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Effect of amino acid supplementation and stress on expression of molecular markers in meagre (*Argyrosomus regius*)

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ABSTRACT

The objectives of this work were: 1) develop of molecular stress biomarkers obtaining sequence data of different transcripts, 2) study the molecular stress response through the expression quantification of key gene involved in it, and 3) assess the effects of dietary amino acid additives on stress response in meagre meagres (*Argyrosomus regius*). Fish batches were fed two experimental diets with tryptophan (Trp) or aspartate (Asp) added for seven days. Before sampling fish were submitted to confinement/netting stress during 1 h, except control fish. Therefore fish were sampled before and after stress (1 h and 6 h post-stress). The sampling consisted of blood and tissues (brain, hypophysis and liver). Several gene expressions related to the stress response were measured in those tissues, and the cloning of corticotropin-releasing hormone (*crh*), corticotropin-releasing hormone binding protein (*crh-bp*), and thyrotropin-releasing hormone (*trh*) has been reported in meagre for the first time. In fact, fish fed an additional Asp diet did not present any *sl, prl* and *gh* expression changes, as for the control group. Contrarily, the Trp diet altered the *prl* and *gh* expressions after stress. For *crh* and *crh-bp* expressions, no significant differences were detected within the Asp diet hence that amino acid improved the stress response. However, Asp feeding, but not Trp, enhanced *pomc-a* expression after stress. Hsp70 expression varied for every treatment, including the control feeding, indicating a late response at 6 h post-stress sampling, where both Asp and Trp treatments increased these expressions significantly. Concluding, the response of molecular stress markers to amino acid enriched diets was diverse. The stressor did not change significantly the relative expression of most analyzed genes for control feeding groups, though the Asp supplemented diet was more effective for attenuating molecular markers than the Trp one.

1. Introduction

The study of fish stress is progressively increasing because of the interest on the maintenance of welfare of captive fish (Schreck and Tort, 2016). Due to its multifactorial nature, it can be assessed from different perspectives. For this reason, stress markers can derive from endocrinological, metabolic, immunological, and behavioral variables, and many others. In this sense, the use of molecular tools to detect changes in the stress-related gene or protein expression is of special interest (Faught et al., 2016).

The physiological stress is usually quantified by means of cortisol concentration since this hormone is released as primary response to stressors, and it is considered the classical stress indicator (Mommsen et al., 1999). Nonetheless, increases, decreases and no changes in this hormone have been described for teleosts fish under different stress situations (Herrera et al., 2009; Sammouth et al., 2009; Santos et al., 2010; Sneddon et al., 2016). This variability has driven the need to develop molecular tools for a better characterization of the stress response, namely the expression of stress-related enzymes or hormone precursors; polypeptides such as corticotropin-releasing hormone (CRH), corticotropin-releasing hormone binding protein (CRH-BP), thyrotropin-releasing hormone (TRH), proopiomelanocortin-derived

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hormones (POMCs) – i.e. adrenocorticotropic hormone (ACTH) originated from POMC A and α-melanocyte stimulation hormone (αMSH) from POMC B, somatolactin (SL), prolactin (PRL), and growth hormone (GH) (Martos-Sitcha et al., 2014; Gorissen and Flik, 2016; Pês et al., 2016; Ruiz-Jarabo et al., 2018; Vargas-Chacoff et al., 2009).

In teleost fish, the stress response is based on the activation of the hypothalamus-pituitary-interrenal (HPI) axis. Briefly, hypothalamic CRH and/or TRH stimulates secretion of ACTH and α-MSH at adenohypophyseal level. ACTH and α-MSH stimulate the production and release of cortisol into the blood stream (Gorissen and Flik, 2016). Similarly to cortisol, plasma SL has been reported to be a stress marker in several fish species (Rand-Weaver et al., 1993; Kakizawa et al., 1996). This adenohypophyseal hormone, along with GH and PRL, play a key role in the homeostasis recovery after stress (Kaneko, 1996). Besides other functions and the hypothetic interaction between stress and thyroid axis, TRH controls the release of PRL and GH (Kagabu et al., 1998; de Groef et al., 2006; Gorissen and Flik, 2016;).

The Heat Shock Proteins (Hsps) have been traditionally linked to the molecular responses to some stressors beyond the temperature variations (Iwama et al., 1998a, 1998b; Basu et al., 2002, Cara et al., 2005; Antonopoulou et al., 2013; Araújo et al., 2020). These proteins are expressed in cells under normal conditions and rapidly increased in response to stress, due to their functions of maintaining cellular homeostasis and protein metabolism (Iwama et al., 1998b, Yamashita et al., 2010). Hsp70 (68-73 kDa) is the family of Hsps most extensively studied in fish known to mediate the repair and degradation of altered or denaturated proteins. Several studies showed that the expression of Hsp70 is altered by the nutritional state of fish (Antonopoulou et al., 2013; Cara et al., 2005;(Feidantsis et al., 2014). In this sense, Hsp70 can be considered an effective biomarker to access nutritional stress in fish.

The meagre (*Argyrosomus regius*) is a commercial fish species, especially in the European Mediterranean countries. As the production of this species is increasing, the study of methods to reduce stress and improve animal welfare are being researched. Recently, it has been demonstrated that amino acid-enriched diets could modulate the stress responses in meagre (Fernández-Alacid et al., 2019), and also enhance immune response in this species (González et al., 2018). The beneficial effects of amino acid supplements are based on specific amino acid functions such as neutrotransmitter or hormone precursors and energy substrates (Yao et al., 2011; Costas et al., 2013; Andersen et al., 2016; Herrera et al., 2019a). For instance, tryptophan (Trp) and phenylalanine (Phe) are precursors of serotonin and catecholamines, hormones involved in the stress response (Herrera et al., 2017). Although the Phe has been scarcely essayed in animals, the effects of Trp additives on fish stress response have been studied in several fish species, reporting that supplements of 0.2–2% (on dry food) can attenuate the stress responses (see review in Herrera et al., 2019a). Therefore it would be probable that 1% Trp supplements in diet could affect the physiological response. Aspartate (Asp) supplements have been scarcely assayed in fish though it is known that has sedative effects in superior animals (Erwan et al., 2014). In fact, only Fernández-Alacid et al. (2019) have reported that Asp additives could modulate the stress response in meagre depending on the stressor type. According to Fernández-Alacid et al. (2019) and Asencio-Alcudia et al. (2019), dietary 1% Asp supplements affected the meagre stress and immune response, although those authors chose that concentration based in other amino acids (i.e. Trp) supplements since no work had been previously published in fish.

However, the effects of amino acid supplemented diets on the molecular stress response have not been reported in meagre yet. In fact, several genes such as *crh, crh-bp*, *pomc a,* and *pomc b* still have not been cloned in this species. Therefore, the objectives of this work were: 1) develop of molecular stress biomarkers obtaining sequence data of Crh-Bp, TRH and CRH transcripts for meagre; 2) study the molecular stress response through the expression quantification of key gene involved in it, and 3) assess the effects of dietary amino acid (Asp and Trp) additives on stress response in meagre.

Table 1
Amino acid composition of the experimental feeds (mg g^{-1} total feed mass; mean ± SE; $N = 3$).

Amino acid	Control		Trp		Asp	
Asparagine	0.24	±0.01	0.25	±0.01	0.24	±0.01
Aspartic acid	27.6	±0.98	26.2	±0.14	50.5	±3.98
Cysteine	0.05	±0.01	0.04	±0.0	0.04	±0.01
Glutamic acid	19.8	±2.34	19.8	±2.35	19.5	±4
Glutamine	0.14	±0.01	0.15	±0.01	0.13	±0.02
Histidine	3.42	±0.16	3.42	±0.16	3.42	±0.19
Isoleucine	6.07	±0.86	5.97	±1.11	5.52	±0.82
Leucine	8.53	±0.32	8.8	±0.25	8.52	±0.58
Lysine	6.42	±0.93	6.85	±1.28	6.76	±0.24
Methionine	1.25	±0.04	1.27	±0.05	1.16	±0.05
Phenylalanine	6.82	±1.08	6.73	±1.14	6.37	±1.76
Proline	9.96	±0.6	10	±0.85	9.82	±1.26
Serine	13.4	±2.11	13.3	±2.1	13.4	±2.58
Threonine	2.28	±0.22	2.13	±0.69	2.16	±0.66
Tryptophan	1.01	±0.1	1.61	±0.02	1.1	±0.1
Tyrosine	1.54	±0.18	1.53	±0.21	1.71	±0.04
Valine	6.57	±0.86	6.39	±1.21	6.2	±1.67

2. Material and methods

2.1. Food making and analysis

Commercial fish feed (Skretting, Burgos, Spain) was used as the control diet. L-tryptophan (Trp) and L-aspartate (Asp) (dry powder) were purchased from Sigma-Aldrich (St. Louis, USA). The commercial control diet was finely ground and then mixed with water (400 mL kg^{-1} dry feed) and Trp or Asp supplementations. The amount of amino acid added was 1% (dry weight) according to Fernández-Alacid et al. (2019). The mixture was thread pelleted into 2 mm diameter and 20–25 cm length strips. These food strips were dried at 60 °C for 24 h. Finally, these were cut to get 2–3 mm size pellets, which were stored at 4 °C.

All experimental diets were later analyzed through gas chromatography–mass spectrometry (GC–MS) to determine the final amino acid content. Feed homogenization was done through basic hydrolysis as reported in Dai et al. (2014). For derivatization, an aliquot (100 μL) of standard solution or sample was placed in a 2-mL vial, adding 400 μL of a water/ethanol/pyridine (60:32:8) mixture and 40 μL of ethyl chloroformate. It was capped and vigorously shaken a vortex mixer for 30 s at room temperature. Then, 200 μL of chloroform (containing 1% ECF) was added and the derivatives were extracted into the organic phase by striking the tube against a pad for about 30 s. The organic phase was dried with anhydrous sodium sulfate. The organic layer was transferred into a new vial with a 300-μL fixed insert. Aliquots (1 μL) of the derived extracts were injected into a Shimadzu GC–MS (GCMS-TQ8030) equipped with an Agilent HP-5MS fused silica capillary column (30 m × 0.25-mm i.d., 0.25-mm film thickness). The gas chromatograph system was equipped with a split/splitless injection port operating in splitless mode. The oven temperature was programmed from 40 °C (5 min) to 270 °C (20 min) by increasing the temperature at 5 °C min^{-1}. The transfer line was heated at 280 °C. The carrier gas was helium with a constant flow of 1 mL min^{-1} (mean velocity 36 cm s^{-1}). The mass spectrometer was performed with electron ionization (EI) at 70 eV, operating in scan mode (75–500 amu). Identification of derived amino acids was achieved comparing the gas chromatograph retention times and mass spectra with those of the pure standard compounds. All mass spectra were also compared with the data system library (NIST 11). Quantification of samples was conducted by the external standard method following the same procedure as that for samples. Amino acid compositions of the experimental feeds are shown in Table 1.

2.2. Fish stock, experimental design and sampling

Meagre (*Argyrosomus regius*) juveniles were obtained at EPPO – IPMA (Aquaculture Research Station, Portuguese Institute for the Ocean and

Table 2
Sequences of primers used for cloning of the partial CDS of *Argyrosomus regius crh-bp*, *trh*, and *crh*.

Gene	Sequence	Primers	
crh-bp	*Sparus aurata* (acc. KC195965)	Sense RTD ATG GAG CGC ACB TTC C	Ext.
	Paralichthys olivaceus (acc. XM_020081828)	Antisense GTT GAG TTT GAT SGT CTG YAR CTC	
	Seriola dumerili (acc. XM_022769490)	Sense TGG AGGGYC ART TCA CCT	Int.
	Lates calcarifer (acc. XM_018694301)	Antisense TGT TDT CRC AGC CRA TCT TC	
trh	*Larimichthys crocea* (acc. XM_010755325)	Sense GAY YTG GAG AAG CGG CAG	Ext.
	Sparus aurata (acc. KC196277)	Antisense CCY GGG TGT TGY CKC TTC T	
	Seriola dumerili (acc. XM_022739244)	Sense TKC AAA RRA GAC AGC ACC C	Int.
	Paralichthys olivaceus (acc. XM_020096263)	Antisense AAG TYG AGC AGC ARR CTG GT	
crh-bp	*Haplochromis burtoni* (acc. NM_001287404)	Sense GCT ACG AAT GTC GGG CTA TTG	Ext.
	Sparus aurata (acc KC195964)	Antisense CGA AGA GCT CCA TCA TTC TTC	
	Maylandia zebra (acc. XM_004546653)	Sense GGG GAC TCT AAC TCT TTC CC	Int.
	Paralichthys olivaceus (acc. KX345138)	Antisense GGA CAT CTC CAT CAT CTCCC	

Atmosphere, Olhão, Portugal) originated from an F1 broodstock. After hatching, fish were reared following the protocol used in EPPO-IPMA for this fish species. Meagre juveniles were transported to IFAPA Centro Agua del Pino (Cartaya, Spain) and kept in recirculating seawater system (600 L), where the experimental study was carried out. The water temperature was maintained at 19 ± 1 °C with a flow rate of 2 m^3 h^{-1} and 33 psu salinity. The photoperiod was natural (12 L:12D) and fish were fed a commercial pellet diet (Skretting, Spain) at a rate of 1% body weight day^{-1} by 24 h belt-feeders. Fish were allowed to acclimatize for 21 days before the start of the experimental trial.

After the acclimatization period, fish ($n = 150$; 105 ± 2.6 g; mean ± SE) were distributed in 6 tanks (25 fish/tank; 4.4 kg m^{-3}) and the experimental design was as follows: fish from Control group received the unsupplementated commercial diet, the other two groups were fed the commercial diet supplemented with aspartate (Asp group) or with tryptophan (Trp group), respectively. The treatments were applied in duplicate. Fish were fed these diets for 7 days prior the stress trials, at a rate of 1% body weight day^{-1}, since the effects of amino acid supplementation on physiological responses are detectable in 7 days or less (Höglund et al., 2005; Tejpal et al., 2009; Hoseini et al., 2012; Basic et al., 2013a, 2013b; Herrera et al., 2017, 2019b). Afterwards, for each one of the three feeding groups of fish, 10 fish (5 from each replicated tank) were firstly sampled as control (non-stressed), and remaining fish were subjected to stress by confinement and netting, which was obtained by decreasing the water level (20 cm; 15 kg m^{-3}) and fish being chased with a net (without exposing them to the air) for 3 min. This last process was repeated every 10 min for one hour.

At sampling, fish ($n = 10$, 5 from each replicated tank) were sacrificed through anesthetics overdose (immersion in >1 mL L^{-1} 2-phenoxyethanol). Blood samples were collected at baseline time (time-zero or non-stressed) as well as 1 and 6 h post-stress (1stress and 6stress), according to Samaras et al. (2016). Samples were obtained from the caudal vein with an insulin syringe and were centrifuged (3600 rpm, 10 min, 4 °C) for obtaining plasma which was stored at −80 °C until analysis. The time between fish collection and blood extraction was lower than 3 min. Brain and hypophysis samples were excised and placed at 4 °C for 24 h in RNAlater® (Invitrogen, Waltham, USA) and then frozen at −80 °C until analysis. A small portion of liver from each individual was dissected, frozen in liquid nitrogen and stored at −80 °C for later use.

The experiment complied with the Guidelines of the European Union Council (2010/63/EU) and the Spanish Government (RD1201/2005; RD53/2013 and law 32/2007) for the use of laboratory animals. All experimental protocols were approved by the Ethical Committee of the IFAPA (Andalusian Institute of Researching and Training on Fisheries and Agriculture), sited in Seville (Spain).

Table 3
PCR steps used for cloning of CRH, CRH-BP and TRH partial CDS.

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	30 s	1
Denaturation	95 °C	15 s	35
Annealing	54 °C (*crh* and *crh-bp*) 56 °C (*trh*)	30 s	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1

2.3. Cloning of crh-bp, trh, and crh partial cDNAs

Gene specific *crh-bp*, *trh*, and *crh* primers were designed from conserved regions of the same genes for other teleost found in NCBI Nucleotide Database (https://www.ncbi.nlm.nih.gov) The sequences used are listed in Table 2. Considering that, it was not possible to design primer pairs with a 100% of consensus among all sequences compared, degeneracies were included. Sequences of degenerate and conserved cloning primers are also shown in Table 2. All primers used were purified by desalting and purchased from IDT (Integrated DNA Technologies, Belgium). All kits were used according to manufacturers' instructions.

Total RNA was extracted with the NucleoSpin® RNA kit, using ~40 mg of brain sample. Every sample was homogenized by an IKA® Ultra-Turrax® T25 with the dispersing tool S25N-8G (IKA-Werke), and including the on-column DNA digestion using the RNase free DNase provided with the kit. RNA quality was checked in the Bioanalyzer 2100 system (Agilent Technologies, Life Sciences), using Agilent RNA 6000 Nano kit for hypothalamus RNA. RNA quantity was measured spectrophotometrically at 260 nm with a Nanodrop ONE (Thermo Scientific). cDNA synthesis proceeded after assuring RNA integrity number (RIN) > 8 and abundant quantity of RNA (> 200 ng μL^{-1}).

cDNA was synthesized from each RNA sample by the SuperScript™ III Reverse Transcriptase (Invitrogen, LifeTechnologies), using ~1 μg of total RNA and Oligo(dT)20 as reverse primer. Finally, 40 ng of cDNA obtained from each tissue was used in a specific PCR reaction (NZY taq II mastermix) applied for the amplification of the target genes in a total volume of 25 μL. The PCRs were accomplished in a Mastercycler® personal (Eppendorf). Different PCR programs were used for gene's amplification (Table 3). Purified PCR products were directly cloned into the p-JET 1.2 cloning vector (CloneJET PCR Cloning Kit, Thermo Scientific) and sequenced in Stab Vida (Portugal). For all putative clones, forward and reverse sequencing was carried out using the dideoxynucleotide chain-termination method with pJET1.2 Forward Sequencing and pJET1.2 Reverse Sequencing as universal primers.

2.4. Total RNA extraction and quantitative reverse transcription polymerase chain reaction (QPCR)

Total RNA from brain was extracted using the NucleoSpin® RNA kit (Macherey-Nagel), whereas total RNA from pituitary was extracted using the NucleoSpin® RNA XS kit (Macherey-Nagel), following the manufacturer's protocol (for all kits in this study only originally supplied components and protocols were used). RNA concentration and quality was assessed using the Nanodrop ONE (Thermo Scientific) and Bioanalyzer 2100 system (Agilent Technologies, Life Sciences). All samples that had RNA integrity number (RIN) values higher than 8. 500 ng (brain) or 50 ng (pituitary) of total RNA were used for cDNA synthesis using qScriptTM cDNA Synthesis Kit (Quanta BioSciences). Generated

Table 4
Sequences of primers used in qPCR amplification for each gene. Efficiency parameters (R^2 and efficiency are showed beside each primer pair).

Gene	Primer	Sequence	Efficiency parameters
sl	arSL-QPCR_fwd1	TGACAAATGGTTGCTCCACT	$R^2 = 0.999$; E = 99.9%
	arSL-QPCR_Rev1	GCCATCGTAGTGATCCATTG	
prl	arPRL-QPCR_fwd3	TGGTTTGACCCCCTGGAA	$R^2 = 0.996$; E = 96%
	arPRL-QPCR_Rev3	GAGTGCTCCTGCAGCTCCTT	
gh	ar-GH-QPCR-Fw	GGAGTTCCCCAGTCGTTCTC	$R^2 = 0.998$; E = 98.1%
	ar-GH-QPCR-Rev	CCCTGATCAGCAGCAGGAT	
crh	arCRH-QPCR_fwd2	AACAGCAGTCTGGTCCCATC	$R^2 = 0.994$; E = 102.3
	arCRH-QPCR_Rev2	CTTATGAGCGCCCTGATGTT	
crh-bp	arCRH-*bp*-QPCR_fwd1	GGCAGGGGACTTCATCAC	$R^2 = 0.998$; E = 101.1%
	arCRH-*bp*-QPCR_Rev1	CATATCGCTCGTACAGAGGCA	
trh	arTRH-QPCR_fwd1	CATGGAGCTCCAGAAAAGGC	$R^2 = 0.995$; E = 104%
	arTRH-QPCR_Rev1	CCGGGTGCTGTCGTTTTG	
pomc-a	arPOMCA-QPCR_fwd1	CCAGCAACATCATAGCGACA	$R^2 = 0.998$; E = 100.7%
	arPOMCA-QPCR_Rev1	GTGCCGTCCTTCTTCTCGT	
pomc-b	arPOMCB-QPCR_fwd2	CAGCGGAAGTGCTCCATG	$R^2 = 0.999$; E = 100.2%
	arPOMCB-QPCR_Rev2	CTCTGTCGCTCCGACCTC	
β-act	arACT-QPCR_fwd1	ATCCACCATGAAGATCAAGA	$R^2 = 0.999$; E = 99.9%
	arACT-QPCR_Rev1	GCTGGAAGGTGGACAGAGAG	

cDNAs were stored at −20 °C for less than one month.

The QPCR primers were designed using the software primer3 (http://frodo.wi.mit.edu/primer3/) based on the cDNA sequences cloned above for *crh-bp, trh*, and *crh*. For *gh, prl, sl, pomc a* and *pomc b*, QPCR primer design cDNA sequences published in GenBank were used (http://www.ncbi.nlm.nih.gov/nuccore). Specifically from *Argyrosomus regius*, the next sequences had been already cloned in our laboratory: GH (acc. no.: KM402037); PRL (acc. no.: KP984534); SL (acc. no.: KX458064); POMC-A (acc. no.: MF565481); POMC-B (acc. no.: MF565482) and β-ACTIN (acc. no.: KM402038) (Mohammed-Geba et al., 2017). The QPCR primers were desalted and purchased from IDT; the designed primers are detailed in Table 4. All QPCR steps were performed using qScript cDNA synthesis kit and PerfeCTaTM SYBR®Green FastMixTM (Quanta BioSciences).

To optimize the QPCR conditions several primer concentrations (100 nM, 200 nM and 400 nM) and temperature gradients (from 50 to 60 °C) were used. Different pituitary glands (*gh, prl, sl, pomc a* and *pomc b*) and hypothalamus (*trh, crh-bp* and *crh*) cDNAs templates concentrations were assayed in triplicate (10 ng μL^{-1}, 1 ng μL^{-1}, 100 pg μL^{-1} and 10 pg μL^{-1} of input RNA) to check the assay linearity and the amplification efficiency. The assay was linear between 10 ng μL^{-1} and 10 pg μL^{-1} of cDNA per reaction (amplification efficiencies and regression coefficients are shown in Table 4).

Relative gene quantification was performed using the ΔΔCT method (Livak and Schmittgen, 2001). QPCR reactions (10 μL), composed of 4 μL cDNA template (final in-well concentration of 10 and 1 ng of cDNA for hypothalamus and pituitary gland samples, respectively). 5 μL PerfeCTaTM SYBR®Green FastMix (2× concentrated, Life Technologies), and 0.5 μL from each primer were analyzed with the Bio-Rad CFX96 touch Real Time PCR (Bio-Rad) and Bio-Rad CFX96 Manager 3.1 control software (Bio-Rad). Reactions, run in triplicate, were incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

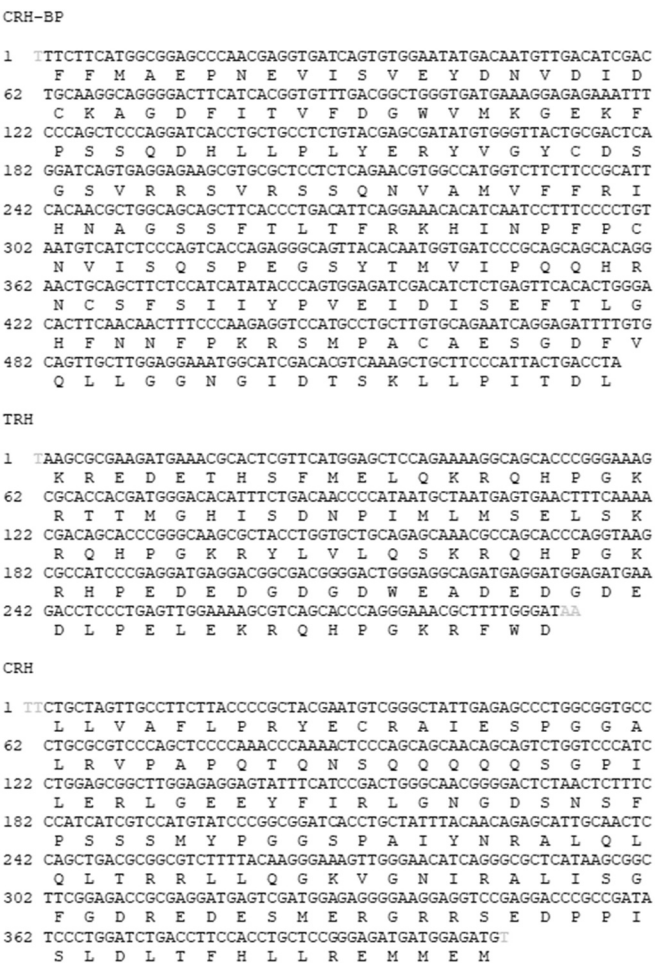

Fig. 1. Nucleotide and predicted amino acid sequence of *crh-bp*, *trh*, and *crh*.

Non-template controls (NTCs) were used as negative controls in every experiment. A single-peak melting curve was used to check for the absence of primer-dimer artifacts and non-specific amplifications. *β-act* was used as the internal reference gene for normalizing mRNA expression data, owing its low CT variability found during the QPCR runs (less than 0.5 CT differences among different salinities).

2.5. Hsp70 analysis

Hsp70 protein expression levels were analyzed by western blot (Matias et al., 2020). Liver samples were homogenized in 3 mL g^{-1} of cold lysis buffer: 20 mM b-glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 10 mM benzamidine, pH 7, 200 μM leupeptin, 10 μM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 5 mM dithiotheitol, 300 μM phenyl methyl sulfonyl fluoride (PMSF), 50 μg mL^{-1} pepstatin,1% *v*/v Triton X-100), and proteins extracted in liquid nitrogen. Samples were centrifuged (15,000 *g*, 10 min, 4 °C) and the supernatants were boiled with 0.33 volumes of SDS/PAGE sample buffer (330 mM Tris–HCl, 13% v/v glycerol, 133 mM DTT, 10% *w/v* SDS, 0.2% w/v bromophenol blue, pH 6.8). Protein concentrations were determined using a commercial kit (997,180, QCA) based on the Biuret method (Gornall et al., 1949) using bovine albumin as reference. Equivalent amounts of proteins (75 μg) were separated on SDS-polyacrylamide (12%) gels and transferred to PVDF membranes (0.45 μm, GE Healthcare Life Sciences). Non-specific binding sites on the membranes were blocked with 5% (*w/v*) non-fat milk in TBST (20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 0.1% (*v*/v) Tween 20) for 30 min at room temperature. Subsequently, the membranes were incubated overnight with Hsp70

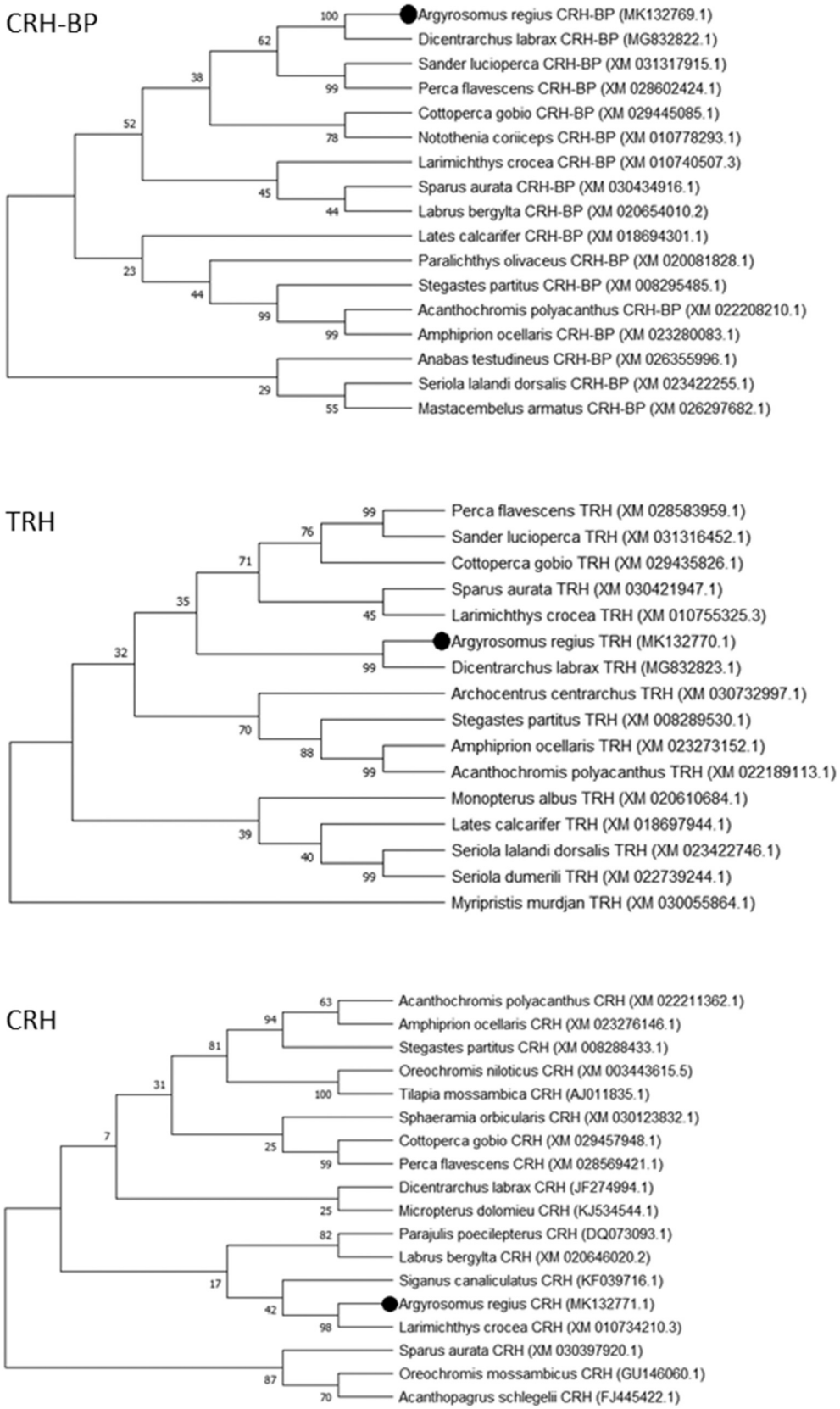

Fig. 2. Neighbor-joining phylogenetic tree of *crh-bp*, *trh*, and *crh* nucleotide sequences. 1000 bootstraps were used to assess the efficacy of the test. Species and accession numbers are shown in the tree. The position of *Argyrosomus regius* genes are marked by the black circle in every case.

Table 5
Plasma markers from every experimental treatment according to Fernández-Alacid et al. (2019). Different superscripts indicate significant differences within every feeding type.

Control	Non-stressed		1stress		6stress	
Glucose (mg dL^{-1})	67.8 ± 5.4	a	114 ± 18	b	99.3 ± 11	b
Lactate (mg dL^{-1})	11.2 ± 1.2	ab	22.9 ± 6.8	a	9.23 ± 1.4	b
Cortisol (ng mL^{-1})	2.01 ± 0.5	a	8.30 ± 0.8	b	5.63 ± 0.8	c
Proteins (g dL^{-1})	4.44 ± 0.3		6.83 ± 1.1		4.39 ± 0.4	

Asp	Non-stressed		1stress		6stress	
Glucose (mg dL^{-1})	77.6 ± 6.2	a	115 ± 13	b	106 ± 13	ab
Lactate (mg dL^{-1})	10.4 ± 1.1		13.9 ± 2.5		13.5 ± 3.9	
Cortisol (ng mL^{-1})	4.01 ± 0.6	a	8.67 ± 0.4	b	6.25 ± 1.2	ab
Proteins (g dL^{-1})	4.19 ± 0.3		6.13 ± 0.8		5.79 ± 1.1	

Trp	Non-stressed		1stress		6stress	
Glucose (mg dL^{-1})	82.3 ± 11		92.6 ± 5.1		88.7 ± 10	
Lactate (mg dL^{-1})	12.1 ± 1.7	a	13.1 ± 1.3	b	6.95 ± 0.9	a
Cortisol (ng mL^{-1})	4.49 ± 0.4	a	9.19 ± 0.4	b	3.73 ± 0.9	a
Proteins (g dL^{-1})	4.42 ± 0.7		5.62 ± 0.3		4.36 ± 0.4	

(monoclonal mouse anti-heat shock protein, 70 kDa, Sigma H5147, 1:4000) or β-actin (monoclonal mouse anti-β-actin, Santa Cruz Biotechnology sc-47,778, 1:1000) primary antibodies. After washing in TBST (3 periods, 5 min each) the blots were incubated with horseradish peroxidase-linked secondary antibody (ThermoFisher Scientific, G21040, 1:5000) for 1 h at room temperature, washed again in TBST (3 periods, 5 min each) and the signals were visualized with enhanced chemiluminescence (Roche, 12,015,196,001) through Chemidoc XRS+, BioRad. Digital images were quantified using Image Lab v6.0 software (Bio-Rad) and normalized to β-actin.

2.6. Statistical analysis

Normality and homoscedasticity of all data sets were checked through the Kolmogorov-Smirnov and Levene tests, respectively. Differences among treatments were detected through a one-way ANOVA (normal variables) or Kruskal-Wallis (non-normal variables) tests, followed by Duncan or U-Mann Whitney post-hoc tests. Data are expressed as mean ± standard error of mean ($n = 10$). The significance level of tests was 0.05.

3. Results

3.1. Cloning

The cloning of partial *Argyrosomus regius crh-bp, trh*, and *crh* mRNA by PCR resulted in PCR products with a size of 538, 294 and 408 base pairs (bp), respectively (Fig. 1). All these sequences were submitted to gene-bank database (acc. no.: MK132771; MK132769 and MK132770). The phylogenetic analysis was developed by comparising with the coincident partial CDS fragments for each gene. The results showed that *crh-bp* and *trh* genes were classified in the same subcluster as *Dicentrarchus labrax,* whereas *crh* gene was classified in the same subcluster as *Larimichthys crocea* (Fig. 2).

3.2. Plasma markers

Table 5 shows the plasma markers analyzed for every experimental group (see Fernández-Alacid et al., 2019 for details).

3.3. Adenohypophyseal sl, prl and gh gene expressions

sl, prl and *gh* relative gene expressions in control groups remained significantly stable among status within the control feeding group (Fig. 3). In the Asp feeding treatment, significant differences towards the control were only detected in the non-stressed status for *sl* (Fig. 3). *sl* expression did not change significantly among treatments in the Trp feeding (Fig. 4). However, *prl* increased significantly 6 h post-stress only while *gh* decreased 1 h post-stress, both within the Trp feeding treatment.

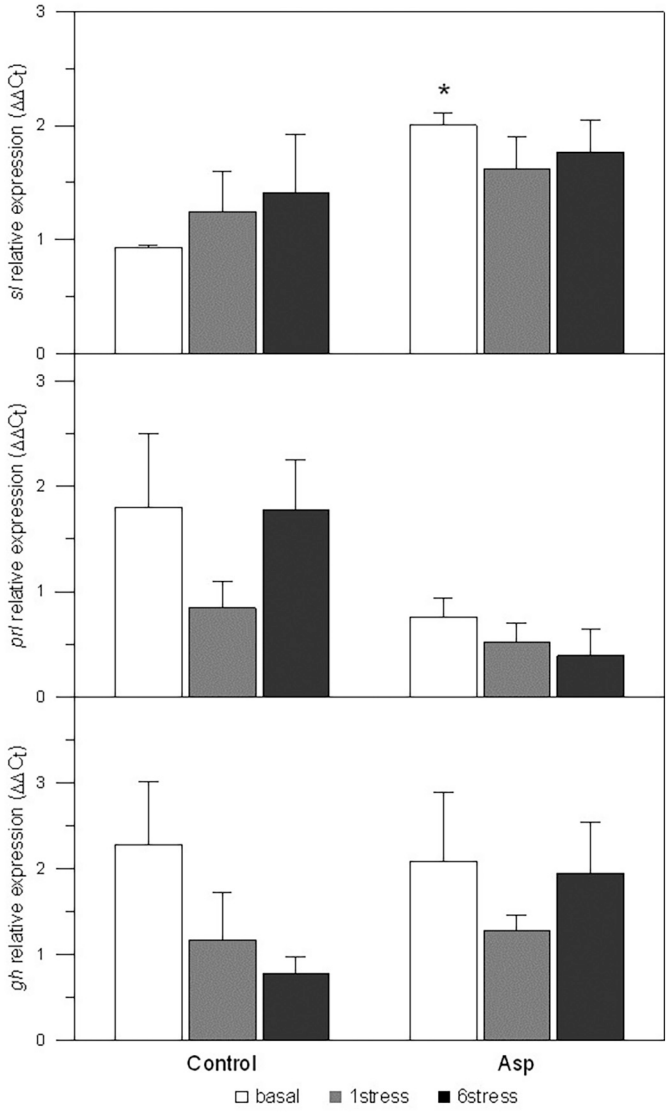

Fig. 3. Adenohypophyseal *sl*, *prl* and *gh* gene expressions for meagre (*Argyrosomus regius*) fed Asp supplements ($n = 10$). Different letters indicate significant differences within every feed group, and asterisks (*) show significant differences from control group for a single status (non-stressed, 1stress or 6stress).

3.4. Brain crh, crh-bp and trh gene expressions

crh, crh-bp and *trh* expressions significantly changed among different status for Trp treatment, whereas no differences were observed for Asp feeding group (Figs. 5 and 6). *crh* and *crh-bp* were also different to controls in the 1 h post-stress status. In the control feeding treatment, only *crh-bp* increased from non-stressed to 6 h post-stress significantly.

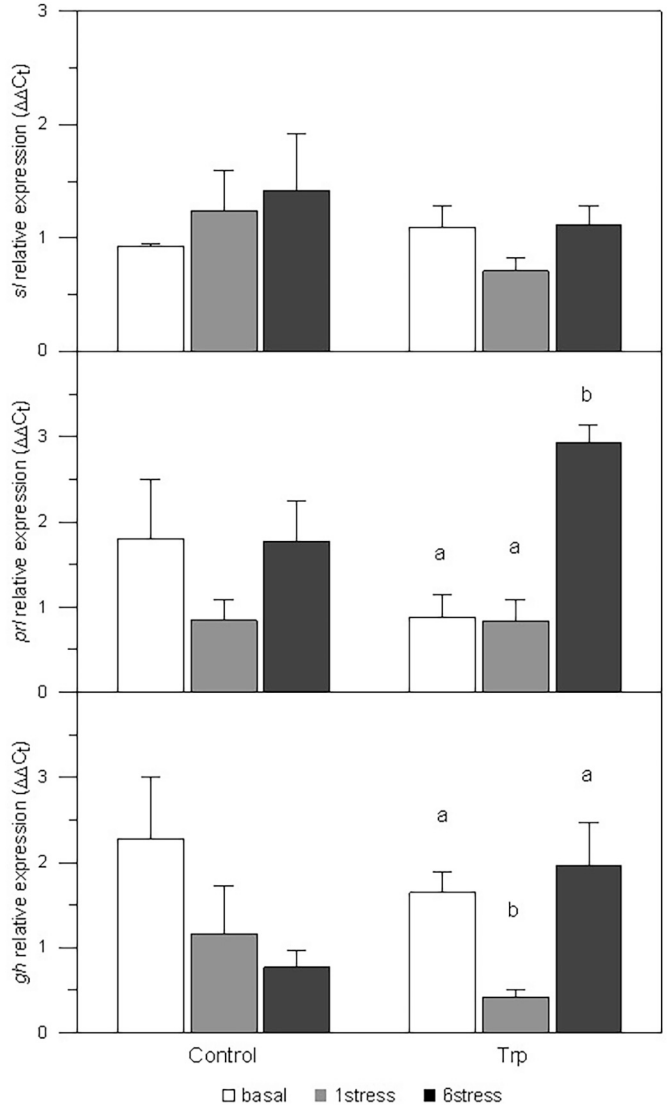

Fig. 4. Adenohypophyseal *sl*, *prl* and *gh* gene expressions for meagre (*Argyrosomus regius*) fed Trp supplements (n = 10). Different letters indicate significant differences within every feed group, and asterisks (*) show significant differences from control group for a single status (non-stressed, 1stress or 6stress).

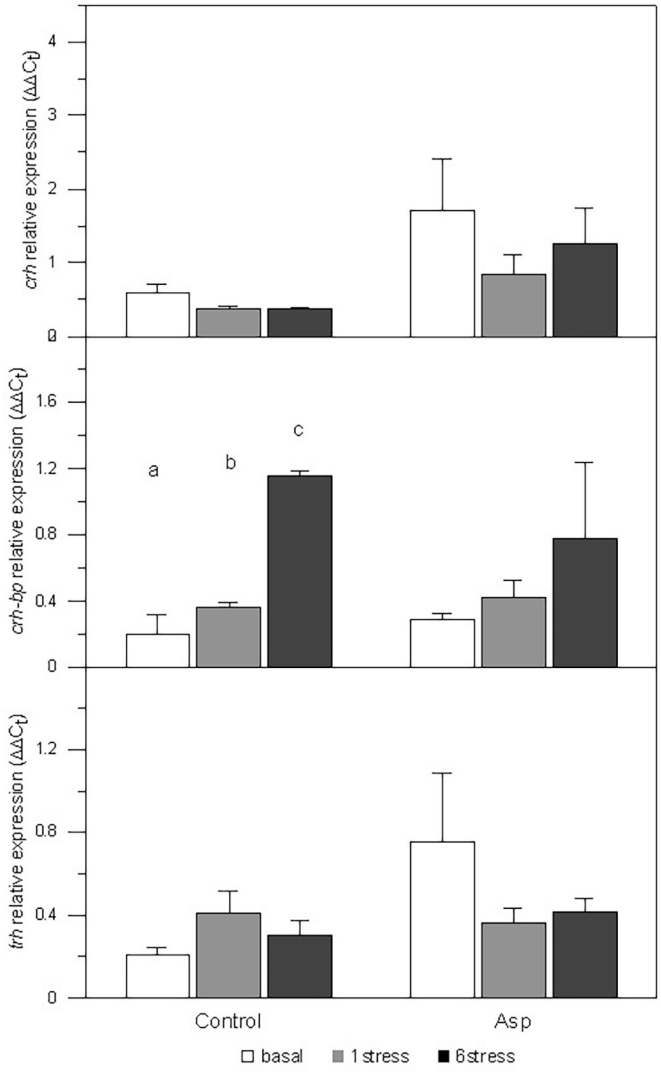

Fig. 5. Brain *crh*, *crh-bp* and *trh* gene expressions for meagre (*Argyrosomus regius*) fed Asp supplements (n = 10). Different letters indicate significant differences within every feed group, and asterisks (*) show significant differences from control group for a single status (non-stressed, 1stress or 6stress).

3.5. Hypophyseal pomc-a and pomc-b gene expressions

The hypophyseal markers, *pomc-a* and *pomc-b*, showed different variation patterns. *Pomc-a* did not change significantly due to stress in the control and Trp treatments, however it increased significantly at 1 h post-stress in fish fed Asp additives (Fig. 8). Compared to non-stressed status, both Trp and Asp treatments enhanced significantly the *pomc-b* expression in 6 h post-stress groups (Fig. 7). The *pomc-b* expression was different between 1 h and 6 h post-stress in the control treatment, and it grew significantly 6 h post-stress in Trp feeding (Fig. 8).

3.6. Hsp70 protein expressions

Hsp relative expression in liver varied among treatments (Fig. 8). Both Asp and Trp feeding groups altered significantly this variable response when compared to control group for every status. Hsp70 decreased strongly from non-stressed to stressed status within the control feeding group. A different pattern was observed both for Asp and Trp feeding groups, where Hsp70 relative expression slightly decreased from non-stressed to 1 h post-stress, growing significantly at 6 h post-stress status. Curiously, the Hsp70 response in meagre seemed to be maintained even 6 h after stress for both feeding groups.

4. Discussion

To our knowledge this is the first time the cloning of the *crh-bp*, *trh* and *crh* and genes in meagre and the expression of these genes under stress has been reported for meagre.

Regards to *crh-bp* cloning, the resulting sequence included a partial open reading frame (ORF) for the *crh-bp* (178 aa). The transcript shared 98% sequence identity with the CDS of *Dicentrarchus labrax crh-bp* (acc. no.: MG832822.1) and 93% with *Perca flavescens crh-bp* (acc. no.: XM_028602424.1), respectively. For *trh* cloning, the sequencing resulted in a *trh* precursor whose nucleotide sequence shared 98% identity with *Dicentrarchus labrax trh* (acc. No.: MG832823.1), and 93% with *Stegastes partitus*. The product derived from *crh* cloning resulted in a high similarity with others *crh* proteins: 99% homology with *Larimichthys crocea crh* (acc. No.: XM_010734210.3), 98% with *Dicentrarchus labrax* (acc. No.: FQ310508.3) and 97% with *Sparus aurata* (acc. No.: LR537139.1). This product included a partial open reading frame for *crh* (134 aa).

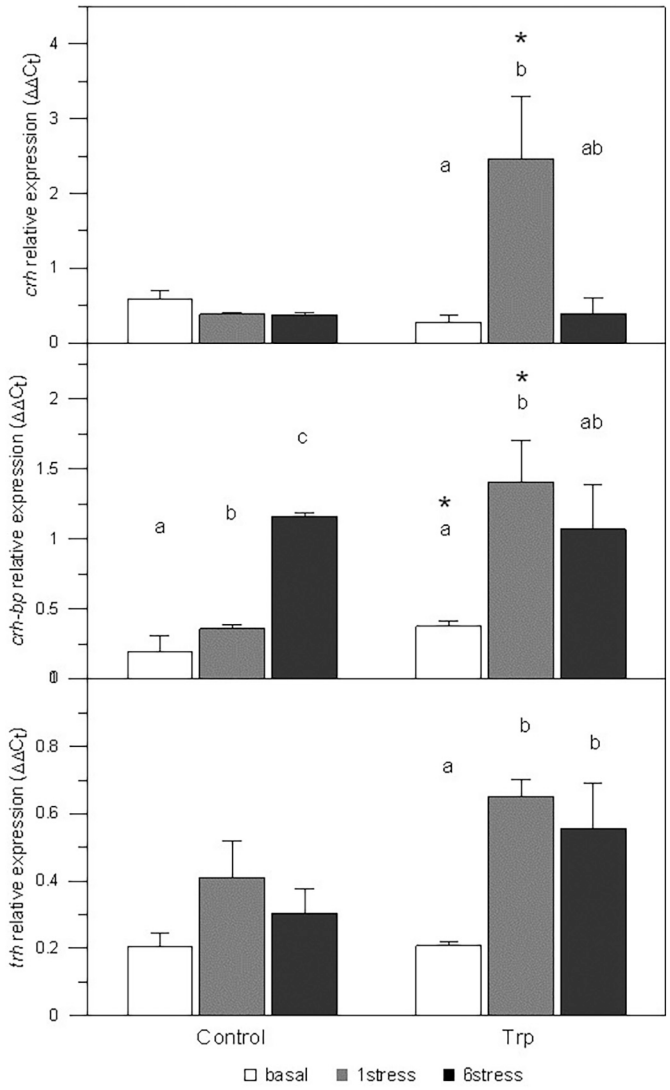

Fig. 6. Brain *crh*, *crh-bp* and *trh* gene expressions for meagre (*Argyrosomus regius*) fed Trp supplements (n = 10). Different letters indicate significant differences within every feed group, and asterisks (*) show significant differences from control group for a single status (non-stressed, 1stress or 6stress).

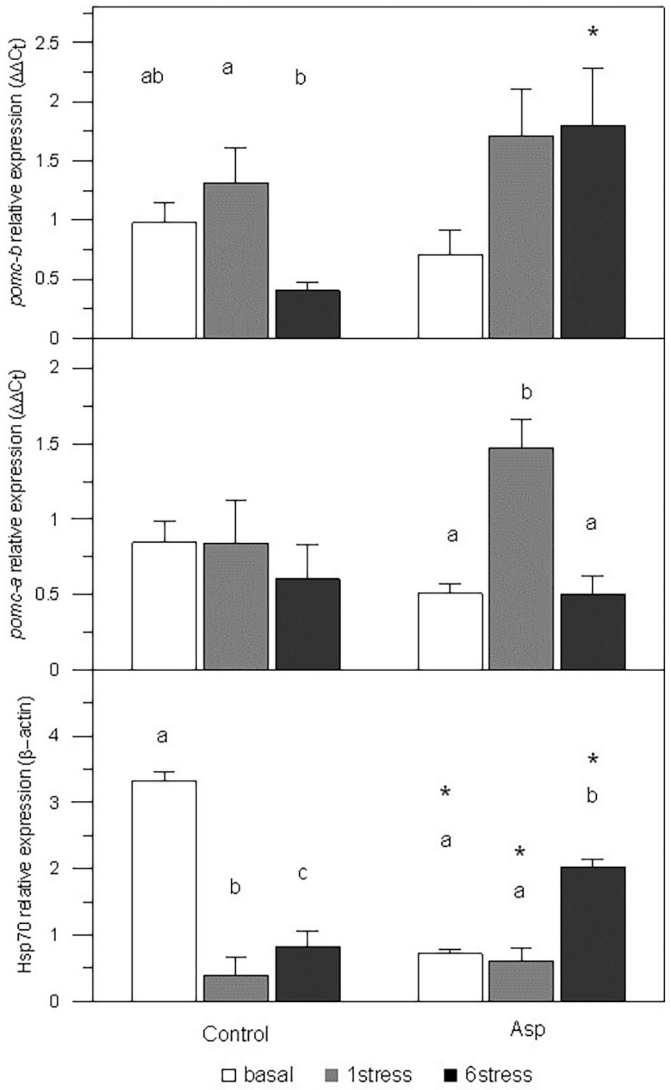

Fig. 7. Hypophyseal *pomc-a* and *pomc-b* gene, and hepatic *Hsp70* protein expression levels for meagre (*Argyrosomus regius*) fed Asp supplements (n = 10). Different letters indicate significant differences within every feed group, and asterisks (*) show significant differences from control group for a single status (non-stressed, 1stress or 6stress).

In a previous study (Fernández-Alacid et al., 2019), it was demonstrated that the experimental diets (1% Trp and 1% Asp), identical to the diets used in the current work, modulated the stress response in meagre, based on plasma and mucus measurements (i.e. cortisol, glucose, lactate, etc.). In fact, the experimental confinement stressor increased the classical plasma stress markers, and the amino acid-enriched diets were effective at attenuating several of those parameters, mainly the Trp supplemented feed.

However, in the current work, analyzing stress responses at molecular level, the results did not follow the pattern described by Fernández-Alacid et al. (2019). Firstly, confinement stressor did not change significantly the relative expression of most analyzed genes for control feeding groups and, secondly, the Asp supplemented diet was more effective for attenuating molecular markers than the Trp one (Figs. 6 and 7; Table 6).

It has been stated that SL, PRL and GH belong to the same hormone family due to their structural similarities (Rand-Weaver and Kawauchi, 1993). Although all of them are related to stress, these relationships are stressor- and species-dependent (Pickering et al., 1991; Rand-Weaver et al., 1993; Laiz-Carrión et al., 2009). In our work, no post-stress changes for these gene expressions were found in the control feeding group, despite the classical plasma markers indicating a stressed status in fish (Fernández-Alacid et al., 2019). However, *sl* expression of meagre fed control diet enhanced with experimental time, suggesting a role of SL in the stress process, as it has been postulated for other fish (Rand-Weaver et al., 1993; Wendelaar Bonga, 1997). Similar to that reported in other fish, *gh* expression was down-regulated, possibly due to the influence of high plasma cortisol levels induced by the applied stressors (Laiz-Carrión et al., 2009; Sadoul and Vijayan, 2016). Probably, the stress procedure employed in our experiment was not so long (1 h) and/or severe to detect expression changes in this species. However, the absence of responses regarding molecular markers has already been reported in other species. For instance, *prl* expression did not vary significantly in *Rhamdia quelen* and *Sparus aurata* submitted to transport and food deprivation stress, respectively (Laiz-Carrión et al., 2009; Saccol et al., 2018).

Meagre juveniles fed an additional Asp diet did not present any *sl, prl* and *gh* expression changes, as for the control group. However, it is interesting to remark that the tendency to up-regulate *sl* and down-

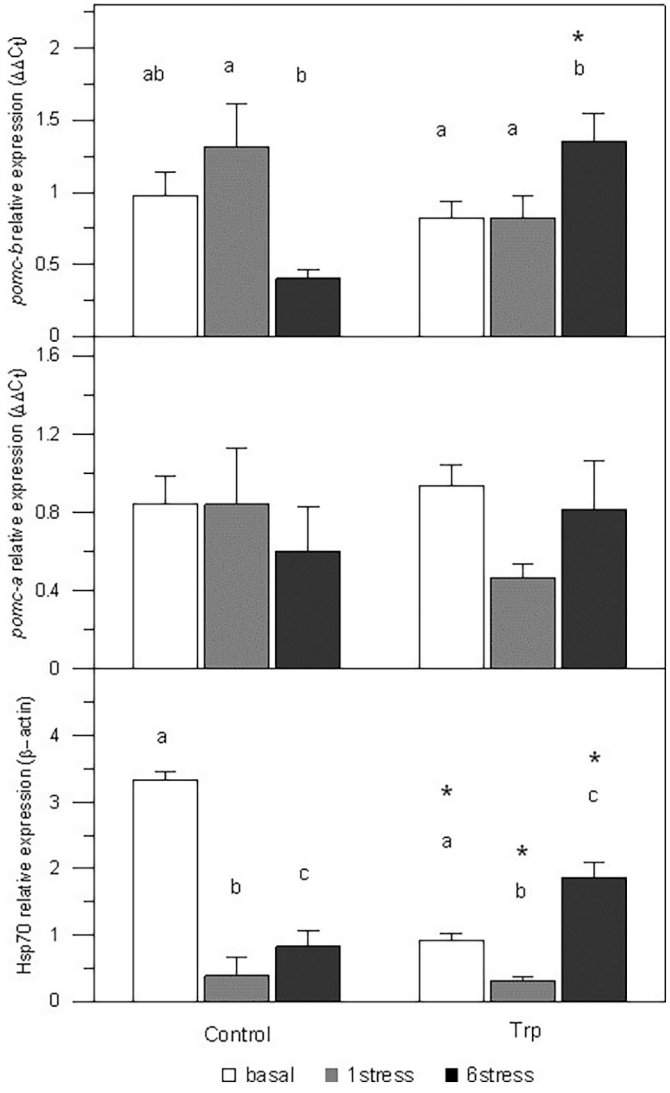

Fig. 8. Hypophyseal *pomc-a* and *pomc-b* gene, and hepatic *Hsp70* protein expression levels for meagre (*Argyrosomus regius*) fed Trp supplements (n = 10). Different letters indicate significant differences within every feed group, and asterisks (*) show significant differences from control group for a single status (non-stressed, 1stress or 6stress).

regulate *gh* expressions detected in the feed control group were not observed in specimens supplied with Asp-enriched diet (Fig. 3).

Contrarily, the Trp diet altered the *prl* and *gh* expressions after stress. It is known that dietary Trp can modulate the cortisol release in fish (Hoseini et al., 2019), and cortisol presents an inhibitory influence on somatotropic axis and growth process (Sadoul and Vijayan, 2016). With respect to *gh* expression, its values are down-regulated at 1 h post-stress, a situation clearly associated to the high cortisol levels observed; however these values are recovered at 6 h post-stress when plasma levels reach values similar to that observed at time 0 pre-stress (non-stressed levels), as Fernández-Alacid et al. (2019) showed (Table 5). These results agree with the negative effects of cortisol on *gh* expression as well as growth process and suggest a stimulatory role of Trp in the somatotropic axis homeostasis recovery (Pérez-Sanchez and Le Bail, 1999; Pickering et al., 1991). The *prl* expression was up-regulated at 6 h post-stress in specimens supplied with the Trp-enriched diet. In several fish, plasma PRL levels and *prl* expression were enhanced with confinement (Avella et al., 1991; Laiz-Carrión et al., 2009). In addition, administration of exogenous PRL increased plasma cortisol levels (Wendelaar Bonga, 1997; Sangiao-Alvarellos et al., 2006). In this way, the *prl* expression enhancement observed at 6 h post-stress could be useful to meagre in combatting a stress situation.

Corticotropin-releasing hormone (CRH) levels are regulated by the CRH-binding protein (CRH-BP) (Gorissen and Flik, 2016). The *crh* expression for the control group did not vary significantly after stress, contrarily to the *crh-bp* one. This difference in responses could be due to the stress system needing to adapt to both factors to cope with acute stress, also demonstrating the possible HPI axis activation by the binding protein without CRH stimulation, as the cortisol data (Table 5) indicate (Skrzynska et al., 2018). However, Trp-fed fish showed different *crh* and *crh-bp* expressions, showing significant high values in 1 h post-stress samplings. In this sense, and taking the cortisol decrease at 6stress into account (Table 5), it is possible to suggest that *crh-bp* expression enhancement observed at 1 h post-stress can counteract the CRH action and decrease plasma cortisol levels at 6 h post-stress. Although there are several previous recent works reporting the effects of dietary Trp on cortisol release in marine fish (Fernández-Alacid et al., 2019; Herrera et al., 2017, 2019b), there is no evidence on how it affects the molecular marker expressions.

The effects of Asp supplements on fish stress have been studied only in a couple of works, though focusing on immune and classical plasma markers (Fernández-Alacid et al., 2019; González et al., 2018). Those works have reported that Asp supplements improve the fish health by maintaining the levels of protease activity under stress. In superior animals, Asp can act as neurotransmitter for stimulating the *N*-methyl-D-aspartate (NMDA) receptor (Chen et al., 2005). In this way, it has been demonstrated that intracerebroventricular injections of Asp attenuate the isolation stress in chicks (Erwan et al., 2012, 2014). In our work, it seems that amino acid improved the stress response measured through *crh* and *crh-bp* expressions since no significant differences were detected within the Asp diet. Despite a clear link among HPI axis stimulation and Asp exists, it is still hardly possible to determine specifically the physiological mechanisms connecting *crh/crh-bp* expressions and Asp metabolism in fish.

trh expression showed a similar pattern to *crh/crh-bp* expressions, with no significant changes in both control and Asp feeding. It has been reported that hypothalamus-pituitary-thyroid (HPT, which TRH is involved in) and HPI axis interact (Geven et al., 2006; Klaren et al., 2008; Ruiz-Jarabo et al., 2018), and that TRH stimulates the POMC secretion (Rotllant et al., 2000). Due to the above mentioned interaction, we expected that stress responses depending on feeding type were similar among HPI and HPT axis markers. In that sense, our results

Table 6
Different stress-response types for every molecular marker analyzed in tissues of meagre, *Argyrosomus regius*, before stressor (non-stressed) and 1 and 6 h after stressor.

	SL	PRL	GH	CRH	CRHBP	TRH	POMCA	POMCB	HSP70
Control	C	C	C	C	LR↑	C	C	QR↑	QR↓ LR↓[a]
Asp	C	C	C	C	C	C	QR↑	C	LR↑
Trp	C	LR↑	QR↓	QR↑	QR↑	QR↑	C	C	QR↓ LR↑[a]

Meagre juveniles were fed control or supplemented diets with Aspartate (Asp) or Tryptophan (Trp) for 7 days. C: no significant differences among stress statuses; QR↑/QR↓: increasing/decreasing Quick Response, significant changes between non-stressed and at 1stress status; LR↑/LR↓: increasing/decreasing Late Response, significant changes between non-stressed and 6stress status.

[a] Only in these cases late and quick responses exist because both 1stress and 6stress statuses are significantly different to non-stressed.

supported it since, overall, Trp feeding altered significantly many markers, meanwhile Asp feeding kept most of them stable.

The end product of POMC-A processing is αMSH, while that for POMC-B is ACTH (Gorissen and Flik, 2016). These hormones, mainly ACTH but also α-MSH, stimulate cortisol synthesis in interrenal cells of the head kidney (Wendelaar Bonga, 2011). In meagre, *pomc-b* expression (Fig. 7) as well as plasma cortisol levels observed (Table 5) are correlated in agreement with the role of POMC B as precursor of ACTH (Gorissen and Flik, 2016). The down regulation observed in *pomc-b* expression at 6 h post-stress could be related to a negative feedback regulation due to high cortisol levels observed at 1 h post-stress (Table 5). Although no previous work has confirmed the influence of Trp or Asp supplements on this molecular marker, Trp could promote a delay in the stress response (*pomc-b* expression peak from 1 h to 6 h). In this sense, Pavlidis et al. (2015) have described that the *pomc* expression shows a peak at 30 min after stress in the zebrafish (*Danio rerio*). However Asp additives attenuated the *pomc-b* response (Fig. 7).

Similarly to other fish species (Toni et al., 2015; Sneddon et al., 2016; Saccol et al., 2018), *pomc-a* expression remained stable in specimens fed control food suggesting that this expression is not affected by our experimental condition (or perhaps, that it is necessary more time to detect any variation in its expression).

However, Asp feeding, but not Trp, enhanced *pomc-a* expression after stress. Both POMC-A and its derived product αMSH are involved in several biological process, include stress responses (Takahashi et al., 2009; Gorissen and Flik, 2016). For instance, α-MSH has been shown to be implicated in stressed fishes when kept in seawater, being related to blood acidosis and pH changes (Lamers et al., 1997; Kobayashi et al., 2011). Therefore, it is possible that, in the Asp feeding, blood pH decreased at 1 h stress hence *pomc-a* expression increased, and later it came back to the initial state (Fig. 7).

It has been stated that Hsp70 increases are related to the exposure to different stressors (Iwama et al., 1998a, 1998b; Vijayan et al., 1998; Ackerman and Iwama, 2001; Feidantsis et al., 2009; Cara et al., 2005), although some works have reported no changes in fish submitted to stress (Carpenter and Hofmann, 2000; Hofmann et al., 2000; Washburn et al., 2002; Zarate and Bradley, 2003; Álvarez et al., 2020). In our work, these proteins varied for every treatment, including the control feeding. It seems that this was a late response, at 6 h post-stress sampling, where both Asp and Trp treatments increased these expressions significantly. In fact, it was already described that Hsp70 levels remain elevated in organisms long after the stressor is removed (Basu et al., 2002). However, both Asp and Trp treatments increased these expressions significantly. Therefore, considering these Hsp70 variations, the experimental diets did not affect the expression changes due to stress.

In conclusion, the response of molecular stress markers to amino acid enriched diets was diverse. It seems that Asp and Trp supplements modulated the molecular responses though it would be difficult to confirm that they attenuated the marker variations. Contrarily to Trp, Asp diet made some markers stable, spite of increasing some markers in previous works (Fernández-Alacid et al., 2019). Previous works have already demonstrated several effects of these diets on the stress response using physiological indicators and short experimental times. Probably the molecular marker responses would require a longer exposure time to the stressor for detecting significant expression changes, hence future works should be focused in it. The results will improve our knowledge on the mechanisms behind the stress response in this important commercial species, and also could be used to develop less stressful aquaculture techniques in order to improve welfare of cultured fish.

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.

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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.2020.736238.

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