/µl) in 10 to 30 µl reaction volume respectively. The amount of total RNA and the reaction volume for DNase treatment was adjusted depending on the amount of total RNA availability per region. For the brain regions first-strand cDNA was synthesized from 1.0 to 2 µg of the DNase treated total RNA sample using

SuperScript III Reverse Transcriptase (Invitrogen, CA, United States) and Oligo(dT)20 (50 µM) primers in a total reaction volume of 20 µl. For the stomach total RNA was isolated using TRI Reagent and 10 µg of total RNA was treated with TURBO DNase-free Kit followed by 2.75 µg of the DNase treated total RNA sample was used to synthesize the cDNA.

3.2.6 Primer design and assay development for qPCR

Primers were designed spanning exon–exon junctions when possible. All primers were analyzed for quantitation cycle (Cq), primers efficiency (E), and melting peaks. All qPCR products were analyzed in a 2% agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and cloned into a pCR4-TOPO vector (Thermo fisher, Scientific, Waltham, MA, United States). Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway), and their identity was confirmed using *blastn* analysis against the Atlantic salmon genome database.

3.2.7 Quantification of mRNA expression using qPCR

In **Paper I**, to quantify the absolute mRNA abundance for each gene, qPCR products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and used to generate a standard curve using a 10-fold dilution series (initial concentration 10^{10} number of copies). For **other papers**, wherever needed a standard curve dilution series (10 - fold) was generated from each target and reference genes (ribosomal protein *s20* and actin beta / beta actin) cloned into a pCR4-TOPO vector. The qPCR was carried out using 10 µl of iTaq Universal SYBR Green supermix (Bio-Rad, CA, United States), 0.6 µl of forward and 0.6 µl of reverse primers each (10 µM), 6.8 ultrapure water (Biochrom, Berlin, Germany), and 2 µl cDNA template (40, 50 or 60 ng/reaction for target gene and 10 or 12 ng/reaction for reference genes). All reactions were run in duplicate, and a non-template control, no-reverse transcriptase control, and a positive between plate controls were always included. The following qPCR protocol was performed: (1) 95°C for 30 s, (2) 95°C for 5 s, (3) 60°C for 25 s, (4) repeating steps 2 and 3 for 39 more times. Melting curve analysis over a range of 65°C – 95°C (increment of 0.5°C for 2 s) allowed the detection of non-specific products and/or primer dimers. The qPCR was performed using 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). Subsequently, the absolute mRNA expression level for each gene was determined based on the respective standard curve using the following equation:

$$Copy\ number = 10^{((Cq - intercept)/slope)}$$

In the **paper I and II** the copy number was normalized using the total ng of RNA used for each target gene whereas in the **papers III, IV and V** the copy number was normalized using the total ng of RNA used in the reaction for each target and reference genes. The ratio of the target gene copy number to the geometric mean copy number of reference genes was used in the plots and statistical analysis. For further details refer to corresponding papers.

4 GENERAL DISCUSSION

The ability to sense and respond to fluctuations in the environmental nutritional cues is a prerequisite for life (Efeyan et al., 2015). The sensing mechanisms regulate specific processes such as neuroendocrine hormone secretion, food intake, digestion and energy expenditure to maintain energy homeostasis (Roh et al., 2016; Rønnestad et al., 2017; Bertucci et al., 2019). The nutrient molecule binding to its sensor and the nutrient abundance is detected through signaling processes in both central and peripheral tissues (Efeyan et al., 2015). At the central level, the brain integrates metabolic information related to nutrient availability, through gut-derived hunger/satiety signals and hormones related to adiposity (Roh et al., 2016; Bertucci et al., 2019).

This PhD study primarily aimed to investigate the key appetite regulators in the gut-brain axis of Atlantic salmon where the study updated and developed assays for known and novel gene paralogs of key appetite regulators (Primers' sequences **Table 2**) for the mRNA expression in qPCR analyses. For the primer blast, the available public genome databases Genbank (ICSASG_v2) and Ensembl were used within the period of 2018 and 2020. Parallelly, a standard dissection protocol for studying spatial distribution of appetite genes in the Atlantic salmon brain was developed and established. Since there were many paralogs/isoforms retrieved for each of gut appetite regulators (**Figure 5**), and extensive work involved in the assay development and analysis of the appetite regulators from gut region, this PhD study aimed to explore the link between stomach and brain in appetite regulation in Atlantic salmon.

4.1 *In silico analysis of key appetite regulators*

The papers underlying this thesis are the first report for the identification and characterization of paralogs of three *npy* (*npya1*, *npya2*, and *npyb*), ten *cart* (*cart1a*, *1b1*, *1b2*, *2a*, *2b1*, *2b2*, *3a1*, *3a2*, *3b* and *4*) and 4 *mc4r* (*mc4ra1*, *a2*, *b1* & *b2*). Previously, two *agrp* (*agrp1* and *agrp2*) and three *pomc* (*pomca1, pomca2* and *pomcb* with one splice variant *pomca2s*) has been identified and characterized in other studies (Murashita et al., 2009a, 2011) in Atlantic salmon (**Figure 5**).

Table 2. The details of assay used for qPCR mRNA expression analysis in Atlantic salmon.

Gene	Ensembl Acc. No.	GenBank Acc. No.	Primer sequences (5'→3')	Size (bp)	Chromosome No.	
					Earlier	Now
agrp1	ENSSSAT00000108840.2	NM_001146677.1 XM_014182676.1 XM_014182677.1	F:ATGGTCATCTCAGTATTCCCAT R:AGAGAGCCTTTACCGATATCTG	152	Un.Sc	ssa11 N/A
agrp2 (known as asip2)	ENSSSAT00000080587.2	NM_001146678.1 XM_045714297.1	F:TGTTTCGCCGAAGACCTGAA R:GTTTCTGAAATGCAACGTGGTG	142	ssa03	ssa03
npya1	ENSSSAG00000040508	NM_001146681.1	F:GAACGCACAGCAGCAGAAAG R:AGGATGCATATTGACTTGAAGGTT	80	ssa14	ssa14
npya2	ENSSSAG00000015791	XM_014178359.1	F:CAGTCCAGGTATGATGAACCGT R:GGCACAGGAGTAACCTCTGG	195	ssa27	ssa27
cart1a	ENSSSAT00000013202.1 (ENSSSAT00000195581.1; ENSSSAT00000187452.1)	XM_014149393.1	F:GGCCAACTCCCACGACTTTC R:CATCAGCATCACACATGGGAACC	170	ssa16	ssa16
cart1b	ENSSSAT00000151185.1 (ENSSSAT00000181493.1; ENSSSAT00000205340.1)	XM_014150559.1	F:GGCCAGCATTCAACTGCTTT R:GGAACTCTAGAGCGCGAGTC	104	ssa16	ssa16
	ENSSSAT00000086533.1 (ENSSSAT00000194663.1)	XM_014151634.1			ssa17	ssa17
cart2a	ENSSSAT00000028631.1	XM_045688783.1	F:GGCAAAACTGCAGGGATTGG R:ACATAGGATGGACAGCAGCG	146	N/A	ssa10
cart2b	ENSSSAT00000074136.1 (ENSSSAT00000074124.2)	NM_001146680.1	F:GTGAGAGACTTCTACCCCAAAGA R:CGTAGGGACTTGGCCGAATT	135	ssa11	ssa11
	ENSSSAT00000019793.1 (ENSSSAT00000034744.2; ENSSSAT00000034654.2)	XM_014176449.1			ssa26	N/A
	ENSSSAT00000019780.1	XM_014183838.1			ssa26	ssa26
	ENSSSAT00000019807.1	XM_014188599.1			Un.Sc	N/A
cart3a	ENSSSAT00000060018.1 ENSSSAT00000060001.1	XM_014177116.1	F:GTGATGTTGGAGAGCGGTGC R:CGGGGTCAGTAACAACGCA	112	ssa27	ssa27
	ENSSSAT00000121230.1	NM_001141227.1			ssa14	ssa14
cart3b	ENSSSAT00000118490.1	XM_014127320.1	F:AGAAAGAACTTGTGGGTGCGA R:ACCACAAGGAGGGATCATGC	95	ssa11	ssa11
cart4	ENSSSAT00000121235.1	XM_014141614.1	F:CGTTCCGTCGTTGGAAACAC R:CCACGTTGGAATTGCACAGA	185	ssa14	ssa14

Table continued in next page.

Gene	Ensembl Acc. No.	GenBank Acc. No.	Primer sequences (5'→3')	Size (bp)	Chromosome No.	
					Earlier	Now
pomca1	ENSSSAT00000219912.1 ENSSSAT00000160584.1	NM_001198575.1	F:ATACTTTTGAAACAGCGTGACGA R:CAACGAGGATTCTCCCAGCA	108	ssa09	ssa09
pomca2	ENSSSAT00000247399.1 ENSSSAT00000124177.2 ENSSSAT00000243720.1	NM_001198576.1	F:TTTGGCGACAGGCGAAGATG R:TCCCAGCACTGACCTTTCAC	91	ssa01	ssa01
pomcb	N/A	NM_001128604.1	F:CAGAGGACAAGATCCTGGAGTG R:TTTGTCGCTGTGGGACTCAG	182	ssa06	ssa06
mc4r-a1	ENSSSAT00000098970.2	XM_014140480.1 XM_014140481.1 XM_014140482.1 (XM_045694109.1)	F:GTCATCGCCGCCATCATTAAG R: CCAATCCCCAGATTTCCGTC	152	ssa14	ssa14
mc4r-a2	ENSSSAT00000050406.2	XM_014190362.1	F:TGGCAACTTGGGTATCGGC R:GGCGCACGGTCATAATGTTG	170	ssa03	ssa03
mc4r-b1	ENSSSAT00000084254.2	XM_014157590.1	F:GGCGGTAATCGTGTGCATCT R:GCACGGCGATCCTCTTTATG	185	ssa19	ssa19
mc4r-b2	ENSSSAT00000103424.2	XM_014180569.1	F: GAGCTCCCCGGGGAAATAGTG R: AGTGCAAATCAGTCCTCACCA	158	ssa29	ssa29
actb	ENSSSAG00000001782.1 ENSSSAT00000187370.1 ENSSSAT00000227977.1 ENSSSAT00000003752.2	BG933897.1 (NM_001123525.1; XM_014194536.2)	F: CCAAAGCCAACAGGGAGAAG R: AGGGACAACACTGCCTGGAT	91	ssa03	ssa03
s20 rps20	ENSSSAT00000130996.1 ENSSSAT00000131003.2	NM_001140843.1 /XM_014180600.1 (splice variant) / XM_014157862.1)	F: GCAGACCTTATCCGTGGAGCTA R: TGGTGATGCGCAGAGTCTTG	85	ssa29	ssa29
ghrl1	N/A	NM_001142709.1	F:CCAGAAACCACAGGTAAGACAGGGTA R:GAGCCTTGATTGTATTGTGTTTGTCT	128	N/A	N/A
ghrl2	ENSSSAG00000045825 (ENSSSAT00000069524.1)	NM_001139585.1 XM_014132734.1	F:TCCCAGAAACCACAGGGTAAA R:GAGCCTTGATTGTATTGTGTTTGTCT	121	ssa12	ssa12
mboat4	ENSSSAG00000079778 (ENSSSAT00000150515.1 ENSSSAT00000150518.1)	XM_014161051.1 (XM_045703012.1)	F:GGGTTGGCAAACATTCTGGC R:ACACTGATAGGAGAAGCCTGG	89	ssa20	ssa20

Sequence accession number (Ensemble and GenBank databases), primer sequences, amplicon sizes(bp), chromosome (Chr) numbers: earlier and now. The gene sequences were retrieved from Genbank between 2018 to 2020 vs 2022. The Gene ID marked in red are not available in the latest version of update in Genbank and Ensembl. Id numbers ending with .1 versions in red are now available as .2 version. Gene IDs in blue are newly added in the database. Un.sc - unplaced scaffold.

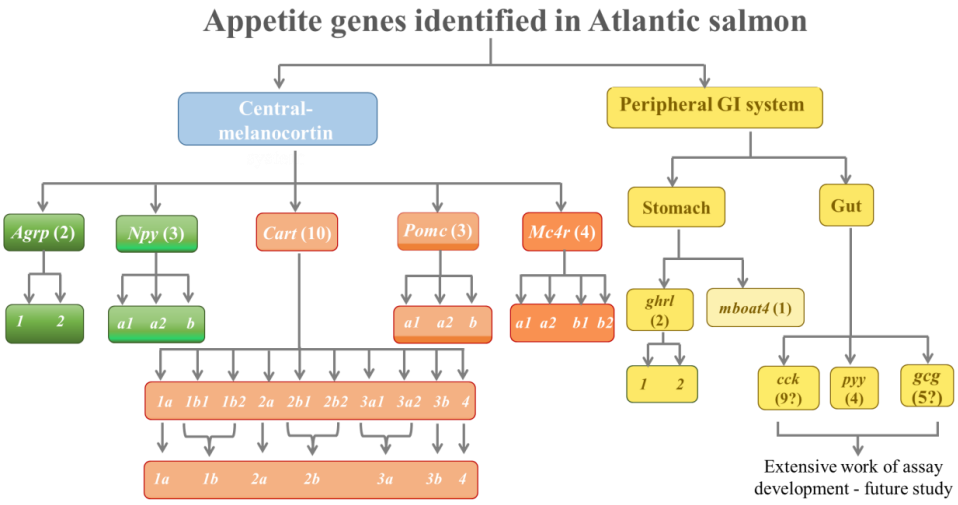

Figure 5. Chart with key appetite genes identified in the central melanocortin system in the brain and in the peripheral gastrointestinal system of Atlantic salmon**.** Major color scheme for the genes corresponds to **Figure 1.**

Murashita et al. (2009a) identified and characterized the Atlantic salmon Agrp and demonstrated that the salmon Agrp2 (also known as Asip2) has a conserved C-terminus 10 cysteine residue which is also seen in the Agrp isolated from other species, on the other hand, salmon Agrp1 lacks the 6th and 7th cysteine residues that make a disulfide bond with 2nd and 8th cysteine residue respectively. Further, the authors observed the fish agouti-family peptides have variation at C-terminus polycysteine domain. Although there are fewer cysteine residues in salmon Agrp1, the predicted three-dimensional structure seems to be strongly conserved between human and salmon and possibly have conserved function.

The Atlantic salmon Npy mature peptide shared at least 77% of AA identity demonstrates a possible conserved orexigenic role as in mammals (**Paper IV**). In teleosts, the Npy peptides named Npya and Npyb presumably have resulted from the 3R WGD in the teleost fish lineage (Sundström et al., 2008). The three proline and two tyrosine residues vital to the conformation of the NPY family were conserved in all three salmon Npy paralogs (Cerdá-Reverter and Larhammar, 2000). The predicted Npy sequence conservation likely points toward a conserved functional role Npy as a regulator of energy metabolism in Atlantic salmon.

The Atlantic salmon predicted mature peptide sequences of Cart were grouped into 4 major clusters in phylogenetic analysis (**Paper III**). Full length of Cart peptide sequences shared from 19 to 50% of identity with the human homolog and between 25 and 90% of sequence identity among paralogs, except for Cart4 which only shared 18 - 23% of identity. Six C-terminal cysteines are conserved in all the Atlantic salmon Cart, except Cart4 where only 5 cysteine residues are present. The absence of C-terminal cysteine bond among the homologs has not been reported in teleosts before. The three disulfide bridges are key residual structures that are essential for the protein 3-dimensional structure and function in biological activities such as food intake (Ludvigsen et al., 2001; Maixnerová et al., 2007). Among Cart paralogs, the potential proteolytic processing sites of dibasic residues KR and KK (Kuhar and Yoho, 1999; Rogge et al., 2008) are conserved only in Cart2, possibly cleaving it into two different size products as previously suggested by Murashita et al. (2009a). The Atlantic salmon Cart sequence alignment clearly shows that Cart4 structure potentially differs from the other salmon's Carts and human CART. This suggests that Atlantic salmon Cart4 may differ in functionality from the other Carts and may likely be ruled out from its role in appetite regulation. It would also be possible that Cart4 is non-functional or has adopted new function and this warrants further studies. In the Atlantic salmon the salmonid specific 4R WGD resulted in 10 *cart* genes, including sub types *cart1b1* and *1b2*, *cart2b1* and *2b2*, *cart3a1* and *3a2*. The divergence of the *cart* gene in salmonids opens the possibility that the gene products might have adopted new functions (neofunctionalization) or partitioning the ancestral function among the paralogs (sub functionalization) during genome evolution reviewed by Volff (2005).

Salmonid Pomc peptide sequences as expected clusters mainly into Pomca and Pomcb in the phylogenetic tree (**Paper I**). Common carp and goldfish have duplicate Pomcs that belong to each of two major clades suggests that the *pomc* duplication to *pomca* and *b* in salmonids would have evolved from teleost specific 3R WGD. Atlantic salmon Pomcs shared 27 - 37% AA identity with human homolog and Pomca1 and a2 shared 84% of AA identity indicate a possible deviation in the conserved function for these peptides from mammals. The **paper I** and previous study by Murashita et al. (2011)

revealed that the core sequences for MSH (HFRW) and β- END (YGGFM) segments were well conserved in the salmon Pomc.

Presence of four *mc4r* paralog genes *mc4ra1*, *mc4ra2*, *mc4rb1*, and *mc4rb2*, clustered into four different groups in all salmonid species analyzed, appears to be the result of the Ss 4R WGD (**Paper I**). All four paralogs are relatively well-conserved with the human homolog, sharing from 63 to 68% of AA sequence identity. There are two putative disulfide bonds in human MC4R, one between Cys271 (TMHVI) and Cys277 (ECL3) and another between Cys40 (N- terminus) and Cys271 (ECL3), whereas our predicted model showed one disulfide bond within the ECL3 for Mc4ra1 (Cys274 and Cys280) and Mc4rb2 (Cys275 and Cys281) in Atlantic salmon. Since the disulfide bonds play a key role in stabilizing protein structures, with disruption strongly associated with loss of protein function and activity (Karimi et al., 2016) the functional role for salmon Mc4rs is unclear.

Murashita et al., (2009b) previously identified and characterized isoforms of Ghrl1 and 2 in Atlantic salmon, while single Mboat4 has been identified in the current study. Atlantic salmon, Mboat4 shares 34% AA identity with human MBOAT4. Only in few teleosts the interlink between Ghrl and Mboat4 has been reported. Goat was first characterized and reported in detail in zebrafish as a non-mammal by Hatef et al., (2015). In Atlantic salmon two splice variants of *mboat4* in chromosome ssa20 were identified retrieving from Ensembl and GenBank. Based on the sequence in the database, primers for qPCR analysis were designed. As in Atlantic salmon two splice variants of *goat* were also reported in agastric goldfish (another species which went through 4R WGD) (Blanco et al., 2017).

4.2 *Regional distribution of key appetite regulators in brain*

The present study updates the current knowledge of the *agrp*, *npy*, *pomc*, *cart* and *mc4r* gene paralogs in Atlantic salmon and demonstrates their distribution profile and abundance in 8 brain regions (**Figure 6**). The current study investigates regional distribution of the mRNA expressions of *mc4r*, *agrp*, *pomc* in **Paper I**, *cart* in **Paper**

III and *npy* in **Paper IV**. The results showed *agrp2*, *npya1*, *cart2b*, *pomcb* and *mc4rb1* the most abundant paralog for each of the key appetite gene analyzed. The regional distribution of the key appetite gene paralogs from anterior to posterior region of Atlantic salmon brain demonstrate a higher expression in OB for *cart2b*, *npya1* & *a2*, in TEL for *npya1*, *cart2b*, *agrp2*, *npyb*, *cart3a, mc4rb1* & *a2*, in MB for *npya1* & *a2, cart2b & 3a* and *mc4rb1*, in HYP for *agrp1*, *npya1* and *npya*2, *cart2b*, *pomca1* and *pomca2* and *mc4rb*1, *b2* and *a2*, in PT for *pomcb*, *a1* & *a2*, *cart1b* and *agrp1*, in SV for *pomca1*, *a2* & *b*, *agrp1* & *2, npya2, mc4rb1 & b2*, in CE for *cart3a*, in BS for *cart3b* & *3a and mc4rb1.* Further, CE seems to be the least appetite key genes expressed region in Atlantic salmon.

In mammals, HYP is the feeding center and the appetite regulator gene expressions are restricted to this region (Hall, 2011). In teleost, ventral part of the tuberal lateral nucleus (NLTv) has been proposed to be homologous to the mammalian ARC in hypothalamus (Peng et al., 1994; Cerdá-Reverter et al., 2000; Conde-Sieira et al., 2018). The gene expression distribution in Atlantic salmon could indicate that, in addition to HYP, brain regions, such as OB, TEL, MB, BS, and PT may contribute to the control of appetite, feeding and energy homeostasis (**Figure 6**). However, a remarkably diverse array of physiological functions has been reported in correlation to the site of gene expression in the teleost's brain. A recent functional analysis in grass carp (*Ctenopharyngodon idellus*) suggested that HYP and TEL might be involved in feeding and reproduction process, OB in immune response and reproduction, optic tectum in vision and feeding, PT in energy metabolism and medulla oblongata in auditory functions (Ye et al., 2020). The physiological role of SV in masu salmon (*Oncorhynchus masou masou*) has been reported to act as a sensor of seasonal changes in day length and SV removed fish had lower gonadosomatic index (Nakane et al., 2013). The expression of *pomcb*, *agrp1* and *npya2* in SV raises the question whether the SV involves in appetite regulation or due to the macrodissection of SV which could have included any trace of hypothalamic neurons. The HYP showed considerable expression levels of these genes while the higher mRNA level of *agrp2* expression in SV (**Paper I**) indicates that it might have another role other than appetite regulation.

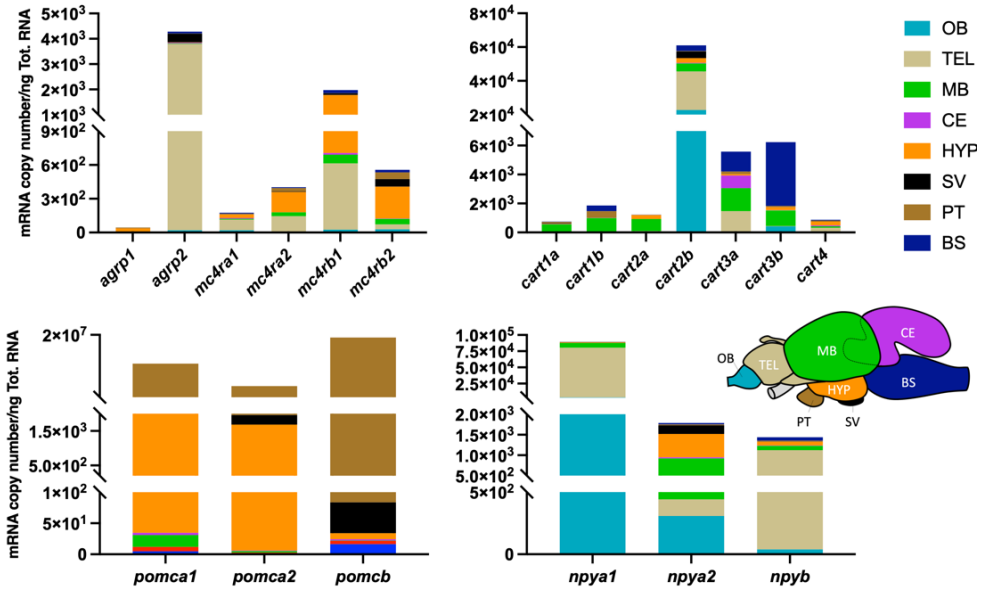

Figure 6. The mRNA expression of *agrp*, *mc4r*, *cart, pomc* and *npy* paralogs in eight brain regions (Insert: Illustration of brain dissection by Prof. Rønnestad) in Atlantic salmon. The genes were grouped according to their expression level to fit in the graphs.

Other studies in teleost also report the spatial distribution of the neuropeptides in the brain in connection to varying functions. For example, Npy signaling in the peripheral olfactory system is correlated with its nutritional state and is both necessary and sufficient for the olfactory perception of food- related odorants (Kaniganti et al., 2021). Similarly, c*art* has been suggested to play a role in processing olfactory information (Akash et al., 2014; Porter et al., 2017) including antiviral immune response (Sepahi et al., 2019). However, the Atlantic salmon appetite genes expression in the OB and a possible link to olfaction and/or immune response is not clear and needs to be further investigated. In goldfish, *npy* in the TEL/preoptic region (POA) was stimulated by the ovarian steroids (Peng et al., 1994) and *npy* in optic tectum (MB) believed to be involved in searching food activity suggesting the multiple functional roles of *npy* in context to the region of expression (Vanegas, 1983).

Another example in our study is higher level of *cart3b* expression in the BS region which contains Mauthner neurons (MN), specific multifunctional neurons in fish. These neurons receive afferent information from vestibular, auditory, and visual

analyzers and can integrate the afferent activity (Santalova et al., 2018). However, in Chinese sleeper (*Perccottus glehni*), the MN responded to severe environmental conditions like deficit of nutrients/energy substrates, oxygen, and temperature changes by modifying the structure of the cell and through different pathways for the maintenance of the energy balance (Santalova et al., 2018). Thus, the differential expressions of the *cart* paralogs in different brain regions suggest that they may have roles in regional integration of appetite signals and are possibly involved in regulating other brain functions in Atlantic salmon; for example, Cart4 with structural difference from other Carts (**Paper III**). The spatial distribution pattern of 7 major *cart* paralogs in brain regions of Atlantic salmon have been quite similar to that was reported in Senegalese sole (Bonacic et al., 2015) suggest that gene products might have adopted to new functions (neofunctionalization) or partitioning the ancestral function among the paralogs (sub functionalization) during genome evolution (Volff, 2005). The fact that salmon has 10 *cart* paralogs, while mammalians only one, opens interesting perspectives for comparative research on evolutionary adaptations of gene function in the control of appetite and energy homeostasis.

How the well-known neuronal centers involved in regulating feeding, in particular the hypothalamic nuclei, are regulated by and connected to superordinate brain centers including the reward system and sensory organs (e.g., the gustatory and olfactory systems) are still not explored well. Higher level of *agrp1* mRNA expression in peripheral tissues like skin and ovary (Murashita et al., 2009a) shows apart from appetite regulation *agrp1* possibly have other functional roles. Whereas, *agrp2* expression high in midgut, red muscle, and reproductive tissues (Murashita et al., 2009a)**,** higher levels in TEL and SV, and low levels in HYP (**Paper I**) believed to have completely different role other than appetite regulation in Atlantic salmon. (Timper and Brüning, 2017). Further, *agrp2* expression in other tissues like pineal gland and preoptic region in brain in zebrafish have been reported possibly with other neuroendocrine functions in stress axis and background adaptation (Shainer et al., 2017, 2019) while in African cichlids (*Haplochromis sauvagei*) *agrp2* expression negatively associated with the presence of stripe patterns across species flocks (Kratochwil et al., 2018). Similarly, most appetite genes are also expressed in the

peripheral tissues like eyes, GI tract, ovaries and potentially have physiological functions that are not fully explored. Moreover, there is a huge knowledge gap in the functional role of neuropetides relevant to the region of expression in brain of Atlantic salmon.

In Atlantic salmon, *mc4rb1* the highest expressed paralog, showed higher levels in TEL and HYP like other paralogs *mc4ra1* and *a2*. The *mc4rs* are also expressed in other regions like MB, and BS considerably which is in line with goldfish homolog *mc4r* expression (Cerdá-Reverter et al., 2003). In Goldfish, *mc4r* expression in peripheral tissues like ovary, gill, spleen and eye indicate that *mc4r* might have other physiological functions (Cerdá-Reverter et al., 2003) while *mc4r* in melanocortin system involve in appetite regulation and energy homeostasis.

4.3 *Impact of short-term (3 and 4 days) fasting on key appetite regulators*

In the 3 or 4 days of fasting studies in Atlantic salmon postsmolts *agrp1* and *cart2b* in hypothalamus responded to fasting and *npya2*, and *pomca2* with a trend of response (**Table 3**) (**Papers II, III and IV**). Our results showed that 3 days of fasting significantly increased hypothalamic *agrp1* with a trend of decline in *pomca2* mRNA expression, suggesting that *agrp1* acts as an orexigenic factor and *pomca2* might have responded as anorexigenic factor in the short-term fasting in Atlantic salmon (**Paper II)**. No differences were observed for *agrp2*, *pomca1* and *pomcb* in the same study. In the 4 days fasting experiment (**Paper I**), there was no significant differences observed for any of the genes *agrp1*, *agrp2*, *pomcs*, and *mc4rs*, where higher individual variations were observed. The variations in the response to fasting in both studies could have been due to differences in duration of fasting. Moreover, in the 3 days fasting study, the mRNA abundance of *agrp1* was negatively correlated with the stomach dry weight content (**Paper II**). Corresponding inverse patterns were observed for *pomca2*, albeit not statistically significant. In this context, *agrp1* seems to be a potential orexigenic neuropeptide providing hunger signal and *pomca2* as satiety signaling factor in Atlantic salmon. The orexigenic role of Agrp1 has been supported by the

upregulation of hypothalamic *agrp1* under fasting conditions, followed by elevated food intake in many teleost including zebrafish larvae (Song et al., 2003), coho salmon (Kim et al., 2015), goldfish (Cerdá-Reverter and Peter, 2003), sea bass (Agulleiro et al., 2014), mouth brooding African cichlid (*Astatotilapia burtoni*) (Porter et al., 2017) and seabream (Koch et al., 2019).

Whereas at 4 days of fasting resulted in a significant decrease of *npya1* mRNA expression in the OB, increased *npya2* mRNA expression in the MB and a trend of increase in HYP (**Paper IV**). These findings imply that Atlantic salmon *npya* is the main paralog involved in feeding regulation as in Nile Tilapia (Yan et al., 2017) and *npyb* is likely not involved in central appetite control (**Paper IV**). In teleost, an orexigenic role of Npy has been reported in many species including goldfish (Narnaware et al., 2000), grass carp (Zhou et al., 2013), zebrafish (Yokobori et al., 2012), rainbow trout (Aldegunde and Mancebo, 2006), chinook and coho salmon (Silverstein et al., 1998) while in few species like Atlantic cod (*Gadus morhua*) (Kehoe and Volkoff, 2007) and in cunner (*Tautogolabrus adspersus*) (Babichuk and Volkoff, 2013) opposite action was reported (**Paper IV**).

In Atlantic salmon, among *cart* paralogs only *cart2b* (the most abundant gene paralog) upregulated after 4 days of fasting in OB, MB, and HYP compared to Fed group (**Paper III**). No other significant effect was observed for other *cart* paralogs, which suggests that the only *cart* responded to fasting is *cart2b*. The currently reported short-term fasting study demonstrated an unexpected, but possible orexigenic role for *cart2b* in Atlantic salmon or a fasting induced stress effect or being anorexigenic factor suppress/inhibit the effect of orexigenic factor *npya2* (**Paper IV**) as shown in goldfish (Volkoff and Peter, 2001)**.**

The results in short-term fasting study (**Paper I**) demonstrated that no response to fasting for *mc4r* paralogs. In mammals, MC4R signaling is an important regulator of feeding and weight gain. MC4R mutation has been most common cause of obesity in humans (Barsh and Schwartz, 2002). This further indicates that the MC4R is important for the anorexigenic pathway in line with few studies in teleost that demonstrated feed

restriction induced downregulation of *mc4r* expression (Wan et al., 2012; Aspirasa et al., 2015). Another study in sea bass suggested that the Mc4r activity to be dependent on the Agrp binding rather than the *mc4r* expression in case of progressive fasting (Sánchez et al., 2009). The presence of 4 *mc4r* paralogs in Atlantic salmon hypothalamus and *mc4rs* not responding to short-term fasting suggests the role on appetite regulation is species specific and depends on the period of fasting. There are more to explore on the functional role of this receptor in Atlantic salmon.

4.4 *Impact of long-term fasting on key appetite regulators*

In the extended fasting trials (**Paper V**), among the genes we analyzed in hypothalamus, *agrp1*, *npya1*, *cart2a* & *2b*, *pomca1* & *a2*, *mc4ra2* and in the stomach, *ghrl1* responded to long-term fasting (**Table 3**). Whereas the data on *agrp1* and *npya1* supported an orexigenic role at 4 weeks of fasting, *agrp1* data alone supported an orexigenic role in extended fasting over 6 weeks. Moreover, the mRNA expression of *cart2b*, most abundant among the *cart* paralogs analyzed, was upregulated along with *pomca1* and *pomca2* in long-term fasting indicate that these neuropeptides play either a vital role in appetite regulation by inducing shutting down hunger and/ or counteract on orexigenic effect /hunger signals (*agrp1* and *npya1*) to save energy from foraging search activity during catabolic conditions or play a fasting induced stress effect. Similar effect has been shown in goldfish that human CART suppresses Npy-induced feeding (Volkoff and Peter, 2001), suggesting that CART has an inhibitory effect on Npy. As previously mentioned, *cart2b* which is highest expressed among *cart* paralogs analyzed in the hypothalamus have the same proteolytic sites as in mammals indicating that this paralog takes major part in appetite regulation. While the functions of *cart3a* and *cart4* are not clear as these did not respond to fasting after 4 or 6 weeks. On the other hand, both *agrp1* and *npya1* responding to 4 weeks of fasting as orexigenic factor and *agrp1* alone acting in orexigenic role at 6 weeks of fasting raises a question whether the orexigenic neuropeptides *agrp1* and *npya1* compensate to overcome the anorexigenic effect of *cart2b, pomca1* and *a2* under fasting at different time points which also need further investigation. However, a recent study conducted in mice

confronted the idea that POMC neurons are solely anorexigenic by showing that activation of the cannabinoid receptor 1 on POMC neurons leads to the release of β-endorphin (but not α-Msh), an opioid neuropeptide, which in turn promotes feeding (Koch et al., 2015). This anorexigenic-to-orexigenic switch of POMC neurons might be responsible for cannabinoid-induced hyperphagia (Timper and Brüning, 2017) and whether this switch is possible under long-term fasting in Atlantic salmon needs to be resolved.

Table 3. A comparison of action of key appetite regulators that responded to fasting in Atlantic salmon (this work) with mammals (Hall, 2011). Color scheme follows the **Figure 1**.

Mammals		Atlantic salmon		
Genes	Action	Genes	Action	
			Short-term study	Long-term study
agrp	hunger signal	*agrp1*	↑; hunger signal	↑; hunger signal
npy	hunger signal	*npya1*	-	↑; hunger signal
		npya2	↑; hunger signal trend	-
cart	satiety signal	*cart2a*	-	↑; ?
		cart2b	↑; ?	↑; ?
pomca	satiety signal	*pomca1*	-	↑; ?
		pomca2	↓; satiety signal trend	↑; ?
mc4r	important for satiety/anorexigenic pathway	*mc4ra2*	-	↑; ?
ghrl	hunger signal	*ghrl1* *ghrl2*	-	↓; adapted satiety signal?

↑ *upregulation of mRNA expression,* ↓ *downregulation*, - *no differences, ? action unclear. Short-term fasting on ghrl from Del Vecchio et al.(2021).*

The available data in Atlantic salmon demonstrates that *ghrl2* mRNA level was the highest among the paralogs expressed in the stomach (Del Vecchio et al., 2021) and that *ghrl1* significantly declined after 6 weeks of fasting, whereas *ghrl2* showed a trend of decline (**Paper V**). This is in line with a study with 2 days of fasting in the same

species (Hevrøy et al., 2011). Based on the drop in mRNA expression of *ghrl* under catabolic conditions, it could be hypothesized that Ghrl might return as hunger signal once feed becomes available in the vicinity. In **Paper V**, the *mboat4* had no differences between control and treated groups at any time points, which contradicts with a study in zebrafish that feed deprivation increased its expression level similar to changes in *ghrl* expression (Hatef et al., 2015).

In goldfish, although *goat* was observed abundant in vagal lobe of brain, other regions including telencephalon, hypothalamic nuclei, pineal gland, optic tectum and cerebellum was also detected with *goat* transcripts (Blanco et al., 2016). Further, the study showed that the time of feeding is a critical external factor for the 24 h rhythmic expression of *goat* transcripts whether feeding takes place in photophase (light phase) or scotophase (dark phase) of the photocycle. In goldfish, the *ghs-r1a* and *2a* mRNAs expressed in vagal lobe which involved in gustatory system of goldfish and responding to feeding under fasting conditions has been demonstrated (Kaiya et al., 2010). Moreover, the presence of Goat immunoreactive cells in the secondary gustatory tract and in vagal lobe suggests Goat participate in feeding (Blanco et al., 2016).

4.5 *Fasting-induced stress in Hypothalamus - Pituitary - Interrenal (HPI) axis.*

In this study it was hypothesized that, under fasting-induced stress conditions, energy reserves are mobilized to cope with increased metabolic demand, and in turn the signaling mechanism linked to appetite may be affected as reviewed in an article on stress response (Conde-Sieira et al., 2018), until the feed becomes available and returning to control conditions. Stress exerts the energy mobilization through primary, secondary, and tertiary responses through endocrine changes such as in measurable levels of circulating catecholamines and corticosteroids. Catecholamines furnish energy in the short-term (through glycogenolysis) whereas cortisol underlies release of energy in the longer term, by stimulating catabolism of glycogen, lipids and protein (reviewed in Van Weerd and Komen, 1998). Under the primary response in the HPI axis, the α-Msh from hypothalamic Pomc neurons stimulate the Crh (cortocotropin

releasing hormone) producing neurons which in turn produces Crh/Crf (cortocotropin releasing factor) that activate the pituitary Pomc neuron to release Acth which act on the interrenal to release the cortisol (principle corticosteroid in actinopterygian) (Wendelaar Bonga, 1997; Barton, 2002; Matsuda et al., 2008) (**Figure 7**). In the secondary response, the presence of glucocorticoids, such as cortisol, increase the availability of blood constituents including metabolites like glucose and major ions to the brain via acting on the liver, muscle, adipose tissue, and pancreas. Glucagon, a polypeptide hormone secreted by pancreatic α-cells has a primary function to prevent hypoglycemia (Barton, 2002; Zhang et al., 2019b). In the liver, high cortisol levels along with glucagon increase gluconeogenesis and decrease glycogen synthesis (Wang and Harris, 2015).

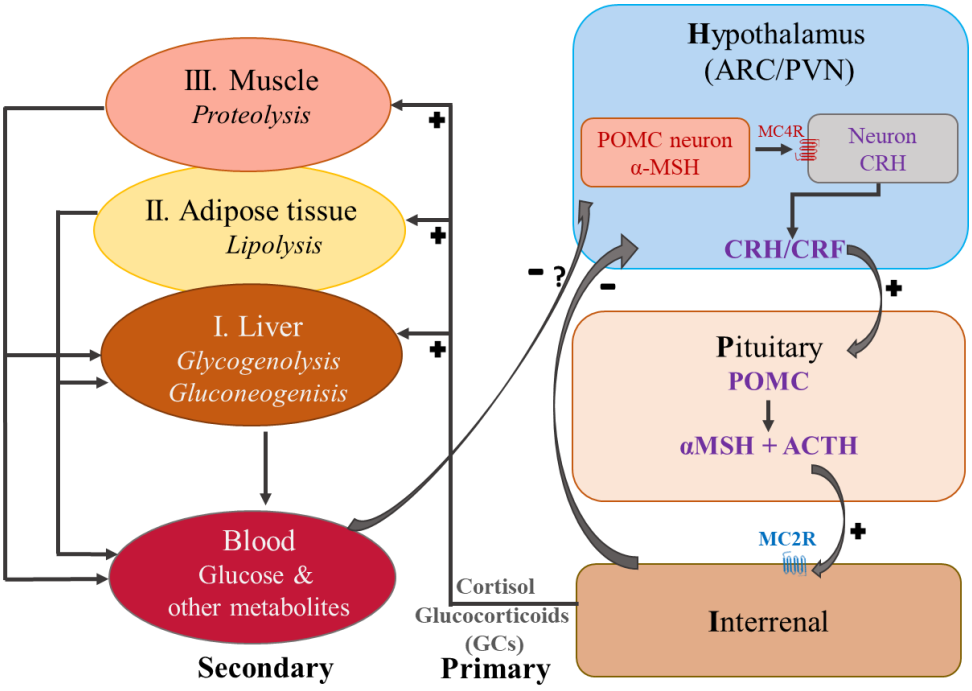

Figure 7. Hypothetical illustration on starvation induced primary and secondary stress response via **H**ypothalamus - **P**ituitary - **I**nterrenal (**HPI**) axis based on literature (Bernier and Peter, 2001; Barton, 2002; Lu et al., 2003; Smart et al., 2007; Bar, 2014; Conde-Sieira et al., 2018; Timmermans et al., 2019; Zhang et al., 2019b). CRH-Corticotropin releasing hormone (CRF- corticotropin releasing factor).

The tertiary response of stress as a consequence of primary and secondary responses can result on whole-animal including changes in performance, such as in growth, disease resistance and behavior, and possibly affect survivorship (Barton, 2002).

The results from the short-term and long-term fasting studies (**Papers III and V**) showed an increase in *cart* and *pomc* expression in the Fasted group (**Table 3**) where a decline for these two genes were expected. The reason being that these genes are believed to serve an anorexigenic action in mammals. However, in mammals, intracerebroventricular injection of CART has increased C-Fos levels in many hypothalamic regions (Pirnik et al., 2010) and administration of CART peptide into cerebrospinal fluid activated CRH neurons in PVN as well as increased the Crh gene expression of both Fasted and Fed rats (Sarkar et al., 2004). Matsuda et al.(1996) demonstrated that chronic social stress exerted persistent *C-fos* expression in brains of mice. This imply that Cart has an important role in the HPI axis under chronic, starvation induced stress, as previously discussed for Pomc in Atlantic salmon.

In our long-term fasting study, decline in both *K* factor and HSI supports that in the Fasted group of fish under catabolic condition, glucagon along with glucocorticoids response to long-term fasting stress possibly stimulated hepatic gluconeogenesis as secondary response in the short transient phase (Bar, 2014). Bar (2014) described, fasting is usually followed by three phases: (I) a short transient phase where both protein tissues and fat reserves (liver) are mobilized, (II) a longer steady state with mobilization of fat (adipose) as the main source of energy and when the fat reserves reached a critical threshold, (III) a shift to mobilization of protein tissue (muscle) as this becomes the main source of energy. In Atlantic salmon, Einen et al. (1998) reported that fish starved for 58 days or more had a lower fat content than fish starved for 7 days or less in whole-fillet fat content and found no significant change of fillet protein level after 86 days starvation. Further, Einen et al. (1998) found a steady decrease in liver fat content with increasing starvation time, indicating liver fat is also an important metabolic fuel during starvation, although the fat loss from liver, quantitatively, was far less than from muscle and viscera. In the same study, there was no significant change of fillet protein level after 86 days starvation while lower liver protein level in

fish starved for 86 days suggests that the liver protein is depleted more rapidly than muscle protein during starvation. In **Paper V**, the fish performance on *K* factor and HSI believed to have been as a result of fasting induced mobilization of lipid reserves from liver and adipose tissues (Love, 1980; Lie and Huse, 1992), and the liver as fuel storage of both glycogen and fat. Hvas et al. (2022), have refed the fish after the 8 weeks of fasting, showed a gradual increase in feed intake over the first month. After three months, the weight difference between the experimental groups (Fed vs. Fasted) 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)(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).

4.6 *Limitations in the field of appetite endocrinology in fish*

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, nutrient composition of the feed, species specific digestive physiology, gut evacuation time, feeding strategy and behavior, and the temperature, and consequently the metabolic rate of the fish. Adding to the mentioned, the feeding regime (continuously or meal-based) and the period in between last meal offered, and sampling will also reflect in the expression of neuropeptides regulating appetite in the brain. Most probably, there is a higher variation in voluntary feed intake when the fish are fed continuously. Further, 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), since the mRNA precursors undergo several regulatory processes including protein cleavage and conformational changes and also depend on other factors like

ribosomal density and occupancy (Maier et al., 2009). A combined study with mRNA and protein expression could provide certain level of knowledge of orexigenic and anorexigenic action of appetite regulators (for each specified paralog/isoform) in response to prolonged fasting in Atlantic salmon. However, there are still unknown post translational factors like stability of protein and protein half-lives, which could influence the final protein expression level.

5 CONCLUSION

This PhD study has identified and characterized the key appetite controlling neuropeptides and the receptor in the melanocortin system and neuroendocrine hormones in stomach revealed the spatial distribution of the appetite regulators in brain; their response to short-term fasting and demonstrated the interaction of stomach-hypothalamic neuroendocrines in response to long-term fasting in Atlantic salmon postsmolt. The key conclusions drawn in this study are:

- In the melanocortin system, *npy*, *cart*, *mc4r* with multiple paralogs such as, three *npy* (*npya1*, *npya2*, and *npyb*), ten *cart* (*cart1a*, *1b1*, *1b2*, *2a*, *2b1*, *2b2*, *3a1*, *3a2*, *3b* and *4*) and 4 *mc4r* (*mc4ra1*, *a2*, *b1* & *b2*) and single *mboat4* from stomach are novel key appetite regulators. Further, the existence of two *agrp* (*agrp1* and *agrp2*) and three *pomc* (*pomca1, pomca2* and *pomcb* with one splice variant *pomca2s*) from melanocortin system and two *ghrl* isoforms from stomach, that were identified and characterized in previous studies, was confirmed.

- Assays for these appetite regulators, that were developed and established by designing primers specific to the gene paralogs wherever possible and validated for qPCR analysis, are still valid for the current updated gene sequences in the GenBank and useful for further studies, even though we retrieved the sequences for the qPCR analysis during the period 2018 to 2020.

- The protocol for macrodissection of Atlantic salmon brain into 8 regions (OB, TEL, MB, CE, HYP, SV, PT and BS) established and validated in the current study, can be used as a standard protocol in spatial distribution analysis of appetite regulating genes in the brain. Further, utilizing the standard protocol will enable to maintain the consistency among the studies on appetite regulation.

- Spatial distribution of mRNA expression of melanocortin in the brain of Atlantic salmon postsmolts revealed a wide distribution pattern with varying range for all genes analyzed. Particularly, HYP, TEL, OB, MB, BS and PT were observed with high level of mRNA expression. This enabled to select the hypothalamus for qPCR analysis when investigating fasting response on particular appetite regulator.

- In short-term fasting in Atlantic salmon hypothalamus, only *agrp1* and *cart2b* upregulated in response to fasting, while *npya2* and *pomca2* showed a trend of upregulation and downregulation respectively. At short-term fasting hypothalamic *agrp1* acted in orexigenic role, and *pomca2* showed a trend of anorexigenic role.

- Among the selected appetite regulating genes, *agrp1*, *npya1*, *cart2a* & *2b*, *pomca1* & *a2*, *mc4ra2* and *ghr1* responded to long-term fasting (4 and 6 weeks). The *agrp1* and *npya1* responded as orexigenic in action, while *cart2a*, *cart2b*, *pomca1* and *pomca2* upregulation demonstrates that the neuropeptides might play either a vital role in appetite regulation, i.e., they either counteract hunger signals (*agrp1* and *npya1*) with fasting to save energy from foraging activity in catabolic conditions or play a fasting induced stress effect in the HPI axis.

- From the findings reported in this study it is postulated that the decline in *ghrl1* mRNA expression under catabolic conditions might return as hunger signal once the food becomes available in the vicinity.

- Taken together the data reported in this study suggest a*grp1* as a potential appetite biomarker gene though the temporal dynamics of the expression in relation to a meal is complicated. Unraveling the physiological roles of the neuropeptides will provide more insight into the appetite regulation in Atlantic salmon, a global key commercial aquaculture species.

6 FUTURE PERSPECTIVES

This PhD study initially focused on the appetite regulation in the gut-brain axis, but it was only possible to study the link between stomach and brain due to the extensive work involved in the *in silico* analysis and assay development for qPCR analysis of gut hormone that could not be completed within the time frame of the PhD study. To unravel the basic mechanism on appetite control via the central and peripheral pathways in the gut-brain axis in Atlantic salmon it is important to develop assays for qPCR and perform the analyses for the key signaling elements from gut. Current study is incomplete in gut-brain axis signaling and lacks the data on gut hormonal control on appetite and feed intake in Atlantic salmon. Therefore, in the future studies gut hormones need to be included on such aspects.

In the **Papers I - V**, it was only possible to study the mRNA expression of the appetite regulators. However, mRNA expression levels may not necessarily contribute to the same trend of protein expression of a specific gene and activity at its corresponding receptors since the mRNA precursors undergo several regulatory processes. Therefore, adding a combined study with mRNA and protein expression could provide significant knowledge of orexigenic and anorexigenic action of appetite regulators (for each specified paralog/isoform) in response to short/prolonged fasting in Atlantic salmon in forthcoming researches.

Among the appetite regulating key genes we analyzed, both in short-term and long-term fasting hypothalamic *agrp1* acts as orexigenic neuropeptide and has potential as appetite biomarker gene. Though the dynamic and development process into biomarker is complex, the hypothalamic *agrp1* gene expression might possibly have correlation to the behaviour of fish in response to feeding, modeling efficient, sustainable, and economical feeding strategy might be beneficial to the Atlantic salmon aquaculture industry. Therefore, efforts on developing *agrp1 as* appetite biomarker may be prioritized in the upcoming studies.

This study focused on Fed or Fasted groups as treatments at particular environmental condition. Conversely, investigating the expression levels of central appetite-

controlling genes such as *pomc*, *cart*, *mc4r*, *agrp*, and *npy* in Atlantic salmon under varying environmental factors like temperature and photoperiod and correlating it with its annual feeding cycle may provide more insight. Thus, in the future projects these could be considered. Further, we utilized salmon smolt from varying size in this study and the smoltification related stress might also influence the feed intake. Investigating the appetite control at different developmental stages particularly during transition from FW, BW to SW would provide more insight. Samples related to stress parameters including metabolites were not performed or collected in the current study. Evaluating the circulating peripheral hormonal appetite regulators and plasma metabolites (including stress related) during photophase and scotophase of a day with single feeding could therefore be included in the future studies.

In the previous studies, only few paralogs of key appetite controlling genes were analyzed in tissue distribution profile in Atlantic salmon. This study indicates multiple paralogs/isoforms exist for some of the key genes and the tissue distribution profile remains to be updated with these gene paralogs to clarify more on the physiological functions. In other species the expression profile of some of these neuropeptides and receptors were found in reproductive organs, interrenal tissues and immune system, which indicates their involvement in other physiological functions like reproduction, stress, and immune resistance. Therefore, it is worthwhile for further studies to expand and update the mentioned tissue distribution profile and the physiological functions of these gene paralogs.

The genes were selected for the **Paper V** from peripheral functional sites only stomach and for central control site hypothalamus. However, some of the key appetite regulator genes are expressed both in the central as well as peripheral system, which makes it more complex to understand their potential role in appetite regulation. For example, in few studies in teleost, higher level of *ghrl* and *mboat4* expressions were observed in brain, and *agrp1* and *npy* expressed in the gut regions. The interlink among these networks are still unclear in Atlantic salmon and it is practically impossible to analyze all paralogs of such key genes using qPCR or *in situ* hybridization (ISH) study. Therefore, utilizing transcriptome study with high throughput RNA sequencing would

provide a better understanding of interactions, the extent of signals and the signaling pathway (Differentially expressed gene analysis (DEG) along with Gene ontology/pathways) from the sensation of meal through olfaction, vision, chemo, and mechanical sensation to feed intake. In addition, transcriptome data will provide extensive knowledge on all paralogs (of each appetite genes, and their receptors at least in the gut-brain axis) that respond to treatment and to identify nonfunctional/multifunctional ones.

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The Melanocortin System in Atlantic Salmon (*Salmo salar* L.) and Its Role in Appetite Control

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The melanocortin system is a key neuroendocrine network involved in the control of food intake and energy homeostasis in vertebrates. Within the hypothalamus, the system comprises two main distinct neuronal cell populations that express the neuropeptides proopiomelanocortin (POMC; anorexigenic) or agouti-related protein (AGRP; orexigenic). Both bind to the melanocortin-4 receptor (MC4R) in higher order neurons that control both food intake and energy expenditure. This system is relatively well-conserved among vertebrates. However, in Atlantic salmon (*Salmo salar* L.), the salmonid-specific fourth round whole-genome duplication led to the presence of several paralog genes which might result in divergent functions of the duplicated genes. In the current study, we report the first comprehensive comparative identification and characterization of Mc4r and extend the knowledge of Pomc and Agrp in appetite control in Atlantic salmon. *In silico* analysis revealed multiple paralogs for *mc4r* (*a1*, *a2*, *b1*, and *b2*) in the Atlantic salmon genome and confirmed the paralogs previously described for *pomc* (*a1*, *a2*, and *b*) and *agrp* (*1* and *2*). All Mc4r paralogs are relatively well-conserved with the human homolog, sharing at least 63% amino acid sequence identity. We analyzed the mRNA expression of *mc4r*, *pomc*, and *agrp* genes in eight brain regions of Atlantic salmon post-smolt under two feeding states: normally fed and fasted for 4 days. The *mc4ra2* and *b1* mRNAs were predominantly and equally abundant in the hypothalamus and telencephalon, the *mc4rb2* in the hypothalamus, and *a1* in the telencephalon. All *pomc* genes were highly expressed in the pituitary, followed by the hypothalamus and saccus vasculosus. The *agrp* genes showed a completely different expression pattern from each other, with prevalent expression of the *agrp1* in the hypothalamus and *agrp2* in the telencephalon. Fasting did not induce any significant changes in the mRNA level of *mc4r*, *agrp*, or *pomc* paralogs in the hypothalamus or in other highly expressed regions between fed and fasted states. The identification and wide distribution of multiple paralogs of *mc4r*, *pomc*, and *agrp* in Atlantic salmon brain provide new insights and give rise to new questions of the melanocortin system in the appetite regulation in Atlantic salmon.

Keywords: melanocortin system, Atlantic salmon, *melanocortin-4 receptor* (*mc4r*), *proopiomelanocortin* (*pomc*), *agouti-related protein* (*agrp*), food intake, brain, appetite control centers

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INTRODUCTION

In vertebrates, food intake is controlled by the synergic actions of central and peripheral signaling pathways which provide information on ingestion and presence of food in the digestive tract and on the nutritional status (Volkoff, 2016; Rønnestad et al., 2017). In mammals, the melanocortin system is a key neuroendocrine network playing a pivotal role in regulating appetite and energy homeostasis. This system is mainly located within the hypothalamus where neurons expressing the melanocortin-4 receptor (MC4R) mediates either anorexigenic or orexigenic signals, thereby controlling the energy homeostasis of the animal (Nuzzaci et al., 2015). The MC4R neurons receive dual innervation from neurons expressing MCR agonists as melanocortin-stimulating hormones (α-, β-, γ-MSHs) and adrenocorticotropic hormone (ACTH), which derive from the posttranslational cleavage of proopiomelanocortin (POMC) peptide and from neurons expressing the antagonist agouti-related peptide (AGRP) (Ghamari-Langroudi et al., 2011; Kim et al., 2014; Nuzzaci et al., 2015). Furthermore, both POMC and AGRP neurons integrate peripheral endocrine signals and information on nutrient levels received through blood circulation or vagal afferent projections. In mammals, leptin was shown to play an anorexigenic role by increasing the excitability of POMC neurons and decreasing AGRP neuron action (Cowley et al., 2001; Baver et al., 2014); on the contrary, ghrelin plays an orexigenic (appetite stimulator) role by directly stimulating AGRP neurons and inhibiting POMC neurons (Riediger et al., 2003). This complex network has been well-described in mammals; however, in other vertebrates, such as teleost, little knowledge still exists.

Many of the neuropeptides and endocrine signals involved in appetite control in mammals have also been identified in teleost, although only a few have been functionally described.

The involvement of the Mc4r receptor in teleost energy balance was demonstrated in salmonids, and modulation of the Mc4r activity with the receptor antagonist (HS024 or SHU9119) or the agonist (MTII) increased or decreased, respectively, food intake in rainbow trout (*Oncorhynchus mykiss*) (Schjolden et al., 2009). In common carp (*Cyprinus carpio*), brain *mc4r* expression declined with fasting, while it surged with refeeding (Wan et al., 2012). Similarly, a winter fasting state also induced a lower expression of *mc4r* in the hypothalamus in Arctic charr (*Salvelinus alpinus*) (Striberny et al., 2015). In an extended study in spotted sea bass (*Lateolabrax maculatus*), incubation of isolated brain cells with α-MSH showed changes at *npy* and *agrp* levels and a downregulation of *mc4r* transcript levels during both short- and long-term fasting (Zhang et al., 2019). Both *in vitro* and *in vivo* experiments demonstrated that a naturally mutated Mc4r in Mexican cave fish (*Astyanax mexicanus*) is responsible for elevated appetite, growth, and starvation resistance as an adaptation to an environment with poor nutrient conditions (Aspiras et al., 2015). In contrast, food deprivation in barfin flounder (*Verasper moseri*) did not induce any changes of *mc4r* transcripts in the brain (Kobayashi et al., 2008).

Several studies have explored the involvement of Pomc in appetite control in teleost, and the results have suggested that its role may be species-specific. Intracerebroventricular administration (ICV) of the Mc4r agonist MTII downregulated *pomc* mRNA levels (Kojima et al., 2010), whereas intraperitoneal injection of cholecystokinin octapeptide (Kang et al., 2010) and leptin (Yan et al., 2016) upregulated *pomc* expression in the diencephalon, favoring enhanced anorexigenic action of Pomc. Starvation of zebrafish (*Danio rerio*) larvae resulted in a decrease in *pomc* expression levels, and refeeding after 2 days of fasting recovered *pomc* to the level of the control group (Liu et al., 2016). Under hyperglycemic conditions, rainbow trout resulted in increased hypothalamic *pomc* mRNA levels (Conde-Sieira et al., 2010; Otero-Rodiño et al., 2015), while 28 days of fasting downregulated *pomc* expression in the same species (Leder and Silverstein, 2006).

In Atlantic salmon (*Salmo salar*), brain *pomc* levels declined from 3 to 6 h of post-feeding of a single meal (Valen et al., 2011). In fully fed growth hormone (GH) transgenic coho salmon (*Oncorhynchus kisutch*), hypothalamic *pomc* mRNA decreased 4 h post-feeding, while there was no difference in the non-transgenic group (Kim et al., 2015). Similar results were also reported in GH transgenic zebrafish fasted for 2 days (Dalmolin et al., 2015). No changes in the *pomc* mRNA expression were observed in zebrafish (Opazo et al., 2019), barfin flounder (Takahashi et al., 2005), and goldfish (*Carassius auratus*) (Cerdá-Reverter et al., 2003b) under fasting regimes. In Atlantic halibut (*Hippoglossus hippoglossus*) larvae, the response of *pomc* to food deprivation and refeeding did not show a consistent expression pattern to explain their contribution to appetite control (Gomes et al., 2015).

The AGRP-mediated action on food intake seen in mammals appears to be conserved in teleost species. Ablation of Agrp1-expressing neurons and knockout of the *agrp1* gene showed that Agrp stimulates food consumption in zebrafish larvae (Shainer et al., 2019) or induces obesity in transgenic zebrafish overexpressing *agrp* (Song and Cone, 2007). An upregulation of *agrp* transcript was described in the larvae of the same species under fasting conditions (Song et al., 2003). GH transgenic common carp (Zhong et al., 2013) and coho salmon (Kim et al., 2015) showed increased hypothalamic *agrp1* mRNA and elevated food intake compared to the wild type. Moreover, fasting upregulated the hypothalamic *agrp* mRNA in goldfish (Cerdá-Reverter and Peter, 2003), coho salmon (Kim et al., 2015), sea bass (Agulleiro et al., 2014), mouth brooding African cichlid (*Astatotilapia burtoni*) (Porter et al., 2017), rainbow trout (Comesaña et al., 2017), seabream (*Sparus aurata*) larvae (Koch et al., 2019), Atlantic salmon (Kalananthan et al., 2020), and Ya-fish embryo (*Schizothorax prenanti*) (Wei et al., 2013). In contrast, an opposite action was described in the brain of common carp (Wan et al., 2012) and Atlantic salmon (Murashita et al., 2009; Valen et al., 2011).

The extensively described variations in the role of Mc4r receptor and neuropeptides Pomc and Agrp in teleost compared to mammals may be due to major physiological and environmental adaptation. Moreover, salmonids went through a salmonid-specific fourth round whole-genome duplication (Ss 4R WGD) around 80 million years ago (mya),

leading to large genomic rearrangements and the presence of several paralog genes, which may result in divergent functions for the different paralogs (Takahashi and Kawauchi, 2006; Warren et al., 2014; Lien et al., 2016).

Atlantic salmon is an economically important species of aquaculture industry in Norway. Periods of 2–4 days' fasting is a common practice during transport, handling, vaccination, and harvest of salmon to ensure a proper evacuation of the gut (Waagbø et al., 2017). Studying the impact of fasting on fish biology is essential to optimize the Atlantic salmon aquaculture practices with regard to the period of recovery, fish welfare, and feed utilization. In this study, we investigated the spatial gene expression of *mc4r, pomc*, and *agrp* genes and their paralogs in the brain of Atlantic salmon post smolts at fed and fasting (4 days) states. Our study provides a foundation for new insights on the role of *mc4r*, *pomc*, and *agrp* genes in appetite, feed intake, and fasting in Atlantic salmon.

MATERIALS AND METHODS

Ethics Statement

The animal experiments were carried out in accordance with Norwegian Animal Research Authority regulations and approved by the local representative of Animal Welfare at the Department of Biological Sciences, University of Bergen, Norway.

Experimental Design

In this study, Atlantic salmon post smolt of ca. 250 g were obtained from Engesund fish farm (Fitjar, Norway) and randomly distributed into two 2,000-L tanks (48 fish per tank) at the Industrial Lab (ILAB) in Bergen (Norway). Fish were reared in tanks supplied with flow through seawater (27 ppt; 16 L/min) at 10°C and oxygen saturation above 80%. Constant light (LD 24:0) was provided in accordance to common practice in commercial aquaculture to promote optimal growth and to inhibit unwanted sexual maturation (Hansen et al., 1992; Endal et al., 2000; Nordgarden et al., 2003; Fjelldal et al., 2012). Fish were fed continuously with commercial dry feed pellets (Biomar intro 75 HH 50 mg Q) using an automatic feeder. Oxygen saturation, temperature, and salinity were measured daily, and the fish were acclimatized for 3 weeks. After the acclimation period, the two tanks were randomly labeled into two experimental groups, fed, and fasted. Thereafter, 21 fish per tank (263 ± 13.06 g and 275.7 ± 15.68 g) were sampled as a baseline control. Next, one tank was kept under continuous feeding (fed group) with the same commercial dry pellet, whereas the other tank was fasted for 4 days (fasted group). After the 4 days, 27 fish were sampled from the fed group (280 ± 12.69 g) and 26 from the fasted group (246 ± 12.88 g). One fish was excluded from this group due to previous mild winter sore mark. Fish from the fed and the fasted group were collected and euthanized using an overdose of 200 mg/L of MS222 (Tricaine methanesulfonate, Scan-Vacc, Hvam, Norway) before and after the 4 days fed/fasted, respectively. Length and weight were recorded. The whole brains were rapidly collected and transferred into RNAlater solution (Invitrogen, Carlsbad, CA, United States), kept at 4°C overnight, and then stored in −80°C until further analysis.

Condition Factor (*K*) Calculation

Condition factor (*K*) was used to analyze the fitness of the fish before (fed $n = 21$; fasted $n = 21$) and after (fed $n = 27$; fasted $n = 26$) the feeding experiment by using weight and length of the fish in the following equation:

$$K = 100\frac{W}{L^3}$$

where W is the weight (g) and L is the length of the fish (cm) (Froese, 2006).

Structural Analysis and Phylogenetic Comparison of Mc4r and Pomc in the Salmonidae Family

Mc4r and Pomc peptide sequences of 17 species representatives of ray-finned fishes (Actinopterygiian) were retrieved from NCBI GenBank[1] and Ensembl[2]: the Lepisosteidae spotted gar (*Lepisosteus oculatus*) as a species before the teleost specific WGD (Ts WGD) (around 320 mya) (Lien et al., 2016), the Osteoglossidae Asian arowana (*Scleropages formosus*) as one of the oldest teleost groups; three Cyprinidae, including goldfish and common carp as species that went through a very recent 4R WGD and zebrafish which did not; one Characidae, cave fish; seven Salmonidae species, including Atlantic salmon, rainbow trout, chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), coho salmon, arctic char, and brown trout (*Salmo trutta*); the Esocidae northern pike (*Esox lucius*) as a sister group of salmonids that diverged before the Ss 4R WGD; and three Neoteleostei Atlantic cod (*Gadus morhua*), medaka (*Oryzias latipes*), and stickleback (*Gasterosteus aculeatus*) (**Supplementary Figures 2** and **3**). The human amino acid (AA) sequences were also included in the analysis. Multiple alignments were generated using MUSCLE from MEGAX (Hall, 2013) and edited using GeneDoc 2.7 software (Nicholas et al., 1997).

Mc4r transmembrane domains/helices (TMHI-TMHVII), extracellular loops (ECL1-ECL3), and intracellular loops (ICL1-ICL3) were retrieved from UniProt[3] database. Potential cleavage sites of Pomc precursor were acquired using UniProt and ProP 1.0[4] by using the full-length AA sequences.

The phylogenetic trees were predicted using the maximum likelihood (ML) method and based on the predicted full-length AA sequence of Mc4r and Pomc. The substitution model used in the phylogenetic analysis was determined by using the best-fit substitution model suggested by MEGAX. A Jones Taylor Thornton (JTT) and gamma distributed (G)

[1]https://www.ncbi.nlm.nih.gov/

[2]http://www.ensembl.org/index.html

[3]https://www.uniprot.org/

[4]http://www.cbs.dtu.dk/services/ProP/

matrix-based model was used to produce the phylogenetic tree for Mc4r, while a JTT and G with invariant sites (I) matrix-based model was used for Pomc (Hall, 2013). Tertiary protein structures of Atlantic salmon Mc4r were predicted using the IntFOLD5 (McGuffin et al., 2019) and human MC4R structure with AGRP (PDB entry 2IQV) was retrieved from UniProt. The images were edited, and disulfide bonds were predicted by PyMOL Molecular Graphics System v 2.3.[5] Searches for Agrp in Atlantic salmon genomic database did not identify any novel paralogs in addition to the ones previously published by Murashita et al. (2009).

Brain Dissection

The Atlantic salmon brain of fed (n = 6) and fasted group (n = 6) was randomly selected and dissected into eight regions for appetite gene expression analysis: olfactory bulb, telencephalon, midbrain, cerebellum, hypothalamus, saccus vasculosus, pituitary, and medulla oblongata/brain stem. To ensure high RNA yield and quality, the brain was placed on an ice block during dissection under a zoom stereomicroscope (Olympus SZ51) and cleaned from blood vessels. The pineal gland, olfactory bulb, telencephalon, brain stem, cerebellum, saccus vasculosus, hypothalamus, and midbrain were separated in this order (**Figure 4A**).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from each section of the brains by using TRI Reagent (Sigma-Aldrich, MO, United States) following the manufacturer's protocol. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, United States) and a 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, CA, United States) were used to assess the quantity and the quality of the extracted total RNA, respectively. To avoid any remnants of genomic DNA, 5 or 10 μg of total RNA was treated with TURBO DNase-free Kit (Ambion Applied Biosystems, CA, United States) with 1 μl of DNase (2 Units/μl) in 10 or 30 μl reaction volume. The amount of total RNA and the reaction volume for DNase treatment was adjusted depending on the amount of total RNA availability per region. First-strand cDNA was synthesized from 2 μg of the total RNA sample using SuperScript III Reverse Transcriptase (Invitrogen, CA, United States) and Oligo(dT)$_{20}$ (50 μM) primers in a total reaction volume of 20 μl.

Quantitative RT-PCR Setup and Primer Design

The salmon mRNA of *mc4r* (*a1*, *a2*, *b1*, and *b2*), *pomc* (*a1*, *a2*, and *b*), and *agrp* (*1* and *2*) was quantified by real-time quantitative RT-PCR (qPCR). The qPCR primers were designed from Atlantic salmon gene sequences retrieved from GenBank database (**Table 1** for accession numbers information). For each gene paralog, primer pairs were designed using Primer3[6] and/or NCBI primer designing tool and synthesized by Sigma-Aldrich (St. Louis, MO, United States). The specific primers were designed spanning exon–exon junctions when possible. All primers were analyzed for quantitation cycle (Cq), primers efficiency (E), and melting peaks. All qPCR products were analyzed in a 2% agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and cloned into a pCR4-TOPO vector (Thermo fisher, Scientific, Waltham, MA, United States). Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway), and their identity was confirmed using blastn analysis against the Atlantic salmon genome database.

Quantitative RT-PCR

To quantify the absolute mRNA abundance for each gene, qPCR products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and used to generate a standard curve using a 10-fold dilution series (initial concentration 10^{10} number of copies).

qPCR was carried out using 10 μl of iTaq Universal SYBR Green supermix (Bio-Rad, CA, United States), 0.6 μl of forward and 0.6 μl of reverse primers each (10 μM), 6.8 ultrapure water (Biochrom, Berlin, Germany), and 2 μl cDNA template (40 or 50 ng/reaction).

All reactions were run in duplicate, and a non-template control, no-reverse transcriptase control, and a positive between plate controls were always included. The following RT-PCR protocol was performed: (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 a range of 65°C–95°C (increment of 0.5°C for 2 s) allowed the detection of non-specific products and/or primer dimers. The qPCR was performed using 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).

Subsequently, the absolute mRNA expression level for each gene was determined based on the respective standard curve using the following equation:

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

The copy number was normalized using the total ng of RNA used for each target gene.

Statistical Analysis

All statistical analyses were performed using GraphPad (GraphPad Software, version 8). Data related to the K were analyzed by two-way ANOVA followed by Sidak posttest. Equal variances and normality of distribution of gene expression were assessed using F-test and Shapiro–Wilk normality test. To achieve normal distribution, data were log-transformed and the analysis of differential expression between the fed and fasted groups was performed with two-tailed t-test. When either the F-test or the normality test failed, the no-parametric Mann–Whitney test was performed. Two-way analysis of variance (ANOVA) followed by the Sidak *post hoc* test was used to examine differences in the expression within the brain regions and the two treatment groups. A $p < 0.05$ was considered

[5]https://pymol.org/2/

[6]http://primer3.ut.ee/

TABLE 1 | Primers sequences used for quantitative RT-PCR (qPCR) mRNA expression analysis in Atlantic salmon.

Gene	Gene Bank ID	Primer Sequence (5′ → 3′)	Amplicon size (bp)	R^2	Efficiency %
pomca1	NM_001198575.1	ATACTTTTGAAACAGCGTGACGA	108	0.9985	103
		CAACGAGGATTCTCCCAGCA			
pomca2	NM_001198576.1	TTTGGCGACAGGCGAAGATG	91	0.999	98
	AB462420.1	TCCCAGCACTGACCTTTCAC			
pomcb	NM_001128604.1	CAGAGGACAAGATCCTGGAGTG	182	0.995	89
		TTTGTCGCTGTGGGACTCAG			
agrp1	NM_001146677.1	ATGGTCATCTCAGTATTCCCAT	152	0.9998	96
	XM_014182676.1	AGAGAGCCTTTACCGATATCTG			
	XM_014182677.1				
agrp2	NM_001146678.1	TGTTTCGCCGAAGACCTGAA	142	0.9986	101
		GTTTCTGAAATGCAACGTGGTG			
mc4ra1	XM_014140480.1	GTCATCGCCGCCATCATTAAG	152	0.9997	95
	XM_014140481.1	CCAATCCCCAGATTTCCGTC			
	XM_014140482.1				
mc4ra2	XM_014190362.1	TGGCAACTTGGGTATCGGC	170	0.9995	98
		GGCGCACGGTCATAATGTTG			
mc4rb1	XM_014157590.1	GGCGGTAATCGTGTGCATCT	185	0.9997	95
		GCACGGCGATCCTCTTTATG			
mc4rb2	XM_014180569.1	GAGCTCCCCGGGAAATAGTG	153	0.9996	97
		AGTGCAAATCAGTCCTCACCA			

Primer sequences, amplicon sizes (bp), R^2, and qPCR efficiency (in %) are indicated for each primer pair. agrp, agouti-related protein; mc4r, melanocortin-4 receptor; pomc, proopiomelanocortin.

significant. All data are presented as mean ± SEM, unless otherwise stated.

RESULTS

Characterization of Mc4r in Atlantic Salmon and Phylogenetic Analysis

In salmonids, four Mc4r protein paralogs (**Supplementary Figure 2**) were identified, showing well-conserved domains with respect to other ortholog sequences within teleost. In Atlantic salmon, Mc4r paralogs were found to be encoded by genes located on chromosomes ss03 (Mc4ra2), ssa14 (Mc4ra1), ssa19 (Mc4rb1), and ssa29 (Mc4rb2). The predicted AA sequence of Atlantic salmon Mc4r varied from 333 to 339 AA in length, and protein weighed from 37.37 to 37.99 kDa (data retrieved from UniProt). The paralogs Mc4ra1 and a2, and paralogs Mc4rb1 and b2, shared 89% identity at the AA level, whereas Mc4ra and Mc4rb shared at least 73% of identity. All four paralogs are relatively well-conserved with the human homolog, sharing from 63 to 68% of AA sequence identity. Atlantic salmon Mc4r paralogs shared from 73 to 90% AA identity with northern pike Mc4r and 73 to 95% of identity with other salmonid species (**Supplementary Table 3**). In the phylogenetic analysis, the teleost Mc4r divided into two clades and the Atlantic salmon Mc4r paralogs clustered into four different groups (**Figure 1**), each containing species belonging to the Salmonidae family. Each cluster, except for the Mc4rb1, branches from the northern pike. According to our phylogenetic analysis, two Mc4r duplicates (Mc4ra and Mc4rb) are present in salmonids and northern pike. In addition, salmonids have two copies of Mc4ra (Mc4ra1, Mc4ra2) and two copies of Mc4rb (Mc4rb1 and Mc4rb2) possibly as a result of the Ss 4R WGD. The alignment of human MC4R and Atlantic salmon Mc4r showed well-conserved seven transmembrane domains with divergent AA residues at N-terminus, ECL1, and C-terminus (**Figures 2**, **3**). Two of three N-terminal asparagine (N) N-glycosylated sites (NxS/T) are also conserved in Atlantic salmon (**Figure 2**). Further, the C-terminal palmitoylation site cysteine (Cys) residue Cys318 in human MC4R is conserved in the Atlantic salmon Mc4ra1 and a2. Importantly, motif DPxIY and C-terminal motif $E(x)_7LL$ for G Protein-Coupled Receptors (GPCRs) [reviewed in Rodrigues et al. (2013)] are conserved in all salmon paralogs. There are two putative disulfide bonds in human MC4R, one between Cys271 (TMHVI) and Cys277 (ECL3) and another between Cys40 (N-terminus) and Cys271 (ECL3). Our predicted model showed one disulfide bond within the ECL3 for Mc4ra1 (Cys274 and Cys280) and Mc4rb2 (Cys275 and Cys281) in Atlantic salmon (**Supplementary Figure 5**).

Characterization of Mature Pomc in Atlantic Salmon and Phylogenetic Analysis

In Atlantic salmon, three previously identified Pomc protein paralogs were located in chromosomes ssa01 (Pomca2), ssa06 (Pomcb), and ssa09 (Pomca1). The predicted AA sequence length of Atlantic salmon Pomc varied from 225 to 232 AA and protein weight from 24.7 to 25.9 kDa (data retrieved from UniProt). Pomca1 and a2 shared 84% of AA identity

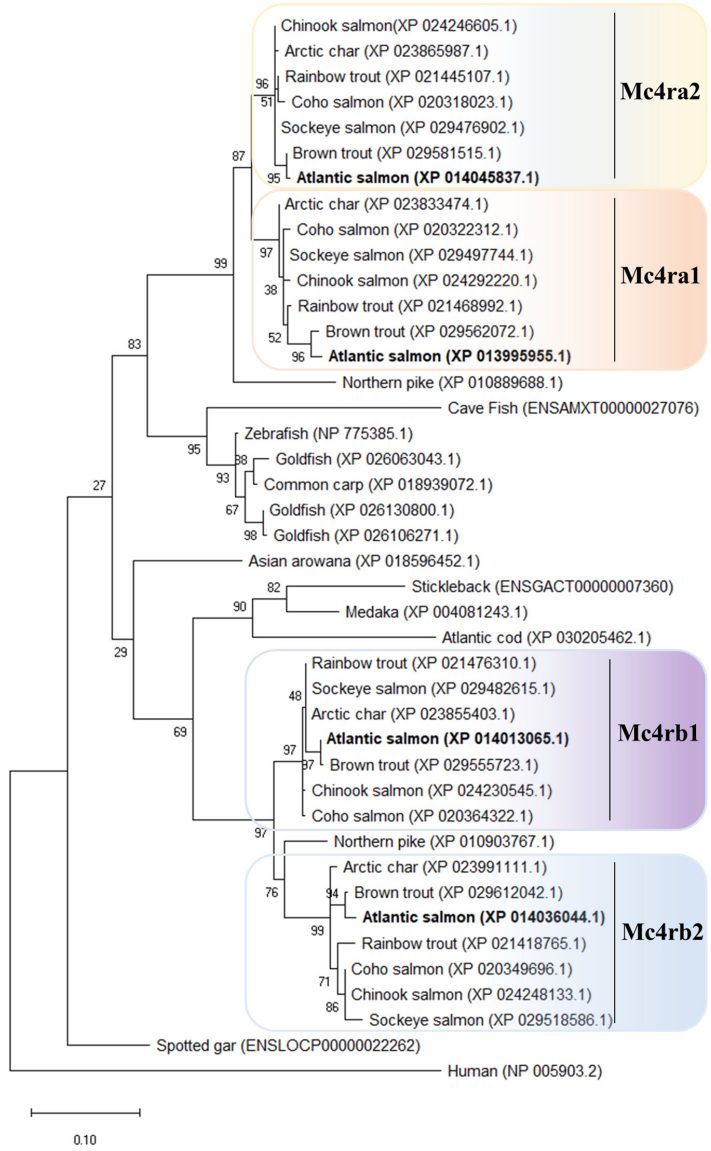

FIGURE 1 | Phylogenetic relationship of melanocortin-4 receptor (Mc4r) in Salmonidae family. The phylogenetic tree was constructed based on the predicted full-length peptide sequences using the maximum likelihood (ML) method, 1,000 bootstraps replicates, and JTT + G matrix-based model in MEGA X. The tree with the highest log likelihood (–3598.40) is shown. Protein ID accession numbers are shown after the species name. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Phylogenetic tree is rooted to the human MC4R sequence. For additional information related to the protein sequence alignment, please refer to **Supplementary Figure 2**.

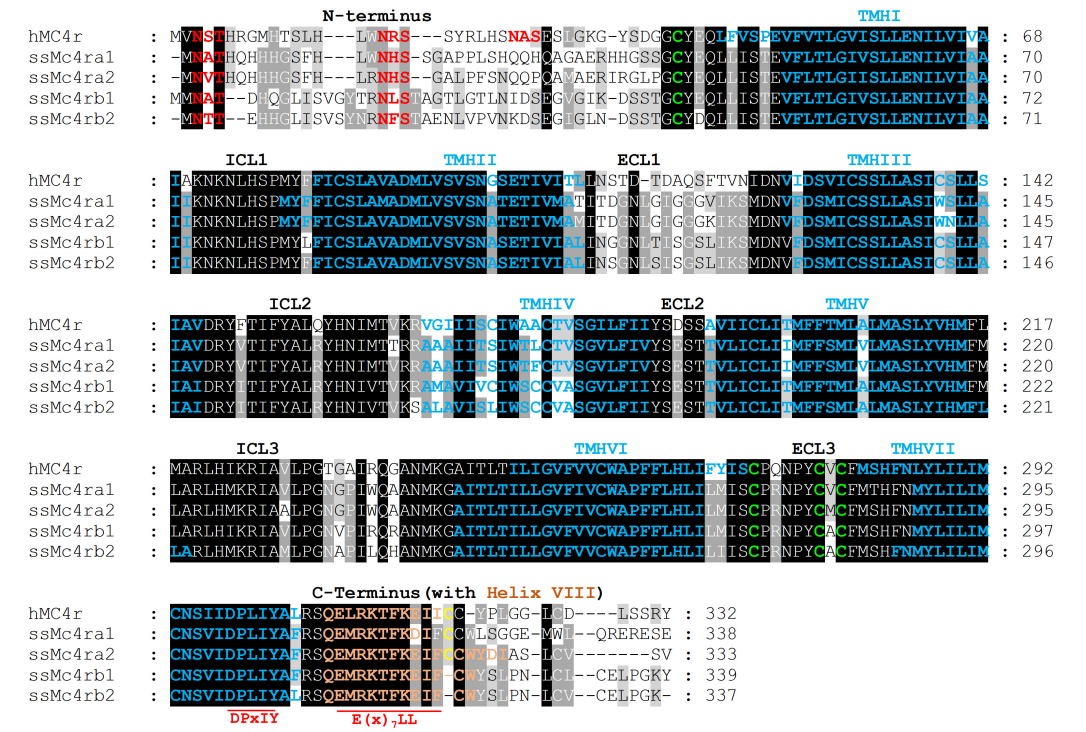

FIGURE 2 | Primary protein sequence alignment of the human melanocortin-4 receptor (hMC4R) and the Atlantic salmon paralogs (ssMc4ra1, a2, b1, and b2). The transmembrane domains for hMC4R (as reviewed in UniProt) and ssMc4r (as predicted in UniProt) are marked in blue. The N-terminal, extracellular loops (ECLs) 1–3, intracellular loops (ICLs) 1 to 3, and C-terminus (with helix VIII) are also shown. The N-terminal glycosylated amino acid residues and the important conserved motifs of GPCRs are marked in red (Rodrigues et al., 2013). C-terminal palmitoylation Cys is shown in yellow. The Cys involved in the disulfide bonds in hMC4R and those conserved in ssMc4r are in green.

(**Supplementary Table 4**). Pomcb AA sequence shared 36% of identity with Pomca1 and a2. Salmon Pomc paralogs shared 27–37% AA sequence identity with the human Pomc and 31–51% with northern pike Pomc. Atlantic salmon Pomc shared from 32 to 98% of AA identity with other species from the Salmonidae family. Atlantic salmon Pomc, as the human homolog, has a signal peptide of 26 AA, with the exception of Pomcb, which has a 21-AA signal peptide (**Supplementary Figure 3**). As expected, the phylogenetic analyses divided the salmonid Pomc peptide sequences in two main clusters Pomca and Pomcb (**Supplementary Figure 4**). The salmonid Pomca and Pomcb clustered with the northern pike and the Neoteleostei Pomca and Pomcb sequences, respectively. The salmonids have two copies of Pomca, whereas common carp and goldfish have duplicate Pomcs that belong to each of two separate clades. The phylogenetic tree suggests that the duplicated *pomc* would have evolved from Ts WGD.

The predicted posttranslational cleavages sites in Atlantic salmon were determined taking a comparative homology approach using the human homolog protein (**Supplementary Figure 4**). The human KR, KRR, and KK cleavage sites lead Pomc into mature peptide hormones: α-, β-, and γ-MSH, ACTH, corticotropin-like intermediate peptide (CLIP), β- and γ-LPH (lipotropin), β-endorphin, and INN (Met-enkephalin). In the teleost species analyzed, the same potential KR, KK, and RR cleavage sites were present in Pomca1 and a2, while in Pomcb, the last KK cleavage site was not present. Moreover, the alignment confirmed the lack of γ-MSH in teleost compared to human.

Brain Distribution of Atlantic Salmon *mc4r*, *pomc*, and *agrp* mRNA

Both the melanocortin receptor *mc4r* and the neuropeptides *pomc* and *agrp* mRNA analyzed in this study showed a wide distribution in the eight brain regions (**Figures 4**, **5**). All Atlantic salmon *mc4r* genes showed high mRNA expression levels in the hypothalamus, whereas *mc4ra1* was more abundant in the telencephalon (**Figure 4**). Interestingly, *mc4ra2* and *mc4rb1* showed a predominant mRNA abundance in the

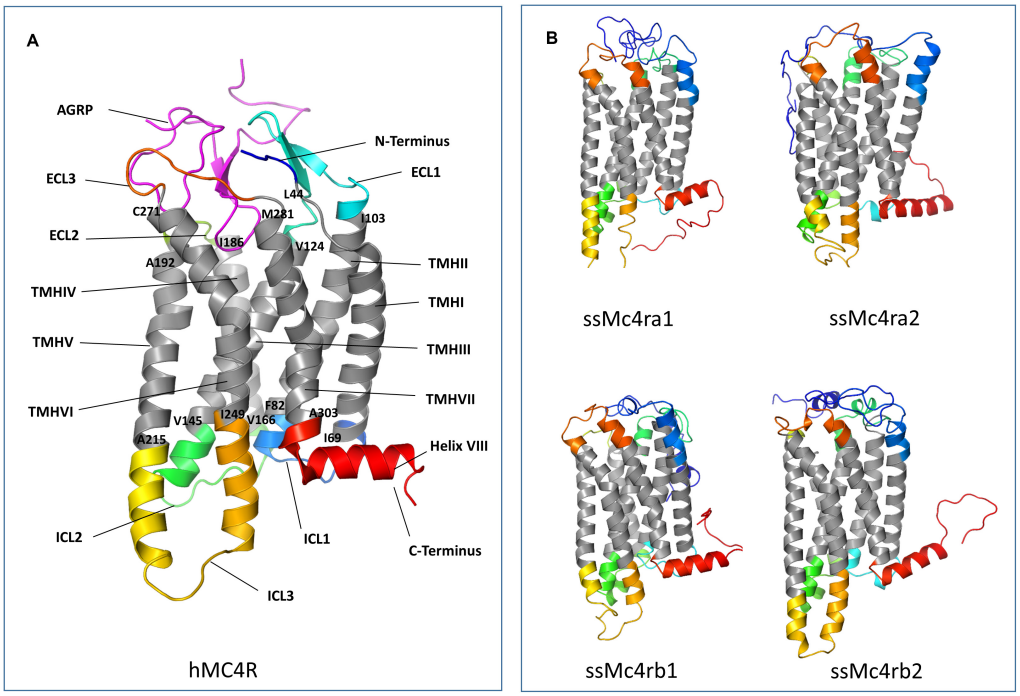

FIGURE 3 | Tertiary structure of human melanocortin-4 receptor (MC4R) and Atlantic salmon Mc4r paralogs. Three-dimensional (3D) protein structures were obtained from IntFOLD and edited in the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC. **(A)** Human MC4R 3D structure with agouti-related protein (AGRP) (PDB entry 2IQV). **(B)** Atlantic salmon Mc4r paralogs 3D structure. N-terminal domains are colored in dark blue, C-terminal domains in red, the seven transmembrane helices (TMH) in gray. The extracellular (ECL) and intracellular (ICL) loops are represented by different colors. The boundary amino acids of TMH are labeled according to the protein sequence alignment.

telencephalon and hypothalamus, and *mc4rb2* was high in the hypothalamus and similar expression level in other regions. The *mc4rb1* was the most abundant paralog in the Atlantic salmon brain. All *mc4r* paralogs show low mRNA expression levels in the olfactory bulb, cerebellum, and saccus vasculosus.

The *pomca1*, *pomca2*, and *pomcb* were predominantly expressed in the pituitary, followed by hypothalamus and saccus vasculosus, but low levels of expression were also found in other brain regions (**Figure 5**). *Agrp1* showed a prevalence gene expression in the hypothalamus, pituitary, and saccus vasculosus (**Figure 5**). On the contrary, *agrp2* was mainly expressed in the telencephalon, saccus vasculosus, and olfactory bulb.

Effects of 4 Days of Fasting in Atlantic Salmon

The *K* factor of fed and fasted Atlantic salmon was significantly different (two-way ANOVA $p = 0.0010$) (**Supplementary Figure 1**). After 4 days of fasting, fish showed a significantly lower *K* factor (1.035 ± 0.011) than the fed group (1.095 ± 0.015) (Sidak posttest $p = 0.0027$) (**Supplementary Table 1**).

No significant differences in the mRNA expressions of *mc4r*, *pomc*, and *agrp* paralogs were observed between the fed and fasted groups (**Figures 4**, **5**) in any highly expressed brain regions, i.e., hypothalamus, telencephalon, pituitary, and saccus vasculosus. On the other side, 4 days of fasting had a significant effect on the expression of *pomca1*, *pomca2*, and *pomcb* (**Figure 5**) in very low expressed regions (**Supplementary Table 2**). A significant decrease was found in the expression of *pomca1* in the olfactory bulb (*t*-test $p = 0.0057$), of *pomca2* in the telencephalon (*t*-test $p = 0.0233$) and cerebellum (*t*-test $p = 0.0340$), and on *pomcb* in the telencephalon (Mann–Whitney $p = 0.0303$). *pomca1* showed a decreased tendency in the telencephalon of the fasted group, although not statistically significant (*t*-test $p = 0.0873$). Both *agrp1* and *agrp2* did not show any significant difference between the fed and fasted groups (**Figure 5**). A high individual variation was observed in the mRNA expression levels of the target genes analyzed.

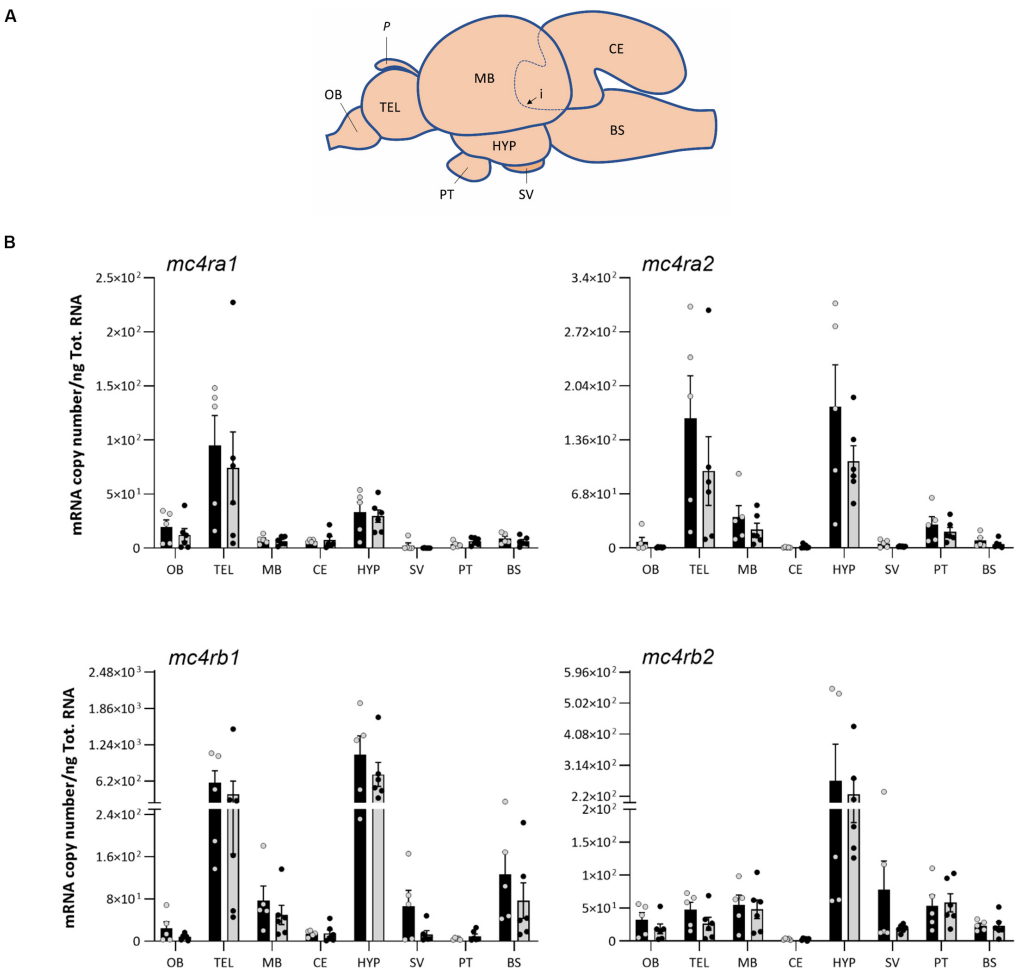

FIGURE 4 | (A) Schematic representation of Atlantic salmon post smolt brain, showing dissection of the eight brain regions for gene expression analysis: olfactory bulb (OB), telencephalon (TEL), midbrain (MB), hypothalamus (HYP), cerebellum (CE), saccus vasculosus (SV), pituitary (PT), and brain stem (BS). Dashed line (i) represents the dissected area of the CE inside the MB, and P represents the pineal gland. **(B)** Effects of 4 days of fasting on the mRNA expression levels of *melanocortin-4 receptor* (*mc4r*) paralog genes in eight regions of Atlantic salmon brain. Black and gray columns represent fed (n = 6) and fasted (n = 6) fish, respectively. Values are expressed as copy number per total RNA used in the reaction. The dots represent the individual fish, and bars represent mean ± SEM. Two-tailed t-test was performed to assess the statistically significant differences between the two groups. Interaction between the brain region response and the treatment was analyzed with two-way ANOVA (**Supplementary Table 1** for detailed information).

DISCUSSION

The role of the melanocortin system on appetite and energy homeostasis in Atlantic salmon is still poorly understood. In addition, the presence of several paralog genes as a result of the Ss 4R WGD has led to possible divergent functions of the key genes involved in this system.

In the current study, we report for the first time the identification and characterization of the Atlantic salmon receptor Mc4r. Our *in silico* analysis revealed the presence of four paralog genes *mc4ra1*, *mc4ra2*, *mc4rb1*, and *mc4rb2*, clustered into four different groups in all salmonid species analyzed. The presence of four Mc4r in Atlantic salmon (and other salmonids) appears to be the result of the Ss 4R WGD. The presence of *mc4ra* and *mc4rb* homologs in Northern pike suggests that the origin of

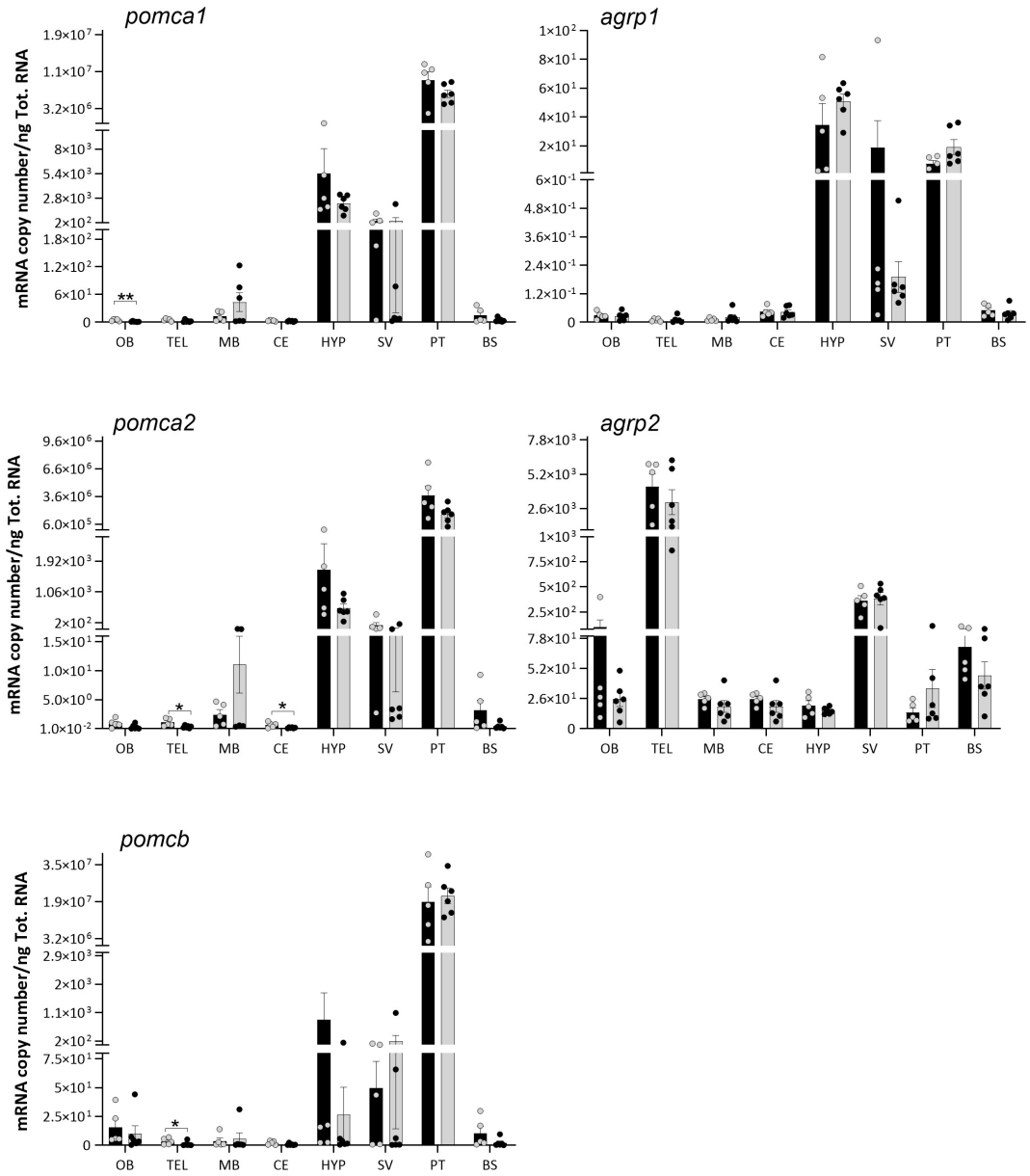

FIGURE 5 | Effects of 4 days of fasting on the mRNA expression levels of *proopiomelanocortin* (*pomc*) and *agouti-related protein* (*agrp*) paralog genes in eight regions of Atlantic salmon brain. Black and gray columns represent fed ($n = 6$) and fasted ($n = 6$) fish, respectively. Values are expressed as copy number per total RNA used in the reaction. The dots represent individual fish, bars represent mean ± SEM, and asterisks show the significant degree (*$p < 0.05$, **$p < 0.01$). Two-tailed *t*-test was performed to assess the statistically significant differences between the two groups. Interaction between the brain region response and the treatment was analyzed with two-way ANOVA, followed by Sidak posttest (refer to **Supplementary Table 1** for detailed information).

these genes occurred either just prior to the divergence between Salmoniformes and Esociformes or it is a result of an independent species-specific duplication. Cypriniformes, such as common carp and goldfish, have also experienced additional 4RWGD around 50–16 mya subsequent to the Ts WGD and Ss 4R WGD. Consequently, we found three Mc4r in goldfish but only one Mc4r in common carp (Larhammar and Risinger, 1994; David et al., 2003). Moreover, the Cypriniformes Mc4r is distantly related to the other analyzed teleost, which is reflected in the separated clade and seems to be the Mc4ra type. The confirmed mRNA sequences of the qPCR amplicons indicated that all four salmon *mc4r* genes are not pseudogenes. The Atlantic salmon showed well-conserved seven hydrophobic transmembrane domains as well as one putative disulfide bond, within the ECL3 for Mc4ra1 (Cys274 and Cys280) and Mc4rb2 (Cys275 and Cys281), as described for human MC4R (Cys271 and Cys277) (Chai et al., 2005; Chapman et al., 2010; Heyder et al., 2019). Even though Cys are also present in the primary sequence of Mc4ra2 and Mc4rb1, a disulfide bond was not present in the predicted tertiary structure in PyMOL. Natural mutation occurring in the human Cys271 (C271R and C271Y) have been linked to severe MC4R functional changes, but this AA substitution was not found in the predicted Atlantic salmon Mc4r sequence. Further analysis is needed to investigate these aspects in this species. The human N-glycosylated site (NxS/T) located in the N-terminus is also present in Atlantic salmon Mc4r paralogs. The glycosylated site and the disulfide bonds are important for the receptor structure folding, stability, and target trafficking (Chai et al., 2005; Tao, 2010; Rodrigues et al., 2013). Furthermore, the palmitoylation site at the Cys residue Cys318 of the C-terminus in human MC4R is also present in the Atlantic salmon Mc4ra1 and a2. The conserved C-terminal Cys318 serving as palmitoylation site might possibly lead to a fourth intracellular loop by anchoring the C-terminus to the cell membrane [reviewed in Tao (2010)]. Importantly, GPCR motifs N/DPxIY and $E(x)_7LL$ [reviewed in Rodrigues et al. (2013)] are also present in all Mc4r salmon paralogs. The N/DPxIY motif acts as an on/off switch with two conformational changes according to the active and inactive states (Chapman et al., 2010; Rodrigues et al., 2013), whereas $E(x)_7LL$ motif seems to be important in anterograde trafficking of MCRs (from endoplasmic reticulum to cell surface) [reviewed by Rodrigues et al. (2013)].

In general, for human MC4Rs, the pocket of aspartic acid Asp122/126 in TMHIII and basic histidine (His) 264 residues in TMHVI (Metz et al., 2006; Chapman et al., 2010; Wen et al., 2017; Heyder et al., 2019) along with ECL2 and ECL3 (Tao, 2010) are essential for ligand binding. β-MSH has been shown to the have the highest affinity to human MC4R, followed by α-MSH and ACTH (Tao, 2010). The same pocket seems to be conserved in Atlantic salmon; however, future studies are necessary to explore the ligand–Mc4r interactions in this species.

In the mammalian hypothalamus, numerous interconnecting nuclei, as the arcuate nucleus (ARC), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), and lateral hypothalamus (LH), are organized into a complex neuronal network that plays a crucial role in the central control of appetite (Bouret et al., 2004; Rønnestad et al., 2017; Soengas et al., 2018). The ARC has been described as the location for neurons expressing POMC and AGRP that project to the hypothalamic PVN where the MC4R is located (Ghamari-Langroudi et al., 2011; Hall, 2011). In teleost, the lateral tuberal nucleus (NLT) in the hypothalamus has been described as the homolog of the mammalian ARC (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003a,b). In goldfish and spotted sea bass, *in situ* hybridization showed neurons expressing *mc4r* in the telencephalon, thalamus, preoptic area, and hypothalamus (NLT and hypothalamic inferior lobe) (Cerdá-Reverter et al., 2003a; Zhang et al., 2019). Similarly, *agrp* and *pomc* were found in the NLT and in rostral hypothalamus of gold fish and rainbow trout (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003b; Otero-Rodiño et al., 2019).

Our results showed that all the Atlantic salmon *mc4r* paralogs were predominantly expressed in the hypothalamus and telencephalon, even though to a lesser extent expressed in other regions of the brain. It seems therefore that the hypothalamus and telencephalon are the major functional sites for the central *mc4r* in Atlantic salmon, but their role in appetite regulation is still unclear. These results are in line with the study of Zhang et al. (2019), where high *mc4r* levels were detected in the telencephalon and diencephalon of spotted sea bass. Among the Atlantic salmon *mc4r* paralogs, *mc4rb1* had the highest levels of expression, particularly in the hypothalamus, but its role in appetite regulation it is still unclear.

In this study, we have extended the current knowledge on Atlantic salmon *pomc* and *agrp* on appetite regulation, which was previously based on the analysis of the whole brain (Murashita et al., 2009, 2011) or on the hypothalamus (Kalananthan et al., 2020). Murashita et al. (2011, 2009) identified and characterized three *pomc* gene paralogs (*pomca1*, *a2*, and *b*) and one splice variant (*pomca2s*) and two *agrp* paralogs (*agrp1* and *2*). In goldfish, *in situ* hybridization studies demonstrated *pomc* mRNA cell bodies exclusively expressed within the mediobasal hypothalamus, in the NLT, and in the medial region of the lateral recess nucleus (Cerdá-Reverter et al., 2003b). In our spatial analysis, we found a clear dominant expression of *pomca1, pomca2,* and *pomcb* in the pituitary, followed by the hypothalamus and saccus vasculosus. The pituitary is an important site of *pomc* expression, where it is further post-translated into ACTH and α-MSH, responsible for the biosynthesis of glucocorticoids (e.g., cortisol) from the adrenal cortex (Dunn and Berridge, 1990). On the other hand, α-MSH in the hypothalamus activates the MC4R, leading to reduced food intake and increased energy expenditure (Anderson et al., 2016). The α-MSH has been described as the most well-conserved posttranslated forms of Pomc, underlining the strong functional constraint along the vertebrate lineage (Takahashi and Kawauchi, 2006).

The *agrp* paralogs showed different spatial distributions in the brain of Atlantic salmon. The *agrp1* was mainly expressed in the hypothalamus, as also described in previous studies in the ventral neurons of the NLT and rostral hypothalamus in goldfish (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2003a), sea bream (Koch et al., 2019), and rainbow trout (Otero-Rodiño et al., 2019). The *agrp1* mRNA was also detected in other regions of the brain as saccus vasculosus and pituitary.

Agrp2 showed high expression levels in telencephalon and saccus vasculosus. Recently, Agrp1 was reported to be involved in the control of food consumption in zebrafish, while the Agrp2 in the preoptic neurons was suggested to act as a neuroendocrine regulator of stress response by downregulating cortisol secretion (Shainer et al., 2019).

The role of AGRP, POMC, and MC4R on appetite regulation have been suggested to be evolutionarily conserved across vertebrates (Ghamari-Langroudi et al., 2011; Kim et al., 2014; Nuzzaci et al., 2015). However, in the present study, no significant differences in mRNA expression of *mc4r*, *agrp*, or *pomc* paralogs between fed and fasted states were observed in the hypothalamus, which has been described as the central area in the control of appetite in mammals and teleost fishes (Nuzzaci et al., 2015; Volkoff, 2016; Rønnestad et al., 2017). Similar results were also observed in sea bass, where 4 days of food deprivation did not affect the *mc4r* expression in the hypothalamus or *pomc* mRNA expression in the hypothalamus and pituitary (Sánchez et al., 2009). Few studies have described that Mc4r was downregulated when feeding was restricted in teleost species (Wan et al., 2012; Aspiras et al., 2015; Striberny et al., 2015; Zhang et al., 2019). In barfin flounder, in contrast, the *mc4r* was downregulated in the liver, but no changes in expression were detected in the brain by fasting (Kobayashi et al., 2008). However, a previous study in sea bass showed the Mc4r activity to be dependent on the Agrp binding rather than the *mc4*r expression in case of progressive fasting (Sánchez et al., 2009).The importance of Mc4r in the regulation of appetite in fish is emphasized by naturally occurring mutations in Mexican cavefish (Aspiras et al., 2015). In this species, coding mutations in conserved residues reduces the signaling efficiency and basal activity of the Mc4r probably due to adaptation to long-term starvation and sporadic food availability (Aspiras et al., 2015).

On the other hand, 4 days of fasting had a significant effect on the expression of *pomca1*, *pomca2*, and *pomcb* in other regions of the Atlantic salmon brain. A significant decrease was found at the mRNA level of *pomca1* in the olfactory bulb, *pomca2* in the telencephalon and cerebellum, and *pomcb* in the telencephalon. However, it is important to underline that *pomc* was very lowly expressed in these regions, and it is not yet clear if these brain regions actually contribute to the appetite regulation in Atlantic salmon. In coho salmon, the posttranslational Pomc-derived α-Msh exhibits an anorexigenic effect (White et al., 2016). These authors showed that intraperitoneal injections of α-Msh suppressed feed intake, acting as an anorexigenic factor. However, in rainbow trout, 14 days of fasting did not have any effect on the mRNA expression of the three *pomc* paralogs (*pomca1*, *a2*, and *b*), but 28 days of fasting favored the decrease in hypothalamic *pomca1*, but not in *pomca2* or *pomcb* (Leder and Silverstein, 2006). Furthermore, hyperglycemic conditions increased the hypothalamic *pomca1* mRNA expression levels in rainbow trout (Conde-Sieira et al., 2010; Otero-Rodiño et al., 2015). In the whole-brain analysis of Atlantic salmon, the upregulation of *pomca1* (3 h post-feeding) and *pomcb* (0.5 and 6 h post-feeding) was suggested to represent a role in short-term feeding regulation (Valen et al., 2011).

In teleost, Agrp shows different functions depending on the region of the brain where it is expressed. In zebrafish, hypothalamic *agrp1* was proposed to have a similar function in the control of appetite and food intake as in mammals, whereas *agrp2* in the preoptic region acted as a stress regulator (Shainer et al., 2017, 2019). These authors also found *agrp2* to be expressed in the pineal and proposed that it may have a novel function rather than a neuroendocrine role involved in the regulation of the stress axis. An upregulation of Agrp was observed in short-term fasting of early larvae of Ya-fish (Wei et al., 2013). This was supported by Song et al. (2003) in zebrafish and Koch et al. (2019) in gilthead sea bream larvae, where starving increased *agrp1* expression. In another study, Agulleiro et al. (2014) reported the involvement of both *agrp* paralogs in appetite regulation by showing an increase of hypothalamic *agrp1* and decrease of *agrp2* in sea bass when subject to progressive fasting. In rainbow trout, Comesaña et al. (2017) showed that ICV of leucine decreased feed intake with a decrease in mRNA abundance of *agrp*. In Atlantic salmon, Murashita et al. (2009) reported that whole-brain *agrp1* was downregulated after 6 days of fasting, while *agrp2* was not affected, indicating that *agrp2* may not play a role on the control of appetite. A similar effect was described in common carp (Wan et al., 2012). In contrast, in our recent study, hypothalamic *agrp1* was upregulated after 3 days of fasting in Atlantic salmon (Kalananthan et al., 2020). However, in the current study, no differences were observed in the hypothalamus or other regions between the fed and fasted groups for either *agrp1* or *agrp2*.

The identification and basic characterization of the multiple paralogs of the appetite-regulating genes provide an essential groundwork to elucidate their functional role in the central control of food intake in Atlantic salmon. Minimal or no effects of fasting on the mRNA expression of the investigated genes suggest that they play a minor role in the central control of appetite in the short 4 days' fasting. However, a high individual variability was observed in both fed and fasted experimental groups, which might have led to a possible deviation in the results from our recent study on hypothalamic *agrp1* in Atlantic salmon (Kalananthan et al., 2020). Among the factors that contribute to the differences in feed intake, the feeding rate, frequency and time, and social relationships between conspecifics are the result of stimulating competition for a feed resource among individuals (Attia et al., 2012). Differences in physiology, life stages, feeding requirements, living environments, and individual variability might be at the base of the species-specific responses (Volkoff, 2016; Soengas et al., 2018). In salmonids as in other teleost species, the presence of dominant individuals can increase aggression and inhibition and limit food viability toward subordinate fishes, leading to differences in feeding behavior (Gilmour et al., 2005). Moreover, the difference in the sampling protocols and methodology used increases the complexity and variability of the data when comparing across and in between species.

The genome of teleost, compared to mammals, is the result of a third round (3R) or 4R (e.g., Salmonidae) of WGD. This evolutionary duplication make the teleost a potentially more complex model to study the function of feed-regulating factors

in comparison to the mammalian homologs (Volkoff, 2016). This fact should be taken into consideration when comparing studies in teleost *versus* mammals.

In conclusion, the present study provides new understanding of the still limited information available on the appetite regulation in Atlantic salmon. The identification of the multiple paralogs of *mc4r*, *pomc*, and *agrp* and their wide distribution in Atlantic salmon brain provide novel insights and lay the groundwork for experimental studies. Fasting did not affect the mRNA expression levels of melanocortin system players in the hypothalamus compared with fed fish. Further studies exploring the mRNA and/or protein localization within the brain areas and functional characterization are needed to elucidate the role of the melanocortin system in the central control of food intake in Atlantic salmon.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by local representative of Animal Welfare (at Department of Biological Sciences, University of Bergen, Norway) in accordance with Norwegian Animal Research Authority regulations.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the study, contributed to the writing of the manuscript, and read and approved the submitted version. TK, SH, IR, and FL contributed to the sampling. KM, FL, and AG did the preparatory lab work. TK and FL performed the qPCR analysis. IR made the schematic illustration of Atlantic salmon brain. TK performed the tertiary protein structures. FL did the statistical and phylogeny analyses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnana.2020.00048/full#supplementary-material

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II

frontiers in Physiology

BRIEF RESEARCH REPORT
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Hypothalamic *agrp* and *pomc* mRNA Responses to Gastrointestinal Fullness and Fasting in Atlantic Salmon (*Salmo salar*, L.)

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The orexigenic agouti-related protein (AgRP) and the anorexigenic pro-opiomelanocortin (POMC) are crucial players in the control of feed intake in vertebrates, yet their role in teleosts has not been fully established. Triplicate groups of Atlantic salmon (*Salmo salar*) post smolts were subjected to (1) fasting for 3 days (fast) and (2) normal feeding (fed), resulting in a significant ($p < 0.05$) upregulation of hypothalamic *agrp1* transcripts levels in the fast group. Moreover, the mRNA abundance of *agrp1* was significantly ($p < 0.05$) correlated with the stomach dry weight content. Corresponding inverse patterns were observed for *pomca2*, albeit not statistically significant. No significant differences were found for the other paralogues, *agrp2* and *pomca1* and *b,* between fed and fast groups. The significant correlation between stomach fullness and *agrp1* mRNA expression suggests a possible link between the stomach filling/distension and satiety signals. Our study indicates that hypothalamic *agrp1* acts as an orexigenic signal in Atlantic salmon.

Keywords: Atlantic salmon, hypothalamus, *agrp*, *pomc*, fullness, fasting, gastrointestinal tract

INTRODUCTION

Food intake and appetite are controlled by the integration of peripheral and central signals in the hypothalamus of vertebrates (Volkoff, 2016; Delgado et al., 2017; Rønnestad et al., 2017). Appetite-stimulating (orexigenic) and appetite-inhibiting (anorexigenic) factors are key drivers of feeding, and several studies have suggested that their functional role have been evolutionary conserved across vertebrates, including teleosts (Volkoff, 2016; Rønnestad et al., 2017; Soengas et al., 2018). In many teleost species, two agouti-related protein (*agrp*) paralogous genes (*agrp1* and *agrp2*) have been identified (Agulleiro et al., 2014; Shainer et al., 2019), including in Atlantic salmon (*Salmo salar*) (Murashita et al., 2009a). In addition, the orexigenic role of hypothalamic AgRP appears to be conserved in some of the teleost species studied, such as goldfish (*Carassius auratus*) (Cerda-Reverter and Peter, 2003), zebrafish (*Danio rerio*) (Song et al., 2003; Shainer et al., 2019), coho salmon (*Oncorhynchus kisutch*) (Kim et al., 2015), and gilthead seabream

(*Sparus aurata*) (Koch et al., 2018). However, in Atlantic salmon, previous results indicated that *agrp1* may have an anorexigenic effect based on analyses of the whole brain mRNA expression after 6 days of fasting, while *agrp2* had no effect on the control of appetite (Murashita et al., 2009a).

Proopiomelanocortin (POMC) is a precursor peptide which is post-transcriptionally cleaved into melanocyte-stimulating hormones (α-, β- and γ-MSH) and the adrenocorticotropic hormone (ACTH) (Castro and Morrison, 1997). In Atlantic salmon, three *pomc* paralogous genes (*pomca1*, *pomca2*, and *pomcb*) and one splice variant (*pomca2s*) have been previously identified and characterized (Murashita et al., 2011). In mammals, MSHs are involved in appetite control (Saneyasu et al., 2011), while in teleosts their functional role in appetite control remains to be clarified. For example, fasting did not change *pomc* expression in goldfish (Cerdá-Reverter et al., 2003), but intracerebroventricular administration of α-MSH showed an anorexigenic effect for this species (Matsuda et al., 2008; Kojima et al., 2010). In zebrafish, a cyprinid species as goldfish, it has been shown that *pomca* expression decreased in starved larvae (Liu et al., 2016). In salmonids, fasting has triggered a decrease in *pomca1* (but not *pomca2* or *pomcb*) expression in the hypothalamus of rainbow trout (Leder and Silverstein, 2006) and in whole brain of Atlantic salmon (Valen et al., 2011). These results are consistent with the anorexigenic role reported for mammals.

Signals from the gastrointestinal tract, such as sense of *fullness*, are important for appetite control and contribute to regulate food intake on a meal-to-meal basis (Sam et al., 2012). After a meal, the distension of the stomach and interactions between nutrients and the gut wall trigger the secretion of several peptide hormones, communicating the filling along with luminal nutrient status to the hypothalamus. This applies also to salmonids, as satiety signals from the gastrointestinal tract have a major impact on appetite (Grove et al., 1978). In rainbow trout, for instance, appetite returned (i.e., fish restarted feeding) when 80–90% of the stomach content from the previous meal was transferred downstream into the proximal gut (Ware, 1972).

In the Atlantic salmon aquaculture production, a period of fasting that lasts for 2–4 days is a common practice prior to handling, transportation, and harvest (Waagbø et al., 2017). This practice allows complete evacuation of the gut and an empty digestive tract, which minimizes impacts on fish welfare and ensures proper hygiene after harvest (Einen et al., 1998; Robb, 2008). Fasting also suppresses any postprandial elevation of metabolic rate, thereby permitting the fish to allocate more energy towards swimming and stress handling (Waagbø et al., 2017). Uncovering the impacts of these fasting periods on appetite and food intake control is therefore essential to optimizing their recovery. In this study, we investigated the effect of 3 days of fasting on hypothalamic *agrp* (*1* and *2*) and *pomc* (*a1, a2* and *b*) and explored the relationship between appetite and gastrointestinal filling.

MATERIALS AND METHODS

Ethical Treatment of Animals

The research and sampling were conducted in accordance with the Norwegian Animal Research Authority regulations and was approved by the local representative of Animal Welfare at the Department of Biological Sciences, University of Bergen (Norway).

Experimental Setup and Sampling

We obtained Atlantic salmon individuals from Bremnes Seashore's RAS facility (Trovåg, Norway). Fish were randomly assigned to tanks (5 fish per tank) and acclimatized to the experimental setting consisting of six freshwater indoor 150 L tanks and water temperature at 8.5°C. Continuous day light was used to mimic the standard commercial procedures and to stimulate optimal growth (Hansen et al., 1992). During the 18 days of acclimation period, all tanks were fed daily *ad libitum* from 9:00 to 16:00 h with commercial dry fish pellets (Biomar 3 mm) using automatic fish feeders.

To evaluate the effect of the fasting, 14 Atlantic salmon post smolts (average body weight 214.7 ± 61.7 g and length 26.8 ± 2.4 cm) were sampled from two groups that were either fed (sampled 2 h after feeding) or fasted for 3 days. In total, seven fish per group were sampled (2 or 3 fish per tank). Atlantic salmon were euthanized with a lethal dose of 200 mg/l of MS222 (Tricaine methanesulfonate, Sigma-Aldrich, MO, USA). The whole brain was removed from the skull, and the hypothalamus sampled and stored in RNAlater (Thermo Fisher Scientific, MA, USA). The Fulton's condition factor (*K*) was determined at the sampling time using the equation:

$$K = 100\left(\frac{W}{L^3}\right)$$

where, *W* is the weight (g) and *L* is the length of the fish (cm) (Froese, 2006).

Gastrointestinal Tract Compartments Filling

We dissected and carefully divided the gastrointestinal tract into three compartments (see **Supplementary Figure 1A**): stomach (ST), midgut (MG), and hindgut (HG), using surgical clamps to avoid loss or transfer of content between compartments. Next, each segment was emptied of food and digesta by gently stroking the content out onto pre-weighed pieces of aluminium foil. The weight of contents in each segment was first measured on a wet weight basis, and thereafter, dry weight was obtained after incubating in an oven at 110°C for at least 3 h, until it was completely dried.

mRNA Abundance Analysis by RT-qPCR

Total RNA was isolated from the hypothalamus using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Samples were treated with TURBO DNA-free (Thermo Fisher Scientific) to eliminate possible genomic DNA contamination. Quality of DNase treated total RNA was assessed on all samples using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). All samples had a RNA integrity number (RIN) equal or higher than 9 (scale 1–10). cDNA was synthesized from 1.0 μg of DNase treated total RNA using oligo (dT) primer from SuperScript III First-Strand Synthesis system for RT-PCR kit (Thermo Fisher Scientific).

Specific primers spanning an exon-exon junction were designed for all the target genes (**Table 1**). qPCR reactions were performed in duplicates using iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) in a 20 µl final reaction volume. The qPCR reactions were performed in a Bio-Rad CFX96™ Real-Time System with the following cycling conditions: 95°C for 30 s; 40 cycles

TABLE 1 | Sequence of the specific primers used for qPCR mRNA expression analysis. Primer sequences, amplicon sizes, R^2, and qPCR efficiency are indicated for each primer pair.

Gene	GenBank acc. no.	Sequence (5′ → 3′)	Amplicon (bp)	R^2	Efficiency (%)
agrp1	NM_001146677.1	F: ATGGTCATCTCAGTATTCCCAT R: AGAGAGCCTTTACCGATATCTG	152	0.9995	93
agrp2	NM_001146678.1	F: TGTTTCGCCGAAGACCTGAA R: GTTTCTGAAATGCAACGTGGTG	142	0.9997	98
pomca1	NM_001198575.1	F: ATACTTTTGAAACAGCGTGACGA R: CAACGAGGATTCTCCCAGCA	108	0.9997	101
pomca2	NM_001198576.1	F: TTTGGCGACAGGCGAAGATG R: TCCCAGCACTGACCTTTCAC	91	0.9949	94
pomcb	NM_001128604.1	F: CAGAGGACAAGATCCTGGAGTG R: TTTGTCGCTGTGGGACTCAG	182	0.9916	103

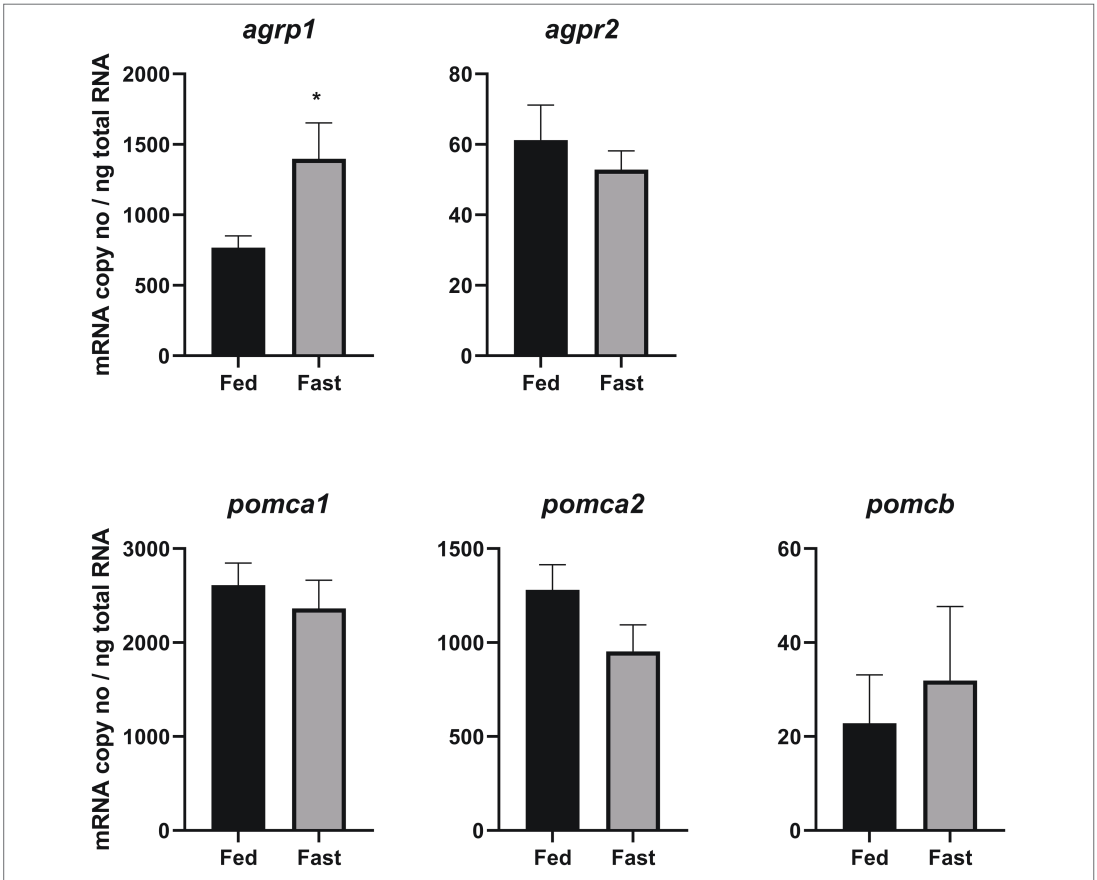

FIGURE 1 | Effects of 3 days of fasting on hypothalamic mRNA expression of *agrp1*, *agrp2*, *pomca1*, *pomca2*, and *pomcb*. Results are presented as mean ± SEM (n = 7). Asterisk (*) indicates statistically significant difference (p = 0.02). For detailed statistical information, refer to **Supplementary Table 1**.

of 95°C for 5 s, 60°C for 25 s. Melting curve analysis over a range of 65–95°C (increment of 0.5°C for 2 s) allowed for the detection of possible nonspecific products and/or primer dimers.

Standard curves relating initial template quantity to amplification cycle were generated from the target gene cloned into pCR4-TOPO vector (Thermo Fisher Scientific) using a 10-fold stepwise dilution series. The standard curves were used to determine the qPCR efficiency for each assay (**Table 1**). The copy number was determined for each gene/sample based on the respective standard curve, using the following equation:

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

Statistical Analysis

All mRNA expression data were tested for normality using Shapiro-Wilk W-test and subsequently log-transformed to ensure that it followed a normal distribution. Correlation analyses were conducted with generalized linear models (GLM) assuming a normal distribution. The effects of treatment (fed versus fast) on condition factor (*K*) and on the mRNA expression levels (copy numbers of each transcript) were evaluated (n = 7 fish per treatment group). It was also tested the effects of gastrointestinal dry weight contents (ST, MG, and HG) on the mRNA levels of each transcript. In addition, it was assessed the relationship between gastrointestinal dry weight contents and wet weight and tested for tank effects on mRNA expression levels by adding the tank as an interaction factor. All statistical analyses were carried out by RCoreTeam (2018), using the package ggplot (Wickham, 2016) to plot graphs. The plot bar graphs (**Figure 1; Supplementary Figure 1**) were produced in GraphPad Prism version 8.2.0 for Windows (GraphPad Software, CA, USA). Statistical significance was considered at $p < 0.05$.

RESULTS

Gastrointestinal Tract Fullness and Condition Factor (*K*)

The condition factor significantly (p = 0.01) decreased in the fast group (1.06 ± 0.03) compared to the fed group (1.11 ± 0.02). The content of the three different regions of the gastrointestinal tract (ST, MG, and HG) was significantly different between the fed and fast group (see **Supplementary Figure 1**). In fact, the fast group had no ST content, and both MG and HG content was much lower than in the fed group. The wet and dry content weight was highly correlated in all the three gut sections (**Supplementary Figure 2**).

Effects of Fasting on *agrp* and *pomc* mRNA Levels and Correlation With Gut Sections Fullness

Fasting significantly (p = 0.02) upregulated hypothalamic *agrp1* mRNA expression (**Figure 1**). Furthermore, *pomca2* showed an opposite trend and appeared to be downregulated by fasting; however, the differences were not statistically significant (**Supplementary Table 1**). No other significant differences were observed for *agrp2*, *pomca1*, and *pomcb* between fed and fast Atlantic salmon (**Figure 1**). Moreover, *agrp1* mRNA levels and stomach filling were significantly (p = 0.03) correlated (**Figure 2A; Supplementary Table 2**), while no other statistically significant correlation was found between *agrp2*, *pomca1*, *pomca2*, and *pomcb* mRNA copy number and gastrointestinal compartments content (**Figures 2B–E**).

There were no statistically significant effects of the tanks on the expression levels of the target genes.

DISCUSSION

The present study adds to and partially revises the existing knowledge on neuroendocrine control of appetite in Atlantic salmon, which was based on analysis of whole brain instead of specific brain regions. Here, we focused on the hypothalamus that is considered the hub for the appetite control in vertebrates. As in mammals, the teleost feeding center seems to reside in the hypothalamic area (reviewed in Peter (1979)). Furthermore, several neuropeptides involved in appetite control, including AgRP and POMC, are present in the hypothalamus of teleost species (Cerdá-Reverter et al., 2003; Cerda-Reverter and Peter, 2003; Otero-Rodiño et al., 2019). Different nutrient status also modulate the expression of teleost hypothalamic neuropeptides (reviewed in Volkoff (2016); Delgado et al. (2017); Rønnestad et al. (2017)). However, we cannot rule out that other areas of the brain might act as feeding centers in teleost fishes, as reviewed in Cerdá-Reverter and Canosa (2009) and Soengas et al. (2018), emphasizing the importance to explore the role of each brain region in appetite control.

Our results show that 3 days of fasting significantly increased hypothalamic *agrp1* mRNA expression, suggesting that *agrp1* acts as an orexigenic factor in Atlantic salmon. This contrasts previous findings for this species (Murashita et al., 2009a), providing novel insights that may revise the current knowledge on orexigenic and anorexigenic factors in salmon. Our data are in agreement with the suggested AgRP orexigenic role for other vertebrates including several fish species, such as goldfish (Cerda-Reverter and Peter, 2003), zebrafish (Song et al., 2003; Jeong et al., 2018; Shainer et al., 2019), seabass (*Dicentrarchus labrax*) (Agulleiro et al., 2014), Ya fish (*Schizothorax prenanti*) (Wei et al., 2013), arctic char (*Salvelinus alpinus*) (Striberny et al., 2015), and coho salmon (Kim et al., 2015). The discrepancies observed between the results obtained in the present study and the previous studies from Valen et al. (2011), which indicated that *agrp1* have an anorexigenic role, are most likely a result from hypothalamus versus whole brain analysis. It is possible that *agrp1* is also abundant in non-hypothalamic regions of the brain (Kurokawa et al., 2006) and offers other functional roles than appetite control (Xiao et al., 2003). This denotes the importance of analyzing individual tissues/organs and the need to revisit previous data in a more

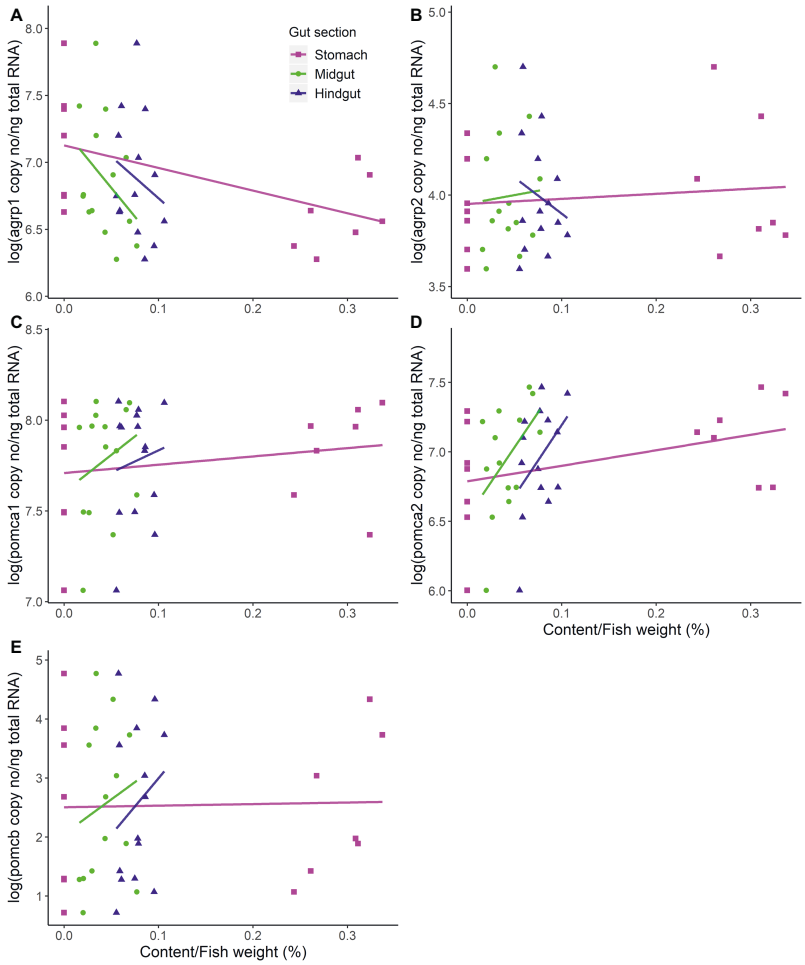

FIGURE 2 | mRNA expression levels versus content in the different compartments of the gastrointestinal tract (stomach, midgut, and hindgut). **(A)** *agrp1*, **(B)** *agrp2*, **(C)** *pomca1*, **(D)** *pomca2*, and **(E)** *pomcb* mRNA expression is presented as the log-transformed copy number (no) per nanograms (ng) of total RNA. Dots represent all individual fish ($n = 14$), while solid lines represent linear regressions estimated by the general linear model (GLMs). The weight of gastrointestinal tract content was standardized by the fish weight. For detailed statistical information, refer to **Supplementary Table 2**.

detailed manner. However, we cannot rule out the hypothesis that the controversial findings may be a consequence of the different sampling times, i.e., 3 days fasting in the present study versus 6 days (Murashita et al., 2009a) or 30 min to 24 h (Valen et al., 2011) fasting periods. Therefore, a future detailed study including several sampling time points will be necessary to ascertain the current hypothesis of this study. Furthermore, *in situ* hybridization and immunohistochemistry studies will be essential to reveal the location of AgRP1 expression within the hypothalamus and investigate its possible co-localization with other neuropeptides, such as neuropeptide Y (NPY), POMC or cocaine- and amphetamine-regulated transcript (CART).

We found a correlation between *agrp1* mRNA levels and stomach filling content, which may support the hypothesis that *agrp1* is also an important orexigenic factor in Atlantic salmon. Previous studies in rainbow trout (150–200 g) have shown that appetite and fullness had an almost perfectly inverse relationship, with appetite return reaching its maximum level when fullness approaches 0 and vice-versa (Grove et al., 1978). Furthermore, ca. 50 h for a complete gastric emptying in fish with a 150–250 g of weight was indicated. Our study is in line with these results,

as 3 days of fasting resulted in a complete empty stomach and increased levels of the orexigenic *agrp1*, supporting the hypothesis proposed by Grove et al. (1978) that the return of appetite is proportional with the emptying of the stomach. In addition, it has been previously reported by Murashita et al. (2009b) that 6 days of fasting induced upregulation of stomach *ghrelin 1* in Atlantic salmon. The orexigenic hormone ghrelin promotes the release of hypothalamic AgRP (reviewed in Nuzzaci et al., 2015). Therefore, we can hypothesize that also in Atlantic salmon fasting induces the increase of ghrelin levels, which, consequently triggers the increase expression of *agrp1* in the hypothalamus. However, this hypothesis needs to be further investigated, particularly because the current study is limited to only two very distinct phases, i.e., full versus empty stomach, and therefore other factors, such as nutritional conditions, may also contribute to the hypothalamic expression of the neuropeptides analyzed. For example, in mammals, it has been shown that increased glucose levels inhibit the NPY/AgRP neurons (reviewed in Marty et al., 2007); however, it seems hypothalamic *agrp* mRNA abundance is not affected by hyperglycaemic treatment in rainbow trout (Otero-Rodiño et al., 2015). The link between appetite (mRNA expression of orexigenic/anorexigenic factors) and stomach fullness, including peripheral hormones, and/or the role of the nutritional status are important issues that require further research.

The analysis of expression of *agrp2* revealed that fasting has no effect on its mRNA levels, confirming the observations by Murashita et al. (2009a). In addition, the hypothalamic *agrp2* expression levels were much lower than the levels of *agrp1* (**Figure 1**). All together, these results suggest that Atlantic salmon *agrp2* may have other functional roles than controlling appetite. This would correspond to findings in zebrafish (Shainer et al., 2017) suggesting that pineal *agrp2* regulates background pigment adaptation for camouflage (Zhang et al., 2010) and pre-optic *agrp2* acts as a neuroendocrine modulator of the stress response (Shainer et al., 2019).

Among the Atlantic salmon *pomc* genes analyzed in this study, only *pomca2* appears to be possibly downregulated after 3 days of fasting, opposite to previous findings in Atlantic salmon whole brain analysis after 6 days of fasting (Valen et al., 2011) where only *pomca1* significantly decreased after fasting. The anorexigenic role of *pomc* have been reported for several fish species (reviewed in Volkoff, 2016), including salmonids, for e.g., in coho salmon intra-peritoneal injections of α-MSH suppressed feed intake (White et al., 2016). However, conflicting data have also been reported for other salmonids: in rainbow trout 14 days of fasting did not affect *pomc* transcripts expression levels, whereas 28 days of fasting resulted in a significant decrease of hypothalamic *pomca1* but not for *pomca2* or *pomcb* (Leder and Silverstein, 2006), and 4 months of fasting (118 days) resulted in a significant increase of both hypothalamic *pomca1* and *pomcb* expression levels. The very low mRNA expression levels here reported for hypothalamic *pomcb* suggests that this gene may not serve as an appetite-controlling factor in the hypothalamus of Atlantic salmon. This hypothesis can be also supported by the fact that intraperitoneal deliver of leptin, a strong anorexigenic hormone, did not affect *pomcb* mRNA expression in Atlantic salmon (Murashita et al., 2011) or in rainbow trout (Murashita et al., 2008). Taken together, it can be hypothesized that hypothalamic *pomca2* and possibly *pomca1* (Valen et al., 2011), but not *pomcb* function as an anorexigenic factor in Atlantic salmon; however, this needs to be further investigated.

In summary, our study demonstrates for the first time a correlation between an appetite related neuropeptide, hypothalamic *agrp1*, and stomach filling in a teleost species. Three days of fasting upregulated hypothalamic *agrp1* mRNA expression levels, suggesting an orexigenic role of this gene in Atlantic salmon and indicating a different role in appetite control than the one proposed for whole brain *agrp1* by Murashita et al. (2009a) and Valen et al. (2011). The *agrp1* response observed in this study suggests that this gene plays a role in the control of appetite in Atlantic salmon, enabling the fish to cope with short-term fasting periods and recovery after fasting. Our study provides a basis to form hypotheses about the differential expression patterns of appetite-controlling factors that need to be further explored. Further research needs to focus on tissue-specific analysis in Atlantic salmon, including central and peripheral signals, and how their interaction affects fish health and welfare during sensitive production stages, such as those requiring short-term fasting periods.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The experiment was conducted in an experimental facility approved to conduct experiments with teleosts and in accordance with the rules and regulations of the Norwegian Animal Research authority. The trial mimicked commonly used practices in the aquaculture industry, including 3–4 days of fasting prior transportation, handling, and harvest. The protocol used was approved by the local representative for animal welfare at the Department of Biological Sciences, University of Bergen (Norway). The responsible senior faculty members responsible for sampling were all accredited by Federation of European Laboratory Animal Science Associations (FELASA).

AUTHOR'S NOTE

This work is a result of an educational Summer School student project hosted by University of Bergen from 17 to 28 June, 2019.

AUTHOR CONTRIBUTIONS

KM, IR, and AG designed the study. KM, MI, KT, MSi, YW, and AG performed the laboratory analysis. All authors contributed to the sampling and data analysis, writing of the manuscript, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00061/full#supplementary-material

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III

frontiers in Marine Science

ORIGINAL RESEARCH
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Brain Distribution of 10 *cart* Transcripts and Their Response to 4 Days of Fasting in Atlantic Salmon (*Salmo salar* L.)

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Cocaine- and amphetamine-regulated transcript (CART) has been known to be involved in feeding and energy balance in mammals, acting as an anorexigenic neuropeptide in hypothalamus. In Atlantic salmon, little is known about Cart brain localization and its function. In this study, *in silico* analysis revealed the existence of 10 *cart* paralogs, here named *cart1a*, *1b1*, *1b2*, *2a*, *2b1*, *2b2*, *3a1*, *3a2*, *3b,* and *4.* The Atlantic salmon Cart sequences shared from 19 to 50% of identity with the human homolog and between 25 and 90% of sequence identity among paralogs, except for Cart4 which only shared 18–23% of identity. We further explored *cart* mRNA expressions in 8 brain regions (Olfactory Bulb-OB, Telencephalon-TEL, Midbrain-MB, Cerebellum-CE, Hypothalamus-HYP, Saccus vasculosus-SV, Pituitary-PT, and Brain Stem-BS) of Atlantic salmon smolt under 4 days of fasting and continuous fed conditions. The *cart* paralogs analyzed were widely distributed among the brain regions and OB, TEL, HYP, MB, and BS seemed to be the major sites of expression. The expression of *cart1a* and *1b* showed quite similar pattern in MB, HYP, and BS. The expression of *cart2a* had the highest in MB followed by HYP and TEL. The *cart3a* transcript was widely distributed in rostrocaudal regions of brain except in OB and SV whereas *cart3b* was predominantly expressed in BS followed by MB. Expression of *cart4* was high in HYP followed by TEL. With regards to effect of feeding status the Atlantic salmon *cart2b*, which is the most abundant among the paralogs, was upregulated after 4 days of fasting in OB, MB, and HYP compared to fed group. This may suggest an unexpected, but possible orexigenic role of *cart2b* in Atlantic salmon or a fasting induced stress effect. No other significant effect was observed. Collectively, the differential expressions of the *cart* paralogs in different brain regions suggest that they may have roles in regional integration of appetite signals and are possibly involved in regulating other brain functions in Atlantic salmon. The fact that salmon has 10 *cart* paralogs, while mammalians only one, opens interesting perspectives for comparative research on evolutionary adaptations of gene function in the control of appetite and energy homeostasis.

Keywords: appetite, Atlantic salmon, brain, CART, fasting, neuropeptides

INTRODUCTION

The cocaine- and amphetamine-regulated transcript (CART), initially discovered as an unknown peptide isolated from ovine hypothalamus by Spiess et al. (1981), was found to be released in response to the psychomotor stimulants cocaine and amphetamine in rat brain by Douglass et al. (1995) and, thus, its name. The mammalian CART is known to be an anorectic peptide in mediating feeding in accordance with energy balance (Kristensen et al., 1998; Yang et al., 2005; Rogge et al., 2008) but is also involved in stress response, reward and drug addiction/abuse (Koylu et al., 2006).

The CART function and its involvement in appetite control is, however, not so clear for other vertebrates, including teleost fishes. In fish, one *cart* gene has been previously identified in several species, such as Atlantic cod (*Gadus morhua*) (Le et al., 2016), winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009a), winter skate (*Raja ocellata*) (MacDonald and Volkoff, 2009b), Mexican tetra/blind cave fish (*Astyanax mexicanus*) (Penney and Volkoff, 2014), rainbow trout (*Oncorhynchus mykiss*) (Comesaña et al., 2018), channel catfish (*Ictalurus punctatus*) (Kobayashi et al., 2008), silver dollar (*Metynnis argenteus*), bucktooth tetra (*Exodon paradoxus*), black widow tetra (*Gymnocorymbus ternetzi*) (Butt et al., 2019), and Atlantic salmon (*Salmo salar*) (Murashita et al., 2009). In other teleost species, multiple *cart* transcripts have been identified in the brain including two genes in goldfish (*Carassius auracius*) (Volkoff and Peter, 2001), three in Ya-fish (*Schizothorax prenanti*) (Yuan et al., 2015), four in zebrafish (*Danio rerio*) (Nishio et al., 2012; Akash et al., 2014; Ahi et al., 2019), five in yellowtail (*Seriola quinqueradiata*) (Fukada et al., 2021), six in medaka (*Oryzias latipes*) (Murashita and Kurokawa, 2011), and seven in Senegalese sole (*Solea senegalensis*) (Bonacic et al., 2015).

The existence of multiple *cart* genes raises questions about their physiological function, and recent studies have suggested that maybe not all the paralogs are involved in the control of feed intake. The *cart* response to feeding has varied among studies, which can possibly be explained by differences in the method of analysis used, like *in situ* vs. qPCR, or varied methodological approaches like use of whole brain vs. specific brain regions and different development stages, and sampling time related with feeding or fasting. However, it might well be that Cart response to feeding is also species-specific. The differential expression patterns of *cart* genes in brain regions have been described in some teleost species such as goldfish (Volkoff and Peter, 2001), zebrafish (Nishio et al., 2012; Akash et al., 2014), yellowtail (Fukada et al., 2021), and Senegalese sole (Bonacic et al., 2015). The anorectic effect of *cart* has been reported for several teleost species, notably in zebrafish where two forms of hypothalamic *cart* mRNA expressions were downregulated after 5 days of fasting (Akash et al., 2014), and all four identified *cart* genes expression decreased in the brain after 3 days of fasting (Nishio et al., 2012). Similar *cart* anorectic effects have been observed in Ya-fish, where the mRNA expression of the three identified *cart* genes decreased in the hypothalamus after fasting and increased with refeeding (Yuan et al., 2015), while in yellowtail, *cart1b* and *2a* mRNA expression decreased in the telencephalon and hypothalamus following 8 days of fasting (Fukada et al., 2021). Furthermore, whole brain analysis of *cart* mRNA expression showed that 6 days of fasting downregulated it's expression in Atlantic salmon (Murashita et al., 2009), while 1 week of fasting decreased *cart* mRNA in silver dollar but did not affect the expression in bucktooth or black widow tetra (Butt et al., 2019). In medaka, *cart2b (*previously named *cart ch3)* expression decreased with 17 days of starvation (Murashita and Kurokawa, 2011).

Atlantic salmon is a key species in Norwegian aquaculture industry and a substantial portion of the production cost, more than 50%, goes to fish feed (Asche and Oglend, 2016). Furthermore, the extensive expansion of the fish industry combined with frequent overfeeding has contributed to potentially negative effects on the ecosystem (Strain and Hargrave, 2005). As such, the efficient utilization of fish feed is vital to ensure sustainable production with regards to the environment, fish welfare, and production costs. Thus, a clear understanding of the biological mechanisms that underly hunger and satiety, and thereby control feed intake, can be of great benefit to the salmon industry. This includes the neuro-endocrine players that modulate feeding and the identification of reliable biomarkers that can be used to assess appetite and potentially growth in fish trials. Moreover, a 2–4 days fasting is implemented prior to aquaculture practices like handling for vaccination, transportation, and harvest (Waagbø et al., 2017). In this regard, learning the impact of fasting will help to optimize the feed utilization, fish growth performance, and welfare. In the current study, we have identified 10 *cart* paralogs in the Atlantic salmon genome databases and analyzed their brain distribution as well as their response to 4 days of fasting. The results provide a basis for a more comprehensive understanding the role of Cart in the appetite control in Atlantic salmon.

MATERIALS AND METHODS

Ethics Statement

The animal experiment was conducted in accordance with the National Animal Research Authority regulations and approved by the local representative of Animal Welfare at Department of Biological Sciences, University of Bergen, Norway.

Experimental Animals and Sampling

To evaluate the effect of 4 days fasting, we used the experimental design described by Kalananthan et al. (2020b). In brief, Atlantic salmon post smolt (mean body weight 183.81 ± 35.89 g, mean length 25.42 ± 1.68 cm) were divided in two groups, one was kept under continuous feeding (fed group), whereas the other tank was fasted for 4 days (fasted group). Fish were sampled 2 h after feeding for the fed group (n = 6, per tank n = 2) and 4 days of fasting for the fasted group (n = 6, per tank n = 2). Fish were euthanized with a lethal dose of 200 mg/l of MS222 (Tricaine methanesulfonate, Sigma-Aldrich, MO, United States). The whole brain including pituitary was removed from the skull, placed in RNA later (Thermo Fisher Scientific, MA, United States) and stored overnight at 4°C before transferring it to −80°C until further use. Because the key regulatory neural

pathways involving CART in appetite control are found in the hypothalamus of mammals, we have also investigated the hypothalamic *cart* mRNA expression using the total RNA from Kalananthan et al. (2020b) study, which analyzed 3 days fasting vs. normally fed fish groups ($n = 6$ per group).

Sequence Comparison and Phylogenetic Analyses of Cart

Putative Atlantic salmon *cart* genes were retrieved from Ensembl[1] and GenBank[2] genome databases (ICSASG_v2) and compared using ClustalX 2.1 (Larkin et al., 2007).

The Atlantic salmon Cart deduced amino acid (AA) sequences and the homolog sequences from human (*Homo sapiens*) and from 15 other fish species, including the Callorhinchidae, elephant shark (*Callorhinchus milii*); Latimeriidae and Lepisosteidae, spotted gar (*Lepisosteus oculatus*) as species before the teleost specific whole genome duplication event (Ts WGD); two Cyprinidae, goldfish (*Carassius auracius*) as species that went through a very recent 4R WGD and zebrafish (*Danio rerio*) which did not; Characidae, Mexican tetra/ blind cave fish (*Astyanax mexicanus*); Clupeidae, Atlantic herring (*Clupea harengus*) as old teleost; another Salmonidae species, rainbow trout (*Oncorhynchus mykiss*); the Esocidae northern pike (*Esox lucius*) as a sister group of salmonids that diverged before the Ss 4R WGD; six Neoteleostei Gadidae Atlantic cod, Adrianichthyidae medaka (*Oryzias latipes*), Gasterosteidae stickleback (*Gasterosteus aculeatus*), Cichlidae Nile tilapia (*Oreochromis niloticus*) and Scophthalmidae turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*), and coelacanth (*Latimeria chalumnae*) were retrieved from Ensembl or GenBank databases.

Cart prepro- and mature peptide sequences were aligned using MUSCLE with the default parameters (UPGMA clustering method, Gap opening penalty −2.90, Gap extension 0.0) from MEGAX (Hall, 2013). The alignments were displayed in GeneDoc 2.7 (Nicholas et al., 1997) and percentages of sequence identity calculated. Putative signal peptides were predicted using PrediSi[3] and proteolytic cleavage sites predicted using Neuropred.[4] Phylogenetic analysis was performed using the predicted mature peptide sequences of Cart. Based on best-fit substitution model analysis in MEGAX, phylogenetic tree was constructed using Maximum Likelihood (ML) with a Jones-Taylor-Thornton (JTT) model (Jones et al., 1992; Kumar et al., 2018) with fixed Gamma distribution (+G) parameter with five rate categories and 500 bootstrap replicates. The tree was rooted to the human CART.

Brain Dissection

Six brains from fed and six from the fasted group of Atlantic salmon were dissected into 8 regions: olfactory bulb (OB), telencephalon (TEL), midbrain (MB), cerebellum (CE), hypothalamus (HYP), saccus vasculosus (SV), pituitary (PT), and medulla oblongata/brain stem (BS) using the same standard dissection protocol as illustrated by Kalananthan et al. (2020a). To ensure high integrity and quality of total RNA, the brain was placed on an ice block during dissection under a zoom stereomicroscope (Olympus SZ51) and cleaned from blood vessels. The brain regions PT, OB, TEL, BS, CE, SV, HYP, and MB were separated in this respective order.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from each dissected brain region using TRI Reagent (Sigma-Aldrich, MO, United States) following the manufacturer's protocol. To avoid any remnants of genomic DNA 5 or 10 μg of total RNA was treated with TURBO DNase-free Kit (Ambion Applied Biosystem, Foster City, CA, United States) using 2 U DNase per reaction. The input amount of total RNA and the reaction volume for DNase treatment was adjusted depending on the available amount of total RNA per region. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, United States) and a 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, CA, United States) were used to assess the quantity, purity and integrity of the extracted total RNA. First strand cDNA was synthesized from 1.1 μg of the total RNA using SuperScript III Reverse Transcriptase (Invitrogen, CA, United States) and Oligo $(dT)_{20}$ (50 μM) primers in a total reaction volume of 20 μl for OB, TEL, MB, CE, HYP, PT, and BS ($n = 6$ per group) and for SV ($n = 4$ per group). The hypothalamic total RNA from 3 days fasting and normally fed groups were also performed with DNase treatment and cDNA as described above.

qPCR Set Up and Primer Design

Atlantic salmon *cart* qPCR primers were designed using NCBI primer designing tool (Primer-BLAST[5]) and synthesized by Sigma-Aldrich (St. Louis, MO, United States) (**Table 1**). Wherever possible specific primers were designed spanning an exon-exon junction. Due to the high degree of identity between *cart1b1* and *1b2*; *2b1* and *2b2*; *3a1* and *3a2* transcripts, common primer pairs were designed for these paralogs (**Table 1**). Further, the primers were designed to amplify alternate splice variants (if existing) of the gene. Two genes *actin beta* (*actb*) and *ribosomal protein s20* (*s20*) were used as reference genes (Olsvik et al., 2005). For each primer pair, quantitation cycle (Cq), primer efficiency and melting peaks were analyzed. All qPCR products were resolved in a 2% agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into a pCR4-TOPO vector (Thermo Fisher, Scientific, Waltham, MA, United States). Sequencing was performed at the University of Bergen Sequencing Facility (Bergen, Norway), and their identity was confirmed using BLASTN[6] analysis against the Atlantic salmon genome database. A standard curve dilution series (10-fold) was generated from each target and reference gene cloned into a pCR4-TOPO vector.

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 2 μl of cDNA template (for

[1]http://www.ensembl.org/index.html

[2]https://www.ncbi.nlm.nih.gov/

[3]http://www.predisi.de/index.html

[4]http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py

[5]https://www.ncbi.nlm.nih.gov/tools/primer-blast

[6]https://blast.ncbi.nlm.nih.gov/Blast.cgi

TABLE 1 | Sequence of specific primers used for reverse transcriptase quantitative PCR (RT-qPCR) analyses.

Transcripts	Ensembl Gene Acc. No.	GenBank Acc. No.	Chr location	Primer sequences (5′→3′)	Amplicon size (bp)	Efficiency (%)	R^2
cart1a	ENSSSAT00000013202.1	XM_014149393.1	ssa16	F: GGCCAACTCCCACGACTTTC R:CATCAGCATCACACATGGGAACC	170	95.96	0.9997
cart1b	ENSSSAT00000151185.1 ENSSSAT00000086533.1	XM_014150559.1 XM_014151634.1	ssa16 ssa17	F: GGCCAGCATTCAACTGCTTT R: GGAACTCTAGAGCGCGAGTC	104	96.55	0.9997
cart2a	ENSSSAT00000028631.1	N/A	ssa10	F: GGCAAAACTGCAGGGATTGG R: ACATAGGATGGACAGCAGCG	146	93.78	0.9996
cart2b	ENSSSAT00000074136.1 ENSSSAT00000019793.1 ENSSSAT00000019780.1 ENSSSAT00000019807.1	NM_001146680.1 XM_014176449.1 XM_014183838.1 XM_014188599.1	ssa11 ssa26 Unplaced Scaffold	F: TGAGAGACTTCTACCCCAAAGA R: CGTAGGGACTTGGCCGAATT	135	98.78	0.9996
cart3a	ENSSSAT00000060018.1 ENSSSAT00000060001.1 ENSSSAT00000121230.1	XM_014177116.1 NM_001141227.1	ssa27 ssa14	F: GTGATGTTGGAGAGCGGTGC R: CGGGGTCAGTAACAACGCA	112	101.31	0.9997
cart3b	ENSSSAT00000118490.1	XM_014127320.1	ssa11	R: AGAAAGAACTTGTGGGTGCGA R: ACCACAAGGAGGGATCATGC	95	97.58	0.9997
cart4	ENSSSAT00000121235.1	XM_014141614.1	ssa14	F: CGTTCCGTCGTTGGAAACAC R: CCACGTTGGAATTGCACAGA	185	97.49	0.9999
actin beta	ENSSSAG00000001782.1	BG933897.1		F: CCAAAGCCAACAGGGAGAAG R: AGGGACAACACTGCCTGGAT	91	100.61	0.9984
s20	ENSSSAT00000130996.1	BG936672		F: GCAGACCTTATCCGTGGAGCTA R: TGGTGATGCGCAGAGTCTTG	85	98.25	0.9962

Sequence accession number (Ensemble and GenBank databases), chromosome location, primer sequences, amplicon sizes, qPCR efficiency (%), and R^2 are indicated for each primer pair.

target genes 40 ng/reaction and for reference genes 5 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 step 2–3 for 39 more times. Melting curve analysis over a range of 65–95°C (increment of 0.5°C for 2 s) allowed for the detection of possible non-specific products and/or primer dimers.

Subsequently, the copy number for each gene/sample were determined based on the respective standard curve and using the following equation:

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

The copy number was normalized using the total ng of RNA used in the reaction for each target and reference gene. The ratio of the target gene copy number to the geometric mean copy number of reference genes was used in for the plots and statistical analysis.

Statistical Analysis

The data analysis was carried out using GraphPad version 8 (GraphPad Software, San Diego, CA, United States). Normality of distribution and equal variances of gene expression were assessed using Shapiro-Wilk normality test and *F*-test, respectively. The data were log transformed to ensure normal distribution before statistical analysis. The analysis of differential expression between the fed and fasted group were performed with two-tail *t*-test. When either the *F*-test or the normality test failed, the non-parametric Mann-Whitney test was performed. A $p < 0.05$ was considered significant. All data are presented as mean ± SEM.

RESULTS

Sequences and Phylogenetic Comparison of Cart

In silico searches identified the presence of 10 *cart* paralogous genes in the Atlantic salmon genomic databases. Comparison of the Atlantic salmon *cart* gene structure indicated that gene organization is conserved with three exons encoding the prepro-Cart sequence separated by two introns (**Figure 1**). The coding regions of the identified *cart* genes were 327, 393, 327, 357, 357, 357, 345, 345, 324, and 372 bp (**Figure 1**), translated into 108, 130, 108, 118, 118, 118, 114, 114, 107, and 123 AA, respectively (**Figure 2**). The Cart paralogs were found to be encoded by genes located on chromosomes (Chr) ssa16, ssa17, ssa16, ssa10 (exclusively predicted in Ensembl database), ssa11, ssa26, ssa14, ssa27, ssa11, and ssa14 (**Table 1**). Two alternate splice variants were found for *cart* located in chromosome ssa26, with coding regions of 267 and 510 bp which translate into 88 AA and 169 AA, respectively. One splice variant of *cart* located in chromosome ssa27 was found with a 381 bp coding region that translate into 126 AA.

Phylogenetic analyses revealed that the predicted Atlantic salmon Cart sequences were grouped into 4 major clusters.

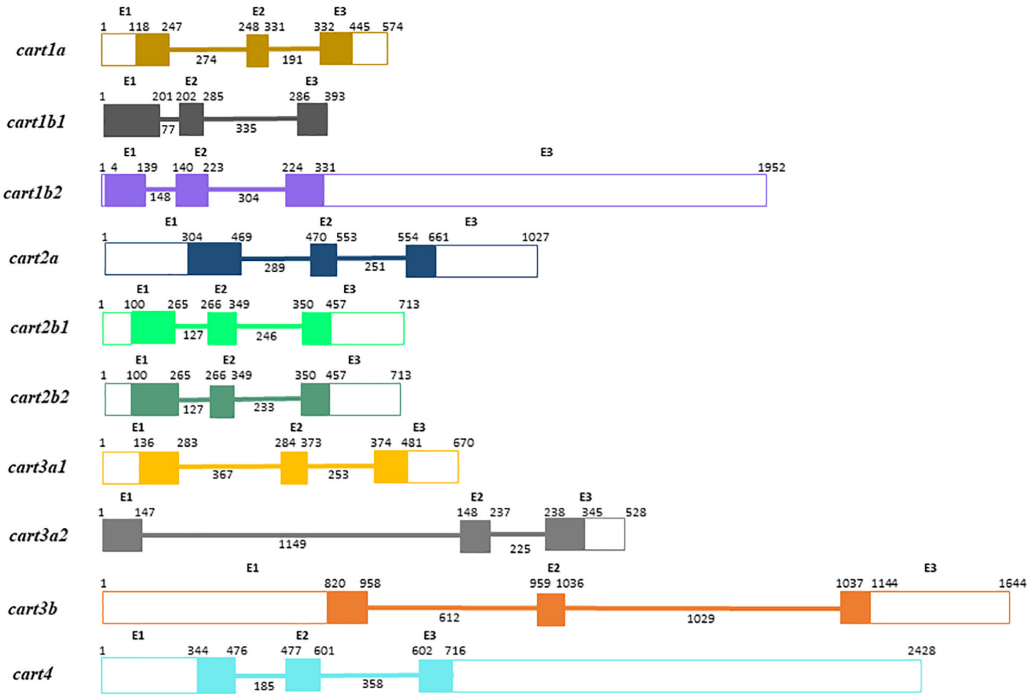

FIGURE 1 | Predicted gene structure of Atlantic salmon *cart* paralogs. Exons (E1–E3) are represented by boxes and connection lines indicate introns. The open reading frames (ORF) are indicated by filled boxes and untranslated regions (5′UTR and 3′UTR) by open boxes. Size of introns and exons (UTRs and ORFs) are given below the lines and above the boxes, respectively, in base pairs (bp).

The Atlantic salmon paralogs were named following the nomenclature proposed by Bonacic et al. (2015): Cart1a, 1b1, 1b2, 2a, 2b1, 2b2, 3a1, 3a2, 3b, and 4 (**Figure 3**). As expected, the sequences of rainbow trout (a very close relative to Atlantic salmon) Cart and Northern pike (a basal sister clade to salmonids) Cart cluster very close with Atlantic salmon Cart. The zebrafish and goldfish (cyprinids that are distant relatives to salmonids) Carts formed only 3 major clusters, lacking Cart4. The Atlantic salmon prepro-Cart sequences shared 19–50% of AA with human homolog sequence. Among the Atlantic salmon Cart paralogs, Cart1 sequences shared between 48 and 81% of identity, Cart2 shared 37–77% sequence identity, and Cart3 35–90% AA identity, whereas Cart4 only shared 18–23% of AA sequence identity with the other Cart paralogs (**Supplementary Table 1**).

All the predicted Cart sequences of Atlantic salmon showed a highly divergent N-terminal AA residues with the signal peptide ranging from 19 to 27 AA long, whereas the C-terminal was highly conserved (**Figure 2** and **Supplementary Figure 1**). The human CART proteolytic processing sites of dibasic residues Lys-Arg (KR), Arg-Arg (KK) were conserved in Atlantic salmon Cart2, but only KK site is conserved in Cart1 and Cart3a, while in Cart3b and Cart4, KK is replaced with other proteolytic dibasic residues KR or RR, respectively (**Figure 2**). Furthermore, the 6 cysteine residues that are critical for disulfide bond in human CART are conserved in Atlantic salmon Cart paralogs except for Cart4 where only 5 cysteines are conserved.

Brain Distribution of *cart* mRNA in Atlantic Salmon and Response to Fasting

The qPCR analysis showed that the *cart* paralogs have a wide distribution pattern in the brain with varying abundance between regions (**Figure 4**). The *cart2b* is the most abundant among the *cart* paralogs followed by *cart3b*, *3a*, *2a*, *1b*, *1a,* and *cart4*. For *cart2b* OB, TEL, MB, HYP, SV, and BS were the regions where high levels of mRNA were found, whereas low expression was found in the CE and PT. The *cart3b* was mainly expressed in BS followed by MB. The *cart2a* was prominently expressed in MB, HYP, and TEL whilst *cart4* showed remarkable expression in HYP and TEL. The *cart3a*, *1a,* and *1b* expression profiles were quite similar with major expression in MB, however, *cart3a* was also expressed considerably in BS, TEL, and CE while PT was the second highest site of expression for *cart1a* and *1b*. In the regions OB, TEL, CE and SV for *cart1b*; OB, CE, SV, and PT for *cart2a*; CE and PT for *cart2b*; OB and SV for *cart3a*, and TEL, CE and SV for *cart3b* only residual mRNA expression levels were found.

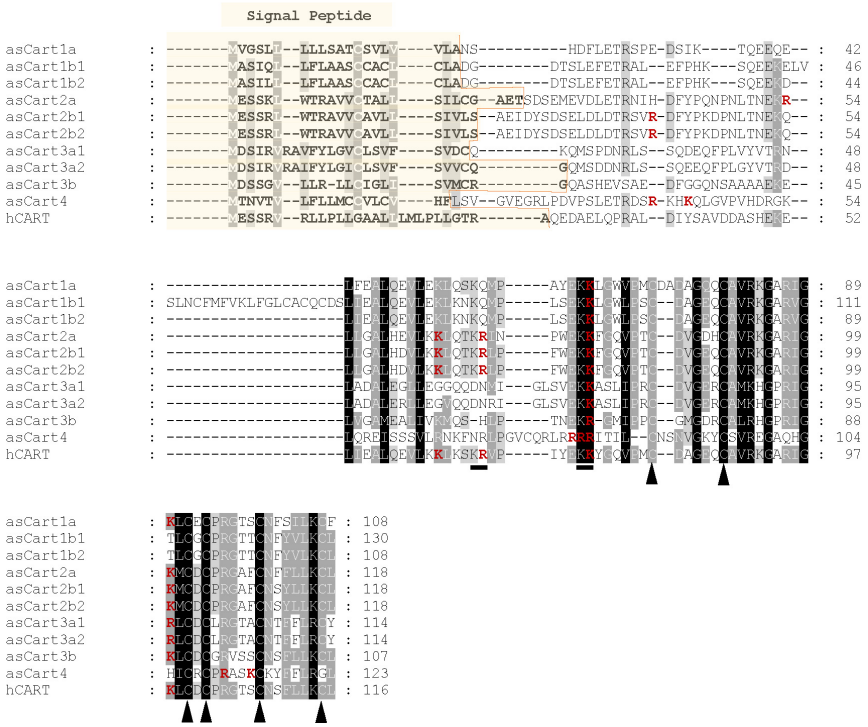

FIGURE 2 | Multiple sequence alignment of Atlantic salmon (as) and human (h) Cart amino acid sequences obtained by using ClustalX 2.1 and edited in GeneDoc 2.7 software. Black areas indicate 100% and shaded areas indicate > 60% conserved amino acid residues among the Cart sequences. The putative signal peptide is highlighted in yellow. Potential proteolytic processing sites KK and KR for hCART are underlined with thick lines and marked in red bold letters are the predicted sites for Cart using Neuropred application. Arrowheads indicate the conserved 6 cysteine residues that forms the disulfide bond in human CART.

Atlantic salmon *cart2b,* which is the most abundant *cart* paralog, was upregulated in OB ($p < 0.05$), MB ($p < 0.01$), and HYP ($p < 0.05$) after 4 days of fasting (**Figure 4**). No effect of fasting was observed for the other *cart* paralogs in any of the highly expressed brain regions (**Supplementary Table 2**).

Among the *cart* paralogs that were tested for the effect of 3 days of fasting in hypothalamus of Atlantic salmon, *cart3a* showed significant ($p < 0.01$) increase in the mRNA expression (**Figure 5**).

DISCUSSION

In this study, we report the identification and characterization of 10 *cart* paralogs in Atlantic salmon, here named *cart1a*, *1b1*, *1b2*, *2a*, *2b1*, *2b2*, *3a1*, *3a2*, *3b,* and *cart4*. This is the first study to analyze all the aforementioned paralogs physiological role in Atlantic salmon appetite control, while the previously reported Atlantic salmon Cart by Murashita et al. (2009) is here denoted as Cart2b (b1 and b2). Furthermore, the mRNA sequences of the qPCR amplicons confirmed that none of salmon *cart* genes are pseudogenes. The deduced amino acid sequences showed a highly divergent N-terminal AA residues whereas the C-terminal showed a high degree of conservation. Among Cart paralogs, the potential proteolytic processing sites of dibasic residues KR and KK (Kuhar and Yoho, 1999; Rogge et al., 2008) are conserved only in Cart2, possibly cleaving it into two different size products as previously suggested by Murashita et al. (2009). In previous studies, Kuhar and Yoho (1999) identified four immunoreactive CART peptides from human and five from rat hypothalamus varying from 4 to 10 kDa. The predicted cleavage sites for Atlantic salmon Cart (**Figure 2**) also suggest possible multiple Cart peptides. The 3 cysteine disulfide bonds between Cys^{82} and Cys^{100}, Cys^{88} and Cys^{108}, Cys^{102}, and Cys^{115} were predicted for human CART C-termini (UNIPROT[7]). These six C-terminal cysteines are conserved in all the Atlantic salmon Cart, except Cart4 where only 5 cysteine residues are present. The absence of C- terminal cysteine bond among the homologs has not been reported in teleosts before. The three disulfide bridges are key residual structures that are essential for the protein 3-dimensional

[7]https://www.uniprot.org/uniprot/Q16568

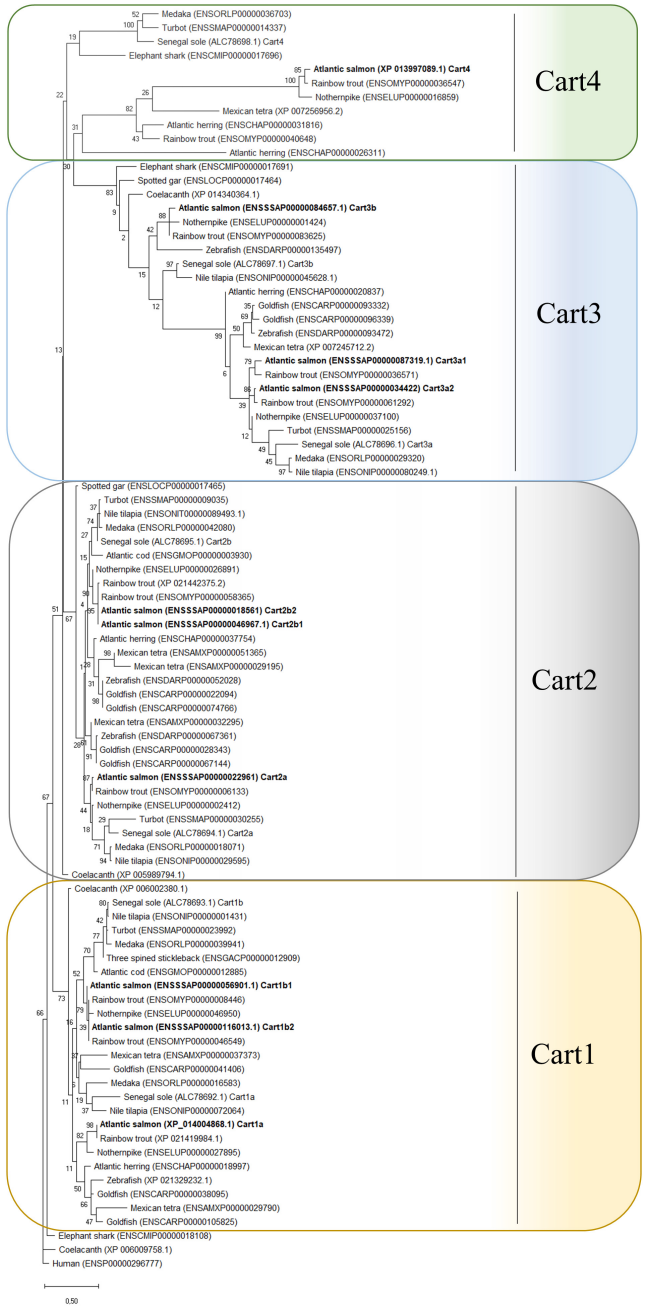

FIGURE 3 | Phylogenetic analyses of Atlantic salmon Cart and other fish species and human based on the predicted mature peptide sequences. The evolutionary history was constructed by using the Maximum Likelihood method, 500 bootstraps replicates and JTT + G matrix-based model in MEGA X. The tree with the highest log likelihood (–4018, 51) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 91 amino acid sequences. The tree is rooted to human CART.

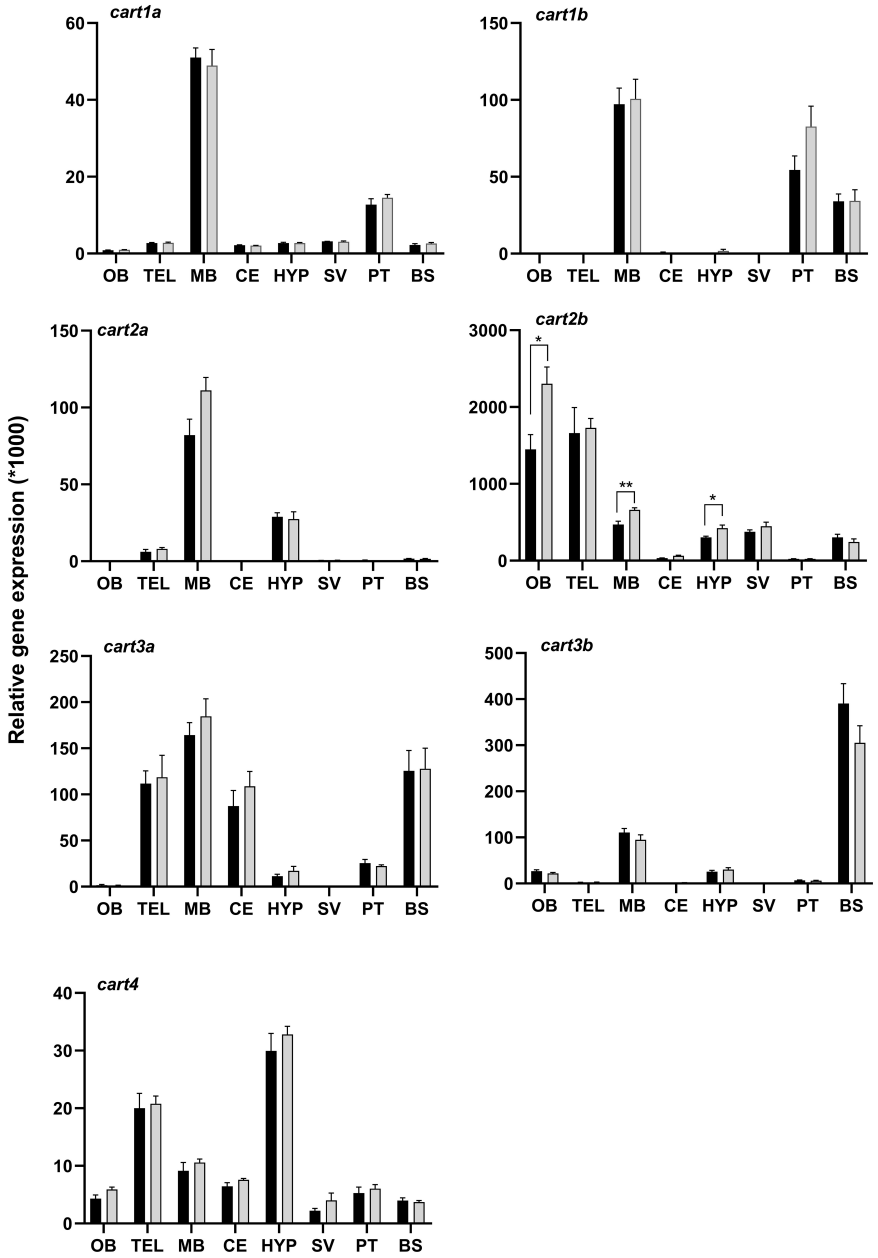

FIGURE 4 | Effect of 4 days of fasting on the mRNA expression of *cart* paralogs in eight brain regions of Atlantic salmon. Black and gray bars represent fed (n = 6 except for SV n = 4) and fasted (n = 6 except for SV n = 4) groups, respectively. The graphs show the normalized *cart* mRNA copy number to the geometric mean copy number of *actin beta* and *s20*. Two-tailed *t*-test and non-parametric Mann-Whitney test was performed to assess the statistically significant differences between the two groups. The error bars represent mean ± SEM and *asterisks* show the significant level (*$p < 0.05$, **$p < 0.01$). OB, olfactory bulb; TEL, telencephalon; MB, midbrain; HYP, hypothalamus; CE, cerebellum; SV, saccus vasculosus; PT, pituitary; BS, brain stem.

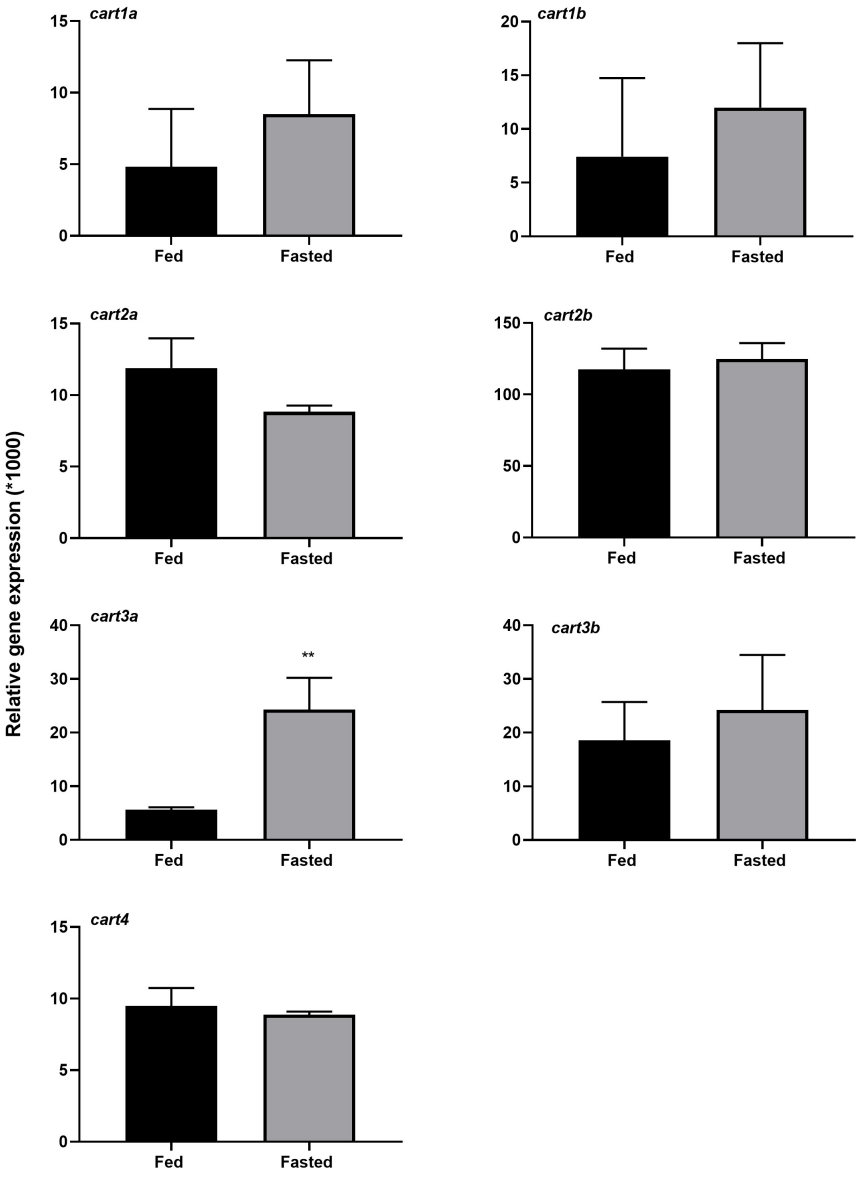

FIGURE 5 | Effect of 3 days of fasting on mRNA expression of *cart* paralogs in hypothalamus of Atlantic salmon. Black and gray bars represent fed (n = 5–6) and fasted (n = 5–6) groups, respectively. The graphs show the normalized *cart* mRNA copy number to the geometric mean copy number of *actin β* and *s20*. Two-tailed t-test and non-parametric Mann-Whitney test was performed to assess the statistically significant differences between the two groups. The error bars represent mean ± SEM and *asterisks* show the significant level (**$p < 0.01$).

structure and its function in biological activities such as food intake (Ludvigsen et al., 2001; Maixnerová et al., 2007). The Atlantic salmon Cart sequence alignment clearly show that Cart4 structure potentially differs from the other salmon's Carts and human CART. This suggests that Atlantic salmon Cart4 may differ in functionality from the other Carts and may likely be

ruled out from its role in appetite regulation. It would also be possible that Cart4 is non-functional or has adopted new function and this warrants further studies. In teleost *cart* mRNA expression in peripheral tissues like eye, gill, ovary, testis has also been reported suggesting Cart may also have other roles (Volkoff and Peter, 2001; Murashita et al., 2009). Interestingly, the multiple sequence alignment of Cart prepropeptides revealed also Cart4 in northern pike, rainbow trout, Atlantic herring and Cart2 in Atlantic cod lack of one or more Cysteine residue in the C-termini (**Supplementary Figure 1**).

The evolutionary history of *cart* depicted in the phylogenetic tree shows that the Ts 3R genome duplication resulted in the presence of 7 *cart* genes in species like Senegalese sole, Mexican tetra, Northern pike, etc., and the Ss 4R genome duplication resulted in 10 *cart* genes in the Atlantic salmon, including sub types *cart1b1* and *1b2*, *cart2b1* and *2b2*, *cart3a1* and *3a2*. The divergence of the *cart* gene in salmonids opens the possibility that the gene products might have adopted to new functions (neofunctionalization) or partitioning the ancestral function among the paralogs (sub functionalization) during genome evolution reviewed by Volff (2005). Furthermore, the fact that Atlantic salmon and other teleosts have multiple *cart* genes while only one is present in mammals', open up interesting perspectives for comparative research on evolutionary adaptations of gene function and the control of appetite and energy homeostasis.

To date, there are only a few studies exploring the full *cart* gene repertoire in teleost species. These studies include the identification of seven *cart* paralogous genes in Senegalese sole (Bonacic et al., 2015), six genes in medaka (Murashita and Kurokawa, 2011), and five yellowtail (Fukada et al., 2021). Thus, a revision of the teleost genome databases and nomenclature of *cart* genes is essential in further studies exploring the functional role of Cart in these species.

Our results revealed that all *cart* paralogs were expressed at different mRNA levels in the eight brain regions analyzed in this study. Among all the *cart* genes, *cart2b* was the most abundant paralog, with predominant expression in the OB followed by TEL, MB, HYP, and SV. A similar pattern has also been reported in Senegalese sole (Bonacic et al., 2015) and yellowtail (Fukada et al., 2021). In teleosts, Cart has been suggested to play a role in processing olfactory information (Akash et al., 2014; Porter et al., 2017) including antiviral immune response (Sepahi et al., 2019). However, the Atlantic salmon *cart2b* expression in the OB and a possible link to olfaction and/or immune response is not clear and needs to be further investigated. The *cart2b* was the only gene that was affected by 4 days of fasting, and a significantly higher expression was observed in the fasted group compared to the fed group in the Atlantic salmon specifically in OB, MB, and HYP. Hypothalamus is considered as a center of appetite control in vertebrates (Volkoff, 2016; Rønnestad et al., 2017; Soengas et al., 2018), and in fish this is supported by Ye et al. (2020) who reported that in grass carp the hypothalamus and telencephalon might be involved in feeding and reproduction. Hence, in Atlantic salmon, hypothalamic *cart2b* might play a vital role in appetite regulation, with fasting maybe inducing shutting down hunger. This is in line with observations in Siberian sturgeon (*Acipenser baerii*) (Zhang et al., 2018), where *cart* mRNA in whole brain increased after 3–17 days of food deprivation. Interestingly, our finding is contrary to several previous studies that demonstrate the anorectic function of Cart. In goldfish, *cart* form I (*alias cart2b*) mRNA level declined with 96 h food deprivation in telencephalon, hypothalamus, and olfactory bulb while *cart* form II (*alias cart2a*) mRNA level declined only in the olfactory bulb. In the same study, *cart2b* transcript levels increased 2 h following a meal in olfactory bulb and hypothalamus whereas no postprandial changes were observed in *cart2a* (Volkoff and Peter, 2001; Volkoff et al., 2005). However, in yellowtail *cart2b* expression in OB, TEL, HYP was upregulated in fish fasted for 8 days, though not significantly, while *cart3a* was significantly upregulated in OB, TEL, and PT of the fasted group (Fukada et al., 2021). The brain *cart* mRNA expression in response to fasting varied depending on species, and even within the same order as for example, in silver dollar, fasting induced a decrease in *cart* expression while for black widow tetra and bucktooth no effect was observed (Butt et al., 2019). Forebrain *cart* mRNA expression levels were significantly higher in cod fish held at 2°C than in those held at either 11 or 15°C (Kehoe and Volkoff, 2008) suggesting Cart mediates temperature- induced changes in appetite during the seasonal changes in the lifespan of this species. In addition, the stomach from fish acclimated to 2°C had less food. Taking into consideration that these fish were handfed to satiety, the results suggested Cart acts as a satiety factor in this species (Kehoe and Volkoff, 2008). Previous studies investigating the role of *cart* in appetite regulation in Atlantic cod (Kehoe and Volkoff, 2007, 2008) and Atlantic salmon (Murashita et al., 2009) using a single *cart* paralog suggested an anorectic role for the neuropeptide. Indeed, in Atlantic salmon, 6 days of fasting resulted in decrease of brain *cart2b* (previously named *cart*) (Murashita et al., 2009). In medaka, 17 days of fasting also downregulated brain *cart2b* (previously named *cart ch3*) (Murashita and Kurokawa, 2011). However, both studies have used whole brain in the gene expression analysis. Accordingly, *cart2b* seemed to be involved in appetite regulation but the available data on its action are contradictory since orexigenic, anorexigenic or no action have been reported depending on whether the analyses were performed at brain region level (for e.g., hypothalamus) or on the whole brain and/or the fasting time used. Besides, two different studies in zebrafish reported that *cart2a* and *cart3a* (previously named *cart2* and *cart4,* respectively, Akash et al., 2014) were downregulated in response to short-term fasting within hypothalamic region and *cart2b* (previously named *cart3*, Ahi et al., 2019) was downregulated in brain after 7 days of fasting. The involvement of Cart2 in appetite regulation in Atlantic salmon is further supported by the fact that this is the only Cart paralog that has the same potential proteolytic sites as in mammals. These studies show the diversity between species and importance of long-term vs. short-term testing of the Cart neuropeptide and emphasizes the need for more sampling time points to uncover the specific role of Cart paralogs in appetite regulation in Atlantic salmon.

The *cart1a* and *1b* show quite similar mRNA expression profile and *cart1*, *cart2a,* and *cart3a* are highly expressed specially in the MB, suggesting that these *cart* paralogs might share the

same functional role. Comparably, in Senegalese sole, Bonacic et al. (2015) observed that *cart1*, *cart2a,* and *cart3a* were highly expressed in mesencephalon which is the equivalent region to the MB in our study. The *cart1a* and *1b* are primarily expressed in MB followed by PT in Atlantic salmon brain, which indicate that these genes might be involved in functions linked to these brain regions, such as vision and feeding for MB, and energy metabolism, organ development and reproduction for PT. Ye et al. (2020) showed that in grass carp the optic tectum seems to play an important role in the visual system and feeding. The potential role of *cart* in reproduction is reported in chicken, where pituitary CART peptide expression and secretion is regulated in the hypothalamic-pituitary-gonadal (HPG) axis through gonadotropin-releasing hormone (GnRH), and GnRH administration increased plasma CART (Mo et al., 2019). In this study, the *cart2a*, *2b,* and *4* mRNA expression in hypothalamus suggests that these might be involved in appetite control in Atlantic salmon. We further speculate that they might relay the signals relevant to feeding at different time points. However, further studies are needed to unravel the actual molecular interaction and functionalization among *cart* paralogs.

The feeding regime (continuously or meal-based) and the period in between last feed and sampling are also reflected in the expression of neuropeptides regulating appetite in the brain. Most probably, there is a higher variation in voluntary feed intake when the fish are fed continuously. The macronutrient composition of the feed and the period of fasting are also important factors as it can influence the species digestive physiology and gut evacuation time, and consequently affect the appetite control. Fukada et al. (2021) reported that in yellowtail, *cart2a* showed a tendency to decrease in hypothalamus with fasting and increased with refeeding when the sampling was performed 3 h after feeding. Similarly, in our study, 3 days of fasting resulted a tendency to downregulation of *cart2a* in HYP in fasted group compared to the fed group (sampled 2 h after last feed).

The *cart3b* expression revealed a completely different profile compared to other paralogs with BS as its primary site of expression of this paralog in the brain. This is in line with particularly high expression levels detected in the spinal cord in Senegalese sole (Bonacic et al., 2015). Though there was no significant difference in *cart3b* mRNA expression between the fed and fasted group, the decreasing trend with fasting in BS shows a potential anorexigenic role. We speculate that satiety information generated during a meal, largely conveyed via afferent fibers of vagus nerves first to the hindbrain from the upper gastrointestinal tract in mammals (reviewed in Schwartz et al., 2000), seems to be conserved in fish species. The BS region which contains Mauthner neurons (MN), specific multifunctional neurons in fish, receives afferent information from vestibular, auditory, and visual analyzers and can integrate the afferent activity. In Chinese sleeper (*Perccottus glehni*), the MN responded to severe environmental conditions like deficit of nutrients/energy substrates, oxygen and temperature changes by modifying the structure of the cell and through different pathways for the maintenance of the energy balance (Santalova et al., 2018). Whether this link between *cart3b* and MN is also seen in Atlantic salmon brain under starvation is unknown and would be an interesting topic for further studies.

Cart has also been suggested to be involved in other physiological processes like stress response, anxiety, depression, reproduction (Koylu et al., 2006), and immune response (Burgos et al., 2019) in mammals.

The interlink between CART peptide and other stress related peptides in the hypothalamic-pituitary-adrenal (HPA) axis have been somewhat studied in mammals and the findings show that intracerebroventricular CART injection increases hypothalamic *corticotropin-releasing factor* (*crf*) mRNA levels and CRF release. In rats, centrally administered CART increased adrenocorticotropic hormone and corticosterone in circulation and exerted dose-dependent increase in anxiety-related behaviors (reviewed in Koylu et al., 2006). Similarly, in fish under stressed conditions the mechanisms involving food intake are deregulated. Consequently, the regular signals controlling appetite in the brain are altered, resulting in changes in the expression of the appetite-related neuropeptides and reduction in food intake (reviewed in Conde-Sieira et al., 2018). In zebrafish, exposure to acute handling stress induced increased expression of *cart* and *pro-opiomelanocortin (pomc)* as central keys in the appetite control (Cortés et al., 2018), but the response of appetite regulating neuropeptides to stress can differ in salmonids (reviewed in Conde-Sieira et al., 2018). In our study, both experimental groups of fish were maintained throughout the experiment under same rearing conditions, even during sampling both groups went through the same procedures. The effect of handling stress in the appetite regulating neuropeptides genes expression in Atlantic salmon brain still remains to be uncovered.

CONCLUSION

This study identifies 10 *cart* paralogs in Atlantic salmon brain with distinct distribution profile. Among the paralogs *cart2b* responded to 4 days of fasting by increase in mRNA expression in hypothalamus, olfactory bulb and midbrain while *cart3b* showed a trend of decline in brain stem. The difference in key residual structure of Cart4 suggesting potentially different function other than appetite regulation. The Cart paralogs 1a, 1b, 2a, and 3a might likely have potential different roles in the regional integration of appetite (relaying) signals in the hypothalamus, telencephalon, and brain stem, and/or other functions in the olfactory bulb and midbrain. To uncover their specific role in appetite regulation in Atlantic salmon more sampling time points will be required. Moreover, the complex molecular interactions with other peptide hormones need to be untangled to understand the potential role of Cart in appetite regulation in Atlantic salmon.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in **Supplementary Table 3**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Representative of Animal Welfare at Department of Biological Sciences, University of Bergen, Norway, in accordance with Norwegian Animal Research Authority regulations.

AUTHOR CONTRIBUTIONS

TK, AG, KM, IT, FL, and IR conceived and designed the study. AG, KM, and IR conducted the experiment. AG, TK, KM, and FL contributed to the sampling. TK and AG did the primers design and phylogenetic analysis. TK and IT did preparatory lab work and qPCR. TK performed statistical analysis and drafted the manuscript. All authors contributed to the interpretation of the data, writing of the manuscript, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.763766/full#supplementary-material

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IV

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BRIEF RESEARCH REPORT
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Regional Expression of *npy* mRNA Paralogs in the Brain of Atlantic Salmon (*Salmo salar*, L.) and Response to Fasting

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Neuropeptide Y (NPY) is known as a potent orexigenic signal in vertebrates, but its role in Atlantic salmon has not yet been fully established. In this study, we identified three *npy* paralogs, named *npya1, npya2, and npyb*, in the Atlantic salmon genome. *In silico* analysis revealed that these genes are well conserved across the vertebrate's lineage and the mature peptide sequences shared at least 77% of identity with the human homolog. We analyzed mRNA expression of *npy* paralogs in eight brain regions of Atlantic salmon post-smolt, and the effect of 4 days of fasting on the *npy* expression level. Results show that *npya1* was the most abundant paralog, and was predominantly expressed in the telencephalon, followed by the midbrain and olfactory bulb. *npya2* mRNA was highly abundant in hypothalamus and midbrain, while *npyb* was found to be highest expressed in the telencephalon, with low mRNA expression levels detected in all the other brain regions. 4 days of fasting resulted in a significant ($p < 0.05$) decrease of *npya1* mRNA expression in the olfactory bulb, increased *npya2* mRNA expression in the midbrain and decreased *npyb* mRNA expression in the pituitary. In the hypothalamus, the vertebrate appetite center, expression of the *npy* paralogs was not significantly affected by feeding status. However, we observed a trend of increased *npya2* mRNA expression ($p = 0.099$) following 4 days of fasting. Altogether, our findings provide a solid basis for further research on appetite and energy metabolism in Atlantic salmon.

Keywords: Atlantic salmon (*Salmo salar* L.), neuropeptide Y, brain, appetite control, fullness, fasting

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INTRODUCTION

Control of food intake and energy metabolism in vertebrates are complex processes involving several neural pathways. In the brain, the hypothalamus integrates central and peripheral signals that either stimulate (orexigenic) or inhibit (anorexigenic) appetite into a coherent physiological and behavioral response (Volkoff, 2016; Rønnestad et al., 2017; Soengas et al., 2018). Among the signaling molecules involved, neuropeptide Y (NPY) plays a key role. In mammals, it has repeatedly been shown that food deprivation induces increased hypothalamic expression of this neuropeptide, and that administration of the 36 amino acid NPY results in increased food consumption and increased growth and body weight (Reviewed by Beck, 2006; Minor et al., 2009; and Mercer et al., 2011). Concurrently, intake of nutrients lowers the activity of

AgRP/NPY neurons, an effect that is proportional to the amounts of calories ingested (Su et al., 2017). In fact, NPY has been reported to be the most potent orexigenic molecule in mammals (Mercer et al., 2011).

Much evidence supports that NPY's functional role as a regulator of energy homeostasis and appetite control is conserved across vertebrates, including in teleosts (Volkoff et al., 2005; Volkoff, 2016; Rønnestad et al., 2017; Soengas et al., 2018). However, fish are the most diversified group of vertebrates with over 34,000 species identified to date (Froese and Pauly, 2019), and teleosts contain more than half of all vertebrate species (Nelson et al., 2016). This large number of species, along with large variations in anatomy, physiology, habitats and feeding and energy allocation strategies is likely to have caused evolvement of species specific appetite control mechanisms (Volkoff et al., 2009). Indeed, the relative importance of NPY in controlling feed intake seems to vary among teleosts. In several species, including goldfish (*Carassius auratus*) (López-Patiño et al., 1999; Narnaware et al., 2000), grass carp (*Ctenopharyngodon idella*) (Zhou et al., 2013), zebrafish (*Danio rerio*) (Yokobori et al., 2012), and rainbow trout (*Oncorhynchus mykiss*) (Aldegunde and Mancebo, 2006), NPY injections increase feed intake, supporting an orexigenic role. In line with this, food deprivation increased *npy* mRNA expression in the brain of goldfish (Narnaware and Peter, 2001), chinook (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*) (Silverstein et al., 1998), zebrafish (Yokobori et al., 2012) and winter skate (*Leucoraja ocellata*) (MacDonald and Volkoff, 2009b). Concomitantly, refeeding normalized *npy* mRNA abundance following food deprivation in goldfish (Narnaware and Peter, 2001). However, 7 days of fasting did not affect *npy* brain expression in the Atlantic cod (*Gadus morhua*) (Kehoe and Volkoff, 2007), and in cunner (*Tautogolabrus adspersus*) 3 weeks of fasting resulted in a decrease in *npy* expression in the telencephalon (Babichuk and Volkoff, 2013).

In Atlantic salmon (*Salmo salar*), *npy* mRNA expression in the brain did not significantly change after 6 days of fasting (Murashita et al., 2009), but increased during the first 9 h after feeding (Valen et al., 2011). These studies suggests that effects of fasting and feeding in Atlantic salmon central *npy* are time-sensitive and that both spatial and temporal response may be different to that found in mammals. However, the authors analyzed whole brain, an approach that does not take into account regional specific *npy* responses. In fact, NPY has several other functions in the central nervous system besides appetite control, including reproductive regulation (Saha et al., 2015), stress regulation (Reichmann and Holzer, 2016), circadian rhythm (Singh et al., 2017), neurogenesis (Agasse et al., 2008; Baptista et al., 2012), cognition (Redrobe et al., 1999; Gøtzsche and Woldbye, 2016), and visual perception (Santos-Carvalho et al., 2015). Furthermore, due to the four whole genome duplication events (4R WGD) in salmonids, it is expected that Atlantic salmon has several *npy* paralogs with potentially divergent roles. Thus, knowledge about the different paralogs, their regional distribution and their responses to different feeding conditions is key to understand the role of Npy in appetite regulation of Atlantic salmon.

In this study, we provide an *in silico* characterization of the three newly identified Npy paralogs in Atlantic salmon, and investigate the regional brain distribution of *npy* in both fed or 4 days fasted salmon. Additionally, we examine the correlation between gastrointestinal filling and hypothalamic mRNA expression of each *npy* paralog to gain further understanding of the Atlantic salmon gut-brain axis. A 2–4 days fasting period prior to handling, transportation and harvest is common practice in Atlantic salmon aquaculture production (Waagbø et al., 2017), and uncovering impact of fasting on farmed fish is essential to safeguard fish welfare and optimize the aquaculture feeding protocols.

MATERIALS AND METHODS

Ethical Treatment of Animals

The research and sampling were conducted in accordance with the Norwegian Animal Research Authority regulations and was approved by the local representative of Animal Welfare at the Department of Biological Sciences, University of Bergen (Norway).

Experimental Setup and Sampling

Atlantic salmon post-smolts (ca 180 g) were obtained from Bremnes Seashore's RAS facility (Trovåg, Norway) and acclimatized in 150 L freshwater tanks at 8.5°C for 18 days. For more details on the experimental setup, please refer to Kalananthan et al. (2020). To evaluate the effect of the fasting, 12 Atlantic salmon post smolts were sampled, 6 from the group that was fed daily *ad libitum* from 9:00 to 16:00 h (sampled 2 h after feeding), and 6 from the 4 days fasted group. The fish were euthanized with an overdose of MS222, and content of the gastrointestinal tract compartments (stomach, midgut, and hindgut) was collected and processed as previously described (Kalananthan et al., 2020). The whole brain was dissected out, and stored in RNAlater (Thermo Fisher Scientific, Waltham, MA, United States). The individual fish weight and length was recorded, and the Fulton's condition factor (K) determined according to Froese (2006).

Sequence and Comparative Analysis

The Atlantic salmon *npy* transcripts were searched using the previously identified salmon *npya1* amino acid sequence (GenBank acc. no. NP_001140153.1) as a query against the Atlantic salmon genome database available in NCBI GenBank[1] and Ensembl[2]. The predicted NPY protein sequences of Atlantic salmon and human NPY were aligned using MUSCLE with the default parameters (UPGMA clustering method, Gap opening penalty −2.90, Gap extension 0.0) from MEGAX (Hall, 2013) and edited using GeneDoc 2.7 software (Nicholas et al., 1997). The percentages of similarity/identity between sequences were calculated using BLASTP[3]. Putative signal peptides were

[1]https://www.ncbi.nlm.nih.gov/genbank/

[2]http://www.ensembl.org/index.html

[3]https://blast.ncbi.nlm.nih.gov/Blast.cgi

predicted by PrediSi[4] (Hiller et al., 2004), and mature peptide sequences were predicted using NeuroPred[5].

Phylogenetic tree was constructed using the deduced amino acid sequences of the full-length NPY from 13 teleost species and the human (*Homo sapiens*) NPY retrieved from NCBI GenBank and Ensembl. Multiple alignments were generated using MUSCLE with the default parameters from MEGAX (Hall, 2013). The sequence alignment was analyzed for the best-fit substitution model in MEGAX to select the best statistical model to study protein family evolution. The phylogenetic tree was constructed using Maximum Likelihood (ML) with a Jones-Taylor-Thornton (JTT) model (Jones et al., 1992) with fixed Gamma distribution (+G) parameter with five rate categories and 1000 bootstrap replicates. The tree was then rooted to the human NPY sequence.

mRNA Abundance Analysis by RT-qPCR

The Atlantic salmon brain of fed (n = 6) and fasted (n = 6) fish were dissected into eight regions: olfactory bulb, telencephalon, midbrain, cerebellum, hypothalamus, saccus vasculosus, pituitary, and brain stem. Total RNA was isolated from each brain region using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Depending on the availability of total RNA per section, 2.5 μg or 10 μg total RNA samples were treated with TURBO DNA-free (Thermo Fisher Scientific) with 1 μl of DNase (2 U/μl) in 10 or 30 μl reaction volume, respectively, to eliminate possible genomic DNA contamination. Quantity and integrity of DNase treated total RNA was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). cDNA was synthesized from 1.1 μg of DNase treated total RNA using oligo (dT) primer from SuperScript III First-Strand Synthesis system for RT-PCR kit (Thermo Fisher Scientific). Specific primers spanning an exon-exon junction were designed for all the target genes (**Supplementary Table 1**). β-*actin* and *ribosomal protein s20* (*s20*) were used as reference genes. qPCR reactions were performed in duplicate using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States) in a 20 μl final reaction volume. The qPCR reactions were performed in a Bio-Rad CFX96^{TM} Real-Time System with the following cycling conditions: 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 25 s. Melting curve analysis over a range of 65–95°C (increment of 0.5°C for 2 s) allowed for the detection of possible nonspecific products and/or primer dimers.

Standard curves were generated by the protocol described by Kalananthan et al. (2020) and used to determine the qPCR efficiency for each assay (**Supplementary Table 1**).

Statistical Analysis

Statistical analyses were performed using GraphPad (GraphPad Software, version 9). Equality of variances and normality of data related to fish weight, length, K, gastrointestinal content, and gene expression were tested using F-test and Shapiro–Wilk normality test, respectively. Grubb's outlier test was used prior to statistical evaluations and outliers were removed. Analysis of differential expression between the fed and fasted group within a brain region and differences between the fed and fasted group pertaining to K and gastrointestinal filling was performed with two-tailed t-test. When either the F-test or the normality test failed, the no-parametric Mann–Whitney test was performed. Pearson's correlation coefficients were calculated to investigate the correlation between wet and dry weight content of gastrointestinal compartments (stomach, midgut, and hindgut), as well as between hypothalamic *npy* mRNA expression and dry weight content from each section of the gastrointestinal tract normalized by fish weight. A $p < 0.05$ was considered significant. All data are presented as mean ± SEM, unless otherwise stated.

RESULTS

Characterization of Atlantic Salmon Npy and Phylogenetic Analysis

In Atlantic salmon, three *npy* genes were found to be located on chromosomes ssa14 (*npya1*), ssa27 (*npya2*), and ssa5 (*npyb*). The predicted full length amino acid (AA) sequences of Atlantic salmon NPY varied from 100 to 167 AA in length (data retrieved from Ensembl, October 2020) (**Supplementary Figure 1**), with predicted protein masses between 11.33 and 18.83 kDa (predicted by Expasy)[6] (**Supplementary Table 2**). The predicted pro-NPY peptides contained putative signal peptides of 28, 95, and 75 AAs for the NPYa1, NPYa2, and NPYb paralogs, respectively [PrediSi (see text footnote 4), Hiller et al., 2004; **Figure 1**]. The potential processing signal (KR) at the C-terminal of the mature peptide was found to be well conserved (**Figure 1**). The predicted 36 AA mature sequences showed a molecular weight around 4.2–4.3 kDa (**Supplementary Table 2**). Based on the predicted mature peptide sequences, NPYa1 and NPYa2 shared 97% identity at the AA level, and both shared 75% identity with NPYb. All three paralogs are relatively well conserved with the human homolog, sharing between 78 and 86% AA sequence identity, with NPYa2 being most similar (**Supplementary Table 3**). The three proline and two tyrosine residues vital to the conformation of the NPY family were conserved in all three salmon NPY paralogs (**Figure 1**; Cerda-Reverter and Larhammar, 2000).

Phylogenetic analysis showed that the NPY peptides encoded by the three Atlantic salmon *npy* genes group with teleost homologs (**Figure 2**). Two major clades are present, one containing the teleost NPYa and the other NPYb. Most teleost species have one *npya* and one *npyb* gene, however, Cypriniformes only have one *npya* (zebrafish) or two *npya* genes (common carp (*Cyprinus carpio*)). In the case of salmonids, all species analyzed have 2 *npya* genes. However, for NPYb, the case is different: two *npyb* genes were found for coho salmon and brown trout (*Salmo trutta*), while only one *npyb* gene was present for Atlantic salmon and rainbow trout and no *npyb* gene was found in the Chinook salmon genome.

[4]http://www.predisi.de/home.html

[5]http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py

[6]https://www.expasy.org/

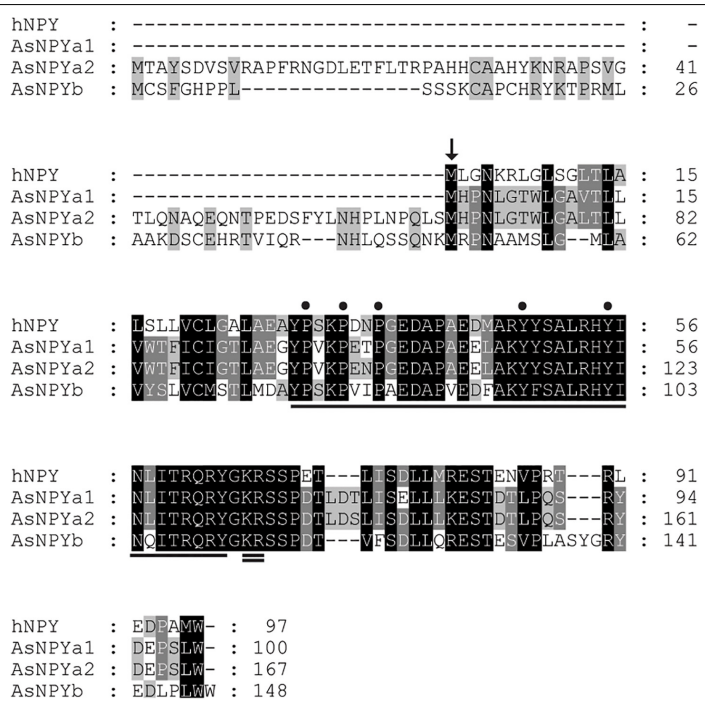

FIGURE 1 | Primary protein sequence alignment of the human neuropeptide Y (NPY) and the Atlantic salmon NPY paralogs (NPYa1, a2 and b). Arrow indicates the beginning of the predicted signaling peptide for the human NPY. Underline indicates the mature NPY sequence and double underline indicates the conserved processing amino acid sites (KR). The three proline (P) and two tyrosine (Y) residues, which are imperative to the conformation of the NPY family, are indicated by dots.

Brain Distribution of Atlantic Salmon *npy* mRNA

The three *npy* paralogs showed a wide distribution in the eight brain regions analyzed (**Figure 3**). *npya1* mRNA was found to be highly expressed in the telencephalon, followed by the midbrain and olfactory bulb. *npya2* mRNA level were highly abundant in the hypothalamus, midbrain, olfactory bulb, saccus vasculosus, and telencephalon, while mRNA expression levels of *npyb* was found to be highest in the telencephalon, with lower levels of expression in the hypothalamus, midbrain and brain stem, and only residual mRNA expression levels in the other brain regions. Overall, the *npya1* was the most abundant paralog in the Atlantic salmon brain, and all *npy* paralogs showed very low mRNA expression levels in the cerebellum, pituitary, and brain stem. Although very low, *npy* detected in the cerebellum is noteworthy as expression found in this region is rare.

Effects of 4 Days of Fasting on Atlantic Salmon *npy*

Both fed and fasted fish groups had a mean K factor of 1.11 (**Supplementary Figure 2A**). As expected, there was a significant ($p < 0.0001$) correlation between wet and dry digesta weight in all gastrointestinal tract compartments (**Supplementary Figure 2B** and **Supplementary Table 4**). The amount of digesta in the stomach and midgut of fasted fish was, as expected, significantly ($p < 0.05$) lower compared to the fed group. However, there were no differences between the two groups in the hindgut (**Supplementary Figure 2C** and **Supplementary Table 4**). Four days of fasting resulted in significantly ($p < 0.05$) decreased expression of *npya1* in the olfactory bulb and *npyb* in pituitary, and increased *npya2* expression in the midbrain. In addition, we also observed a trend ($p = 0.99$) of increased hypothalamic expression of *npya2* in fasted fish compared to fed (**Figure 3** and **Supplementary Table 5**). No statistically significant correlation was found between hypothalamic mRNA expression of *npy* paralogs and the inner content of the gastrointestinal compartments. The highest observed correlation was between stomach content and *npya2* mRNA expression ($p = 0.129$) (**Figure 4** and **Supplementary Table 6**).

DISCUSSION

In the present study, we report for the first time the identification and characterization of three Atlantic salmon *npy* paralogs; namely *npya1*, *npya2,* and *npyb.* All paralogs were highly

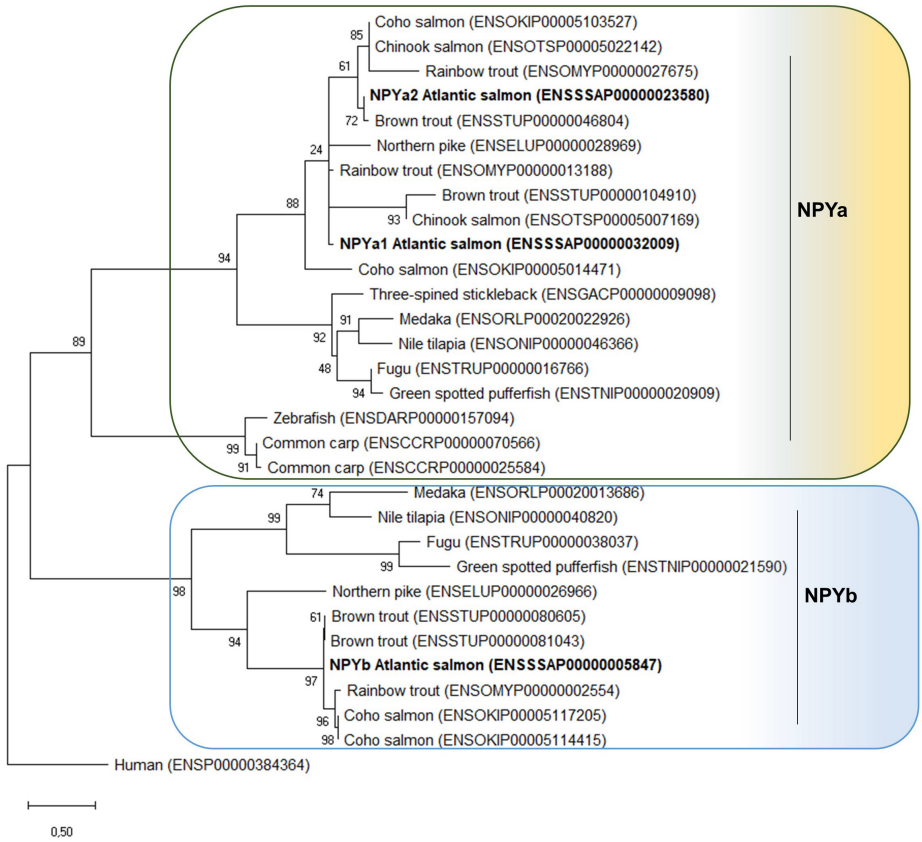

FIGURE 2 | Phylogenetic tree of the NPY family peptide in teleosts. The phylogenetic tree was constructed based on the NPY predicted full-length amino acid sequences using the Maximum Likelihood method and JTT matrix-based model with fixed Gamma distribution parameter with five rate categories, and 1000 bootstrap replicates. The tree was rooted to the human NPY sequence. Evolutionary analyses were conducted in MEGAX (Hall, 2013). Accession numbers are provided in front of the common species name.

conserved at the amino acid level, sharing between 78 and 86% sequence identity with the human homolog. Moreover, in agreement with previous findings (Murashita et al., 2009), each paralog encoded the three proline and two tyrosine residues ($Pro^{2/5/8}$ and $Tyr^{20/27}$) known to be important for maintaining protein conformation (**Figure 1**; Cerda-Reverter and Larhammar, 2000). In teleosts, the NPY peptides so far described have been named NPYa and NPYb (**Figure 2**). Presumably, the a and b duplicates have resulted from the WGD in the teleost fish lineage (Sundström et al., 2008). Differential losses may have occurred, since zebrafish, common carp, three-spined stickleback (*Gasterosteus aculeatus*) and Chinook salmon seems to be missing NPYb. Equally, the additional *npya* gene duplication is present for both salmonids and common carp, as most probably a result of the additional 4R WGD duplication in salmonids and carps (Tang et al., 2014). This was, however, not observed for *npyb* gene, suggesting that *npyb* gene duplication or absence may be a result of a species-specific event. Importantly, this is the first report of NPYb being present in the Atlantic salmon genome and it is likely owed to the recently updated databases.

The predicted NPY protein sequence conservation likely points toward a conserved functional role NPY as a regulator of energy metabolism in Atlantic salmon. However, conclusions from previous studies have been somewhat divergent, possibly due to a temporal response of *npy* expression to feeding status. The results from Murashita et al. (2009) indicated that 6 days fasting did not increase expression levels of *npy,* while Valen et al. (2011) found that feeding status significantly affected *npy* expression levels in the brain, with the increase taking place during the first 9 h after feeding. Importantly, the primers used in these studies only amplified *npya*, and most likely did not differentiate between *npya1* and *npya2.*

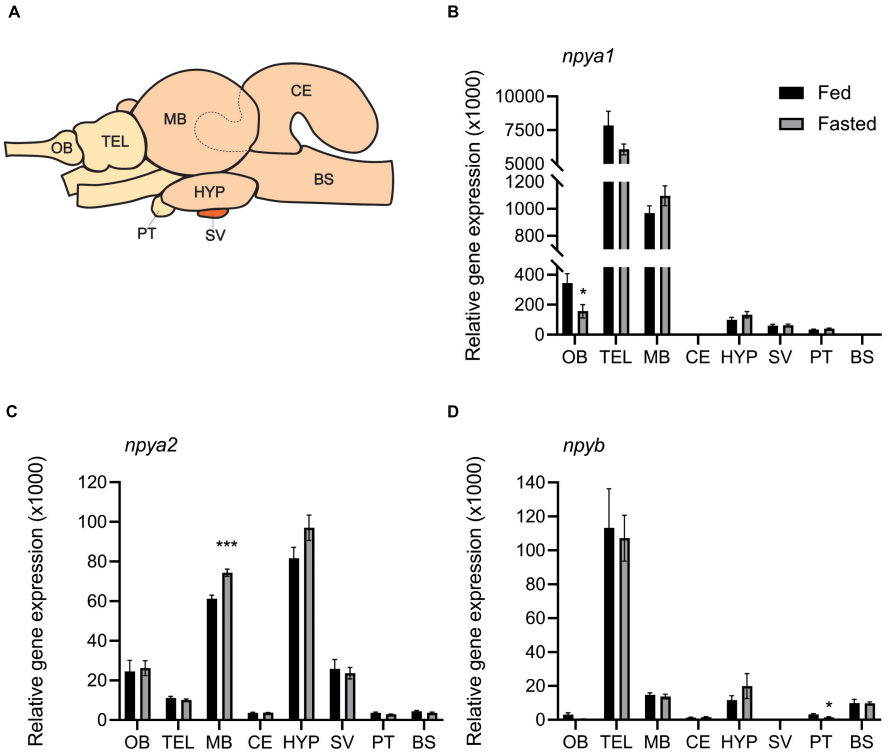

FIGURE 3 | (A) Schematic representation of Atlantic salmon brain, showing dissection of the eight brain regions used for gene expression analysis: olfactory bulb (OB), telencephalon (TEL), midbrain (MB), hypothalamus (HYP), cerebellum (CE), saccus vasculosus (SV), pituitary (PT), and brain stem (BS). The dashed line represents the dissected area of the CE inside the MB. Effects of 4 days of fasting on the mRNA expression levels of **(B)** *npya1*, **(C)** *npya2*, and **(D)** *npyb* in the eight regions of Atlantic salmon brain. Black and gray columns represent fed and fasted fish, respectively. $n = 6$ for both groups and for all regions except in saccus vasculosus ($n = 4$) and in instances where technical or statistical outliers have been removed (refer to **Supplementary Table 5**). Values are expressed as copy number per ng total RNA used in the reaction normalized using the geometric mean of the reference genes s20 and β-actin-actin. Bars represent mean ± SEM. Two-tailed *t*-test was performed to assess whether there were statistically significant differences between the fed and fasted group for each brain region. Asterisks (*) indicate statistically significant difference ($^*p \leq 0.05$, $^{***}p \leq 0.001$).

Moreover, both studies used the whole brain for gene expression analysis, an approach which does not consider potential region-specific responses in *npy* expression or possibly distinct expression pattern of each paralog gene. This is especially important given that the presence of multiple paralogs opens the possibility for neofunctionalization and/or subfunctionalization (Lien et al., 2016).

To overcome this limitation, we investigated the expression pattern of each *npy* gene, and their response to 4 days of fasting in eight brain regions. *npya1* was found to be the most abundant paralog and was highly expressed in the telencephalon, followed by the midbrain, olfactory bulb and hypothalamus (**Figure 3B**). Interestingly, expression in the olfactory bulb decreased significantly following 4 days of fasting. Previous studies have identified the presence of NPY in the olfactory bulb in teleost species such as goldfish (Pontet et al., 1989), the ayu (*Plecoglossus altivelis*) (Chiba et al., 1996) and zebrafish (Kaniganti et al., 2019). In zebrafish it was also demonstrated that NPY signaling increased upon fasting, indicating that NPY levels in the olfactory bulb reflect the energy status in the brain (Kaniganti et al., 2019). Though we observed the opposite effect, our findings may indicate that *npya1* plays a role in the olfactory input related to energy regulation in Atlantic salmon. Moreover, its increased expression in this brain region may account for observations made by Valen et al. (2011). However, we cannot rule out that expression in other brain regions, such as the hypothalamus, may have been affected in the immediate or short-term time frame used in that study. The high expression in the telencephalon is also of interest to the field of appetite control since this brain region has been proposed to be the location of mechanisms involved in hedonic regulation of food intake in fish (O'Connell and Hofmann, 2011; Comesaña et al., 2018; Otero-Rodiño et al., 2018).

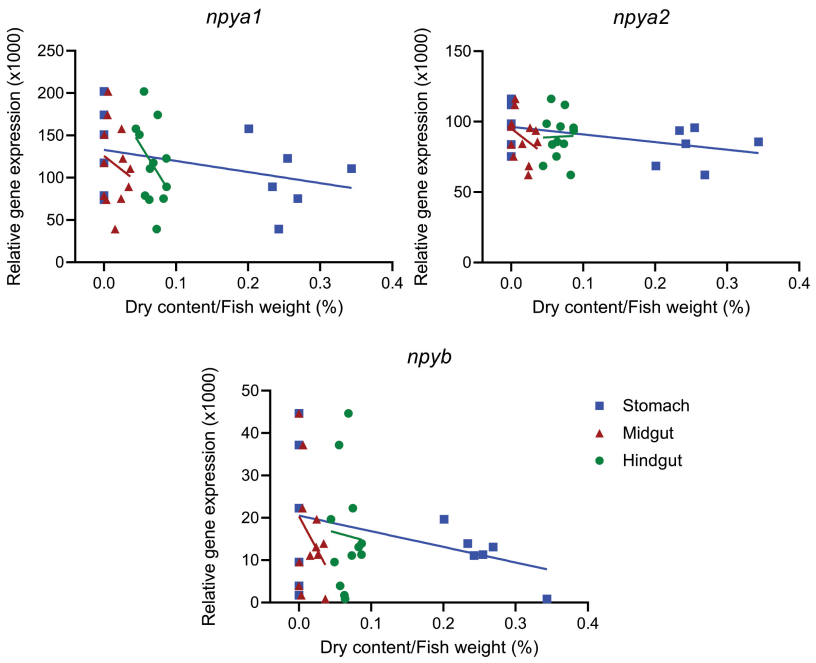

FIGURE 4 | Correlation between dry content in the gastrointestinal tract and hypothalamic expression of *npya1*, *npya2*, and *npyb*. The plots show hypothalamic mRNA expression of *npya1*, *npya2* and *npyb versus* content in the stomach, midgut and hindgut. Dots represent individual fish (n = 12), while solid lines represent the linear regression. The dry weight of the gastrointestinal content was standardized to the fish weight. For detailed statistical information, refer to **Supplementary Table 6**.

npya2 was found to be highest expressed in the hypothalamus and while its expression did not significantly change depending on feeding status, there was a trend of increased expression following 4 days of fasting (p = 0.99) (**Figure 3C**). The hypothalamus is considered the hub for appetite control in vertebrates, and in spite of not being statistically significant, this finding may support the suggested role of NPY as an orexigenic factor in vertebrates, including several fish species, such as goldfish (López-Patiño et al., 1999; Narnaware and Peter, 2001), grass carp (Zhou et al., 2013), zebrafish (Yokobori et al., 2012; Tian et al., 2015), rainbow trout (Aldegunde and Mancebo, 2006), winter skate (MacDonald and Volkoff, 2009b), Atlantic cod (Kehoe and Volkoff, 2007), tiger puffer (*Takifugu rubripes*) (Kamijo et al., 2011), nile tilapia (*Oreochromis niloticus*) (Yan et al., 2017), chinook and coho salmon (Silverstein et al., 1998).

Notably, expression of both *npya1* and *npya2* was found to be high in midbrain, and expression of *npya2* mRNA increased significantly following 4 days fasting (**Figures 3B,C**). The implications of this depends on the area of the midbrain affected. *npy* mRNA has been observed in the *optic tectum* region of midbrain in zebrafish (Söderberg et al., 2000), goldfish (Peng et al., 1994; Narnaware and Peter, 2001), Coho and Chinook salmon (Silverstein et al., 1998), Atlantic cod larva (Le et al., 2016), sea bass (Cerdá-Reverter et al., 2000), winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009a) and winter skate (MacDonald and Volkoff, 2009b). Moreover, NPY-immunoreactive structures have been identified in the *optic tectum* of Atlantic salmon, as well as several other fish species (Vecino and Ekström, 1990; Chiba, 2005; Pirone et al., 2008; Amiya et al., 2011; Magliozzi et al., 2019). The *optic tectum* is the visual center in the non-mammalian brain, and while the function of NPY in this brain region of fish remains to be fully elucidated, the presence of *npya1* and *a2* could indicate a role in modulating retinotectal relay, as proposed in domestic chicks (*Gallus gallus domesticus*) (Székeley et al., 1992) and in toad *Bombina orientalis* (Funke and Ewert, 2006). If the observed increased *npya2* expression occurs in this region, it would be in line with a previous study in goldfish (Narnaware and Peter, 2001) and could indicate a link between feeding status and visual perception in Atlantic salmon. Such a link has previously been demonstrated in zebrafish (Filosa et al., 2016), and may be part of a broader physiological response in which hunger triggers a shift from escape to approach in the case of limited food availability (Barker and Baier, 2015). Another important region of the midbrain is the preoptic area (POA), which is involved in thermoregulation, mating behavior and

orexin detection (Nelson and Prosser, 1979, 1981; Kaslin et al., 2004; Yokobori et al., 2011; Tripp et al., 2020) and is a site for Npy-action in several fish species including goldfish (Narnaware and Peter, 2001), Senegalese sole (*Solea senegalensis*) (Rodríguez-Gómez et al., 2001), pejerrey (*Odontesthes bonariensis*) (Traverso et al., 2003), Cichlid fish (*Cichlasoma dimerus*) (Pérez Sirkin et al., 2013), African lungfish (*Protopterus annectens*) (Trabucchi et al., 2000) and Coho and Chinook salmon (Silverstein et al., 1998). However, expression and possible function of *npya1* and *a2* in the POA of Atlantic salmon remains to be elucidated. If increased expression of *npya2* occurs in this area of the midbrain, it would be in line with findings in goldfish (Narnaware and Peter, 2001) and Chinook and Coho salmon (Silverstein et al., 1998) that the POA is key in orexin detection, thermoregulation and mating behavior. Since these are all tightly intertwined with energy homeostasis and appetite (Peyon et al., 1999; Volkoff and Rønnestad, 2020), a link between feeding status and *npy* expression level in this region would be unsurprising. While these are alluring speculations, tailored studies will be required to assess the plausibility.

For *npyb* (**Figure 3D**) fasting only affected mRNA expression levels in the pituitary. Given the very low expression of *npyb* in the pituitary and that there is no known correlation between pituitary and appetite regulation, this is likely an artifact. In sum, our findings therefore suggest that Atlantic salmon *npyb* is likely not involved in central appetite control. This is in agreement with previous studies in Nile Tilapia, where it was found that *npya* is the main paralog involved in feeding regulation (Yan et al., 2017), as well as in tiger puffer, where it was found that *npya*, and not *npyb*, in the hypothalamus is involved in regulating feed intake (Kamijo et al., 2011). Previous studies in fish have indicated that Npy has a widespread distribution, with expression found in the brain, gastrointestinal tract (summarized by Volkoff, 2016) and eye (Kurokawa and Suzuki, 2002; Chen et al., 2005; Murashita et al., 2009). As such, the NPYb might serve its main function in one of these tissues. We also cannot rule out NPYa1 and NPYa2 having different functions in unexamined tissues, and a study exploring *npy* expression in peripheral tissues would be of high interest.

Another important aspect to consider when studying central appetite control is its relationship to peripheral signaling. Distention of the stomach and interactions between nutrients and receptors on the gut wall regulates secretion of peptide hormones that communicate the degree of stomach and gut fullness as well as nutritional content to the central system. The gut-brain axis is key in control of food intake during a meal (Grove et al., 1978; Sam et al., 2012; Kalananthan et al., 2020), and we therefore investigated the relationship between hypothalamic expression of the *npy* paralogs and content of the gastrointestinal compartments. While no statistically significant correlation was found, the highest observed correlation was that between stomach content and hypothalamic *npya2* expression (**Figure 4** and **Supplementary Table 6**). Given the inverse relationship between appetite and fullness (Grove et al., 1978; Sam et al., 2012; Kalananthan et al., 2020), this could support the hypothesis that *npya2* encodes an important orexigenic factor in Atlantic salmon.

In summary, we identified three *npy* paralogs in the Atlantic salmon genome, including *npyb,* and demonstrated a significant effect of fasting on expression of *npya1* in the olfactory bulb, *npya2* in the midbrain and a trend toward increased expression of *npya2* in the hypothalamus. These findings support a conserved role of NPY in appetite control.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/ **Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The experiment was conducted in an experimental facility approved to conduct experiments with teleosts and in accordance with the rules and regulations of the Norwegian Animal Research authority. The experiment was approved by the local representatives for animal welfare at the Department of Biological Sciences, University of Bergen (Norway). The personnel conducting the experiment and sampling were accredited by Federation of European Laboratory Animal Science Associations (FELASA).

AUTHOR CONTRIBUTIONS

IT, TK, FL, KM, IR, and AG designed the study. TK, FL, and AG did the preparatory lab work. IT performed the qPCR analysis. IT and FL did the statistical analysis. AG did the phylogeny analysis. IR made the basis for the schematic illustration of the Atlantic salmon brain and oversaw the project. All authors contributed to data analysis and writing of the manuscript, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.720639/full#supplementary-material

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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 (1.2kg, ~46cm), 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 weeks 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 antagonist AGRP and NPY peptides in the ARC of mammalian species. In turn, AGRP and NPY act on the 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 POMC/CART-expressing neurons and inhibits feed intake also through the MC4R receptors. Many of the neuropeptides and endocrine signals involved in appetite control in mammals have also been identified in teleost fishes. 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, feeding behaviors, 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 extended feed deprivation (fasting or starving) on the brain/hypothalamic appetite key regulators. These fasting studies have ranged from of one week of feed deprivation in Atlantic cod (*Gadus morhua*) (Kehoe and Volkoff, 2007) and in arctic charr (*Salvanius alpinus*) (Striberny and Jørgensen, 2017), two weeks in winter skate (*Raja ocellata*) (MacDonald and Volkoff, 2009b), 17 days of starvation in medaka (*Oryzias latipes*) (Murashita and Kurokawa, 2011), 3 weeks of fasting in cunner (*Tautogolabrus adspersus*) (Babichuk and Volkoff, 2013), 29 days in sea bass (*Dicentrarchus labrax*) (Agulleiro et al., 2014), one month feed deprivation in arctic charr (Striberny and Jørgensen, 2017) and in winter flounder (*Pseudopleuronectes americanus*) (MacDonald and Volkoff, 2009a), 8 weeks fasting in spotted sea bass (*Lateolabrax maculatus*) (Zhang et al., 2019), to 4 months feed deprivation in rainbow trout (*Oncorhynchus mykiss*) (Jørgensen et al., 2016).

To date, studies have reported the effects of long-term feed deprivation on Atlantic salmon 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, however the control mechanisms for appetite in the stomach and hypothalamus have not been investigated in this species. Atlantic salmon (*Salmo salar*) 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. 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*), *mc4r* (*a2 and b1*) in the hypothalamus. We also analyzed the mRNA expression of *ghrl* (*1* and *2*) and *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 Figure 1A-D** for dissection procedure). The whole brain of each fish was dissected out (see **Supplementary Figure 2A & 2B** 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**):

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

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

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 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 Figure 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 Figure 1D**), rinsed in 1x PBS (phosphate buffered saline), flash frozen, and stored at -

80 ºC 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 Figure 2C**) 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* (Primers 5′-3′ F:GGGTTGGCAAACATTCTGGC, R:ACACTGATAGGAGAAGCCTGG and the amplicon size is 89bp), 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 to 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.

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

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 less than 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 (**Figure 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 (**Figure 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 (**Figure 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.

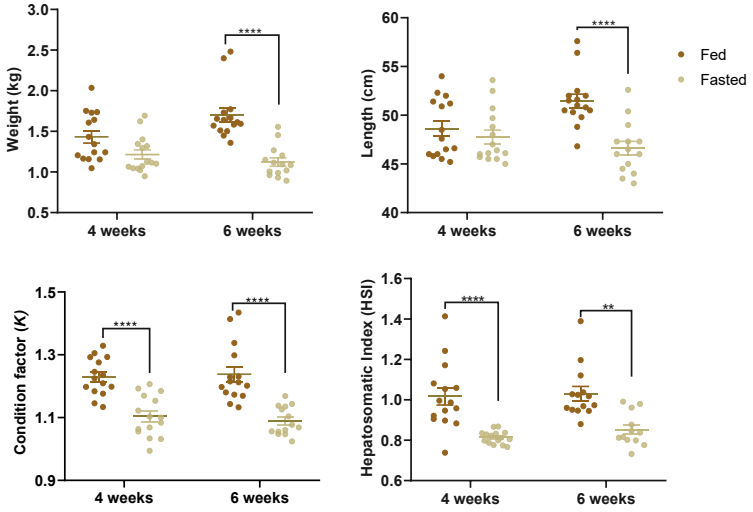

Figure 1. The weight (kg), length (cm), Fulton's condition factor (*K*) (4 weeks: Fed $n = 15$; Fasted $n = 15$; 6 weeks: Fed $n = 15$; Fasted $n = 14$) and Hepatosomatic Index (HSI) of fish sampled at 4 weeks (Fed $n = 15$; Fasted $n = 15$) and 6 weeks (Fed $n = 14$; Fasted $n = 12$) of experimental 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 $p < 0.0001$.

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 Figure 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 Figure 3 & Supplementary Table 2)**.

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 ($p < 0.01$) (**Figure 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 (**Figure 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.

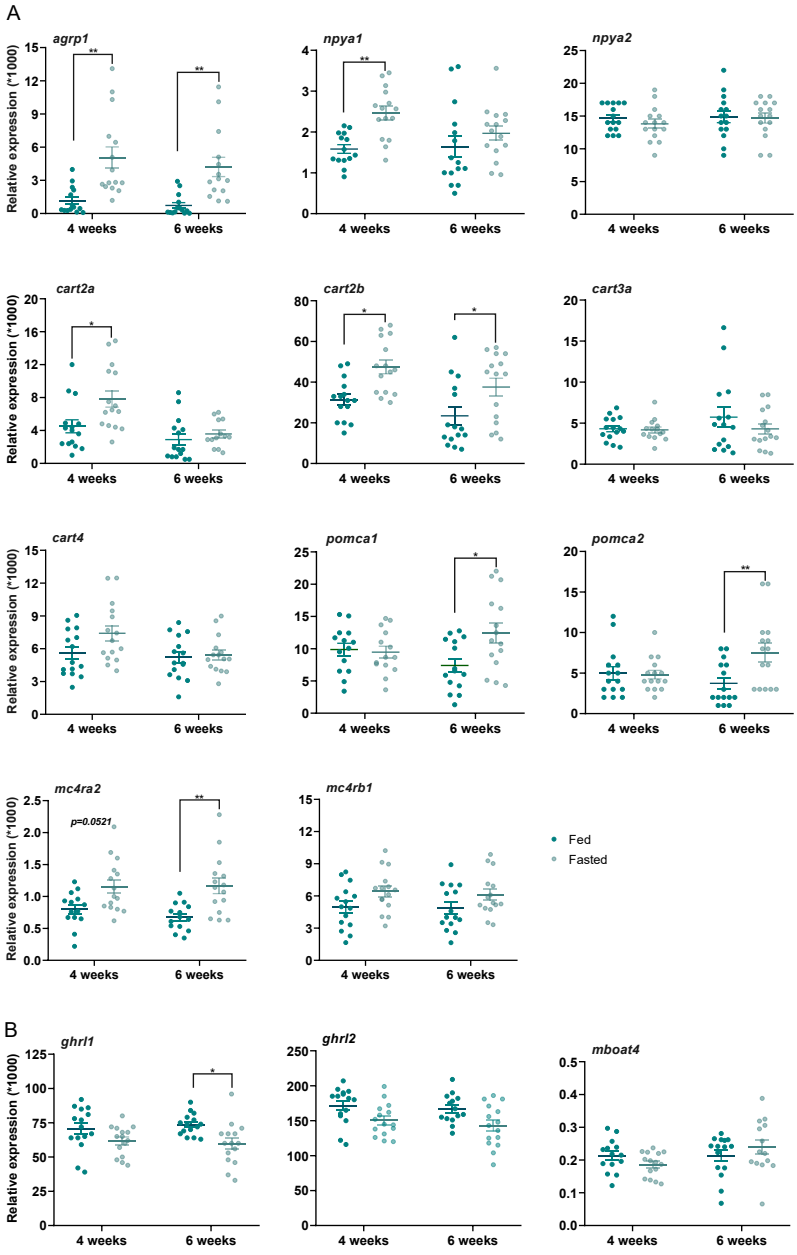

Figure 2. Effect of 4 and 6 weeks of fasting on the mRNA expression of **A.** *agrp1*, *npya1*, *npya2*, *cart2a*, *cart2b*, *cart3a*, *cart4*, *pomca1*, *pomca2, mc4ra2* and *b1* in hypothalamus and **B.** *ghrl1*, *ghrl2* and *mboat4* in stomach of Atlantic salmon. Dark and light blue dots represent 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 (*$p < 0.05$, **$p < 0.01$).

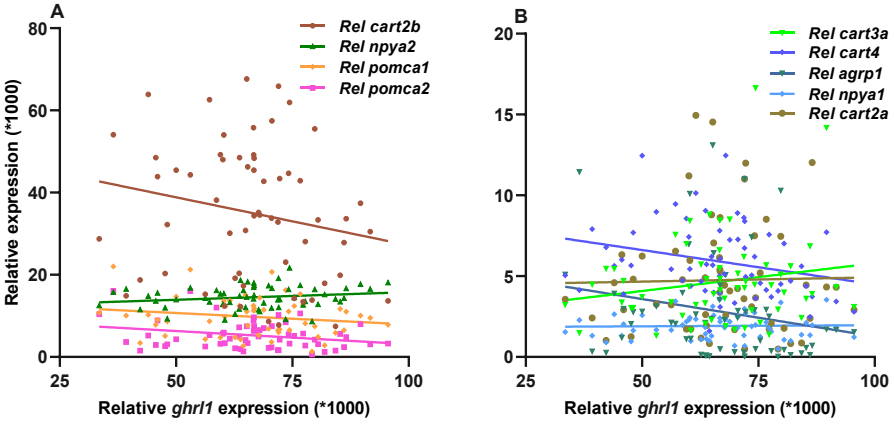

Figure 3. A. Relative mRNA expression of abundant hypothalamic melanocortin neuropeptides *cart2b*, *npya2*, *pomca1* and *pomca2* versus relative mRNA expression of *ghrl1*. **B**. The relative expression of less abundant hypothalamic melanocortin neuropeptides *cart3a, cart4, agrp1, npya1* and *cart2a*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

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 (**Figure 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, in which the neurons from the hypothalamic arcuate nucleus (feeding center) 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 (**Figure 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 observation that the HSI decline at 4 weeks of fasting is greater than that at 6 weeks of fasting 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. A parallel study conducted in the same experiment here presented continued the fasting period until 8 weeks, showing that the fasted fish had lost 7.4% of their start weight (Hvas et al., 2022) vs. 5% as here after 6 weeks, indicating a relative high rate of weight loss between 6 to 8 weeks of feed deprivation. A similar decrease in weight after long-term fasting was found by Einen et al. (1998) where large Atlantic salmon (4.9 kg, *K* factor > 1.5) were fasted at ~4 °C during 86 days. Einen et al. (1998) 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

297 viscera and liver. A significant decline in HSI (related to glycogen reduction) with
298 starvation has also been reported in brown trout (*Salmo trutta*) (Bayir et al., 2011),
299 Siberian sturgeon (*Acipenser baerii*) (Ashouri et al., 2013; Babaei et al., 2016), gilthead
300 seabream (*Sparus aurata*) (Metón et al., 2003) and common dentex (*Dentex dentex*)
301 (Pérez-Jiménez et al., 2012). In our study, HSI data suggest that the liver as fuel storage
302 of both glycogen and fat has possibly been utilized during 4 to 6 weeks fasting. (Hvas
303 et al., 2022), have refed the fish after the 8 weeks of fasting, showing a gradually
304 increased feed intake over the first month. After three months, the weight difference
305 between the experimental groups (Fed vs. Fasting) was minor, while the *K* factor was
306 highest in the Fasted/refed fish. Full compensatory growth was found after 7 months of
307 refeeding and no effect of fasting was found on welfare parameters (Hvas et al., 2022).
308 This highlights the flexibility of growth trajectories and compensatory mechanisms in
309 Atlantic salmon, similar to reported effects of photoperiod manipulation on seasonal
310 growth rate and *K* factor (Stefansson et al., 1991; Oppedal et al., 1999).

311 **4.2 Stomach regulation of appetite**

312 In long-term fasted fish (4 and 6 weeks), each compartment of GI- tract was, as expected,
313 almost empty (**Supplementary Figure 4**) with a minor presence of bright yellow fluid
314 (most likely bile) in the gut. The GI-tract likely had reduced functionality after the long-
315 term fasting as previous studies in salmon showed that fasting led to decreased GI mass
316 and enzyme capacities of the GI-tract by 20–50% within two days, and 40 - 75% after
317 40 days (Krogdahl and Bakke-McKellep, 2005). In this study, the correlation between
318 the stomach content (% dry weight of content to BW) to the relative mRNA expression
319 resulted in no clear correlation (data not shown). Here, the Fed group of each sampling
320 received their last meal on the previous day between 18-24h before sampling. Therefore,
321 the results were incomparable to Kalananthan et al., (2020b) where the fish was fed 2 h
322 prior to sampling and where the fish had a significant stomach filling. From both a
323 physiological and a behavioral point of view and compared to continuous active feeding,
324 feed anticipation, as induced by a mealtime schedule which allows appetite build-up
325 between meals, is highly beneficial both under experimental and farming conditions to
326 maximize the feed utilization of fish (Kulczykowska and Sánchez Vázquez, 2010).

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 membrane-bound O-acyltransferase domain-containing 4 (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). Our investigation in Atlantic salmon demonstrates that *ghrl2* mRNA level (**Figure 2B**) was the highest among the paralogs expressed in the stomach (Del Vecchio et al., 2021) and that *ghrl1* significantly declined after 6 weeks of fasting, whereas *ghrl2* was not affected by 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 up-regulated 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 feed deprivation (14 days) in stomach mRNA level for both paralogs in Atlantic salmon (Hevrøy et al., 2011) and with one month starvation and 5 days of refeeding in single *ghrl* mRNA level in Atlantic cod (Xu and Volkoff, 2009). The mRNA expression level of appetite regulators 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, length of the fasting period, subdivision of stomach region and differences in energy metabolism among species. After short-term fasting in Atlantic salmon, no effect was observed in *ghrl* paralogs when the anterior and posterior part of the stomach was analyzed for mRNA expression (Del Vecchio et al., 2021). The plasma Ghrl level elevated in 24 h post feeding in rainbow trout (Pankhurst et al., 2008), 5 days of starvation in goldfish (Unniappan et al., 2004), 2 weeks of fasting in tilapia (*Oreochromis mossambicus*) (Fox et al., 2009) and in Atlantic salmon (Hevrøy et al., 2011), showing its orexigenic action, whereas in burbot held at 2 ºC, fasting for 2 weeks (Nieminen et al., 2003) and in rainbow trout with fasting up to 3 weeks, plasma Ghrl level declined showing 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. In Atlantic salmon two splice variants of *goat* were identified, retrieved from *rapid.ensembl.org*[1] in chromosome ssa20. Based on the sequence in the database, primers for qPCR analysis were designed. Goat was first characterized and reported in detail in zebrafish as a non-mammal by Hatef et al., (2015). As in Atlantic salmon two splice variants of *goat* were also reported in agastric goldfish (another species which went through a 4th round of whole genome duplications) (Blanco et al., 2017). Furthermore, *goat* colocalization with *ghrl* in the gut of agastric fish confirmed the potential functional interactions in regulation of feed intake (Hatef et al., 2015; Blanco et al., 2017), and there was no *mboat4* detected in the agastric ballan wrasse (*Labrus bergylta*) which also lacks *ghrl* (Lie et al., 2018). We observed low mRNA level of *mboat4* in stomach and no significant effect on the level of expression (**Figure 2B**) in response to long-term fasting, though Mboat4 has been reported as orexigenic peptide in appetite regulation in zebrafish (Hatef et al., 2015). The same study in zebrafish showed that in the gut, *goat* 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 GOAT mRNA in rat stomach mucosa (González et al., 2008). 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.

[1] https://rapid.ensembl.org/Salmo_salar_GCA_905237065.2/Gene/Summary?db=core;g=ENSSSAG00000079778;r=20:17727570-

4.3 Hypothalamic regulation of appetite

The significant increase of hypothalamic *agrp1* mRNA levels (**Figure 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 (**Figure 2A**), in line with its proposed role as an orexigenic factor. Studies in winter skate induced an increase in telencephalon *npy* after two weeks of fasting (MacDonald and Volkoff, 2009b), and in winter flounder hypothalamic *npy* increased after one month fasting (MacDonald and Volkoff, 2009a). 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), two weeks in winter skate (MacDonald and Volkoff, 2009b), 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 for example, variations in duration of fasting since the last meal, temperature differences and species specificity. 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 (**Figure 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, 2009a) 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* (**Figure 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 differ with the period of fasting among or even within the species. 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 (**Figure 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). 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 is involved 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 (*Danio rerio*) (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 is involved in feed intake.

In our study of Atlantic salmon, the observed upregulation of hypothalamic *cart2a, cart2b, pomca1, pomca2* during long-term fasting seems contradictory to their role as anorexigenic/satiety factors described in mammals. However, we hypothesize that these neuropeptides might 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 (**Figure 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* paralogs 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 fasting.

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 paralogs of *ghrl* mRNA expression under catabolic conditions might return as a hunger signal when there is feed available in the vicinity.

We propose *agrp1* as a potential appetite biomarker gene under feed deprived conditions.

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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 AG 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.

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841

Supplementary Material for the paper

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

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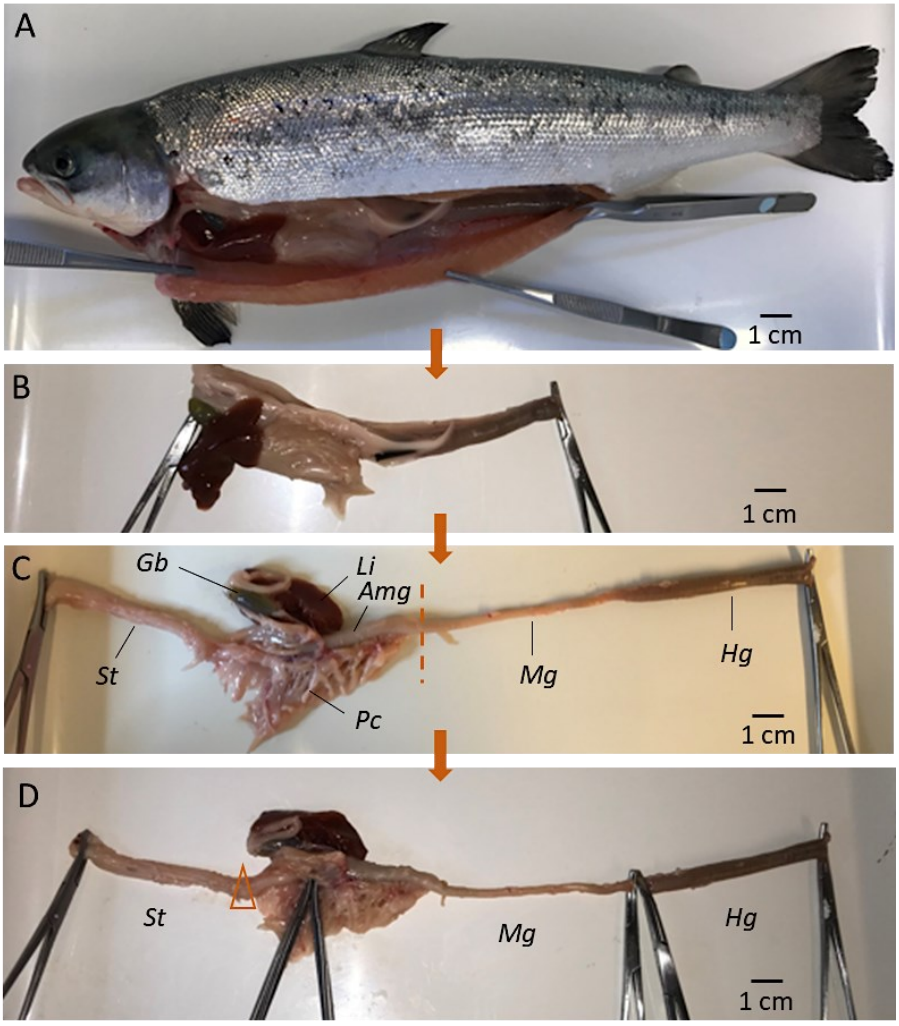
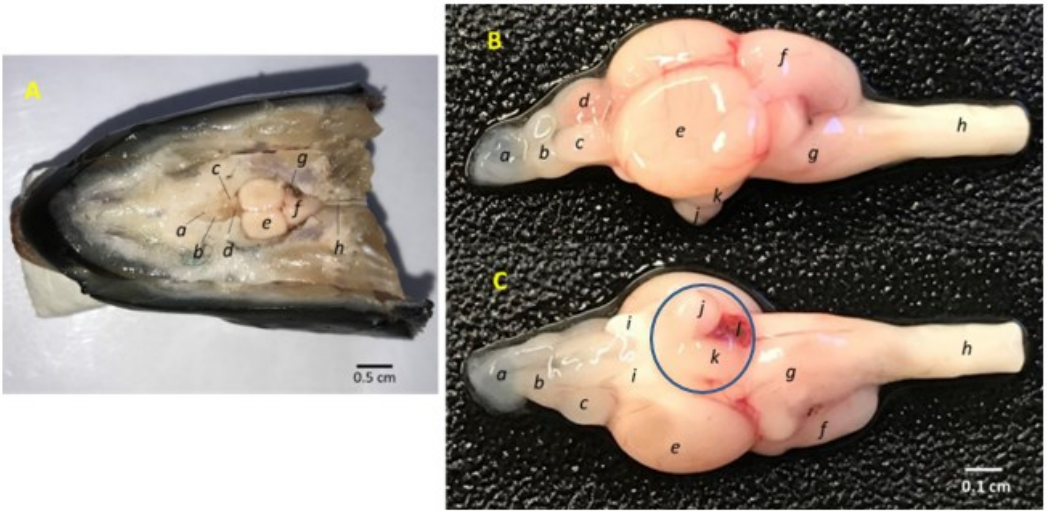
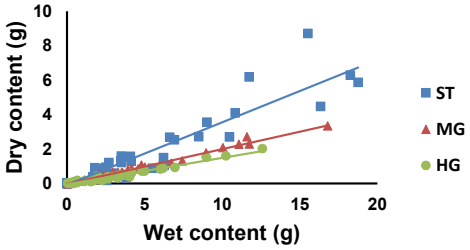


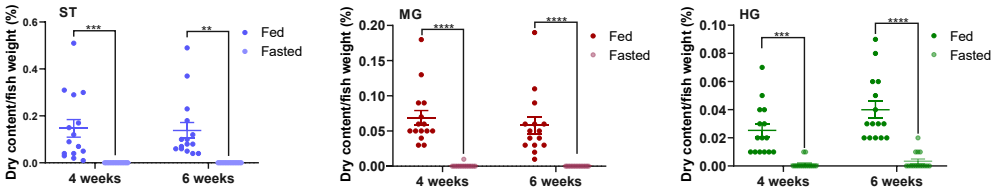
Supplementary Figure 1. Protocol for collection of gut content and stomach tissue for gene expression analyses. A. Initial cut opening of Atlantic salmon postsmolt exposing the gastrointestinal (GI) tract. **B**. Removal of GI tract using surgical clamps at both ends of the tract. **C**. Identification of the different organs/tissues of Atlantic salmon digestive system. Dashed line indicate separation of *Amg* and *Mg*. **D**. Preparation to collect digesta/inner contents from each GI tract compartments using surgical clamps. The triangle shows the stomach ('U' region) tissue sampled for gene expression analysis. *St* - stomach, *Gb* - gallbladder, *Li* - liver, *Amg* - anterior midgut, *Pc* - pyloric cecae, *Mg* - midgut and *Hg* - hindgut.



Supplementary Figure 2A. Upper view of Atlantic salmon postsmolt brain in the fish skull **B**. Upper (/side) view and **C**. Bottom (/inferior) view of Atlantic salmon postsmolt brain. ***a*** - olfactory nerves, ***b*** - olfactory bulb, ***c*** - telencephalon, ***d*** - pineal gland, ***e*** - optic lobe, ***f*** - cerebellum, ***g*** - medulla oblongata/brain stem, ***h*** - spinal cord, ***i*** - optic nerves, ***j*** - pituitary, ***k*** – hypothalamus, ***l*** - saccus vasculosus. The hypothalamus region is encircled in blue (when the pituitary and saccus vasculosus is removed).



Supplementary Figure 3. Correlation between the wet inner content (g) and dry inner content (g) of gastrointestinal tract compartments stomach (ST), midgut (MG) and hindgut (HG). Each point represents one individual (n=60) and lines represent linear regression. For all gut compartments the p value is <0.0001 and confidence intervals are ST ± 0.034, MG ± 0.006 and HG ± 0.007.



Supplementary Figure 4. Dry content of the gastrointestinal compartments (ST-stomach, MG-midgut, and HG- hindgut) to fish weight (%). Darker dots represent the Fed groups and lighter dots represents the Fasted groups. The data is presented as mean ± SEM and asterisks show the significant level (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Supplementary Table 1. Statistical analysis information of biometrical data and dry weight (%) of gastrointestinal content of the experimental fish.

Parameter (average)	4 weeks			6 weeks			Fed 4 weeks vs Fed 6 weeks (p-value)	Fasted 4 weeks vs Fasted 6 weeks (p-value)
	Fed	Fasted	p-value	Fed	Fasted	p-value		
Weight (kg)	1.433 ± 0.076	1.215 ± 0.058	0.121	1.70 ± 0.082	1.12 ± 0.052	<0.0001	0.037	0.77
Length (cm)	48.63 ± 0.775	47.77 ± 0.701	0.831	51.47 ± 0.687	46.6 ± 0.721	<0.0001	0.034	0.669
Condition factor (K)	1.229 ± 0.015	1.104 ± 0.017	<0.0001	1.238 ±0.024	1.089 ±0.012	<0.0001	0.987	0.936
Hepatosomatic index (HSI)	1.017 ± 0.165	0.814 ± 0.031	<0.0001	1.031 ± 0.13	0.852 ± 0.084	0.0013	0.987	0.825
ST_Dry innercontent to fish weight (%)	0.147 ± 0.037	0	0.0007	0.138 ± 0.034	0	0.0016	0.995	>0.999
MG_Dry innercontent to fish weight (%)	0.069 ± 0.010	0	<0.0001	0.058 ± 0.0115	0	<0.0001	0.768	>0.999
HG_Dry innercontent to fish weight (%)	0.025 ± 0.005	0	0.0003	0.04 ± 0.006	0	<0.0001	0.048	0.983

Significance when *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Supplementary Table 2. Statistical results from the linear regression analysis of wet content (g) to dry content (g) of gastrointestinal tract. Region, R^2, correlation coefficient, 95% confidence interval (CI) and p-values are given.

Region	R^2	Coefficient	95% CI	p-value
ST	0.887	0.363	± 0.034	<0.0001
MG	0.987	0.202	± 0.006	<0.0001
HG	0.972	0.153	± 0.007	<0.0001

Significance when****p<0.0001

Supplementary Table 3. Statistical analysis results of relative mRNA expression data.

Genes_HYP	number (*n*)		Normality test	Lognormality test	Shapiro-Wilk test	number (*n*)		Normality test	Lognormality test	Shapiro-Wilk test	Two-Way ANOVA (Tukey's multiple comparisons) *p* -value			
	4 weeks fed	4 weeks fasted				6 weeks fed	6 weeks fasted				Fed vs Fasted (4 weeks)	Fed vs Fasted (6 weeks)	4 weeks vs 6 weeks (Fed)	4 weeks vs 6 weeks (Fasted)
agrp1	14	15	not passed	passed	passed	14	14	not passed	passed	passed	**0.0011	**0.0048	0.9707	0.8232
npya1	14	15	passed		passed	15	15	not passed	passed	passed	**0.008	0.5831	0.9969	0.2331
npya2	15	15	not passed	not passed	D'Agostino & Pearson test & Kolmogorov-Smirnov test	15	15	passed		passed	0.8274	0.9972	0.9972	0.8274
cart2a	15	15	not passed	passed	passed	15	13	not passed	passed	passed	*0.0157	0.9256	0.4453	**0.0019
cart2b	15	15	passed		passed	15	15	not passed	not passed	D'Agostino & Pearson test	*0.0169	*0.0465	0.4501	0.2475
cart3a	15	14	passed		passed	15	15	not passed	passed	passed	0.9991	0.4991	0.5036	0.9993
cart4	15	15	passed		passed	15	15	passed		passed	0.1132	0.9906	0.9583	0.0725
pomca1	14	14	passed		passed	15	15	passed		passed	0.9944	*0.0142	0.4273	0.2809
pomca2	15	15	not passed		passed	15	15	not passed	not passed	D'Agostino & Pearson	0.9981	**0.0088	0.6899	0.0932
mc4ra2	14	15	passed		passed	14	15	passed		passed	0.0521	**0.0028	0.7781	0.9997
mc4rb1	15	15	passed		passed	15	15	passed		passed	0.2192	0.3481	0.9984	0.9706
Genes_ST	**number (*n*)**		**Normality test**	**Lognormality test**	**Shapiro-Wilk test**	**number (*n*)**		**Normality test**	**Lognormality test**	**Shapiro-Wilk test**	**Two-Way ANOVA (Tukey's multiple comparisons) *p* -value**			
	4 weeks fed	**4 weeks fasted**				**6 weeks fed**	**6 weeks fasted**				**Fed vs Fasted (4 weeks)**	**Fed vs Fasted (6 weeks)**	**4 weeks vs 6 weeks (Fed)**	**4 weeks vs 6 weeks (Fasted)**
ghrl1	15	15	passed		passed	15	15	passed		passed	0.2691	*0.0235	0.9156	0.9663
ghrl2	15	15	not passed	not passed	D'Agostino & Pearson test test	14	15	passed		passed	0.1221	0.0645	0.957	0.8298
mboat4	14	15	passed		passed	15	14	not passed		D'Agostino & Pearson test	0.6203	0.6478	>0.9999	0.0917

Significance when *p<0.05, **p<0.01

Supplementary Table 4. Results from the Pearson correlation for the relative mRNA expression of hypothalamic neuropeptides and receptors *versus ghrl1* & *2* relative mRNA expression. Genes, R^2, correlation coefficient, 95% confidence interval (CI) and *p*-values are given.

Genes	*Rel ghrl1*				*Rel ghrl2*			
	R^2	Coefficient	95% CI	*p* -value	R^2	Coefficient	95% CI	*p* -value
Rel agrp1	0.043	-0.207	-0.443 to 0.057	0.123	0.026	-0.161	-0.407 to 0.106	0.235
Rel npya1	0.001	0.024	-0.233 to 0.279	0.856	0.009	0.094	-0.169 to 0.344	0.484
Rel npya2	0.036	0.189	-0.0679 to 0.423	0.147	0.027	0.163	-0.097 to 0.402	0.218
Rel cart2a	0.000	0.020	-0.239 to 0.277	0.879	0.012	0.108	-0.157 to 0.358	0.426
Rel cart2b	0.038	-0.195	-0.428 to 0.062	0.136	0.000	0.010	-0.247 to 0.265	0.942
Rel cart3a	0.030	0.173	-0.0868 to 0.411	0.190	0.084	0.290	0.034 to 0.509	*0.027
Rel cart4	0.067	-0.258	-0.481 to -0.004	*0.047	0.082	-0.286	-0.505 to -0.032	*0.028
Rel pomca1	0.028	-0.169	-0.409 to 0.094	0.206	0.022	-0.150	-0.395 to 0.115	0.266
Rel pomca2	0.069	-0.263	-0.485 to -0.010	*0.042	0.070	-0.264	-0.487 to -0.009	*0.043
Rel mc4ra2	0.091	-0.301	-0.519 to -0.047	*0.022	0.034	-0.186	-0.426 to 0.079	0.167
Rel mc4rb1	0.020	-0.142	-0.382 to 0.116	0.279	0.002	-0.042	-0.294 to 0.217	0.755

Significance when *$p<0.05$

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