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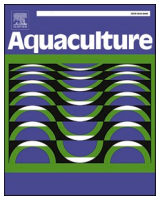

Anorectic role of high dietary leucine in farmed Atlantic salmon (*Salmo salar* L.): Effects on feed intake, growth, amino acid transporters and appetite-control neuropeptides

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ABSTRACT

Leucine has been identified to modulate feed intake and energy homeostasis in fish as in other vertebrates. Under allostatic conditions, energy expenditure may change, and adjustments to the processes that govern the energy homeostatic system may be necessary. We investigated the responsiveness of appetite-related neuropeptides involved in feed intake regulation in Atlantic salmon (*Salmo salar*) reared with high (35 g/kg leucine) or control (27.3 g/kg leucine) leucine-supplemented diets and/or under chronic stressor conditions (chasing) for eight weeks. We also analysed the response of amino acid transporters potentially involved in uptake of branched-chain amino acids (BCAA), including leucine, into areas of the brain where nutrient sensors may signal locally or to other areas involved in appetite control. At the end of the experiment, all fish were subjected to a novel-acute stressor (confinement). Our results show that fish fed with high leucine diet had a lower feed intake, growth, and hepatosomatic index (HSI) when compared to fish fed control leucine diet. In addition, increased mRNA expression of amino acid *solute carrier* (*slc*) genes in the diencephalon, and genes related to appetite control, such as *proopiomelanocortin a1* (*pomca1*), in both the diencephalon and telencephalon, imply their involvement in leucine anorectic effect.

Stress, as high leucine, reduced feed intake, growth and HSI of fish fed control or high leucine diet and antagonized the high leucine effect on the *slc* genes mRNA expression. An increase of *neuropeptide y a1* (*npya1*) was observed both due to high dietary leucine and/or stress treatment which may represent a compensatory regulatory mechanism with the aim to reverse the decrease in feed intake. In summary, our results confirm an anorectic role of high dietary leucine via the activation of amino acid sensing mechanisms in the brain. Further, *corticotropin-releasing hormone 1 b1* (*crh1b1*) and *npya1* showed to play a role in the regulation of appetite in Atlantic salmon under stress conditions and/or high leucine levels.

1. Introduction

In fish, regulation of feed intake and energy expenditure is dependent upon interaction of homeostatic and hedonic signalling pathways (Soengas et al., 2018). The homeostatic signalling is mainly integrated in the hypothalamus (Rønnestad et al., 2017; Volkoff, 2016) and regulates feed intake following the depletion of energy stores. On the other hand, the hedonic or reward-based signalling primarily occurs in the telencephalon and results from pleasure and/or sensory perception of feed consumption, independently of energy homeostasis (Delgado et al., 2017; Soengas, 2021). Sensing of nutrient levels is a crucial step in the homeostatic control of feed intake and circulating branched-chain

Abbreviations: BCAA, branched-chain amino acid; RGR, relative growth rate; SGR, specific growth rate; HSI, hepatosomatic index; K, condition factor.

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amino acids (BCAA), as leucine, have increasingly been recognized as regulators of the amino acid sensing systems in the teleost brain (Comesaña et al., 2021a, 2021b; Comesaña et al., 2018a; Comesaña et al., 2018b; Soengas, 2021; Soengas et al., 2018). The evidence on the presence of amino acid sensing mechanisms in fish has started to emerge (Gomes et al., 2019; Rønnestad et al., 2016), and in rainbow trout (*Oncorhynchus mykiss*) rising in leucine levels through intracerebroventricular (ICV) or intraperitoneal (IP) treatments resulted in a general decrease in feed intake and modulation in expression of appetite-related neuropeptides (Comesaña et al., 2018a, 2018b), inferring that leucine functions as a signal of nutrient or energy availability. Leucine has indeed been described as a key BCAA in the control of energy homeostasis and growth in fish (Abidi and Khan, 2007; Ahmad et al., 2021; Rollin et al., 2003; Soengas, 2021; Soengas et al., 2018). It has been shown that leucine is a potent anorectic factor, by regulating the amino acid sensing system through the orexigenic neuropeptides *neuropeptide y* (*npy*), *agouti-related protein* (*agrp*), and the anorexigenic peptide *proopiomelanocortin a1* (*pomca1*) (Comesaña et al., 2018a, 2018b). The mechanisms controlling amino acid sensing in fish are still poorly understood, but available evidence in rainbow trout suggests comparable mechanisms dependent on BCAA and glutamine metabolism, mechanistic target of rapamycin (*mtor*), taste receptors, and general control nonderepressible 2 (*gcn2*) kinase signalling, and specific amino acid carriers as in mammals (Conde-Sieira and Soengas, 2017; Soengas et al., 2018). As for the latter, amino acid carriers have been shown to play a pivotal role in amino acid sensing and several members of the *solute carrier family 7* (*slc7*) and *38* (*slc38*) to be involved in leucine sensing (Comesaña et al., 2018a, 2018b). Moreover, members of the *slc* family have been shown to be involved in the anorectic effect of leucine in Atlantic salmon (*Salmo salar*) (Comesaña et al., 2021a).

Loss of appetite is a general indicator of homeostatic imbalance during allostatic periods (Bernier and Peter, 2001; Conde-Sieira et al., 2018). This seems to be partially regulated by the Corticotropin-releasing hormone (Crh), also known as Corticotropin-releasing factor (Crf), one of the key players in the coordination and regulation of the physiological stress response in the Hypothalamic-Pituitary–Interrenal (HPI) axis (Bernier and Peter, 2001; Doyon et al., 2003). In teleost species, the corticotropin-stress related action has been localised in both telencephalon and diencephalon (Alderman and Bernier, 2009; Aruna et al., 2012; Lai et al., 2021; Olivereau and Olivereau, 1987), same brain regions that have been described as local sites for the regulation of appetite and feed intake (Kalananthan et al., 2020a; Otero-Rodiño et al., 2019; Tolås et al., 2021; Zhang et al., 2019). In these brain regions, stress stimulus such as hypoxia, handling or isolation can result in an increased *crh1* mRNA abundance and lead to a sustained decrease in feeding and growth in teleost (Bernier, 2006; Conde-Sieira et al., 2018; Lai et al., 2021). The feeding suppressing effect under allostatic periods have been documented in several teleost species and could relate to the interaction of the HPI axis with the nutrient sensing mechanisms. In fact, a readjustment of the glucosensing system was observed in rainbow trout under high stocking density, impairing the hypothalamic integration of the metabolic signals to regulate feeding (Conde-Sieira et al., 2010a, 2010b), and Crh could be mediating this interaction (Conde-Sieira et al., 2011). However, no studies have assessed the impact of stress on amino acid sensing mechanisms and their involvement in feed intake regulation in teleosts.

Increasing levels of leucine seems to result in decreased feed intake and growth in fish (Ahmad et al., 2021; Espe et al., 2021; Soengas, 2021; Soengas et al., 2018). We hypothesised that such responses occur via the activation of amino acid sensing mechanisms inducing alterations in the mRNA abundance of hypothalamic and telencephalic key genes. It also remains unclear if the addition of a stressor would have an additive, synergistic, or an antagonistic interaction to the anorectic function of leucine. In fact, during regular production cycles, salmon may encounter episodes of stress such as confinement, handling, food deprivation and diseases. As a consequence of these new or prolonged stress conditions, differential physiological-stress related responses affect fish performance, as recently showed in studies in Atlantic salmon (Lai et al., 2021; Madaro et al., 2015, 2016b; Madaro et al., 2016a). Thus, Atlantic salmon post-smolt were fed with either a control (27.3 g/kg) or high (35 g/kg) leucine-supplemented diet, and half of each exposed to a chronic stressor (chasing) three times a week for 8 weeks, while the other half was left undisturbed. At the end of the experiment, all fish were exposed to a novel-acute stressor, confinement. We analysed feed intake, growth, plasma levels of free amino acids, and the telencephalic and diencephalic mRNA abundance of key amino acid carriers involved in leucine sensing (*slc7a5*, *slc38a2,* and *slc38a9*), and regulators of appetite (*npy, agrp1, pomca1*) and stress response (*crh1*).

2. Materials and methods

2.1. Ethics statement

The experiment complied with the guidelines of the Norwegian Regulation on Animal Experimentation and have been approved by the National Animal Research Authority in Norway (FOTS ID 20799).

2.2. Animals and experimental design

This study is part of a larger experiment in which only two of the four diet groups from Espe et al. (2021) were used to investigate the role of amino acid transporters and appetite regulating factors in leucine's anorexigenic action under control or stress conditions. In brief, Atlantic salmon post-smolt of approximately 500 g were randomly distributed into 16 tanks (volume ca. 470 L) with 50 fish each at Cargill Innovation Center (Dirdal, Norway) and acclimatized for four weeks. Tanks were supplied with seawater at 9 ± 0.01 °C, salinity 28.39 ± 0.03 ‰ and oxygen levels 88 ± 0.42%. Fish were fed with a commercial pellet (Adapt Marine 80) by using an automatic feeder (Hølland Technology, Sandnes, Norway). Continuous light (LD 24:0) was provided in accordance to standard commercial procedures to promote optimal growth and inhibit unwanted sexual maturation (Endal et al., 2000; Fjelldal et al., 2012; Hansen et al., 1992; Nordgarden et al., 2003).

After the acclimation period, fish were PIT-tagged, and weight and fork length were measured. Fish were fed with either a control leucine diet (containing 27.3 g/kg leucine as typical level in farmed Atlantic salmon), or a high leucine diet (containing 35 g/kg leucine which is approximately 30% in excess from the control leucine diet). Four replicated tanks per dietary treatment were used. The diets were formulated with common ingredients used in the fish farm industries and in agreement with the expertise at the Cargill Innovation Center [see Espe et al., 2021 for diet composition and amino acid content details]. After four weeks, half of the tanks (two replicated tanks per dietary/stress treatment) were exposed to a chasing stressor, while the rest of the tanks were only subjected to routine practice of tank maintenance and left undisturbed (Fig. 1) [see Espe et al., 2021 for more details]. At the end of the 8 weeks, all experimental tanks were exposed to a novel-acute stressor, confinement, which consisted of collecting the fish with a net from the experimental tank and carefully transferring them into a small tank with 50 L seawater for 15 min (Fig. 1). An oxygen probe was placed inside the small tank to monitor the oxygen saturation and a net/lid placed on top. At the end of the confinement exposure, fish were placed back in their experimental tank and left undisturbed. During the experimental trial uneaten feed was collected in each tank to calculate the feed intake.

2.3. Sampling protocol

Two samplings were performed during the trial (Fig. 1): ten fish per tank were sampled as the conclusion of the chronic stress period (sampling I); and ten fish per tank were sampled after the novel-acute stressor exposure (sampling II). The fish were sampled 55 and 45 min after the

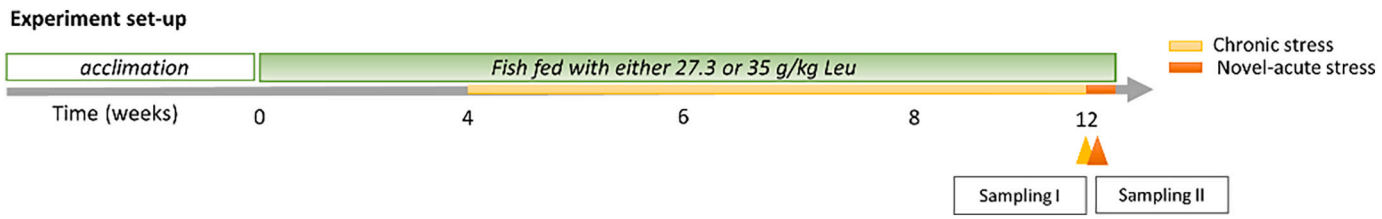

Fig. 1. Schematic representation of the experiment design and sampling points. Two enriched diets, control (27.3 g/kg) and high leucine (35 g/kg), were used to feed Atlantic salmon post-smolt for 12 weeks. After the acclimation period (4 weeks) and 4 weeks of feeding with the experimental diets, half of the tanks per diet were exposed to a chronic stressor (chasing) three days a week for 8 weeks. At the end of the experimental trial (12 weeks) all fish were subjected to a novel-acute stressor (confinement). Ten fish per tank were sampled from all experimental groups at the end of the chronic stress exposure (sampling I), and ten fish per tank sampled after exposure to a novel-acute novel stressor on that same week (sampling II).

chronic and the novel-acute stressor were applied, respectively, following previous studies on Atlantic salmon (Lai et al., 2021; Madaro et al., 2016b; Madaro et al., 2016a; Madaro et al., 2015). Fish were feed deprived for 24 h before the sampling.

Fish were anesthetized with a lethal dose of Tricaine (PHARMAQ Ltd., Hampshire, UK), and blood collected from the caudal vein using a vacuum syringe and BD Vacutainer® set (Ref. 367,869, Becton Dickinson, Plymouth, United Kingdom) with heparin. The blood was stored at 4 °C until centrifuged later the same day at 4000 RPM (2325 RCF) for 10 min using a Hettich Zentrifugen Universal 320R (Hettich®, Tuttlingen, Germany). Subsequently, plasma was collected and stored at −80 °C. PIT-tag numbers, fork length and weight were recorded, and liver was weighed using a OHAUS Pioneer PA2102 Precision Balance (OHAUS Corporation, USA) with an accuracy of two significant numbers. The fish whole brain was collected on week 12 (both end of chronic and novel-acute time points) and transferred into tubes containing RNA later (Invitrogen, Carlsbad, CA, USA). Brains were refrigerated for one day and stored at −80 °C until RNA isolation was performed.

2.4. Growth rate, condition factor, hepatosomatic index and feed intake

Fish growth was calculated as Relative Growth Rate (RGR) and Specific Growth Rate (SGR) as follow:

$$RGR = (w\_t - w\_i)/w\_i \times 100 \quad (1)$$

$$SGR = (ln(w\_t) - ln(w\_i))/t \times 100 \quad (2)$$

where w_i is the initial weight, w_t is the final weight and t equals the numbers of days between.

To determine the fitness of the fish after the experimental treatments, the condition factor (K) was calculated using eq. 3,

$$K = 100 \times w/l^3 \quad (3)$$

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

To provide an indication of the energy status and the metabolic activity of the fish, the hepatosomatic index (HSI) was calculated using the equation cited by Chellappa et al. (1995):

$$HSI = w\_l/w \times 100 \quad (4)$$

in which w_l is the liver weight (g), and w is the weight of the whole fish (g).

Feed conversion rate (FCR) was calculated as follow:

$$FCR = \frac{\text{kg feed consumed}}{(\text{kg final biomass} - \text{initial biomass} + \text{removed biomass} + \text{dead fish})} \quad (5)$$

2.5. Plasma free amino acid

Free amino acids levels were determined from a pool of three fish plasma per tank using a Biochrom 20 plus amino acid bioanalyzer (Amersham Pharmacia Biotech, Sweden) and a post column derivatization with ninhydrin as described by Fontaine et al. (2001).

2.6. Brain dissection

Prior to gene expression analysis, twelve brain samples (six replicates/tank) from each experimental group and sampling point (sampling I and II) were dissected and the telencephalon (TEL) and diencephalon (DN) collected (Supplementary Fig. 1). The diencephalon included hypothalamus, part of the preotic region and the thalamus.

2.7. RNA extraction and cDNA synthesis

Total RNA was extracted from the TEL and DN using TRI Reagent (Sigma-Aldrich, MO, USA). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and a 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, CA, USA) were used to assess the quantity, quality and integrity of the extracted total RNA, respectively. To avoid any remnants of genomic DNA, 10 μg of total RNA was treated with TURBO DNase-free Kit (Ambion Applied Biosystem, CA, USA). Two batches of first strand cDNA were synthesized from 1.8 μg of the total RNA using SuperScript III Reverse Transcriptase (Invitrogen, CA, USA) and Oligo(dT)$_{20}$ (50 μM) primers in a total reaction volume of 20 μL and then merged. The protocols were carried out accordingly to the manufacturer's instructions.

2.8. Real-time PCR (qPCR)

Specific primers were used for Atlantic salmon *npya1* and *npya2* (Tolås et al., 2021), *agrp1*, *pomca1* and *pomca2* (Kalananthan et al., 2020b), *slc7a5aa*, *slc7a5ab*, *slc7a5ba*, *slc7a5bb*, *slc7a5ca/b*, *slc38a2a*, *slc38a2b* and *slc38a9* (Comesaña et al., 2021a), and *crh1a1*, *crh1a2*, *crh1b1* and *crh1b2* (Lai et al., 2021) (Supplementary Table 1). Two genes, *beta actin* (*actb*) and *ribosomal protein s20* (*rps20*), were used as reference genes (Olsvik et al., 2005). The primers were analysed for quantification cycle (Cq), primers efficiency (E) and melting peaks to detect potential nonspecific products and/or primer dimers. Primers efficiency was determined using a 10-fold dilution standard curve (from 1.00E+07 to 1.00E+02 copies amplicon/μL) from the target gene cloned into a pCR4-TOPO vector (Thermo Fisher Scientific). qPCR was carried out using 10 μL of SYBR Green I Master Mix (Roche Diagnostic, Basel, Switzerland), 0.6 μL of each forward and reverse primers (10 mM), 6.8 μL Ultra-Pure Water (Biochrom, Berlin, Germany) and 2 μL cDNA template (for reference genes 24 ng/reaction, for *slc* genes 40 ng/reaction, for *npya1* and *a2* 12 ng/reaction, for *pomca1* and *a2* 80 ng/reaction, for *agrp1* 60 ng/reaction, and for *crh1* genes 30 ng/reaction). All reactions were run in duplicate into 96-well plates (Bio-Rad Laboratories, CA,

USA). Two negative controls, no-template and no-reverse transcriptase, and one positive control (between plate control) were included in all plates. 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 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 the detection of nonspecific products and/or primer dimers. The qPCR was performed using a CFX96 Real-Time System (Bio-Rad Laboratories, CA, USA) in connection to CFX Manager Software version 3.1 (Bio-Rad, Laboratories, CA, USA).

Subsequently, the copy number for each target gene was determined based on the respective standard curve slope and intercept using eq. (6):

$$Copy\ number = 10^{\left(\frac{Cq-intercept}{slope}\right)} \quad (6)$$

The copy number was normalized to the total ng of RNA used for each target gene. The ratio of the target gene copy number to the geometric mean copy number of the reference genes (*rps20* and *actb*) was used for the plot bar graphs and statistical analysis (Hellemans et al., 2007; Vandesompele et al., 2002).

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, version 9.3.1, San Diego, USA). All datasets were tested for normality and equal variance using D'Agostino-Pearson test and F-test ratio, respectively. Grubb's outlier test was run prior to statistical evaluations. Comparison between groups was carried out using two-way ANOVA with diets (control or high leucine) and stress (unstressed and stressed) as explanatory variables. For the analysis of RGR, SGR, K and feed intake, control and stressed groups from samplings I and II were pooled, whereas for the gene expression analysis control and stressed groups from samplings I and II were used as single groups. A *post-hoc* Sidak's multiple comparisons test was used to analyse differences between the experimental groups. For gene expression analysis, an additional three-way ANOVA followed by Sidak's multiple comparisons was performed to analyse the response to the novel-acute stress based on the previous experimental condition. All data are presented as mean ± SEM, unless otherwise stated.

3. Results

3.1. Effects of dietary leucine and stress on feed intake and growth performance

The inclusion of higher amounts of leucine (HL) in the diet and exposure to stress has negatively affected feed intake, growth performance and energy storage of Atlantic salmon (Table 1) (Supplementary Table 2).

Feed intake, weight, RGR, and SGR was reduced in fish fed high leucine diet (HL) compared to the fish fed with control leucine diet (CL) (*post-hoc* test $p = 0.0201$, $p = 0.0356$, $p = 0.0034$ $p = 0.0027$ respectively). CL fish subjected to chronic stress had a lower feed intake compared to CL unstressed fish (*post-hoc* test $p = 0.0122$). A similar reduction was observed for the chronic stressed HL, which did not differ from the unstressed HL. FCR was not affected by diets and/or stress. The chronic stressed HL showed a comparable decline in HSI, which was not different from the unstressed HL. Stress also significantly reduced HSI for CL (*post-hoc* test $p = 0.0022$). No differences were observed in the K among the groups.

Table 1
Feed intake and growth performance of unstressed and stressed Atlantic salmon fed with control leucine (CL; 27.3 g/kg) or high leucine (HL; 35 g/kg). * indicates significant dietary effects at the same unstressed/chronic stress exposure condition. # indicates significant stress effects at the same diet condition.

	Control		Stressed	
	CL	HL	CL	HL
Feed eaten (g)	2827 ± 100	2322.5 ± 17.5*	2245.5 ± 41.5$^{\#}$	2312 ± 110
FCR	0.80 ± 0.05	0.81 ± 0.01	0.82 ± 0.03	0.80 ± 0.02
Weight (g)	1521.20 ± 39.75	1460.30 ± 42.25*	1410.82 ± 37.026$^{\#\#}$	1413.78 ± 35.46
Lenght (cm)	48.34 ± 0.38	47.15 ± 0.48	46.54 ± 0.3 $^{\#\#}$	46.98 ± 0.36
SGR %	1.27 ± 0.02	1.17 ± 0.02**	1.21 ± 0.03	1.18 ± 0.02
RGR %	186.49 ± 4.42	164.62 ± 4.42**	176.30 ± 5.90	167.52 ± 4.39
K	1.40 ± 0.01	1.38 ± 0.01	1.39 ± 0.01	1.35 ± 0.01
HSI %	1.44 ± 0.03	1.33 ± 0.03*	1.31 ± 0.03$^{\#\#}$	1.25 ± 0.03

3.2. Plasma free amino acid

Analysis of free amino acids in the plasma showed leucine levels to be affected by dietary leucine ($p = 0.0387$) (Supplementary Table 3 and 4). Under chronic stress, fish fed high leucine diet showed a higher plasma leucine level compared to the control leucine diet fish under the same stress regime (*post-hoc* test $p = 0.0282$). In fish fed control leucine diet, stress caused a tendency of lower leucine levels compared to the unstressed control group, however the differences were not significant ($p = 0.0624$). No dietary or stress effects were observed on the other BCAA valine and isoleucine levels.

3.3. Effect of leucine diets and stress on key genes expression in Atlantic salmon brain

The increased levels of leucine (HL) did affect the mRNA expression of the amino acid transporters analysed (Figs. 2 and 3) (Supplementary Table 5). In the diencephalon, all the genes related to amino acid transporters showed a higher expression in the high leucine diet compared to the control diet (*post-hoc* test *slc7a5aa,* $p = 0.0018$; *slc7a5ab,* $p = 0.0029$; *slc7a5ba,* $p = 0.0025$; *slc7a5bb,* $p = 0.0022$; *slc7a5aca/cb,* $p = 0.0252$; *slc38a9,* $p = 0.0122$; *slc38a2a,* $p = 0.0006$; *slc38a2b,* $p = 0.0006$), while in the telencephalon a rise in expression was only observed for the *slc38a2b* (*post-hoc* test $p = 0.0278$). However, the differences observed were mitigated by the exposure of the groups to chronic or novel-acute stress (Figs. 2 and 3) (Supplementary Table 6 and 7). Exposure to chronic stress induced a lower abundance of *slc7a5ab*, *slc7a5ba* and *slc38a2b* in HL (*post-hoc* test $p = 0.0299$, $p = 0.0387$, $p = 0.0251$ respectively). While the novel-acute stress reduced the expression of *slc7a5ba*, *slc7a5bb slc38a9*, *slc38a2a* and *slc382b* in the unstressed HL (*post-hoc* test $p = 0.0056$, $p = 0.0377$ $p = 0.0455$, $p = 0.0002$, and $p = 0.0008$ respectively), and of *slc7a5bb* in both experimental diets (*post-hoc* test $p = 0.0269$ for CL, and $p = 0.0377$ for HL). Thus, these differences lead to a higher expression of *slc38a2a* and *2b* in the chronic stressed HL compared to unstressed HL (*post-hoc* test $p = 0.002$ and $p = 0.0233$ respectively). A stress effect was also observed for *slc7a5aca/cb* in the telencephalon, where its expression was upregulated in the control leucine diet after exposure to the novel-acute stressor, while on contrary *slc7a5ba* was downregulated (*post-hoc* test $p = 0.0145$, and $p = 0.0284$ respectively).

Analysis of the neuropeptides involved in appetite regulation in Atlantic salmon revealed that high leucine diet increased mRNA abundance of *pomca1* in comparison to fish fed with control leucine diet (*post-hoc* test diencephalon $p = 0.0368$, and telencephalon, $p = 0.0181$) (Figs. 4 and 5). Despite the trend, the significant differences disappeared after exposure to the novel-acute stressor (Supplementary Table 6). A significant response to high leucine diet was observed for *npya1* mRNA levels in the diencephalon (*post-hoc* test $p = 0.0292$) (Fig. 4). In addition, exposure to the chronic stress significantly increased *npya1* mRNA levels in the group fed CL diet (*post-hoc* test $p = 0.0058$). A similar response was observed between the unstressed and chronic stressed groups fed with control leucine after exposure to the novel-acute stressor (*post-hoc*

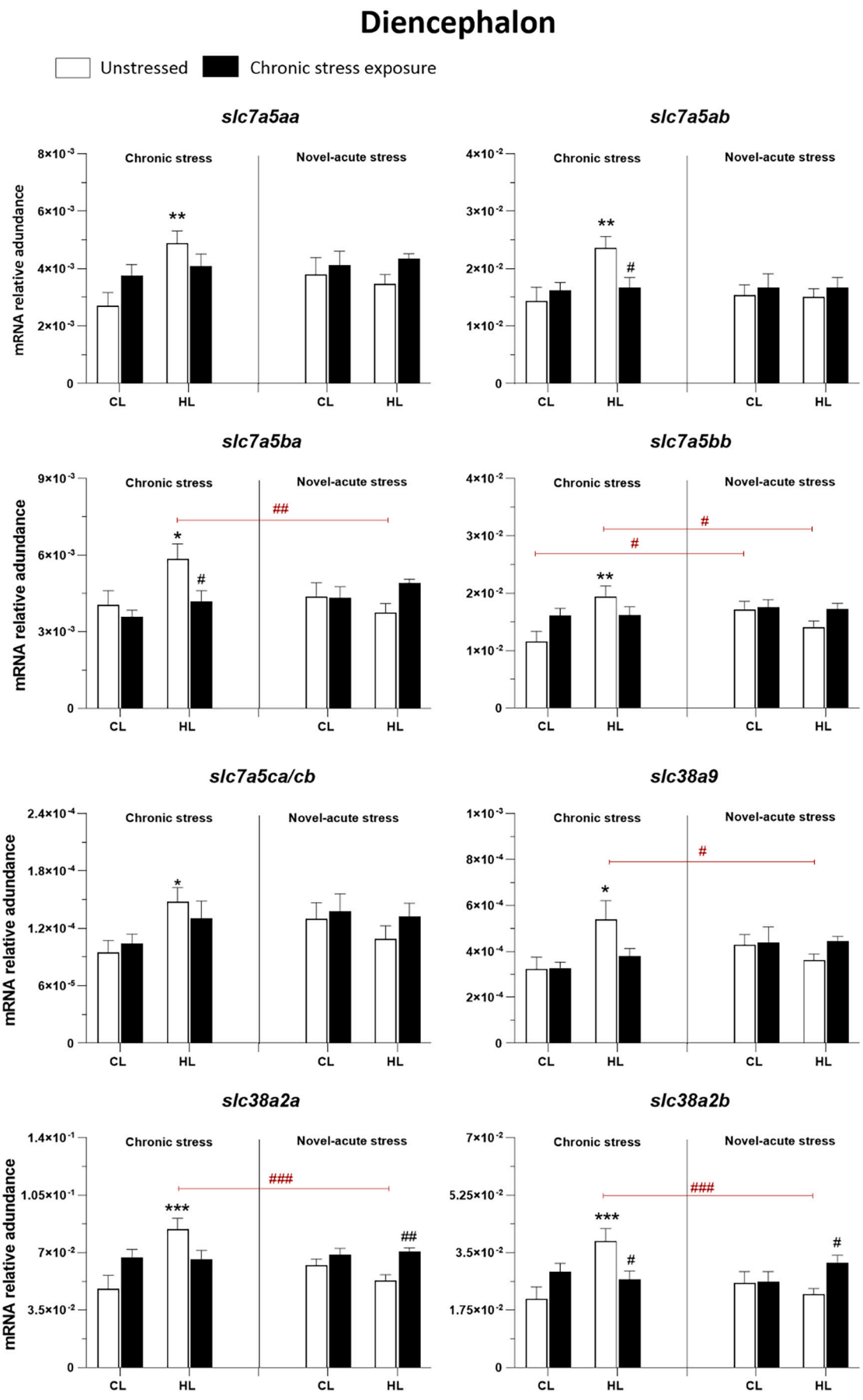

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Fig. 2. mRNA expression levels of amino acid solute carrier genes in the diencephalon of Atlantic salmon. Fish were fed with either control (CL; 27.3 g/kg) or high (HL; 35 g/kg) leucine diets. Half of the tanks were exposed to a chronic stressor (chasing) three days a week for 8 weeks, and all the tanks to a novel-acute stressor (confinement) at the end of the experiment. Bars represent mean ± SEM (n = 9–12/group) of normalized mRNA copy number to the total ng of RNA used for each target gene, and to the geometric mean copy number of *actb* and *rps20*. * indicates significant dietary effects at the same unstressed/chronic stress exposure condition. # indicates significant stress effects at the same diet condition.

test $p = 0.0211$). In addition, exposure from unstressed to novel-acute stress increased the expression of *npya1* (*post-hoc* test $p = 0.0023$). For *npya2* and *agrp1,* no statistically significant changes were observed (Figs. 4 and 5).

Among the stress response related genes analysed in the diencephalon, *crh1b1* expression showed to respond to the leucine diet (*post-hoc* test $p = 0.038$) (Fig. 6**).** High leucine induced a tendency to increase in the *crh1b1* expression from the control leucine diet, albeit not significantly different. Additionally, exposure to the novel-acute stressor confinement induced an increased *crh1b1* level in the chronic stressed fish fed with control leucine compared to the unstressed group (*post-hoc* test $p = 0.0025$). Diencephalic *crh1a2* showed a higher mRNA abundance in fish fed high leucine compared to control leucine, which disappeared after exposure to the novel-acute stress (*post-hoc* test $p = 0.0328$). Changes in *crh1* paralogs expression were observed in the telencephalon. A decrease of *crh1b2* was observed after chronic exposure in HL group compared to the unstressed, and a decrease of *crh1a2* was observed in the unstressed HL compared the CL after novel-acute exposure (*post-hoc* test $p = 0.0032$, and $p = 0.0208$). Based on their previous experimental condition, introduction of the novel-acute stressor induced an increase of *crh1a1* and *b2* in the CL group (*post-hoc* test $p = 0.0271$ and $p = 0.0141$), and of *crh1b1* in the chronic stressed HL group (*post-hoc* test $p = 0.0092$). No other changes in expression were registered for *crh1* in the telencephalon; however, after exposure to the novel-acute stressor, all experimental groups showed a high *crh1b1* individual response variability. The same response profile was also observed for *crh1b2* (Fig. 7).

4. Discussion

Among the BCAA, leucine has been described as a key amino acid in the control of feed intake, and homeostatic energy control in fish (Ahmad et al., 2021; Soengas, 2021; Soengas et al., 2018). However, energy expenditure may differ under allostatic conditions, and adjustment on the mechanisms that control the homeostatic energy system may apply. Thus, we evaluated the responsiveness of feed intake, appetite-control neuropeptides, brain amino acid transporters and growth in Atlantic salmon fed two leucine-enriched diets, as well as their modulation under stressful conditions.

4.1. Effects of dietary leucine in Atlantic salmon

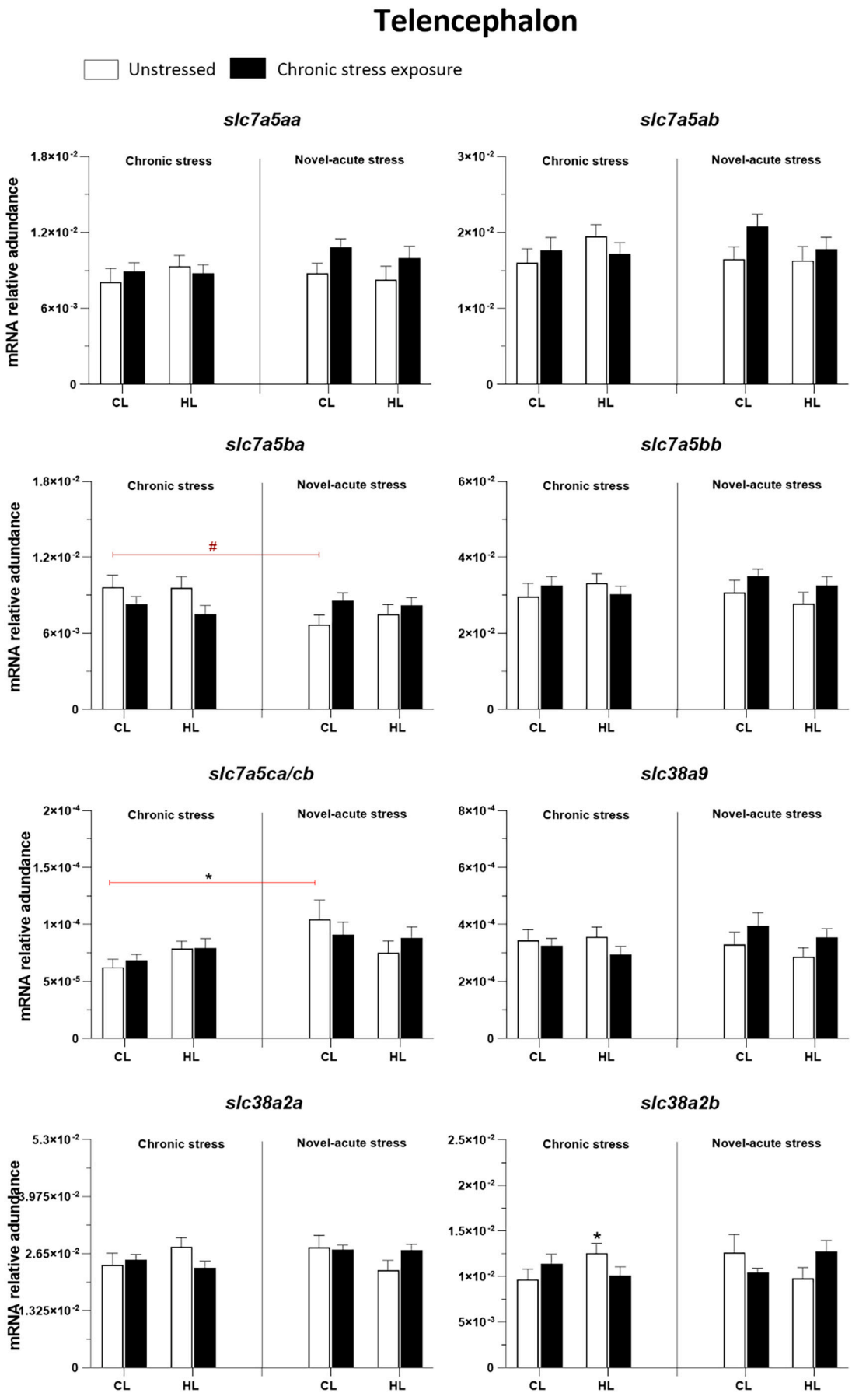
In this study, the high dietary leucine significantly reduced feed intake and growth performance in Atlantic salmon. Twelve weeks of the dietary treatment (control 27.3 g/kg versus supplemented with high leucine 35 g/kg) resulted in lower fish weight, RGR and SGR in the fish fed with high leucine. These observations agree with previous findings in fish, indicating that leucine acts as an anorectic factor. For instance, excess of dietary leucine caused reduced growth in mrigal carp (*Cirrhinus mrigala*), (15–20 g/kg-diet leucine; Ahmed and Khan, 2006), Nile tilapia (*Oreochromis niloticus*) (29.3–30.4 g/kg-diet leucine; Xu et al., 2022), rainbow trout (34.6 g/kg-diet leucine; Choo et al., 1991) and Roho labeo (*Labeo rohita*) (17.5–20 g/kg-diet leucine; Abidi and Khan, 2007). It has been suggested that the negative effect on growth can be attributed to the build-up of toxic metabolites and metabolic dysfunction as a result of the energy allocation directed at removing the BCAA in excess (Ahmed and Khan, 2006; Choo et al., 1991; Xu et al., 2022). This might explain the lower growth and HSI observed in the Atlantic salmon given the high leucine diet. The HSI differences between the two dietary groups might imply that the two experimental groups use energy storage differently. Thus, more research into hepatic glycogen levels and body composition can give a better understanding of the fish's energy status. The high leucine diet significantly increased the mRNA abundance of amino acid transporters *slc7a5*, *slc38a2,* and *slc38a9* in the diencephalon. The diencephalic changes in mRNA abundance support the involvement of leucine in the regulation of feed intake through sensing mechanisms dependent on *slc* transporters. Indeed, other studies have demonstrated that increased dietary levels of leucine promotes the activation of amino acid sensing systems in rainbow trout (Comesaña et al., 2018a, 2018b), Chinese perch (*Siniperca chuatsi*) (Chen et al., 2021), and Atlantic salmon (Comesaña et al., 2021b). The activation of these sensing systems is probably related to the control of feed intake through changes in the mRNA abundance of the neuropeptides related to appetite (Soengas, 2021; Soengas et al., 2018), however diverse regulatory mechanisms may occur during protein translation. Here, we observed that increased leucine levels induced a rise in mRNA abundance of *pomca1* in the diencephalon (area containing the hypothalamus) and telencephalon. These results are comparable to those observed in rainbow trout after intracerebroventricular (Comesaña et al., 2018a) or intraperitoneal (Comesaña et al., 2018b) leucine injections. A rise in the levels of this anorexigenic neuropeptide is a typical response observed in fish under conditions of reduced feed intake (Rønnestad et al., 2017; Soengas et al., 2018), thus confirming that increased levels of leucine function as an anorectic factor. In both humans and mice, the anorectic effect of leucine is directly correlated to the neuropeptides involved in central regulation of feed intake. Consistent with L-leucine's anorexigenic role, 25% of POMC mediobasal neurons were activated by L-leucine, while 10% NPY mediobasal neurons were inhibited. Leucine also rapidly reduced AGRP release, indicating a mechanism for rapid leucine-induced modulation of foraging behaviour in rodents (Heeley et al., 2018).

In line with this, the rise in brain leucine in rainbow trout (Comesaña et al., 2018a, 2018b) and Chinese perch (Chen et al., 2021) also resulted in a general decrease in *npy* and *agrp* genes; however, we did not observe this in our study. On the contrary, a surprising increase of *npya1* mRNA abundance was observed in response to the anorectic leucine action in the HL group, which does not correlate with its putative orexigenic role or the decrease in feed intake observed in this group. However, a similar response was observed in our recent study on the same species, where fasting increased the expression of *npya1* (Kalananthan et al., 2023). This response implies the possible *npya1* role in the attempt to compensate for the lack of appetite and/or feed intake, however not sufficient. Interestingly, regardless of the dietary leucine provided, the *npya1* mRNA increase was observed in the stressed groups, supporting the involvement of *npya1* in counteracting the decrease in feed intake stress response.

As far as we are aware, no studies have evaluated the impact of dietary leucine on the Crh system in fish. In this study, no changes occurred in the mRNA abundance of the different *crh1* transcripts assessed in diencephalon, except for *crh1a2* which showed a higher expression in the high leucine diet. This could indicate that high levels of leucine stimulate downstream metabolic responses resembling stress.

4.2. Effects of stress in Atlantic salmon

Exposure to stress induced a clear reduction in feed intake and growth in fish fed with the control leucine diet. Comparable results have been reported in prior studies in Atlantic salmon under stressful events



(caption on next page)

Fig. 3. mRNA expression levels of amino acid solute carrier genes in the telencephalon of Atlantic salmon. Fish were fed with either either control (CL; 27.3 g/kg) or high (HL; 35 g/kg) leucine diets. Half of the tanks were exposed to a chronic stressor (chasing) three days a week for 8 weeks, and all the tanks to a novel-acute stressor (confinement) at the end of the experiment. Bars represent mean ± SEM (n = 9–12/group) of normalized mRNA copy number to the total ng of RNA used for each target gene, and to the geometric mean copy number of *actb* and *rps20*. * indicates significant dietary effects at the same unstressed/chronic stress exposure condition. # indicates significant stress effects at the same diet condition.

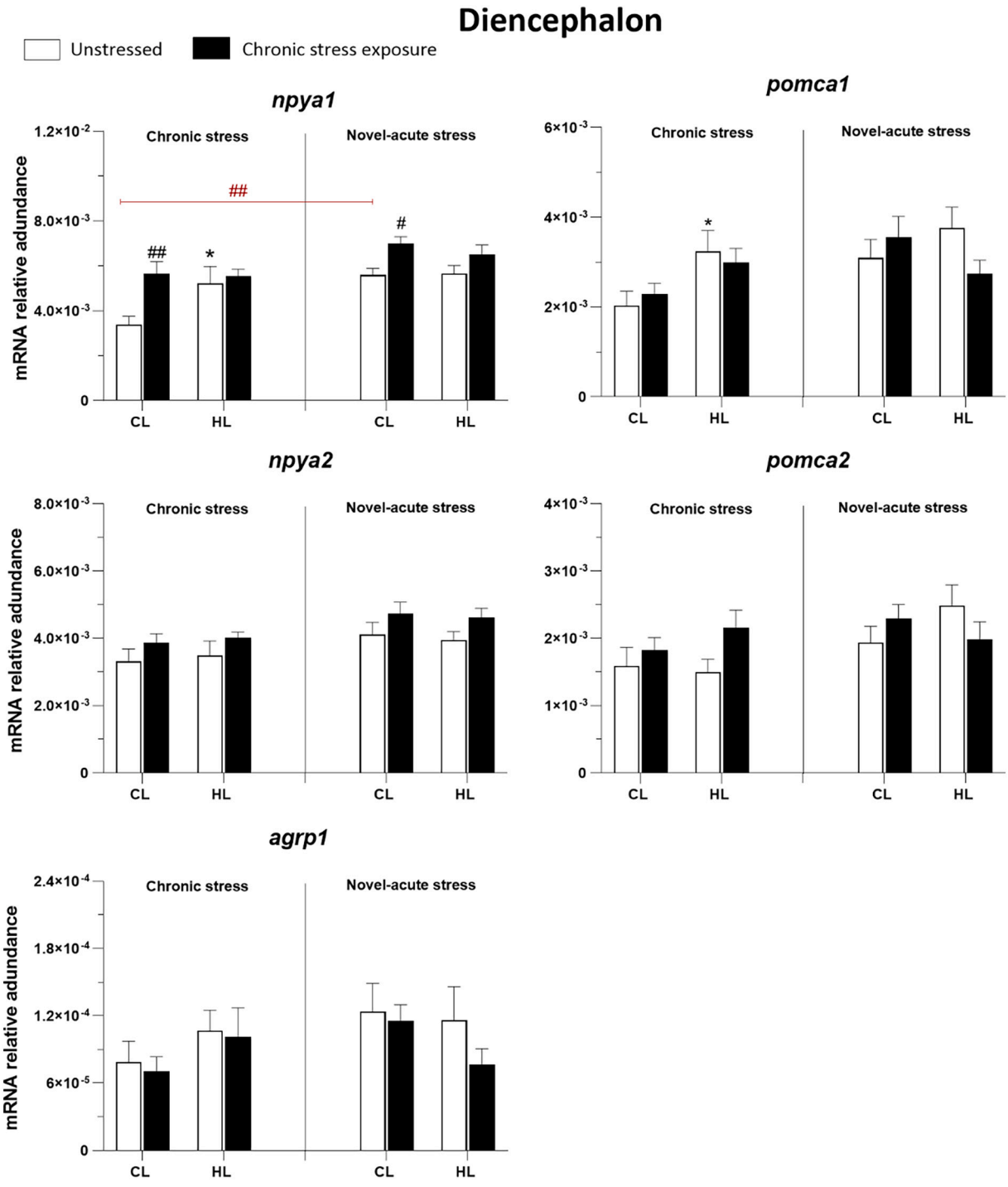


Fig. 4. mRNA expression levels of appetite-regulators genes in the diencephalon of Atlantic salmon. Fish were fed with control (CL; 27.3 g/kg) or high (HL; 35 g/kg) leucine diets. Half of the tanks were exposed to a chronic stressor (chasing) three days a week for 8 weeks, and all the tanks to a novel-acute stressor (confinement) at the end of the experiment. Bars represent mean ± SEM (n = 9–12/group) of normalized mRNA copy number to the total ng of RNA used for each target gene, and to the geometric mean copy number of *actb* and *rps20*. * indicates significant dietary effects at the same unstressed/chronic stress exposure condition. # indicates significant stress effects at the same diet condition.

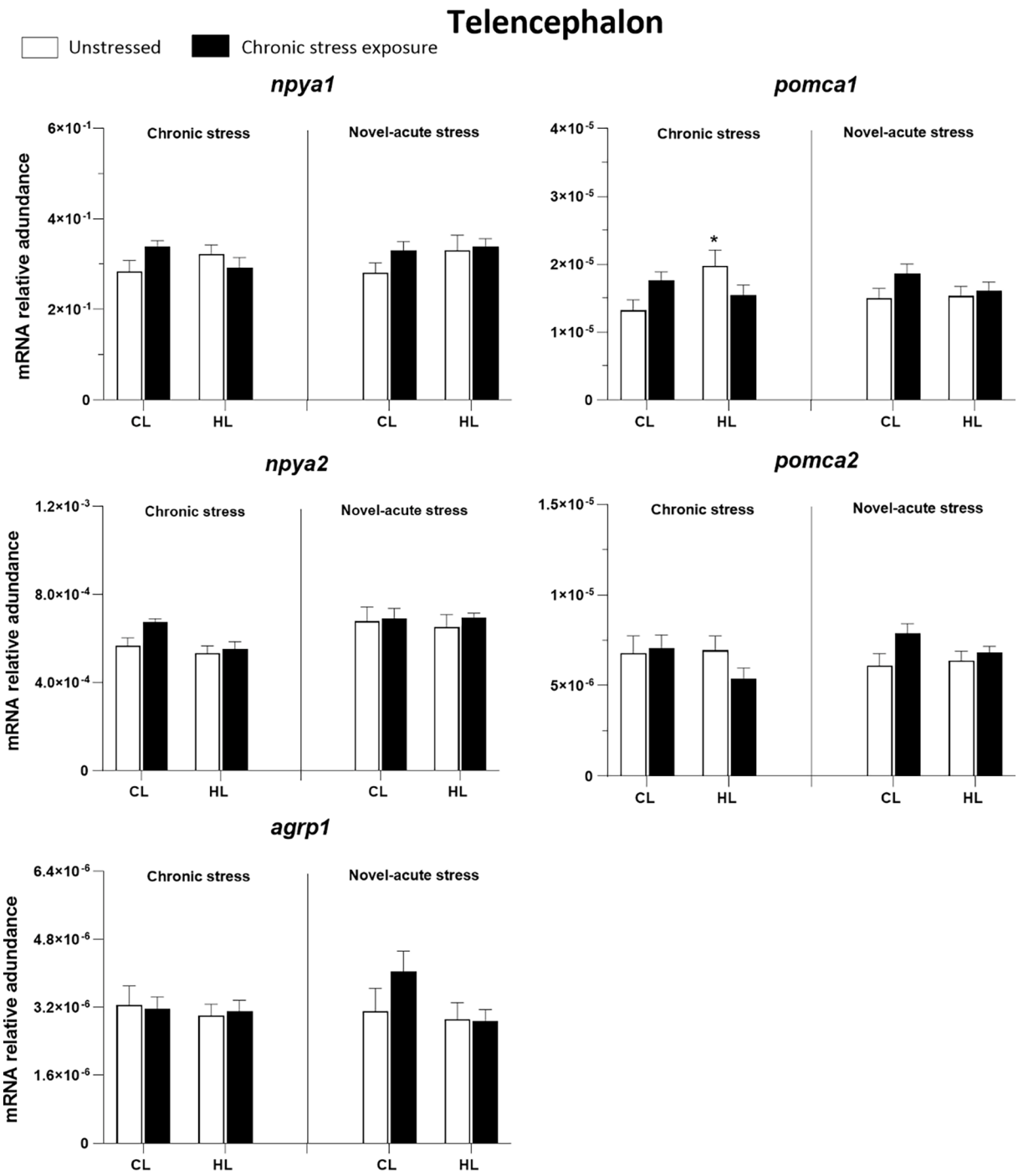


Fig. 5. mRNA expression levels of appetite-regulators genes in the telencephalon of Atlantic salmon. Fish were fed with either control (CL; 27.3 g/kg) or high (HL; 35 g/kg) leucine diets. Half of the tanks were exposed to a chronic stressor (chasing) three days a week for 8 weeks, and all the tanks to a novel-acute stressor (confinement) at the end of the experiment. Bars represent mean ± SEM (n = 9–12/group) of normalized mRNA copy number to the total ng of RNA used for each target gene, and to the geometric mean copy number of *actb* and *rps20*. * indicates significant dietary effects at the same unstressed/chronic stress exposure condition. # indicates significant stress effects at the same diet condition.

as repeated chasing (Lai et al., 2021), hypoxia (Lai et al., 2021; Vikeså et al., 2017), unpredictable chronic stress (Madaro et al., 2015), and elevated sea temperatures (Hevrøy et al., 2012).

In this study, no changes were observed in the mRNA expression of the *slc* transporters, indicating that these are not involved in the pathways leading to decreased feed intake under stress conditions. The anorectic effect of stress might be directly related to the neuropeptides involved in central regulation of feed intake in fish (Soengas et al., 2018).

In Atlantic salmon diencephalon we observed a rise in the mRNA levels of *npya1* in fish fed control leucine, both as a response to chronic and novel-acute stress. Considering the *npy* hypothetical orexigenic role commonly described in fish, such change does not seem to agree with the decrease observed in feed intake in salmon. However, increased mRNA levels of hypothalamic *npy* were reported in zebrafish and rainbow trout submitted to stress, and after cortisol administration in goldfish, whereas no changes were found in stressed tilapia (Conde-Sieira et al., 2010b; Cortés et al., 2018; Naderi et al., 2018).

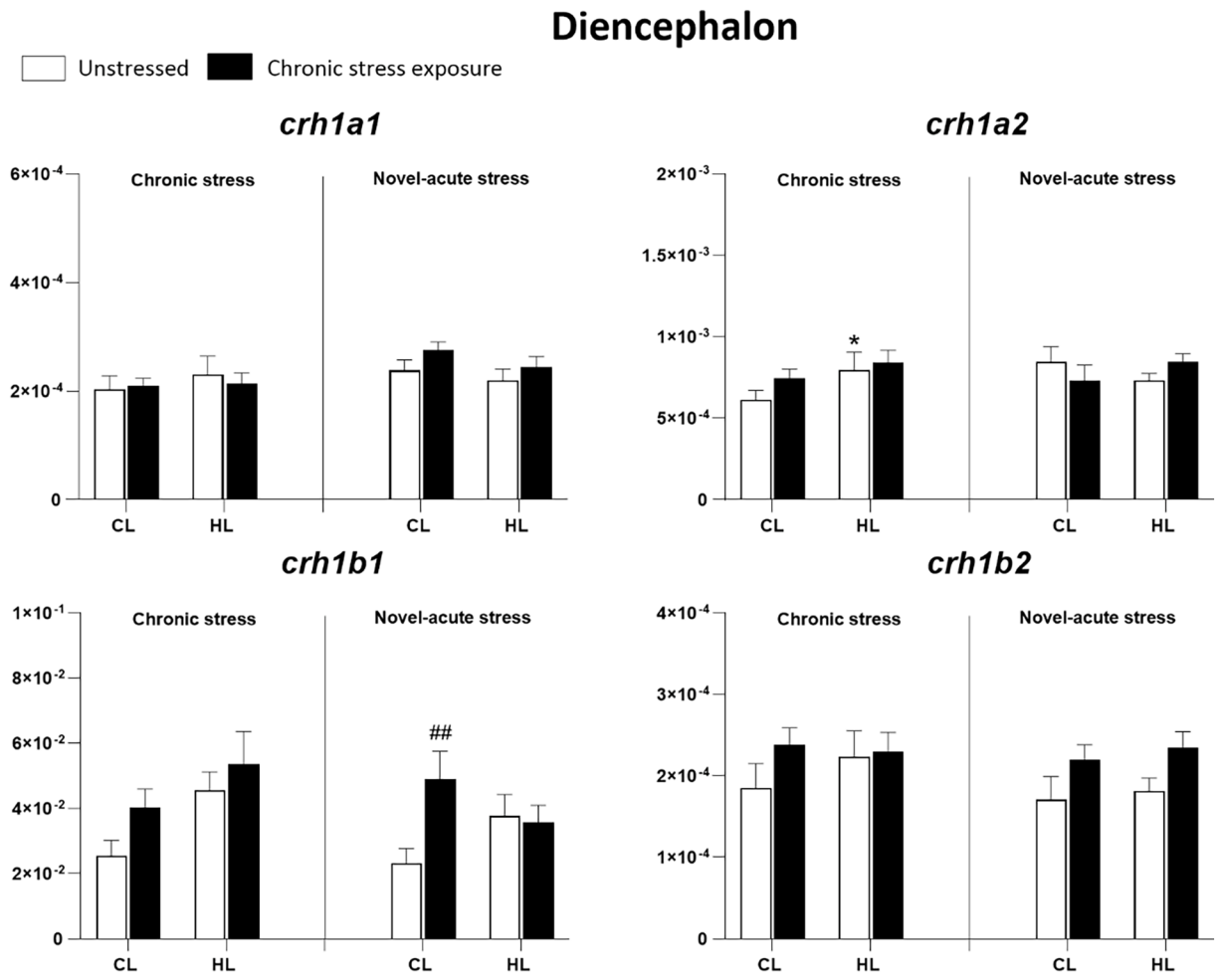


Fig. 6. mRNA expression levels of *crh1* genes in the diencephalon of Atlantic salmon. Fish were fed with either control (CL; 27.3 g/kg) or high (HL; 35 g/kg) leucine diets. Half of the tanks were exposed to a chronic stressor (chasing) three days a week for 8 weeks, and all the tanks to a novel-acute stressor (confinement) at the end of the experiment. Bars represent mean ± SEM (n = 10–12/group) of normalized mRNA copy number to the total ng of RNA used for each target gene, and to the geometric mean copy number of *actb* and *rps20*. * indicates significant dietary effects at the same unstressed/chronic stress exposure condition. # indicates significant stress effects at the same diet condition.

Additionally, *npy* mRNA abundance decreased in the telencephalon and increased in preoptic area and forebrain in socially subordinate rainbow trout (Doyon et al., 2003; Janzen et al., 2012). Also, an increased gene expression of *agrp* was reported after acute handling stress in in the whole brain of zebrafish (Cortés et al., 2018). As mentioned above, the increased levels of the mRNA abundance of the putative orexigenic *npy* and *agrp* neuropeptides do not match with the anorectic effect of stress, suggesting that other neuronal populations or their interactions, as the connection of Npy neurons with Crh neurons, could be mediating this stress effect (Conde-Sieira et al., 2018). In our study, the similar upregulation of *npya* and *crh1b1* as a response to acute stress, suggest a cross action between these two genes as described in literature (Haas and George, 1989; Suda et al., 1993).

Regarding the anorexigenic peptides, divergent results have been observed depending on the fish species and the type of stress applied. For instance, *pomc* mRNA levels were elevated under acute stress conditions in zebrafish, whereas no changes or decreased levels were found in rainbow trout and Senegalese sole, respectively, after crowding stress (Conde-Sieira et al., 2010b; Naderi et al., 2018; Palermo et al., 2008; Wunderink et al., 2012), i.e. a result comparable to that observed in the present study.

4.3. Interaction of dietary leucine and stress: effects on Atlantic salmon

A detrimental impact of stress on growth was observed in the Atlantic salmon reared with a high leucine diet. The combination of high leucine diet with stress resulted in higher reduction of feed intake, growth indices, and hepatosomatic index. These results appear to be more accentuated than in the stress group fed the control leucine diet implying a different use of energy storage across the experimental groups and a higher energy cost to remove the excess leucine.

The rise in mRNA abundance of *slc* transporters reported in the diencephalon of Atlantic salmon fed a high dosage of leucine in unstressed fish did not occur when the fish were stressed. This lack of responsiveness to leucine suggests that the selected transporters might be inhibited under stressed conditions. It should be noted that amino acids, including leucine are required for all living cells and organisms and play fundamental roles in a range of functions including protein synthesis, cellular growth, hormone metabolism, nerve transmission, energy metabolism, nitrogen metabolism, nucleoid synthesis, and more (Bröer and Bröer, 2017). The *slc* transporters mediate the transfer of amino acids across plasma membranes to serve these functions, and their role is thus much broader than acting in nutrient sensing; even more, it is nowadays reckoned that their functional cooperation at the cellular and intracellular membranes (through a combination of secondary active transporters, functionally acting as loaders, harmonizers

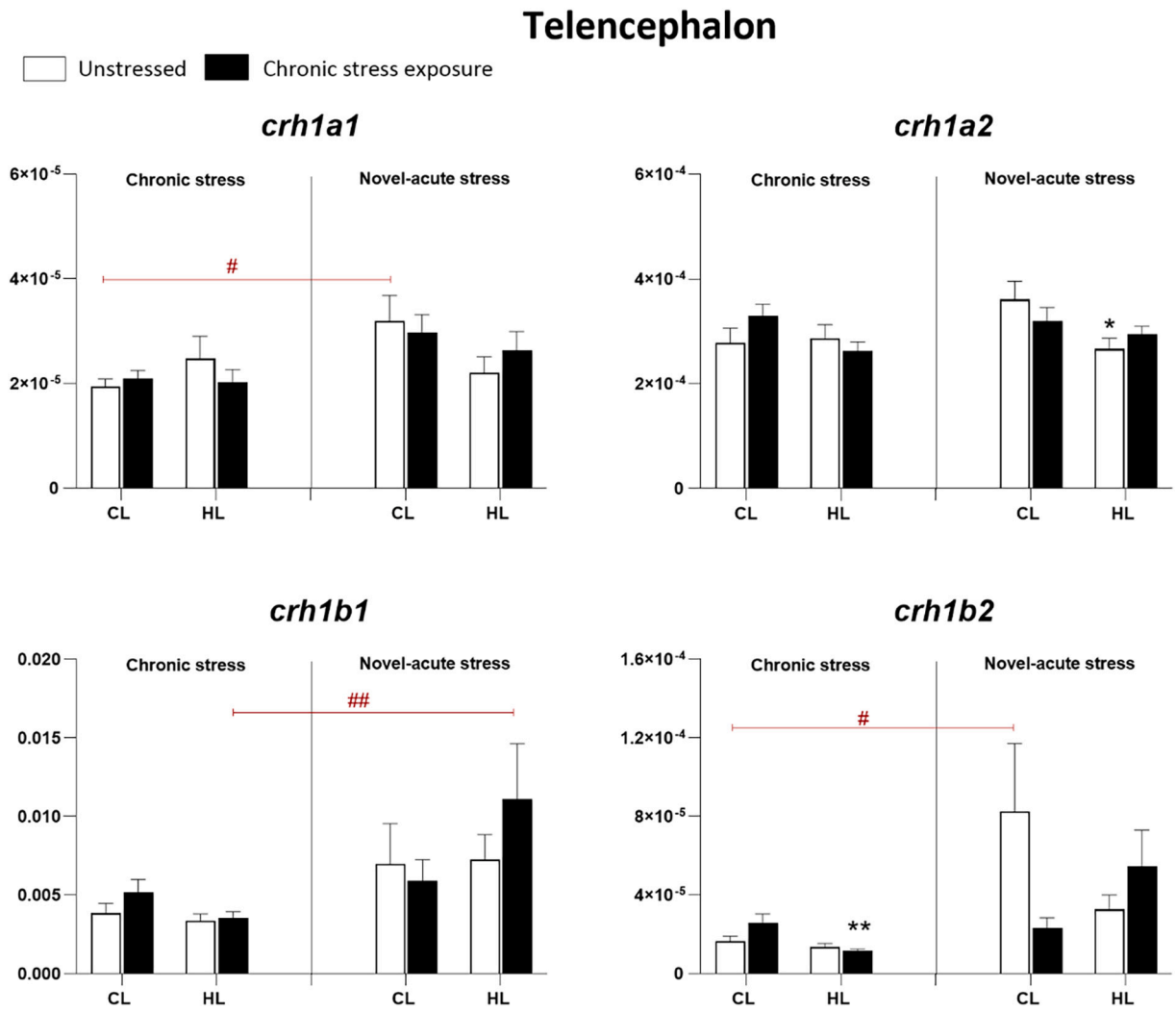


Fig. 7. mRNA expression levels of *crh1* genes in the telencephalon of Atlantic salmon. Fish were fed with either control (CL; 27.3 g/kg) or high (HL; 35 g/kg) leucine diets. Half of the tanks were exposed to a chronic stressor (chasing) three days a week for 8 weeks, and all the tanks to a novel-acute stressor (confinement) at the end of the experiment. Bars represent mean ± SEM (n = 10–12/group) of normalized mRNA copy number to the total ng of RNA used for each target gene, and to the geometric mean copy number of *actb* and *rps20*. * indicates significant dietary effects at the same unstressed/chronic stress exposure condition. # indicates significant stress effects at the same diet condition.

and controller transporters) generate a stable equilibrium of all amino acid concentrations inside the cell. In this framework, Slc7a5-, Slc38a2- and Slc38a9-type transporters are considered essential for establishing intracellular BCAA (and thus leucine) flux and cellular levels via a combination of Na^+/glutamine cotransport processes (through e.g., Slc38a2-type proteins taking glutamine up to the cell) functionally linked to BCAA/glutamine exchange processes (through e.g., Slc7a5-type proteins taking up to the cell extracellular BCAA in exchange with intracellular glutamine) (for details see e.g., Gauthier-Coles et al., 2021; Bröer and Gauthier-Coles, 2022). Moreover, Slc38a9-type proteins do play a major role as components of the lysosomal amino acid sensing machinery that controls Mtorc1 (see e.g., Rebsamen et al., 2015; Scalise et al., 2019). Thus, our data also suggest altered leucine metabolism in the brain of stressed salmon. In rainbow trout brain stress is known to impact the responsiveness of glucosensing processes (Conde-Sieira et al., 2010a, 2010b), but the effects on brain glucose metabolism is not known.

When we analysed the neuropeptides involved in the central control of feed intake, diencephalic *pomca1* mRNA expression increased as a results of high leucine in unstressed fish, but again, this was not observed when the fish were stressed. On the other hand, *npya1* mRNA was upregulated in the stressed group both for fish fed control or high leucine diets, suggesting that the interaction with stress resulted in alterations in the central regulation of feed intake. A putative candidate for this interaction is Crh, which is being expressed and produced under stressed conditions also in salmon (Lai et al., 2021), and is known to interact with the mechanisms involved in the central regulation of feed intake (Conde-Sieira et al., 2018). An interaction of Crh with amino acid sensing mechanisms, comparable to those elicited when interacting with glucosensing mechanisms (Conde-Sieira et al., 2011), could be the responsible of the changes herein observed. And indeed, mRNA abundance of *crh1b1* has increased in stressed fish fed with high leucine levels.

In several fish species, the preoptic area has been described as another Npy-like action region (Tolås et al., 2021), which is also well known for containing Crh-cell bodies (Ando et al., 1999). In rats, NPY in the preoptic area is involved in the increase of CRH mRNA levels and protein release (Haas and George, 1989; Suda et al., 1993). In this study, higher *npya* and *crh1b* mRNA levels were observed in the diencephalon of the stressed group for both experimental diets. However, the higher levels of *npya*, also known as potent orexigenic factor in several fish species (Volkoff, 2016), do not correlate to the lower feed intake registered in these groups. Thus, the low feed intake in these groups could be just the result of the elevated *crh1b* mRNA expression.

Considering these reports, our results suggest a possible cross action between *npya* and *crh1* mRNA abundance in the diencephalic brain area in Atlantic salmon and we can hypothesise that the high *npya1* levels in the stressed groups could be a counter regulatory mechanism to reverse the decrease of feed intake due to stress and/or high dietary leucine. Besides Crh, we cannot discard the possibility that other factors related to stress response are involved in this interaction. The fact that most changes observed due to a stress response are related to the chronic rather than novel-acute treatment allow us to suggest that those are not related to the short-term stress, but rather involved in the long-term response (Conde-Sieira et al., 2018).

In conclusion, our findings support an anorectic role of high dietary leucine in the control of energy homeostasis via the amino acid sensing system. Elevated leucine levels negatively impacted feed intake, growth and HSI via regulation of the amino acid *slc* and the anorexigenic *pomca1* neuropeptide. On the other hand, the same mechanisms do not seem to be responsible for such disruption under stressed conditions. Instead, *crh* or other stress related factors seem to be modulated by high dietary leucine and stress interactions. Interestingly, higher levels of *npya1* mRNA were observed as a response to high leucine supplement diets and/or stress. This upregulation might operate as a counter-regulatory mechanism to minimise the reduction in feed intake caused by stress and/or excessive dietary leucine, however, not sufficiently to restore appetite to control levels. Thus, further studies are needed to better understand the mechanisms that control the regulation of the feed intake in Atlantic salmon reared under allostatic conditions. The current findings also highlight the importance of identifying the most suitable amino acid supplementation concentration for fish diets, since seemingly little variations in the content of a certain essential amino acid like leucine generate considerable changes in signalling pathway regulation of feed intake and growth.

Availability of data and materials

All data generated or analysed during this study are included in the Supplementary Material “data experiment”.

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Author contributions

Conceptualization: FL, ASG, ME, CDS, TV and IR; Data curation and formal analysis: FL; Funding acquisition: IR and JLS; Investigation and methodology: FL, SC, ASG, DF, IT, ME, CDS, MBH and IR; Project administration IR; Resources: IR and JLS; Writing - original draft: FL; Writing - review & editing: FL, SC, ASG, IT, ME, CDS, MBH, TV, JLS and IR.

Declaration of Competing Interest

All authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.739204.

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