General and Comparative Endocrinology 313 (2021) 113894

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

The stress response in Atlantic salmon (*Salmo salar* L.): identification and functional characterization of the corticotropin-releasing factor (*crf*) paralogs

Floriana Lai [a,*], Muhammad R. Royan [a,1], Ana S. Gomes [a], Marit Espe [b], Anders Aksnes [c], Birgitta Norberg [d], Virginie Gelebart [a], Ivar Rønnestad [a]

[a] Department of Biological Sciences, University of Bergen, Bergen, Norway
[b] Institute of Marine Research, Bergen, Norway
[c] Cargill Innovation Center, Dirdal, Norway
[d] Institute of Marine Research, Austevoll, Norway

ARTICLE INFO

Keywords:
Crf
Cortisol
HPI
Atlantic salmon
Genome duplication
Stress

ABSTRACT

Corticotropin-Releasing Factor (CRF) is one of the main mediators of the Hypothalamic-Pituitary–Interrenal (HPI) axis to stress response. In Atlantic salmon, a comparative understanding of the *crf1* paralogs role in the stress response is still incomplete. Our database searches have identified four *crf1* genes in Atlantic salmon, named *crf1a1*, *crf1a2*, *crf1b1* and *crf1b2*. Brain distribution analysis revealed that the four *crf1* paralogs were widely distributed, and particularly abundant in the telencephalon, midbrain, and hypothalamus of Atlantic salmon post-smolts. To increase the knowledge on *crf1*-mediated response to stress, Atlantic salmon post-smolts were exposed to either repeated chasing, hypoxia or a combination of chasing and hypoxia for eight days, followed by a novel-acute stressor, confinement. Cortisol, glucose, lactate, and creatinine levels were used as markers for the stress response.

The *crf1* paralogs mRNA abundance showed to be dependent on the stress exposure regime. Both *crf1* mRNA levels in the telencephalon and *crf1a1* mRNA levels in the hypothalamus showed similar response profiles to the serum cortisol levels, i.e., increasing levels during the first 24 h after stress exposure followed by a decline during the eight-day exposure. The similar trend between *crf1* and cortisol disappeared once exposed to the novel-acute stressor. There was a minor response to stress for both *crf1b1* and *crf1b2* in the hypothalamus, while no changes at mRNA level were observed in the hypothalamic *crf1a2* under the different stress conditions. No or weak relationship was found between the *crf1* paralogs mRNA expression and the other serum stress-indicators analysed. In summary, our data provide novel insights on the dynamic of the HPI axis activation in Atlantic salmon, and thus underline the involvement of the *crf1* paralogs as additional factors in the regulation of the stress response in this species. Likewise, the data highlight the importance of analysing all *crf1* paralogues response to a stress-condition, in particular in this premature knowledge stage of their functionality. Further analysis and a more detailed time-point series will help to elucidate the response of the HPI axis and the link of *crf1* paralogs in the stress response mechanism.

Abbreviations: ACTH, Adrenocorticotropic Hormone; CRF, Corticotropin-Releasing Factor; CRH, Corticotropin-Releasing Hormone; HSI, Hepatosomatic Index; HPI, Hypothalamic-Pituitary–Interrenal; NLT, nucleus lateralis tuberis; POA, Preoptic Area; PVN, Paraventricular Nucleus; qPCRquantitative, Real-time PCR; WGD, Whole Genome Duplication.

* Corresponding author at: University of Bergen, Department of Biological Sciences, Pb 7803, 5020 Bergen, Norway.
E-mail addresses: floriana.lai@uib.no (F. Lai), muhammad.rahmad.royan@nmbu.no (M.R. Royan), ana.gomes@uib.no (A.S. Gomes), marit.espe@hi.no (M. Espe), anders_aksnes@cargill.com (A. Aksnes), birgitta.norberg@hi.no (B. Norberg), virginie.gelebart@uib.no (V. Gelebart), ivar.ronnestad@uib.no (I. Rønnestad).
[1] Physiology Unit, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway.

https://doi.org/10.1016/j.ygcen.2021.113894
Received 16 February 2021; Received in revised form 10 August 2021; Accepted 28 August 2021
Available online 31 August 2021

1. Introduction

Atlantic salmon (*Salmo salar*) is a valuable teleost species in the global aquaculture industry and Norway is the dominant producer (FAO, 2019; Iversen et al., 2020). As part of the daily management routines, farmed fish often encounter various types of stressful conditions including handling, transportation, crowding, low oxygen levels, disease treatments, etc. Such stress will, to various degrees, compromise fish performance, health, and welfare. Therefore, acquiring new knowledge on the Atlantic salmon responsiveness and sensitivity to stress continues to be an important area of research.

The corticotropin-releasing factor (CRF), also known as corticotropin-releasing hormone (CRH), is one of the key players in the coordination and regulation of the physiological stress response in teleosts mediated by the Hypothalamic-Pituitary–Interrenal (HPI) axis (Balasch and Tort, 2019; Faught et al., 2016). In the preoptic area (POA), the release of CRF stimulates the secretion of adrenocorticotropic hormone (ACTH) in the pituitary and the following biosynthesis and release of glucocorticoids (e.g. cortisol) from the interrenal gland (Faught et al., 2016; Hostetler and Ryabinin, 2013), leading to mobilization of energy reserves to handle and compensate for the stress. The role of the CRF in the stress response mediated by the HPI axis has been described in several teleost species including Atlantic salmon (Madaro et al., 2016b; Madaro et al., 2015; Vindas et al., 2017). However, there is still limited information on the *crf1* paralogs originating from the salmon-specific 4th whole genome duplication (4R WGD), including their potential evolving divergent functions in the HPI axis activation. Early in the vertebrate lineage, an ancestral *crf* gene gave rise to two paralogs via the 2nd round (2R) WGD, the *crf1* and *crf2*, but the *crf2* gene seems to have been independently lost in both placental mammals and in teleosts. The additional 3rd round (3R) WGD in teleosts expanded the number of *crf1* genes into *crf1a* and *crf1b* (Cardoso et al., 2016; Grone and Maruska, 2015a; Lovejoy et al., 2014; Lovejoy and Balment, 1999). Furthermore, some teleost lineages, such as Salmoniformes and Cypriniformes, experienced the 4R WGD event leading to a second copy of both *crf1a* and *crf1b* (Cardoso et al., 2016; Grone and Maruska, 2015b). To date, the studies on Atlantic salmon have focused on the functional characterization of the *crf1b* genes (Madaro et al., 2016b; Madaro et al., 2015; Vindas et al., 2017), however the investigation of the published Atlantic salmon genome (Lien et al., 2016) opens an opportunity to investigate the functional specialization of *crf1* paralogs in the HPI axis response to stress.

The magnitude, duration and consistency of a stressor are important factors when assessing the stress response and activation of the HPI axis. The fish ability to cope efficiently with stress implies adjustments of stress mediator responses that activate when necessary and shuts it down when no longer needed. For instance, when fish experiences a stressful condition for a significant period of time, a fading of the neuroendocrine responsiveness may occur due to the desensitization and habituation of the stress axis to the stressor (Kristiansen et al., 2020; Martinez-Porchas et al., 2009). Furthermore, previous exposure to stress can induce adjustments of the physiological-stress related response to optimise the fish performance. However, if the stressful condition persists, a stress-response that was initially considered adaptive may become maladaptive (reviewed by Kristiansen et al., 2020). Thus, in this study, we have identified and characterized the Atlantic salmon *crf1* paralogs *in silico* and analysed their mRNA expression distribution in the brain of post-smolts under different stress conditions. The Atlantic salmon post-smolts were exposed to repeated-stress (chasing, hypoxia or a combination of chasing and hypoxia) for eight days followed by a novel-acute stressor (confinement) on day nine. To increase the knowledge on *crf1*-mediated response to stress, we have evaluated the mRNA expression response of each *crf1* paralog in the telencephalon and the hypothalamus subjected to the different stressors. Additionally, to understand the link between *crf1* mRNA expression and the mobilization of energy reserves, we analysed serum levels of cortisol, glucose, lactate, and creatinine, which are commonly used as markers for stress response.

2. Material and methods

2.1. Fish and rearing facility

Atlantic salmon post-smolts of approximately 170 g were randomly distributed into 12 tanks (volume ca. 600 L) of 40 fish each at Cargill Innovation Center (Dirdal, Norway) and acclimatized for four weeks. Tanks were constantly supplied with flow through seawater at temperature of 8.8 ± 0.06 °C, salinity of 28.5 ± 0.09 ‰ and oxygen saturation of 98 ± 0.64%. Constant light conditions (LD 24:0) was used to mimic standard commercial procedures (Hansen et al., 1992). Temperature, salinity, and oxygen saturation were monitored daily. Fish were fed an equal amount of commercial pellets (ø 4 mm, Adapt Marine 80, Cargill) four times a day (19:00–20:15, 22:00–23:15, 01:00–02:15 and 06:00–07:15) using automatic feeders (Hølland Teknologi AS Feeder System, Florø, Norway).

2.2. Experimental design

After the acclimation period, the 12 tanks were randomly divided into four experimental groups (three replicates): control, chasing, hypoxia and chasing plus hypoxia. The stressors and protocols were chosen based on the latest studies on Atlantic salmon (Madaro et al., 2016a; Madaro et al., 2016b; Madaro et al., 2015). Briefly, the chasing group was subjected to a manually stirring of the water with a deep brush stick (spun clockwise and counterclockwise) for five minutes. The hypoxia group was exposed to low oxygen levels by completely closing the water inflow and reducing the tank water level to 2/3. When the oxygen saturation reached 55% (OxyGuard Dissolved Oxygen Probe, Farum, Denmark), the fish were kept for an additional five minutes before the water inflow was opened again. To the third group a combined stressor, chasing plus hypoxia, was applied. Briefly, as soon as the oxygen saturation reached 55%, the fish were chased for five minutes as described above. The stressors were applied twice a day (08:00 and 15:00) for eight days, while the control group was left undisturbed and only subjected to the routine tank maintenance. On day nine, five fish per tank, including the control group, were exposed to a novel-acute stressor by transferring them into a small bucket (40 × 20 × 20 cm) with 12 L of water for 15 min (confinement) (Fig. S1).

2.3. Sampling protocol

Sampling was carried out on day (D) 0, 1, 8, and 9. To assess basal serum cortisol, lactate, glucose, creatinine, and gene expression levels, two fish per tank were sampled on D0 before any stressor was applied (D0 pre-stress). Five fish per tank were then sampled one hour after the first stressor on D0 (D0 post-stress), D1, D8 and D9, to be comparable with previous studies on Atlantic salmon (Madaro et al., 2016a; Madaro et al., 2016b; Madaro et al., 2015). The experimental stressed groups were sampled 55 min after the stressor was applied, and 45 min after applying the novel-acute stressor on D9.

Fish were euthanized with 300 mg/L of Tricaine Pharmaq (PHARMAQ Ltd., Hampshire, UK) in 12 L of seawater. Blood was collected by a caudal venous puncture using a vacuum syringe and BD Vacutainer set (Becton Dickinson, Plymouth, UK). After overnight at 4 °C, blood was centrifuged at 1000 g for 10 min (4 °C) (Hettich Zentrifugen Universal 320R, Tuttlingen, Germany) and the collected serum stored at −80 °C until further analysis. Fork length and weight were recorded from each individual fish. For the gene expression analysis, the whole brain of each fish was collected and transferred into tubes containing RNAlater (Invitrogen, Carlsbad, CA, USA), refrigerated for one day and then stored at −80 °C until RNA isolation was performed.

2.4. Atlantic salmon crf1 sequence retrieval and synteny analysis

The nucleotide sequences of Atlantic salmon *crf1* paralogs were retrieved from the Atlantic salmon genomic database available in GenBank (https://www.ncbi.nlm.nih.gov/) by using the already public available *crf1* sequence NM_001141590.1 and tBlastn tool. The sequences identified were further confirmed by searching the newly Atlantic salmon genome database in Ensembl (https://www.ensembl.org/).

To investigate if the *crf1* genome region was conserved between Atlantic salmon paralogs and other teleost species, Northern pike (*Esox lucius*) and zebrafish (*Danio rerio*), searches for homologues of neighbouring genes were performed using the chromosome annotation available in Ensembl and Region Comparison view tool (Yates et al., 2020).

2.5. Sequence comparison and phylogenetic analysis

For CRF1 phylogenetic analysis, 14 teleost species were included: seven salmonid species, including Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*) , chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), coho salmon (*Oncorhynchus kisutch*), arctic char (*Salvelinus alpinus*) and brown trout (*Salmo trutta*); three cyprinids, the goldfish (*Carassus auratus*), common carp (*Cyprinus carpio*), and zebrafish; also included medaka (*Oryzias latipes*), Northern pike, Atlantic cod (*Gadus morhua*), and Nile tilapia (*Oreochromis niloticus*). In addition, the cartilaginous fish, Elephant shark (*Callorhinchus milii*) was also included in the analysis. All data was retrieved from GenBank (2020).

Multiple sequence alignments of the CRF1 deduced peptide precursor were performed using MUSCLE in MEGAX with the default parameters (UPGMA clustering method, Gap opening penalty −2.90, Gap extension 0.0). The alignment was subject to the analyses of the best-fit substitution model in MEGAX (Hall, 2013) to select the best-fit model. Phylogenetic analysis was conducted using the Maximum Likelihood method and Jones Taylor Thornton (JTT) matrix-based model with fixed Gamma distribution (+G) with invariant sites (+I) parameter with five rate categories and 1000 bootstrap replicates. The elephant shark CRF2 was used as outgroup.

The deduced CRF1 mature peptide sequence of Atlantic salmon, Northern pike and zebrafish was aligned using ClustalX 2.1 with the default parameters (Gonnet series matrix, Gap opening penalty 10, Gap extension 0.2). The mature peptide sequences were predicted using NeuroPred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py). The alignment was displayed in GeneDoc 2.7 (Nicholas et al., 1997) and percentages of identity and similarity calculated.

2.6. Brain dissection

For *cfr1* brain distribution analysis, nine brain samples (three per tank) from the control group at D1 were dissected into six regions following an in-house protocol: telencephalon (containing the POA), midbrain, cerebellum, hypothalamus (containing part of the POA in the anterior hypothalamus), pituitary and medulla oblongata/brain stem (Fig. 3). To ensure high total RNA yield and quality, the brain was placed on an ice block during dissection and cleaned from blood vessels and the pineal gland was removed. Saccus vasculosus was directly removed using forceps, while the olfactory bulb was cut using a scalpel to separate it from the telencephalon. Brain stem and cerebellum were carefully collected, and the hypothalamus separated from the midbrain and optic nerve. For the *crf1* responsiveness to stress only the telencephalon and the hypothalamus from all experimental groups (n = 2 per tank, D0 pre-stress; n = 3 per tank, D0 post-stress, D1, D8 and D9) were used.

2.7. RNA extraction and cDNA synthesis

Total RNA was extracted from the brain regions using RNeasy Mini Kit with On-column DNase Digestion (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The total RNA concentration and purity were measured using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and the integrity assessed by a 2100 BioAnalyser with RNA 6000 Nano Lab Chip kit (Agilent Technologies, CA, USA). To avoid any remnants of genomic DNA contamination, the total RNA samples were treated with TURBO DNase-free Kit (Ambion Applied Biosystem, CA, USA). First strand cDNA was synthesised from 1.5 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen, CA, USA) and Oligo$(dT)_{20}$ (50 μM) in a total reaction volume of 20 μl. The protocols were carried out accordingly to the manufacturer's instructions.

2.8. Real-time PCR (qPCR) primer design

Specific primers spanning an exon-exon junction were designed for Atlantic salmon *crf1a1*, *cfr1a2*, *crf1b1* and *crf1b2* using Primer3 (http://primer3.ut.ee/) and NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Sigma-Aldrich (Saint-Louis, Missouri, USA) (Table 1). Primers efficiency was determined using a 10-fold dilution series from the target gene cloned into a pCR4-TOPO vector (Thermo Fisher Scientific). The primers were analysed for quantification cycle (Cq), primers efficiency (E) and melting peaks. The resulted qPCR products were resolved on a 2% agarose gel, purified using a QIAquick Gel extraction Kit (Qiagen, Hilden, Germany) and sequenced at the University of Bergen sequencing facility. Its identity was confirmed using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the Atlantic salmon genome database.

2.9. Quantitative qPCR

qPCR analyses were 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 (15 ng/μl). 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 were included in all plates, in addition to the 10-fold dilution series of the target gene cloned. 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 sec) 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 absolute mRNA expression level for each target gene was determined 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.

2.10. Hepatosomatic index (HSI)

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):

$$HSI = 100\frac{LW}{BW}$$

Table 1
Atlantic salmon qPCR primers.

Gene	Chr location	GenBank ID	Ensembl acc. no	Primer Sequence (5′ → 3′)	Amplicon (bp)	Efficiency (%)	R^2
crf1a1	***ss14***	XM_014139989.1 XM_014139988.1	ENSSSAG00000069223	F: TGGACATATTCGGGAAATGAA R: GTCAACGGGCTATGTTTGCT	229	90	0,999
crf1a2	***ss03***	NM_001141590.1	ENSSSAG00000079049	F: GCACTTGATCCATTCCACAA R: ACCGATTGCTGTTACCGACT	232	95	0,999
crf1b1	***ss29***	XM_014181363.1	ENSSSAG00000080751	F: TCCATCACTCGTGGAAAAGGA R: CAGGGGTTCAACGAGATCTTCA	91	92	0,999
crf1b2	***ss19***	XM_014159556.1	ENSSSAG00000052094	F: AACACTTGTCGCGGGTCTTG R: GTCGGGATCAACAGGAATCTTCA	174	95	0,999

Primer sequences used for qPCR in Atlantic salmon. Amplicon sizes, qPCR efficiency and R^2 are listed for each primer pair. F = forward; R = reverse. The efficiency and R^2 values refer to qPCR performance on the brain distribution profile analysis.

where *LW* is liver weight (g), and *BW* is fish body weight (g).

2.11. Serum analysis

Serum cortisol, lactate, glucose and creatinine concentrations were analysed by using standard enzymatic colorimetric methods at the Insitute of Marine Research, Norway. Cortisol was extracted with ethyl acetate from 100 µl of serum (Pankhurst and Carragher, 1992). Serum cortisol was quantified by enzyme-linked immunosorbent assay (ELISA; (Cuisset et al., 1994)). Anti-cortisol, acetylcholine esterase-labelled tracer, and microplates precoated with monoclonal mouse anti-rabbit IgG were supplied by Cayman Chemicals (USA). Standard cortisol was purchased from Sigma-Aldrich (Saint-Louis, Missouri, USA). A Maxmat PL II spectrophotometer (MaxMat, Montepellier, France) was used to quantify lactate by a lactate dehydrogenase assay (Dialab, Neudorf, Austria). Glucose levels were determined using a peroxidase-antiperoxidase kit (MaxMat, Montepellier, France) and creatinine was determined by Jaffé kinetic method on creatinine-picric complex (Medicon, Attiki, Greece).

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, version 9.0.0, 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. The interaction between stress-treatments and observations in weight, length, serum cortisol, metabolites levels, and gene expression levels were assessed using a Two-Way ANOVA test. Gene expression data were log transformed prior to analysis to better fit the assumption of the parametric Two-Way ANOVA test. A *Post-hoc* Sidak's multiple comparisons test was used to analyse differences in weight and length. Whereas cortisol, lactate, glucose, creatinine, and *crf1* mRNA levels in the different experimental groups was assessed using Tukey's multiple comparisons test. Correlation analysis between serum cortisol, metabolites, and gene expression levels was conducted using Person r correlation. A $p < 0.05$ was considered significant. p value less than 0.001 are reported as $p < 0.001$ in the text (for detailed statistical analysis see Table S1). All data are presented as mean ± SEM, unless otherwise stated.

3. Results

3.1. Sequence comparison, phylogeny, and nomenclature of crf1 in Atlantic salmon

Sequence searches in the Atlantic salmon genomic database revealed four *crf1* paralogs, named *crf1a1, crf1a2, crf1b1* and *crf1b2* located on chromosomes (Chr) ssa14, ssa03, ssa29 and ssa19, respectively (Table 1). The *crf1* genomic region was duplicated by the teleost-specific 3R WGD resulting in two paralogs (*crf1a* and *crf1b*) as observed for zebrafish and Northern pike , and further duplicated during the salmonid-specific 4R WGD resulting in four paralogs in the Atlantic salmon (Fig. 1). The number of *crf1* paralogs is identical between the salmonid species investigated, i.e., two *crf1a* (*crf1a1* and *crf1a2*) and two *crf1b* (*crf1b1* and *crf1b2*) (Fig. 2 and Table S2).

The CRF1a peptides have a higher degree of sequence variability within and between species than the CRF1b peptides (Fig. S2). This is also true for Atlantic salmon, CRF1a1 and CRF1a2 full-length peptide sequences share only 63% pairwise identity, with 88% pairwise identity in the predicted 44 amino acid mature peptide region (Fig. S2). Atlantic salmon CRF1b1 and CRF1b2 full-length peptide sequences share 92% identity, and 100% pairwise identity in the predicted 41 amino acid mature peptide region. Additionally, Atlantic salmon CRF1a and CRF1b shared at least 44% and 65% pairwise identity at the predicted full-length sequence and at the mature peptide region level, respectively. In Northern pike, which belongs to Esociformes order, the closest related order to the Salmoniformes, CRF1a mature peptide region shared 100% pairwise identity with salmon CRF1a1 and 88% with CRF1a2. Additionally, pike CRF1b shared 100% identity with both Atlantic salmon CRF1b1 and CRF1b2 putative mature peptide sequence. These findings are also supported by the phylogenetic tree analysis, which shows that teleosts CRF1b protein sequences form a distinct clade (Fig. 2), whereas teleosts CRF1a protein sequences, are more divergent in sequence and do not form such a well-defined clade. The putative Atlantic salmon CRF1a and CRF1b clustered, as expected, with the other salmonids CRF1a and CRF1b protein branches, respectively, and the Northern pike was the closest related to the salmonid sequences. Additionally, the cyprinids formed a separated cluster both for CRF1a and CRF1b.

Searches for genes located up- (10 genes) and downstream (10 genes) of *crf1* paralogs revealed a high degree of conserved gene synteny of *crf1* genome region across teleosts and notably between Atlantic salmon and Northern pike (Fig. 1 and Table S3). Particularly, *crf1b* shows a high degree of conservation, both between salmon *crf1b1* and *crf1b2* with 14 linked genes conserved, and with Northern pike *crf1b* where at least 15 linked genes are conserved with the salmon *crf1b*. Interestingly, the genes located upstream salmon and pike *crf1b* where highly conserved with zebrafish *crf1b*, but no synteny was found downstream. However, for *crf1a*, three adjacent genes downstream and two genes upstream were conserved between zebrafish and Atlantic salmon and Northern pike. Besides *crf1*, three genes downstream (*arfgef1*, *cspp1*, *mybl1*) and five genes upstream (*trim55*, *dnajc5b*, *pde7a*, *armc1*, *bhlhe22*) were well-conserved between *crf1a* and *crf1b* and across the species investigated (Fig. 1 and Table S3).

3.2. crf1 mRNA expression distribution in the Atlantic salmon brain

The analysis of the mRNA expression levels of the *crf1a* paralogs showed a widespread distribution profile in the six brain regions of Atlantic salmon analysed (Fig. 3). Telencephalon, midbrain, and hypothalamus represent the three brain regions where the *crf1* genes were generally highly expressed, except for *crf1a1* which was more abundant in the brain stem than in the telencephalon area, while *crf1a2* showed a similar expression abundance in the midbrain and brain stem. Both

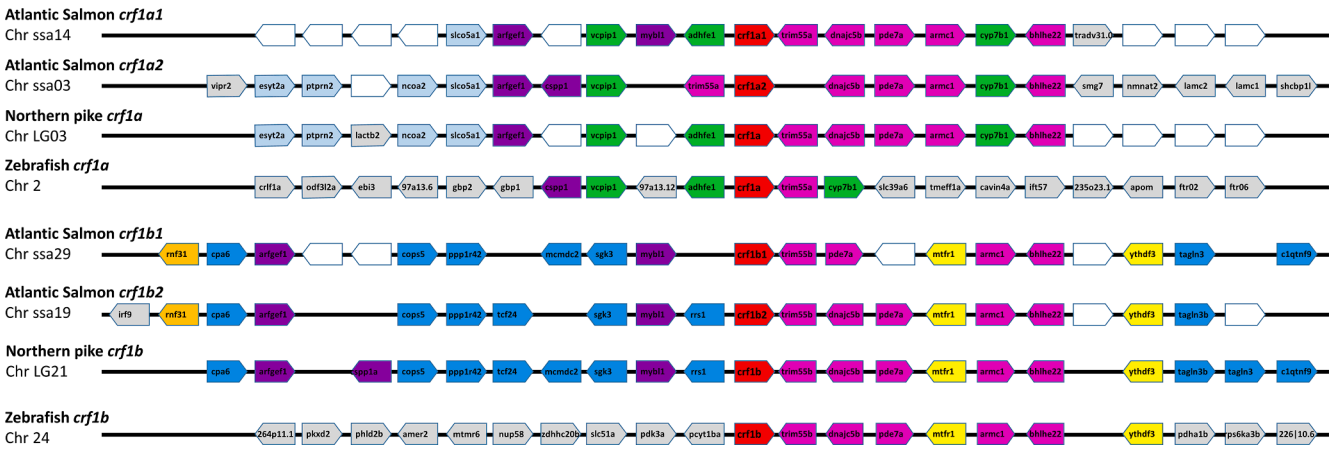

Fig. 1. Synteny analysis of *crf1a* and *crf1b* genomic region in teleosts. From top, *crf1a* genes from Atlantic salmon (*Salmo salar*), Northern pike (*Esox lucius*) and zebrafish (*Danio rerio*). Below, Atlantic salmon, Northern pike, and zebrafish *crf1b* genes. The chromosome (Chr) number is indicated below each species name. The central pentagons in red indicates the *crf1* genes. For each *crf1* gene, 10 flanking genes up- and downstream are represented by different coloured pentagons. Each colour identifies sets of orthologous genes based on the degree of conservation between species and between the chromosomes within species. The pentagons point in the direction of transcription, and only protein coding genes are indicated. Information regarding genes location, full names, and accession numbers of *crf1* and neighbouring genes are given in Table S3.

crf1a1 and *crf1a2* showed their highest expression levels in the hypothalamus and midbrain, and telencephalon and hypothalamus, respectively. *crf1b1* was mainly expressed in the telencephalon and midbrain, and *crf1b2* was more abundant in the midbrain, followed by telencephalon and hypothalamus. *crf1a1, crf1b1* and *crf1b2* had low mRNA expression levels in the pituitary and *crf1b1* and *crf1b2* also in the cerebellum. Among the *crf1* paralogs, *crf1b1* was the most abundant and the *crf1b2* the least.

3.3. Effects of stress on growth

Atlantic salmon post-smolts exposed to a repeated stressor (chasing, hypoxia or a combination of chasing and hypoxia) for eight days, followed by the exposure to a novel-acute stressor (confinement) resulted in a significantly lower growth compared to the control group (undisturbed for eight days) at the end of the experiment, day 9 (Fig. S3). Exposure to a repeated stressor (time effect) affected both weight ($p = 0.036$) and length ($p < 0.001$) of the fish (for detailed statistical information see Table S1). While the control group had a 17.58% increase in body weight ($p = 0.021$) at the end of the experiment, no significant increase was observed for the fish exposed to repeated stress. Interestingly, fish length increased in both the groups exposed to chasing 8.54% ($p < 0.001$) and hypoxia 8.54% ($p < 0.001$), but not for the control (4.73%) or the chasing plus hypoxia (1.28%) groups (Fig. S3).

A strong interaction was observed between the type of stressor and time in the HSI ($p < 0.001$) (Fig. S4). The HSI was higher in the chasing group compared to the other experimental groups at D9 (chasing vs. control $p < 0.001$; chasing vs. hypoxia $p < 0.001$; chasing vs chasing plus hypoxia $p = 0.001$). Additionally, the chasing group had a higher HSI at D9 compared to D0 ($p < 0.001$) and D1 ($p < 0.001$) (for detailed statistical information see Table S1).

3.4. Cortisol, lactate, glucose, and creatinine levels in Atlantic post-smolts under stress conditions

To assess the physiological response to stress, we analysed serum cortisol, glucose, lactate, and creatinine throughout the entire experiment. At the start of the experiment (D0 pre-stress), the serum cortisol levels in the experimental groups varied from 4.87 ± 1.72 ng/ml to 35.84 ± 14.49 ng/ml, but the differences were not significant (Fig. 4). Subsequently, the cortisol levels showed a general increase, in particular in the chasing group, both at D0 post-stress (88.71 ± 8.50 ng/ml) and D1 (85.06 ± 9.17 ng/ml) (D0 pre-stress vs. D0 post-stress, $p = 0.008$; D0 pre-stress vs. D1 $p = 0.0104$). On D8 the magnitude of the cortisol response declined despite the fact that all stressed groups were continuously exposed to the repeated stressors. A high response in cortisol levels was observed in all the experimental groups when exposed to the novel-acute stressor on D9. The novel-acute stressor also induced a higher cortisol response compared to the D0-post stress for all groups (D9 vs D0 post-stress $p < 0.001$). The response at D9 appeared to be highest in the control group albeit not significantly different (Table S1).

No changes in glucose levels were observed during the first 24 h of stress exposure (Fig. S5, statistical details on Table S1). At D0 pre-stress, glucose serum levels were ranging from 4.60 ± 0.24 to 5.66 ± 0.26 mmol/l. Glucose levels remained stable at D0 post-stress, while showed a decreasing tendency on the stressed groups on D1 albeit not statistically significant, except in the chasing plus hypoxia group ($p = 0.009$). On D8 the control group showed lower glucose levels (3.76 ± 0.27 mmol/l) compared to D1 ($p < 0.001$), while in the chasing and chasing plus hypoxia groups the glucose levels increased (5.91 ± 0.34 mmol/l, 5.82 ± 0.17 mmol/l, and $p < 0.001$, $p = 0.001$, respectively). On D9, glucose levels only increased in the control (6.35 ± 0.26 mmol/l, $p < 0.001$) and hypoxia (6.35 ± 0.16 mmol/l, $p < 0.001$) groups compared to the D8 levels. As observed for the cortisol, the increase in glucose levels after confinement was higher than at D0-post-stress, but only for the control ($p = 0.030$) and the chasing group ($p = 0.004$) (Fig. S5). Glucose levels showed a weak correlation with cortisol levels (Fig. S6, statistical details on Table S1).

Lactate baseline levels at D0 pre-stress ranged from 35.69 ± 3.07 to 40.53 ± 1.87 mg/dl and no differences were observed among the experimental groups (Fig. S5, statistical details on Table S1). A short term-increase in lactate levels was observed in the chasing group at D0-post stress compared the D0 pre-stress baseline (70.29 ± 6.91 mg/dl, $p < 0.0001$). Exposure to the novel-acute stressor induced an increase in lactate levels in all experimental groups when compared to D8 ($p < 0.001$). The control group had the highest lactate levels in response to the confinement (83.02 ± 2.88 mg/dl). The novel-acute stressor also induced a significant difference in lactate levels between the control and chasing ($p < 0.001$), the control and hypoxia ($p = 0.002$), and the control and chasing plus hypoxia ($p < 0.001$). The changes in cortisol levels were followed by changes in lactate levels as shown in the positive linear correlation between the two variables (Fig. S7). Instead, lactate showed a weak correlation with the glucose levels (Fig. S8).

Creatinine levels, in contrast to what was observed for cortisol,

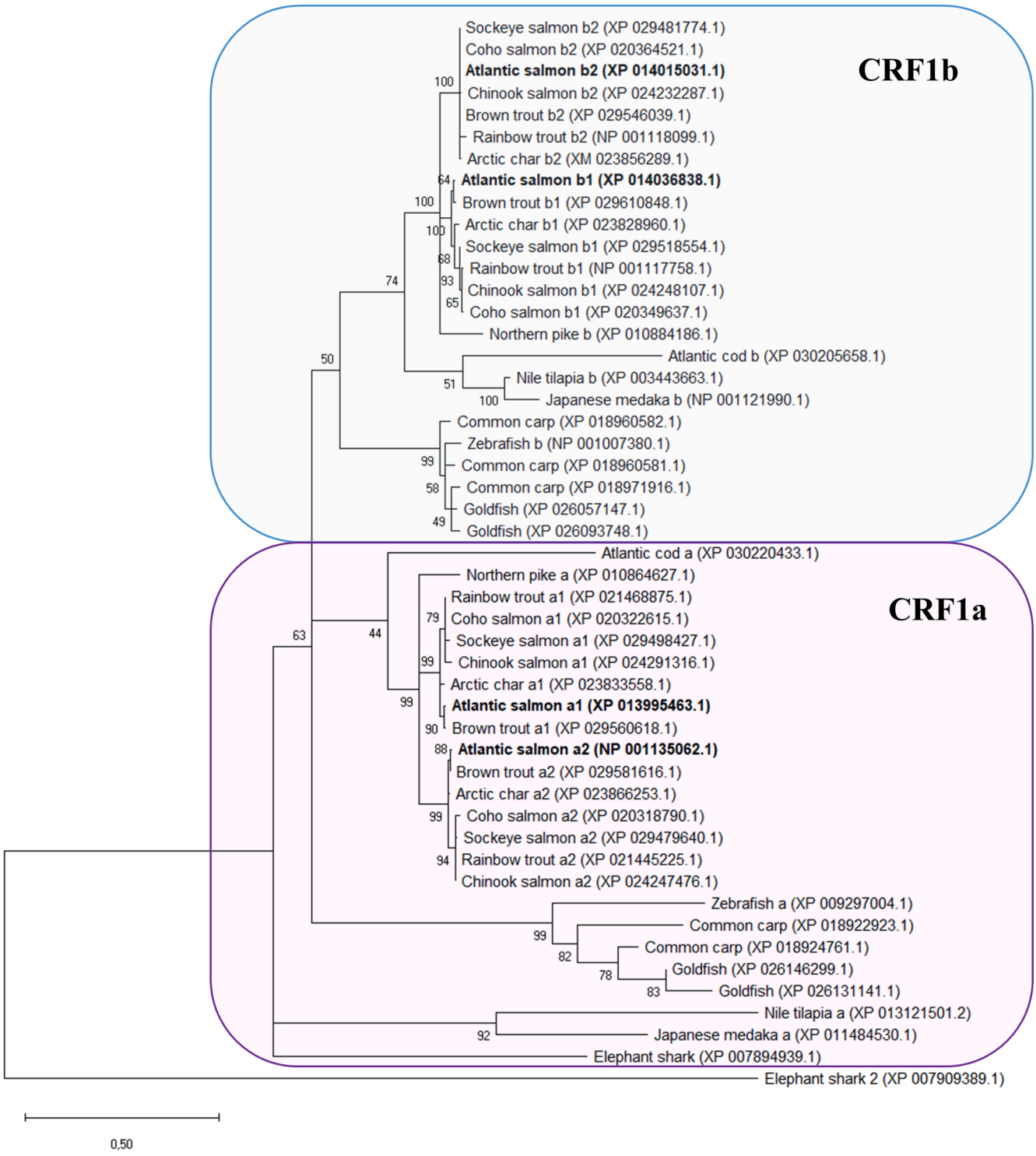

Fig. 2. Phylogenetic relationship of fish CRF1a, CRF1b and CRF2 based on predicted protein sequences. The phylogenetic tree was constructed based on deduced CRF peptide sequences using the Maximum Likelihood (ML) method, 1000 bootstraps replicates, and JTT + G + I matrix-based model in MEGA X. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Phylogenetic tree is rooted to the Elephant shark CRF2 sequence. For additional information related to the protein sequences used please refer to Table S2.

glucose and lactate levels, decreased in the stressed groups during both the repeated stress exposure and in response to the novel-acute stressor (Fig. S5, statistical details on Table S1). At D0 pre-stress creatinine levels ranged from 136.7 ± 13.91 μmol/l to 155.1 ± 2.89 μmol/l. In all stress-induced groups there was a tendency for a decrease in creatinine levels at D0 post-stress, albeit not statistical different. A significant drop in creatinine levels was observed on D1, D8 and D9 when compared to D0 pre-stress for all stressed groups ($p < 0.05$). On D8, creatinine levels in the control group (163.65 ± 9.29 μmol/l) were higher in comparison to D0 post-stress ($p = 0.003$) and D1 ($p < 0.001$), which did not differ from the basal levels registered on D0 pre-stress. No correlation was found between creatinine and lactate, glucose, or cortisol levels.

No correlation was found between HSI and the serum stress indicators analysed.

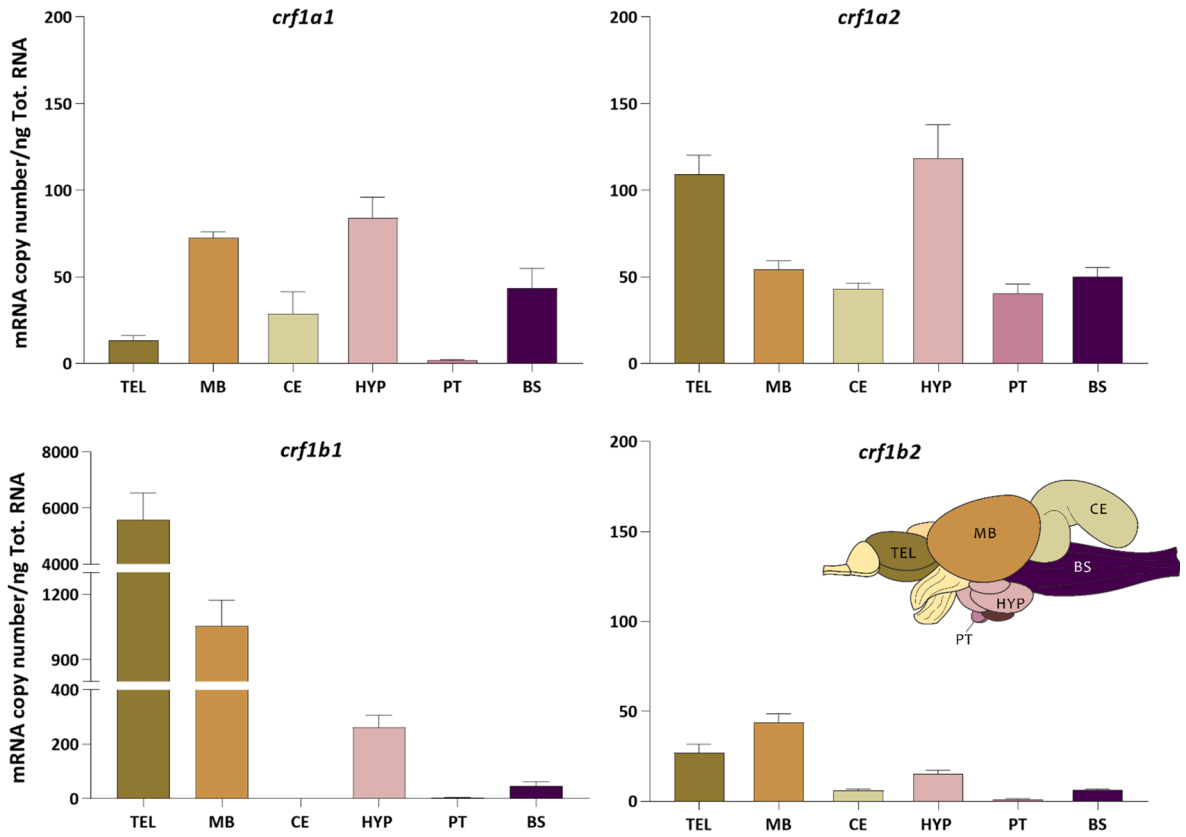

Fig. 3. Distribution of *crf1* paralogs in Atlantic salmon post-smolts brain. mRNA abundance and distribution of *crf1* paralogs in six dissected brain regions: telencephalon (TEL), midbrain (MB) cerebellum (CE), hypothalamus (HYP), pituitary (PT) and medulla oblongata/brain stem (BS). Bars represent mean ± SEM (n = 9).

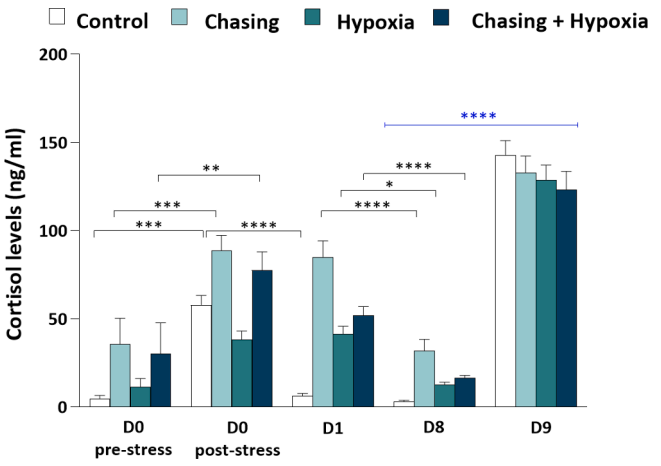

Fig. 4. Serum cortisol levels in Atlantic salmon post-smolts exposed to stress. Bars represent mean ± SEM (n = 6/group on D0 pre-stress, n = 12–15/group on D0 post-stress, D1, D8 and D9). Due to several significant changes in the cortisol levels, only the more relevant significant differences are indicated. For detailed statistical information, please refer to Table S1.

3.5. *Effects of different stressors on the telencephalic and hypothalamic crf1 mRNA expression in the Atlantic salmon*

The mRNA expression of *crf1a1*, *crf1a2*, *crf1b1* and *crf1b2* was analysed in the telencephalon and hypothalamus of the control, chasing, hypoxia, and chasing plus hypoxia groups. A significant ($p < 0.001$) response of *crf1* to the chronic stressors exposure (time effect) was observed in all paralogs in the telencephalon (Fig. 5), and on *crf1a1* in the hypothalamus (Fig. 6). An increasing tendency in *crf1* mRNA levels was observed in all paralogs on D0 post-stress and D1 in the telencephalon (Fig. 5), while a similar trend was observed only on *crf1a1* in the hypothalamus (Fig. 6). Thereafter, a decrease in expression was observed for most of the transcripts on D8 and D9. In the telencephalon, the group exposed to chasing showed a significant up-regulation on *crf1a2* ($p = 0.0416$), *crf1b1* ($p = 0.0117$) and *cr1b2* ($p = 0.0096$) on D0 post-stress compared to the control on D0 post-stress, and *crf1a1* was marginally significant ($p = 0.0719$). Differently from the *crf1b* response in the telencephalon, both *crf1b1* and *crf1b2* in the hypothalamus showed a significant upregulation at D0 post-stress, D8 and D9 compared to D0 pre-stress in the chasing plus hypoxia group ($p < 0.05$). No significant differences in mRNA levels were found for *crf1a2* in the hypothalamus.

The mRNA responses of *crf1* and *crf1a1* in the in the telencephalon and hypothalamus, respectively, showed a response pattern similar to cortisol although not significantly correlated (Table S1). In addition, no or weak correlations were found between the *crf1* paralogs mRNA expression and the serum metabolites (see Table S1).

4. Discussion

Numerous studies in literature have explored the role of the HPI axis on Atlantic salmon stress response. However, the understanding of the *crf1* paralogs function in response to stress is still not complete. In the current study, we have investigated the brain distribution of the four *crf1* paralogs identified in the Atlantic salmon genome and analysed their responsiveness to repeated stressors and a novel-acute stressor. The different mRNA abundance of the *crf1* paralogs in the brain of salmon post-smolts and responsiveness to stress in the telencephalon and hypothalamus suggest that there might be differential regulatory functions between paralogs.

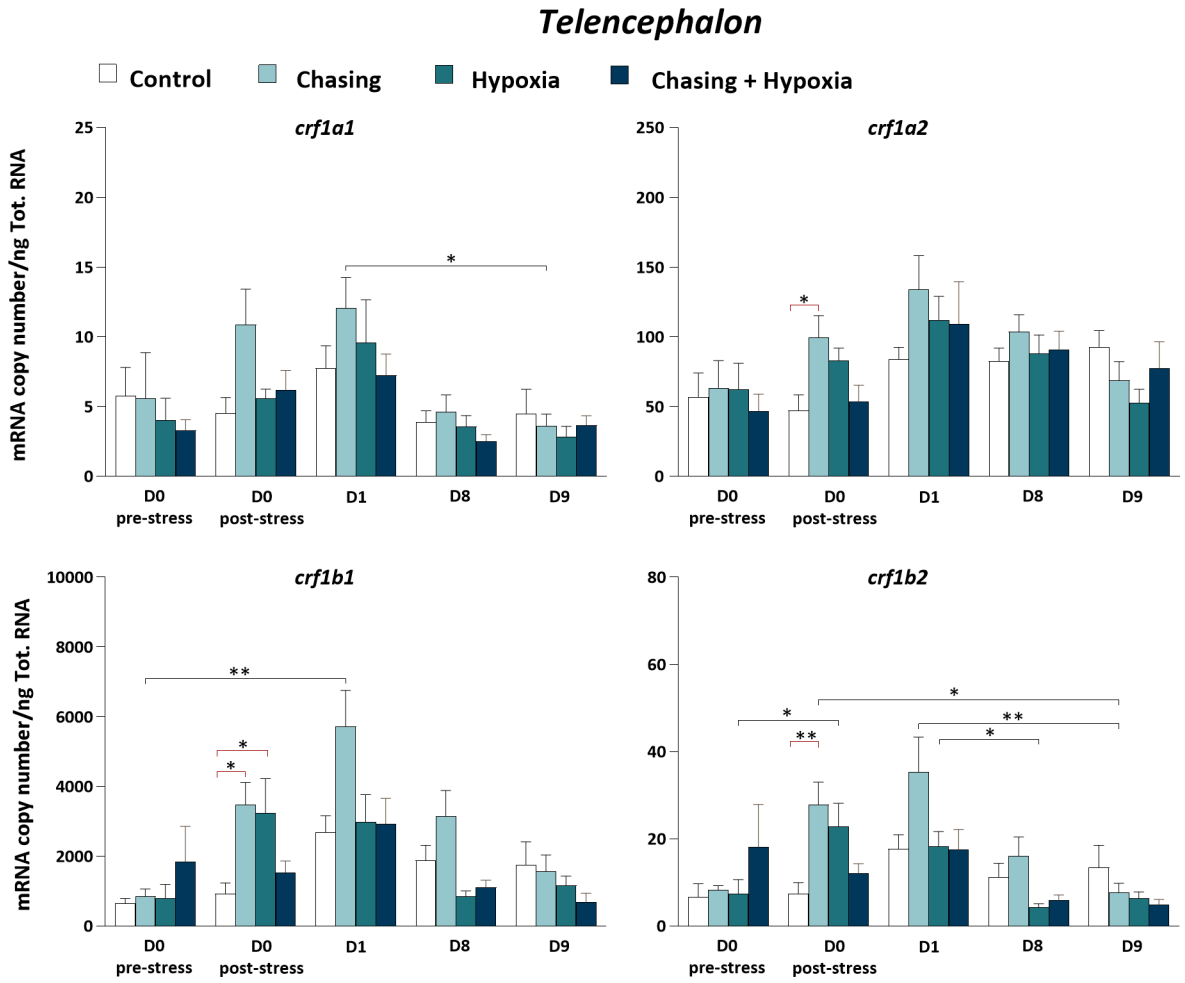

Fig. 5. mRNA expression levels of *crf1* paralogs in the telencephalon of Atlantic salmon exposed to stress. Bars represent mean ± SEM (n = 5–6/group on D0 pre-stress, n = 7–9/group on D0 post-stress D1, D8 and D9). Due to several significant changes in the mRNA expression levels, only the most relevant changes are represented. For detailed statistical information refer to Table S1.

4.1. Atlantic salmon crf1 paralogs

In Atlantic salmon, the presence of four *crf1* paralogs (*crf1a1*, *crf1a2*, *crf1b1* and *crf1b2*) have previously been reported by Grone and Maruska (Grone and Maruska, 2015b), however the functional characterization have only focused on the *crf1b* genes so far (Madaro et al., 2016b; Madaro et al., 2015; Vindas et al., 2017). The origin of *crf1a* and *crf1b* genes appears to be a teleosts-specific WGD event (Cardoso et al., 2016; Grone and Maruska, 2015b). Additionally, species-specific duplication events have occurred for the salmonids and, as a result of the tetraploidization event in the Salmoniformes lineage (Macqueen and Johnston, 2014), two *crf1a* genes (*crf1a1* and *crf1a2*) and two *crf1b* genes (*crf1b1* and *crf1b2*) are present. Cypriniformes, such as common carp and goldfish, have also experienced an additional WGD event around 16 million years ago subsequent to the teleosts-specific WGD (David et al., 2003; Larhammar and Risinger, 1994). Consequently, in common carp we found a total of three *crf1a* and two *crf1b*, adding new information to the previous two *crf1* genes characterized by Flik et al. (2006) and Huising et al. (2004), which were sometimes misunderstood to have arisen in the teleosts WGD (Alsop and Vijayan, 2009). In goldfish, two *crf1a* and two *crf1b* genes were found in the genomic database. Interestingly, and this has previously also been observed for zebrafish (Grone and Maruska, 2015b), Cypriniformes CRF1 are distantly related to the other analysed teleosts, which is reflected in the separated phylogenetic tree branches for both CRF1a and CRF1b (Fig. 2). Notably, *crf2* gene was lost in both teleosts and eutherian mammals (Cardoso et al., 2016; Grone and Maruska, 2015a), suggesting that early in the teleost lineage, the presence of *crf1a* and *crf1b* provided sufficient opportunity for the multiple specialized functions of CRF.

The high degree of sequence identity between the *crf1a* and *crf1b* duplicated genes in salmonids is not surprising, as its origins lie in a recent lineage-specific genome duplication. Nevertheless, the factors explaining the persistence of the duplicate of both *crf1a* and *crf1b* genes in the salmonid genomes have not yet been explored. Our sequence and synteny analyses clearly shows that *crf1b* genes in the Atlantic salmon are more similar to each other than the *crf1a* genes. Notably, the Atlantic salmon and Northern pike genomic regions flanking both *crf1a* and *crf1b* are also particularly syntenic, which is expected due to their relatively close evolutionary proximity (Rondeau et al., 2014). The persistence of the *crf1* paralogs originated from the salmon-specific 4R WGD might have resulted in divergent functions in the HPI axis activation or evolved into differential expression (Glasauer and Neuhauss, 2014). To gain insight into these, we have explored the Atlantic salmon *crf1* paralogs mRNA distribution profile in the brain and investigated their responsiveness to a novel-acute and/or repeated stress.

4.2. Expression of crf1 paralogs in the Atlantic salmon brain

In the mammalian brain, the parvocellular region of the paraventricular nucleus (PVN) in hypothalamus is the principal center of the CRF neurons (Herman et al., 2003; Rivier and Plotsky, 1986; Zhou and Fang, 2018). The disturbance of the steady state homeostasis of the CRF

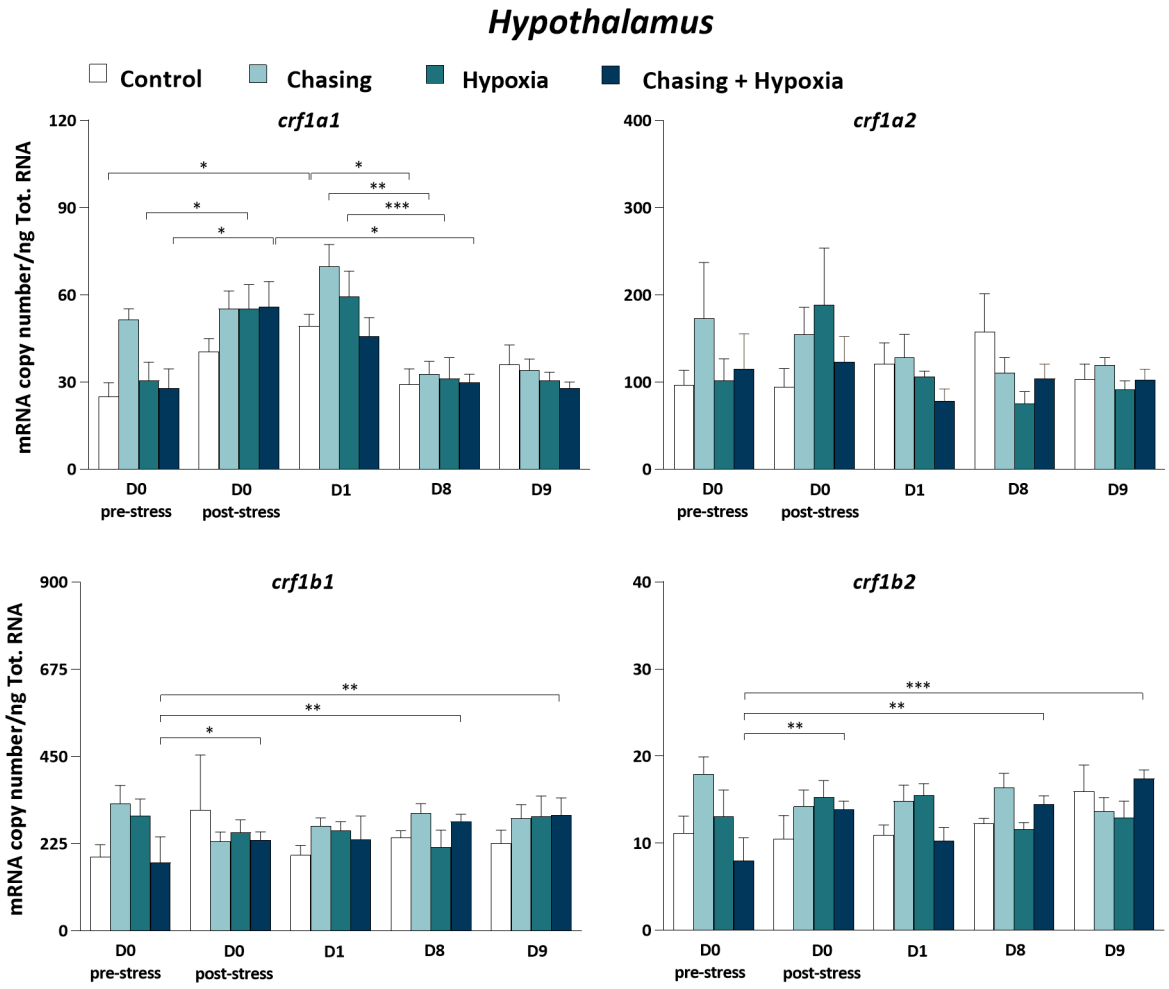

Fig. 6. mRNA expression levels of *crf1* paralogs in the hypothalamus of Atlantic salmon exposed to stress. Bars represent mean ± SEM (n = 6/group on D0 pre-stress, n = 7–9/group on D0 post-stress D1, D8 and D9). Due to several significant changes in the mRNA levels, only the more relevant changes are indicated. For detailed statistical information refer to Table S1.

neurons in the PVN will induce a rapid activation of the stress axis (HPA, Hypothalamic-Pituitary-Adrenal) by projections in the median eminence in the hypothalamus and hypophyseal portal system and, thereby, activate pituitary adrenocorticotropin responses (Herman et al., 2003; Rivier and Plotsky, 1986). In teleosts, the system is slightly different and the hypophysiotropic role of CRF in the HPI axis seems to be mediated by extended CRF neurons in the nucleus preopticus in the POA, where axons directly innervate the pituitary cells (Ando et al., 1999; Matz and Hofeldt, 1999; Olivereau et al., 1988; Olivereau et al., 1984). Despite these differences, both mammals and fish show a widespread distribution of CRF neuronal populations in the brain, supporting that CRF is involved in many other physiological functions that include reproduction, feeding behaviour and food intake, locomotion and that CRF also has a broader role in anxiety and depression in mammals (Backström and Winberg, 2013; Bernier and Craig, 2005; Clements and Schreck, 2001; Hostetler and Ryabinin, 2013; Lovejoy and Balment, 1999; Rivest and Rivier, 1995). Our results showed that the *crf1* paralogs are widely expressed in Atlantic salmon brain supporting the hypothesis of other possible functional roles of *crf1* than an activator of the stress axis (Fig. 3). According to our brain dissection, the telencephalon, midbrain, and hypothalamus represent the three main brain regions where the *crf1* paralogs were highly expressed, suggesting that these may be the three major functional sites for *crf1* in Atlantic salmon. The widespread expression pattern of *crf1* in the Atlantic salmon brain is consistent with findings in other teleosts. For example, in zebrafish *crf1b* mRNA was distributed from the forebrain to the hindbrain, with distinct nuclei regions localised in both telencephalon and diencephalon (Alderman and Bernier, 2009, 2007; Chandrasekar et al., 2007), while *crf1a*-expressing cells were restricted to the hypothalamus (Grone and Maruska, 2015b). In tilapia, *crf1* mRNA levels were observed in all three regions analysed: forebrain, midbrain and hypothalamus (Aruna et al., 2012), as well as in the forebrain and diencephalon in rainbow trout (Bernier and Craig, 2005). The *crf1* localization in the POA was confirmed in Atlantic salmon (Madaro et al., 2016b; Madaro et al., 2015), rainbow trout (Ando et al., 1999; Doyon et al., 2005), common carp (Huising et al., 2004), zebrafish (Fuzzen et al., 2010) and white sucker (*Catostomus commersonii*) (Okawara et al., 1992). Interestingly, in the spotted gar (*Lepisosteus oculatus*), a representative species of an early divergent lineage in vertebrate evolution, the conserved *crf1* (the ancestral gene to *crf1a* and *crf1b*) had a wide distribution from the forebrain to the hindbrain, including expression in telencephalon, diencephalon and midbrain (Grone and Maruska, 2015a), while the *crf2* mRNA expression was restricted to the secondary gustatory/visceral nucleus of the hindbrain and was absent in the hypothalamus (Grone and Maruska, 2015a, 2015b). The remarkable CRF expression in the stress-linked nuclei in the POA, as well as its wide distribution in the brain, has also been confirmed by numerous immunohistochemistry studies in teleost species (Chandrasekar et al., 2007; Matz and Hofeldt, 1999; Olivereau et al., 1988; Olivereau et al., 1984; Olivereau and Olivereau, 1987; Pepels et al., 2002; Yulis et al., 1986; Yulis and Lederis, 1987; Zupanc et al., 1999).

4.3. Effects of stress in Atlantic salmon, serum cortisol and metabolites response

Exposure of Atlantic salmon to different stressors impacted growth, serum levels of cortisol, glucose, lactate and creatinine, and the mRNA expression of *crf1* paralogs in the telencephalon and hypothalamus. All stressors suppressed the fish weight gain compared to the control group and induced a significant gradual increase of HSI in the chased fish compared to the other groups after 9 experimental days. At the beginning of the experiment serum cortisol and metabolites were shown to be within the range of unstressed salmon post-smolt (Iversen et al., 1998; Madaro et al., 2016b; Pankhurst et al., 2008; Skjervold et al., 2001). As the primary response of the HPI axis activation, cortisol showed an immediate increase in serum levels one hour after the fish was first exposed to the stressor, followed by a gradual decrease during the next eight days, despite the repeated stressor application (Fig. 4) as results to either exhaustion of the endocrine system or habituation to the stressor conditions (Aerts et al., 2015; Fast et al., 2008; Kiilerich et al., 2018; Madaro et al., 2016b; Madaro et al., 2015; Martinez-Porchas et al., 2009; Shrimpton and Randall, 1994; Wendelaar Bonga, 1997). Evaluation of the cortisol response showed that its levels were dependent on the stimuli. Among the stressors, repeated chasing induced a higher and long-lasting cortisol response compared to repeated hypoxia and repeated chasing plus hypoxia. However, exposure to a novel-acute stressor (confinement) on D9 induced the highest cortisol response in all experimental groups compared to the first stress exposure on D0 and the repeated stress events. A similar response was observed in the lactate levels but only in the chasing group, suggesting anaerobic glycolysis in the muscle under higher energy demand, which was recovered during the following 24 h (Fig. S5). Interestingly, the same group had the least lactate response after the novel-acute phase, possibly due to HPI axis exhaustion /habituation. Indeed, the control group, which did not receive any stressor during the previous eight days, showed the highest lactate response. The dependent response of lactate to the HPI axis activation was confirmed by the correlation between cortisol-lactate levels (Fig. S7). A higher response in lactate was however expected particularly for the groups exposed to low O_2 due to temporary oxygen debt. For both cortisol and lactate, the repeated hypoxia group had minor or no changes compared to the repeated chasing group, reflecting a tolerance of Atlantic salmon to low oxygen levels (Remen et al., 2013). On the other hand, glucose levels were stable during the first stressing phase most likely as the result of a rapid turnover rate in the blood which did not lead to its depletion after 24 h. Thereafter, due to high energy demand and exhaustion/habituation during the stress exposure, fish probably responded with an increase in glucose availability at both D8 and D9. This is in line with previous studies in several teleost species (reviewed by Martinez-Porchas et al., 2009), in which the glucose response was not as fast as cortisol, and its levels were changed after some minutes or after several days of exposure to stressors (from 0.17 to 6.1 nmol/l (pre-stress) to 0.23 to 10.5 nmol/l (post-stress)). However, in contrast to our results, glucose levels increased in parallel with increasing cortisol levels in Atlantic salmon subject to chronic stress during the first six hours (from 60 mg/dl to >70 mg/dl) (Fast et al., 2008). Thus, the glucose levels observed in our study could be also a result of factors related to metabolic and/or energy homeostasis of the fish rather than stress-response, and more than a single individual measurement would be required over time to have a clear overview of the dynamics of this important metabolite. As an indirect index of energy reserves (gluconeogenesis and glycogenlysis) (Chellappa et al., 1995; Sloman et al., 2001), we correlated glucose levels with the HSI, but no correlation was found among the experimental groups, the stressors or exposure time, and further studies analysing the hepatic glycogen content could provide a deeper insight into the energy status of the fish. On the contrary, serum creatinine levels decreased in the stressed groups both during the repeated and novel-acute stress exposure. Creatinine is the waste product of creatine and creatine phosphate metabolism, which is used as energy substrate in muscle aerobic metabolism (Kashani et al., 2020). Physiological response to exhaustive exercise in fish commonly leads to depletion of energy stores (ATP, glycogen, creatine phosphate) and, anaerobic exercise activity in fish require a post-exercise recovery process longer than in mammals (Kieffer, 2000). Therefore, the decreased levels of serum creatinine may indicate a depletion of energy stores consumed during the exhaustive swimming exercise in the chasing and chasing plus hypoxia groups, and during swimming under low O_2 levels in the hypoxia group.

4.4. Effects of different stressors on the crf1 mRNA expression in the Atlantic salmon brain

The *crf1* paralogs mRNA expression in response to stress is not consistent between teleost species and their response depend on the stressor used. The first results obtained by *in situ* hybridization showed enhanced staining of *crf1* mRNA-containing neurons in the POA of rainbow trout stressed by confinement (Ando et al., 1999). When different intensities of stress (repeated chasing and confinement methods) were applied to the same species, POA *crf1* mRNA increased (Doyon et al., 2005), and this also occurred in the forebrain following hypoxia (Bernier and Craig, 2005). Similarly, the common carp showed an increase of hypothalamic *crf1* mRNA levels after being acutely restrained in a net for 24 h, but no significant differences were observed in the first 30 min of stress exposure (Huising et al., 2004). In addition, *crf1* also increased in response to chasing in the POA region of zebrafish (Fuzzen et al., 2010), to handling and changes in salinity in the forebrain and hypothalamus of tilapia (Aruna et al., 2012), and to high rearing density in the whole brain of Senegalese sole (*Solea senegalensis*) (Wunderink et al., 2011). In contrast, *crf1* levels declined in whole brain of zebrafish after 90 min of acute confinement stress (Ghisleni et al., 2012) and in whole brain of cichlid fish (*Astatotilapia burtoni*) following one month of social stress (Chen and Fernald, 2008). No changes in *crf1* expression levels were observed after exposure to rapid increase in water temperature in the whole brain of Senegalese sole (Benítez-Dorta et al., 2017) nor to chronic hypoxia in the POA area of the common carp (Bernier et al., 2012). To date, the few studies available for Atlantic salmon showed increase in *crf1b* levels in the POA of parr exposed to either a mix of different chronic stressors or novel-acute stressors (Madaro et al., 2016b; Madaro et al., 2015). However, no changes in *crf1b* expression were observed in the POA of both parr and post-smolt exposed to chronic chasing followed by a mix of acute stressors (netting, air exposure or confinement) (Madaro et al., 2016b). In the telencephalon, *crf1b* mRNA abundance showed no difference between proactive and reactive individuals under hypoxia (Vindas et al., 2017). In agreement with its role in activating the HPI axis, *crf1* mRNA levels changed in parallel with the raise in cortisol levels in several telosts species (Aruna et al., 2012; Doyon et al., 2005; Fatsini et al., 2020; Huising et al., 2004; López-Olmeda et al., 2013; Madaro et al., 2015; Volkoff et al., 2005; Wunderink et al., 2012; Wunderink et al., 2011). However, this expected response is not always consistent (Benítez-Dorta et al., 2017; Bernier et al., 2012; Ghisleni et al., 2012; Madaro et al., 2016b).

In the current study, *crf1* mRNA abundance showed to be dependent of the stress exposure regime. For instance, the *crf1* mRNA abundance in the telencephalon and the *crf1a1* mRNA levels in the hypothalamus increased during the first 24 h similarly to the serum cortisol levels. Consistently, the same trend is also present after one week of stress exposure, in which there was a reduction of mRNA abundance on D8 as well as cortisol levels. However, on D9 the proportional trend seems to be absent in both telencephalon and hypothalamus, suggesting that an imminent *crf1* transcript regulation was not involved in the acute stress response. Both *crf1b1* and *crf1b2* in the hypothalamus responded to the combination of chasing plus hypoxia but not to these stressors alone, while no changes at mRNA levels were observed in the hypothalamic *crf1a2* under the different stressor conditions. Moreover, in the

hypothalamus the *crf1* genes expression data showed basal mRNA levels variance among the groups at D0 before the stress experiment started. This variation might be a result of differences in the physiological and metabolic status or sampling-induced stress. However, if the last, the same trend should have been observed at the serum cortisol and metabolite levels as well. Further studies are needed to clarify this issue. Our hypothalamic section also includes the nucleus lateralis tuberis (NLT), which has been shown to be another principle CRF site in the brain of some teleosts species (Matz and Hofeldt, 1999; Pepels et al., 2002; Yulis and Lederis, 1987). As the neurosecretory nuclei in the POA, the CRF nuclei in the NLT control the pituitary hormonal regulation, however, the NLT may have different functional roles in the regulation of the ACTH release. Indeed the NLT has an important role in nutrient metabolism and feeding (review by Rønnestad et al., 2017). Therefore, CRF in the NLT may have a preferential control in the biosynthesis of ACTH linked to appetite control, while the POA is specially involved in stimulating the release of ACTH (Lederis et al., 1982). It can be only speculated that the differential expression response to stress observed in the hypothalamus in our study are a combined response to both POA and NLT. However, the molecular pathway behind this process is still not totally explored, in particular in Atlantic salmon, and more studies are required.

It has been argued that mild stressors not necessarily induce changes at the *crf1* mRNA level, as the rapid HPI response is achieved by enough stored neuropeptides, whilst up-regulation responses occur during persistent chronic conditions (Huising et al., 2004). Evaluation of the different stressors showed that repeated chasing induced the highest cortisol and lactate response. However, considering the different response in cortisol, glucose, and lactate between the repeated stressors and the novel-acute stressor, it seems that confinement is a stronger stressor compared to chasing, hypoxia or chasing plus hypoxia, and therefore enhance a higher HPI response. Yet, none of the *crf1* paralogs changed at the mRNA level in response to the novel-acute stressor. Similar responses were observed in Atlantic salmon subject to chronic chasing for 23 days, and where the fish were exposed to air as the final novel-acute stressor without showing any changes in *crf1b* expression in the POA of both parr and post-smolt (Madaro et al., 2016b).

5. Conclusions

The 4R WGD in Atlantic salmon has resulted in four *crf1* paralogs, which are widely and variably expressed in the brain. *crf1* mRNA is mostly abundant in the telencephalon, midbrain, and hypothalamus, and the least in the pituitary and cerebellum. Among the four paralogs, *crf1b1* was the most abundant in Atlantic post-smolts brain. Exposure to repeated stress over a period of eight days suppressed growth and resulted in lower weight gain in Atlantic salmon compared to a control non-stress group. Among the serum stress indicators analysed, cortisol was best linked with the stress-response, followed by lactate. For glucose there was no clear correlation with time or stressor, suggesting a complex response as part of the dynamic adaptation of the metabolism related to the mobilization of energy reserves following cortisol release and compensation. Serum creatinine levels decreased as energy stores were depleted during the stress conditions. In this study, the mRNA expression of *crf1* paralogs showed a similar stress-response pattern to cortisol. All *crf1* paralogs expression in the telencephalon, but only *crf1a* in the hypothalamus, increased in parallel with cortisol levels under the repeated stress exposure regime. Differently, hypothalamic *crf1b1* and *crf1b2* response to stress was restricted to one experimental group (chasing plus hypoxia group), while no changes in mRNA levels were observed for *crf1a2* in the same brain section due to the stressors. Despite the limitation of having the POA in two distinct brain dissections, our findings suggest the importance of the four *crf1* paralogs identified in response to stress in Atlantic salmon. This provides an important steppingstone for future research on expression analysis and highlights the importance of analysing individual gene paralogs. Furthermore, our study improves the understanding of the stress system response of Atlantic salmon with the potential impact to improve the welfare of this farmed species, by assessing individual *crf1* paralogs in Atlantic salmon and their different responses as a tool for the interpretation of the HPI response to different stressors and environmental conditions.

Funding

This study was supported by the Research Council of Norway (RCN) (LeuSENSE grant No. 267626).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Cargill Innovation Center and its entire staff for fish rearing, assistance with sampling, and water chemistry. In particular, the authors are grateful for the technical assistance provided by Dr. Mali Bjerkhaug Hartviksen and Werner Nedrebø during their stay at the facility. Thanks also to Malaiamaan Joseph Martin, Dr. Neda Gilannejad and Tharmini Kalananthan for assistance during sampling, and to Sara Karolina Olausson for cortisol, and Karen Anita for glucose, lactate and creatinine analysis at the Institute of Marine Research.

Ethic statement

The research facility at Cargill Innovation Center, Dirdal, Norway has an approval to conduct experiments on Atlantic salmon (VSID 1759). The animal handling and procedures described in this study have been approved by the National Animal Research Authority in Norway (FOTS ID 14984). The participants responsible for the sampling were all accredited by Federation of European Laboratory Animal Science Associations (FELASA).

Availability of data and materials

All data generated or analysed during this study are included in the Table S1.

Author contributions

The study was conceived by AA, ME, ASG, and IR. AA, ME and IR designed the experiment. Experiment and sampling were executed by FL and MRR. FL, VG and MRR performed preparatory lab work and qPCR analysis. BN analysed serum. Schematic illustrations of Atlantic salmon brain and experimental stressors made by MRR. Synteny and phylogeny analysis by ASG. Statistical analysis and related graphs done by FL. All authors contributed to the writing of the manuscript, read, and approved the submitted version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113894.

References

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G., De Saeger, S., Crocker, D.E., 2015. Scales tell a story on the stress history of fish. PLoS ONE 10 (4), e0123411. https://doi.org/10.1371/journal.pone.0123411.

Alderman, S.L., Bernier, N.J., 2009. Ontogeny of the corticotropin-releasing factor system in zebrafish. Gen. Comp. Endocrinol. 164 (1), 61–69. https://doi.org/10.1016/j.ygcen.2009.04.007.

Alderman, S.L., Bernier, N.J., 2007. Localization of corticotropin-releasing factor, urotensin I, and CRF-binding protein gene expression in the brain of the zebrafish, Danio rerio. J. Comp. Neurol. 502 (5), 783–793. https://doi.org/10.1002/cne.21332.

Alsop, D., Vijayan, M., 2009. The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event. Gen. Comp. Endocrinol. 161 (1), 62–66. https://doi.org/10.1016/j.ygcen.2008.09.011.

Ando, H., Hasegawa, M., Ando, J., Urano, A., 1999. Expression of salmon corticotropin-releasing hormone precursor gene in the preoptic nucleus in stressed rainbow trout. Gen. Comp. Endocrinol. 113 (1), 87–95. https://doi.org/10.1006/gcen.1998.7182.

Aruna, A., Nagarajan, G., Chang, C.F., 2012. Involvement of corticotrophin-releasing hormone and corticosteroid receptors in the brain-pituitary-gill of tilapia during the course of seawater acclimation. J. Neuroendocrinol. 24, 818–830. https://doi.org/10.1111/j.1365-2826.2012.02282.x.

Backström, T., Winberg, S., 2013. Central corticotropin releasing factor and social stress. Front. Neurosci. 7, 1–10. https://doi.org/10.3389/fnins.2013.00117.

Balasch, J.C., Tort, L., 2019. Netting the stress responses in fish. Front. Endocrinol. (Lausanne) 10, 1–12. https://doi.org/10.3389/fendo.2019.00062.

Benítez-Dorta, V., Caballero, M.J., Betancor, M.B., Manchado, M., Tort, L., Torrecillas, S., Zamorano, M.J., Izquierdo, M., Montero, D., 2017. Effects of thermal stress on the expression of glucocorticoid receptor complex linked genes in Senegalese sole (*Solea senegalensis*): Acute and adaptive stress responses. Gen. Comp. Endocrinol. 252, 173–185. https://doi.org/10.1016/j.ygcen.2017.06.022.

Bernier, N.J., Craig, P.M., 2005. CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. Am. J. Physiol. – Regul. Integr. Comp. Physiol. 289 (4), R982–R990. https://doi.org/10.1152/ajpregu.00668.2004.

Bernier, N.J., Gorissen, M., Flik, G., 2012. Differential effects of chronic hypoxia and feed restriction on the expression of leptin and its receptor, food intake regulation and the endocrine stress response in common carp. J. Exp. Biol. 215, 2273–2282. https://doi.org/10.1242/jeb.066183.

Cardoso, J.C.R., Bergqvist, C.A., Félix, R.C., Larhammar, D., 2016. Corticotropin-releasing hormone family evolution: Five ancestral genes remain in some lineages. J. Mol. Endocrinol. 57, 73–86. https://doi.org/10.1530/JME-16-0051.

Chandrasekar, G., Lauter, G., Hauptmann, G., 2007. Distribution of corticotropin-releasing hormone in the developing zebrafish. J. Comp. Neurol. 505, 337–351. https://doi.org/10.1002/cne.21496.

Chellappa, S., Huntingford, F.A., Strang, R.H.C., Thomson, R.Y., 1995. Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. J. Fish Biol. 47 (5), 775–787.

Chen, C.-C., Fernald, R.D., 2008. Sequences, expression patterns and regulation of the corticotropin releasing factor system in a teleost. Gen. Comp. Endocrinol. 157 (2), 148–155. https://doi.org/10.1016/j.ygcen.2008.04.003.

Clements, S., Schreck, C.B., 2001. The GABAa agonist muscimol enhances locomotor activity, but does not alter the behavioural effects of CRH in juvenile spring chinook salmon (*Oncorhynchus tshawytscha*). Fish Physiol. Biochem. 24, 41–48. https://doi.org/10.1023/A:1011102420877.

Cuisset, B., Pradelles, P., Kime, D.E., Kühn, E.R., Babin, P., Davail, S., Le Menn, F., 1994. Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as laberl: application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. Comp. Biochem. Physiol. Part C Pharmacol. 108 (2), 229–241. https://doi.org/10.1016/1367-8280(94)90035-3.

David, L., Blum, S., Feldman, M.W., Lavi, U., Hillel, J., 2003. Recent duplication of the common carp (*Cyprinus carpio* L.) Genome as revealed by analyses of microsatellite loci. Mol. Biol. Evol. 20, 1425–1434. https://doi.org/10.1093/molbev/msg173.

Doyon, C., Trudeau, V.L., Moon, T.W., 2005. Stress elevates corticotropin-releasing factor (CRF) and CRF-binding protein mRNA levels in rainbow trout (*Oncorhynchus mykiss*). J. Endocrinol. 186, 123–130. https://doi.org/10.1677/joe.1.06142.

FAO, 2019. Fishery and Aquaculture Statistics 2017/FAO annuaire. Rome., FAO yearbook.

Fast, M.D., Hosoya, S., Johnson, S.C., Afonso, L.O.B., 2008. Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long-term stress. Fish Shellfish Immunol. 24 (2), 194–204. https://doi.org/10.1016/j.fsi.2007.10.009.

Fatsini, E., Rey, S., Ibarra-Zatarain, Z., Boltaña, S., Mackenzie, S., Duncan, N.J., 2020. Linking stress coping styles with brain mRNA abundance of selected transcripts for Senegalese sole (*Solea senegalensis*) juveniles. Physiol. Behav. 213, 112724. https://doi.org/10.1016/j.physbeh.2019.112724.

Faught, E., Aluru, N., Vijayan, M.M., 2016. The Molecular Stress Response, Fish Physiology. Elsevier Inc. https://doi.org/10.1016/B978-0-12-802728-8.00004-7.

Flik, G., Klaren, P.H.M., Van den Burg, E.H., Metz, J.R., Huising, M.O., 2006. CRF and stress in fish. Gen. Comp. Endocrinol. 146 (1), 36–44. https://doi.org/10.1016/j.ygcen.2005.11.005.

Fuzzen, M.L.M., Van Der Kraak, G., Bernier, N.J., 2010. Stirring up new ideas about the regulation of the hypothalamic-pituitary- interrenal axis in zebrafish (*Danio rerio*). Zebrafish 7 (4), 349–358. https://doi.org/10.1089/zeb.2010.0662.

Ghisleni, G., Capiotti, K.M., Da Silva, R.S., Oses, J.P., Piato, Á.L., Soares, V., Bogo, M.R., Bonan, C.D., 2012. The role of CRH in behavioral responses to acute restraint stress in zebrafish. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 36 (1), 176–182. https://doi.org/10.1016/j.pnpbp.2011.08.016.

Glasauer, S.M.K., Neuhauss, S.C.F., 2014. Whole-genome duplication in teleost fishes and its evolutionary consequences. Mol. Genet. Genomics 289 (6), 1045–1060. https://doi.org/10.1007/s00438-014-0889-2.

Grone, B.P., Maruska, K.P., 2015a. A second corticotropin-releasing hormone gene (CRH2) is conserved across vertebrate classes and expressed in the hindbrain of a basal Neopterygian fish, the spotted gar (*Lepisosteus oculatus*). J. Comp. Neurol. 523 (7), 1125–1143. https://doi.org/10.1002/cne.23729.

Grone, B.P., Maruska, K.P., 2015b. Divergent evolution of two corticotropin-releasing hormone (CRH) genes in teleost fishes. Front. Neurosci. 9, 1–13. https://doi.org/10.3389/fnins.2015.00365.

Hall, B.G., 2013. Building phylogenetic trees from molecular data with MEGA. Mol. Biol. Evol. 30 (5), 1229–1235. https://doi.org/10.1093/molbev/mst012.

Hansen, T., Stefansson, S., Taranger, G.L., 1992. Growth and sexual maturation in Atlantic salmon, *Salmo salar* L., reared in sea cages at two different light regimes. Aquac. Fish. Manag. 23, 275–280. https://doi.org/10.1111/j.1365-2109.1992.tb00770.x.

Herman, J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., Cullinan, W.E., 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front. Neuroendocrinol. 24 (3), 151–180. https://doi.org/10.1016/j.yfrne.2003.07.001.

Hostetler, C.M., Ryabinin, A.E., 2013. The crf system and social behavior: a review. Front. Neurosci. 7, 1–15. https://doi.org/10.3389/fnins.2013.00092.

Huising, M.O., Metz, J.R., van Schooten, C., Taverne-Thiele, A.J., Hermsen, T., Verburg-van Kemenade, B.M.L., Flik, G., 2004. Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. J. Mol. Endocrinol. 32, 627–648. https://doi.org/10.1677/jme.0.0320627.

Iversen, A., Asche, F., Hermansen, Ø., Nystøyl, R., 2020. Production cost and competitiveness in major salmon farming countries 2003–2018. Aquaculture 522, 735089. https://doi.org/10.1016/j.aquaculture.2020.735089.

Iversen, M., Finstad, B., Nilssen, K.J., 1998. Recovery from loading and transport stress in Atlantic salmon (*Salmo salar* L.) smolts. Aquaculture 168 (1-4), 387–394. https://doi.org/10.1016/S0044-8486(98)00364-0.

Kashani, K., Rosner, M.H., Ostermann, M., 2020. Creatinine: from physiology to clinical application. Eur. J. Intern. Med. 72, 9–14. https://doi.org/10.1016/j.ejim.2019.10.025.

Kieffer, J.D., 2000. Limits to exhaustive exercise in fish. Comp. Biochem. Physiol. – A Mol. Integr. Physiol. 126 (2), 161–179. https://doi.org/10.1016/S1095-6433(00)00202-6.

Kiilerich, P., Servili, A., Péron, S., Valotaire, C., Goardon, L., Leguen, I., Prunet, P., 2018. Regulation of the corticosteroid signalling system in rainbow trout HPI axis during confinement stress. Gen. Comp. Endocrinol. 258, 184–193. https://doi.org/10.1016/j.ygcen.2017.08.013.

Kristiansen, T.S., Fernö, A., Pavlidis, M., Vis, H. van de, 2020. The welfare of fish, Diseases of Aquatic Organisms.

Larhammar, D., Risinger, C., 1994. Molecular genetic aspects of tetraploidy in the Common carp *Cyprinus carpio*. Mol. Phylogenet. Evol. 3, 59–68. https://doi.org/10.1006/mpev.1994.1007.

Lederis, K., Letter, A., McMaster, D., Moore, G., Schlesinger, D., 1982. Complete amino acid sequence of urotensin I, a hypotensive and corticotropin-releasing neuropeptide from Catostomus. Science (80-. 218 (4568), 162–165. https://doi.org/10.1126/science.6981844.

Lien, S., Koop, B.F., Sandve, S.R., Miller, J.R., Kent, M.P., Nome, T., Hvidsten, T.R., Leong, J.S., Minkley, D.R., Zimin, A., Grammes, F., Grove, H., Gjuvsland, A., Walenz, B., Hermansen, R.A., von Schalburg, K., Rondeau, E.B., Di Genova, A., Samy, J.K.A., Olav Vik, J., Vigeland, M.D., Caler, L., Grimholt, U., Jentoft, S., Inge Våge, D., de Jong, P., Moen, T., Baranski, M., Palti, Y., Smith, D.R., Yorke, J.A., Nederbragt, A.J., Tooming-Klunderud, A., Jakobsen, K.S., Jiang, X., Fan, D., Hu, Y., Liberles, D.A., Vidal, R., Iturra, P., Jones, S.J.M., Jonassen, I., Maass, A., Omholt, S. W., Davidson, W.S., 2016. The Atlantic salmon genome provides insights into rediploidization. Nature 533 (7602), 200–205. https://doi.org/10.1038/nature17164.

López-Olmeda, J.F., Blanco-Vives, B., Pujante, I.M., Wunderink, Y.S., Mancera, J.M., Sánchez-Vázquez, F.J., 2013. Daily rhythms in the hypothalamus-pituitary-interrenal axis and acute stress responses in a teleost flatfish, *Solea senegalensis*. Chronobiol. Int. 30 (4), 530–539. https://doi.org/10.3109/07420528.2012.754448.

Lovejoy, D.A., Balment, R.J., 1999. Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. Gen. Comp. Endocrinol. 115 (1), 1–22. https://doi.org/10.1006/gcen.1999.7298.

Lovejoy, D.A., Chang, B.S.W., Lovejoy, N.R., del Castillo, J., 2014. Molecular evolution of GPCRs: CRH/CRH receptors. J. Mol. Endocrinol. 52, T43–T60. https://doi.org/10.1530/JME-13-0238.

Macqueen, D.J., Johnston, I.A., 2014. A well-constrained estimate for the timing of the salmonid whole genome duplication reveals major decoupling from species diversification. Proc. R. Soc. B Biol. Sci. 281 (1778), 20132881. https://doi.org/10.1098/rspb.2013.2881.

Madaro, A., Fernö, A., Kristiansen, T.S., Olsen, R.E., Gorissen, M., Flik, G., Nilsson, J., 2016a. Effect of predictability on the stress response to chasing in Atlantic salmon (Salmo salar L.) parr. Physiol. Behav. 153, 1–6. https://doi.org/10.1016/j.physbeh.2015.10.002.

Madaro, A., Olsen, R.E., Kristiansen, T.S., Ebbesson, L.O.E., Flik, G., Gorissen, M., 2016b. A comparative study of the response to repeated chasing stress in Atlantic salmon (*Salmo salar* L.) parr and post-smolts. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 192, 7–16. https://doi.org/10.1016/j.cbpa.2015.11.005.

Madaro, A., Olsen, R.E., Kristiansen, T.S., Ebbesson, L.O.E., Nilsen, T.O., Flik, G., Gorissen, M., 2015. Stress in Atlantic salmon: response to unpredictable chronic stress. J. Exp. Biol. 218, 2538–2550. https://doi.org/10.1242/jeb.120535.

Martinez-Porchas, M., Martinez-Cordova, L.T., Ramos-Enriquez, R., 2009. Cortisol and Glucose: reliable indicators of fish stress? Panamjas. J. Aquat. Sci. 4, 158–178.

Matz, S.P., Hofeldt, G.T., 1999. Immunohistochemical localization of corticotropin-releasing factor in the brain and corticotropin-releasing factor and thyrotropin-stimulating hormone in the pituitary of chinook salmon (*Oncorhynchus tshawytscha*). Gen. Compar 114 (1), 151–160. https://doi.org/10.1006/gcen.1999.7253.

Nicholas, K.B., Nicholas, H.B.J., Deerfield, D.W., 1997. GeneDoc : analysis and visualization of genetic variation. EMBNEW. NEWS 4, 14.

Okawara, Y., Ko, D., Morley, S.D., Richter, D., Lederis, K.P., 1992. In situ hybridization of corticotropin-releasing factor-encoding messenger RNA in the hypothalamus of the white sucker, *Catostomus commersoni*. Cell Tissue Res. 267 (3), 545–549.

Olivereau, M., Moons, L., Olivereau, J., Vandesande, F., 1988. Coexistence of corticotropin-releasing factor-like immunoreactivity and vasotocin in perikarya of the preoptic nucleus in the eel. Gen. Comp. Endocrinol. 70 (1), 41–48. https://doi.org/10.1016/0016-6480(88)90092-5.

Olivereau, M., Olivereau, J., 1987. Localization of CRF-like immunoreactivity in the brain and pituitary of teleost fish. Peptides 9 (1), 13–21. https://doi.org/10.1016/0196-9781(88)90004-6.

Olivereau, M., Ollevier, F., Vandesande, F., Verdonck, W., 1984. Immunocytochemical identification of CRF-like and SRIF-like peptides in the brain and the pituitary of cyprinid fish. Cell Tissue Res. 237 (2), 379–382. https://doi.org/10.1007/BF00217162.

Pankhurst, N.W., Carragher, J.F., 1992. Oocyte maturation and changes in plasma steroid levels in snapper Pagrus (=*Chrysophrys*) *auratus* (Sparidae) following treatment with human chorionic gonadotropin. Aquaculture 101 (3-4), 337–347. https://doi.org/10.1016/0044-8486(92)90036-K.

Pankhurst, N.W., Ludke, S.L., King, H.R., Peter, R.E., 2008. The relationship between acute stress, food intake, endocrine status and life history stage in juvenile farmed Atlantic salmon, Salmo salar. Aquaculture 275 (1-4), 311–318. https://doi.org/10.1016/j.aquaculture.2008.01.001.

Pepels, P.P.L.M., Meek, J., Wendelaar Bonga, S.E., Balm, P.H.M., 2002. Distribution and quantification of corticotropin-releasing hormone (CRH) in the brain of the teleost fish *Oreochromis mossambicus* (tilapia). J. Comp. Neurol. 453 (3), 247–268. https://doi.org/10.1002/(ISSN)1096-986110.1002/cne.v453:310.1002/cne.10377.

Remen, M., Oppedal, F., Imsland, A.K., Olsen, R.E., Torgersen, T., 2013. Hypoxia tolerance thresholds for post-smolt Atlantic salmon: dependency of temperature and hypoxia acclimation. Aquaculture 416–417, 41–47. https://doi.org/10.1016/j.aquaculture.2013.08.024.

Rivest, S., Rivier, C., 1995. The role of corticotropin-releasing factor and interleukin-1 in the regulation of neurons controlling reproductive functions. Endocr. Rev. 16 (2), 177–199. https://doi.org/10.1210/edrv-16-2-177.

Rivier, C., Plotsky, P.M., 1986. Mediation by corticotropin releasing factor (CRF) of adenohypophysial hormone secretion. Annu. Rev. Physiol. 48, 475–494. https://doi.org/10.1146/annurev.physiol.48.1.475.

Rondeau, E.B., Minkley, D.R., Leong, J.S., Messmer, A.M., Jantzen, J.R., von Schalburg, K.R., Lemon, C., Bird, N.H., Koop, B.F., Yin, T., 2014. The genome and linkage map of the northern pike (*Esox lucius*): Conserved synteny revealed between the salmonid sister group and the neoteleostei. PLoS ONE 9 (7), e102089. https://doi.org/10.1371/journal.pone.0102089.

Rønnestad, I., Gomes, A.S., Murashita, K., Angotzi, R., Jönsson, E., Volkoff, H., 2017. Appetite-controlling endocrine systems in teleosts. Front. Endocrinol. (Lausanne) 8, 1–24. https://doi.org/10.3389/fendo.2017.00073.

Shrimpton, J.M., Randall, D.J., 1994. Downregulation of corticosteroid receptors in gills of Coho salmon due to stress and cortisol treatment. Am. J. Physiol. 267 (Pt 2), R432–R438. https://doi.org/10.1152/ajpregu.1994.267.2.R432.

Skjervold, P.O., Fjæra, S.O., Østby, P.B., Einen, O., 2001. Live chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). Aquaculture 192 (2-4), 265–280.

Sloman, K.A., Taylor, A.C., Metcalfe, N.B., Gilmour, K.M., 2001. Effects of an environmental perturbation on the social behaviour and physiological function of brown trout. Anim. Behav. 61 (2), 325–333. https://doi.org/10.1006/anbe.2000.1567.

Vindas, M.A., Gorissen, M., Höglund, E., Flik, G., Tronci, V., Damsgård, B., Thörnqvist, P. O., Nilsen, T.O., Winberg, S., Øverli, Ø., Ebbesson, L.O.E., 2017. How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. J. Exp. Biol. 220, 1524–1532. https://doi.org/10.1242/jeb.153213.

Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Bernier, N.J., Kelly, S.P., Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. Comp. Endocrinol. 142 (1-2), 3–19. https://doi.org/10.1016/j.ygcen.2004.11.001.

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77 (3), 591–625. https://doi.org/10.1152/physrev.1997.77.3.591.

Wunderink, Y.S., Engels, S., Halm, S., Yúfera, M., Martínez-Rodríguez, G., Flik, G., Klaren, P.H.M., Mancera, J.M., 2011. Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): The involvement of cortisol, CRH and CRH-BP. Gen. Comp. Endocrinol. 171 (2), 203–210. https://doi.org/10.1016/j.ygcen.2011.01.010.

Wunderink, Y.S., Martínez-Rodríguez, G., Yúfera, M., Martín Montero, I., Flik, G., Mancera, J.M., Klaren, P.H.M., 2012. Food deprivation induces chronic stress and affects thyroid hormone metabolism in Senegalese sole (*Solea senegalensis*) post-larvae. Comp. Biochem. Physiol. 162 (4), 317–322. https://doi.org/10.1016/j.cbpa.2012.03.023.

Yates, A.D., Achuthan, P., Akanni, W., Allen, James, Allen, Jamie, Alvarez-Jarreta, J., Amode, M.R., Armean, I.M., Azov, A.G., Bennett, R., Bhai, J., Billis, K., Boddu, S., Marugán, J.C., Cummins, C., Davidson, C., Dodiya, K., Fatima, R., Gall, A., Giron, C. G., Gil, L., Grego, T., Haggerty, L., Haskell, E., Hourlier, T., Izuogu, O.G., Janacek, S. H., Juettemann, T., Kay, M., Lavidas, I., Le, T., Lemos, D., Martinez, J.G., Maurel, T., McDowall, M., McMahon, A., Mohanan, S., Moore, B., Nuhn, M., Oheh, D.N., Parker, A., Parton, A., Patricio, M., Sakthivel, M.P., Abdul Salam, A.I., Schmitt, B.M., Schuilenburg, H., Sheppard, D., Sycheva, M., Szuba, M., Taylor, K., Thormann, A., Threadgold, G., Vullo, A., Walts, B., Winterbottom, A., Zadissa, A., Chakiachvili, M., Flint, B., Frankish, A., Hunt, S.E., IIsley, G., Kostadima, M., Langridge, N., Loveland, J.E., Martin, F.J., Morales, J., Mudge, J.M., Muffato, M., Perry, E., Ruffier, M., Trevanion, S.J., Cunningham, F., Howe, K.L., Zerbino, D.R., Flicek, P., 2020. Ensembl 2020. Nucleic Acids Res. 48, D682–D688. https://doi.org/10.1093/nar/gkz966.

Yulis, C.R., Lederis, K., 1987. Co-localization of the immunoreactivities of corticotropin-releasing factor and arginine vasotocin in the brain and pituitary system of the teleost *Catostomus commersoni*. Cell Tissue Res. 247, 267–273. https://doi.org/10.1007/BF00218308.

Yulis, C.R., Lederis, K., Wong, K.-L., Fisher, A.W.F., 1986. Localization of urotensin I- and corticotropin-releasing factor-like immunoreactivity in the central nervous system of *Catostomus commersoni*. Peptides 7 (1), 79–86. https://doi.org/10.1016/0196-9781(86)90065-3.

Zhou, J.N., Fang, H., 2018. Transcriptional regulation of corticotropin-releasing hormone gene in stress response. IBRO Rep. 5, 137–146. https://doi.org/10.1016/j.ibror.2018.08.003.

Zupanc, G.K.H., Horschke, I., Lovejoy, D.A., 1999. Corticotropin releasing factor in the brain of the gymnotiform fish, *Apteronotus leptorhynchus*: immunohistochemical studies combined with neuronal tract tracing. Gen. Comp. Endocrinol. 114 (3), 349–364. https://doi.org/10.1006/gcen.1999.7273.