

**Different stressors induce differential responses of the CRH-stress system
in the gilthead sea bream (*Sparus aurata*)**

Juan A. Martos-Sitcha^{1,2*}, *Yvette S. Wunderink*^{1,3}, *Justin Straatjes*³, *Arleta K. Skrzynska*²,
*Juan M. Mancera*¹ and *Gonzalo Martínez-Rodríguez*²

*(1) Department of Biology, Faculty of Marine and Environmental Sciences, University of
Cádiz, 11510, Puerto Real (Cádiz), Spain*

*(2) Department of Marine Biology and Aquaculture, Instituto de Ciencias Marinas de
Andalucía (CSIC), Apartado Oficial, 11510, Puerto Real (Cádiz), Spain*

*(3) Department of Organismal Animal Physiology, Institute for Water and Wetland Research,
Faculty of Science, Radboud University Nijmegen, Heyendaalseweg 135 6525 AJ Nijmegen,
The Netherlands*

*Corresponding author:

Dr. Juan Antonio Martos Sitcha

Department of Marine Biology and Aquaculture

Instituto de Ciencias Marinas de Andalucía (ICMAN-CSIC)

Apartado Oficial, 11510, Puerto Real (Cádiz), Spain

Tel.: +34 956 832612, Fax: +34 956834701

E-mail: juanantonio.sitcha@icman.csic.es

Abstract

The hypothalamus-pituitary-interrenal (HPI) axis, involved in the regulation of the neuroendocrine stress responses, presents important players such as corticotropin-releasing hormone (CRH, generally considered as the initiator of this pathway) and CRH-binding protein (CRH-BP, considered as an antagonist of CRH function). CRH and CRH-BP full-length cDNA sequences were obtained from *Sparus aurata* by screening a brain cDNA library, and their phylogenetic analysis as well as their roles during acute and chronic stress responses were assessed. mRNA expression levels and plasma cortisol concentrations were measured by RT qPCR and ELISA, respectively, in *S. aurata* juveniles submitted to: i) different environmental salinities in a short-time course response; and ii) food deprivation during 21 days. In addition, osmoregulatory and metabolic parameters in plasma corroborated a clear reorganization depending on the stress source/period. Salinity transfer induced stress as indicated by enhanced plasma cortisol levels, as well as by up-regulated CRH and down-regulated CRH-BP expression values. On the other hand, food deprivation did not affect both expression levels, although plasma cortisol concentrations were enhanced. These results suggest that different stressors are handled through different stress pathways in *S. aurata*.

Keywords:

Cortisol, CRH, CRH-binding protein, environmental salinity, food deprivation, *Sparus aurata*

1. Introduction

In teleost fishes, the hypothalamus-pituitary-interrenal (HPI) axis is stimulated under stress situation. This axis starts with the production and release of corticotropin-releasing hormone (CRH) from different hypothalamic nuclei, mainly the *nucleus preopticus* (NPO). CRH stimulates the release of adrenocorticotropin hormone (ACTH), which is cleaved from the precursor protein proopiomelanocortin (POMC), produced in adenohypophyseal corticotroph cells. Subsequently, ACTH activates head kidney interrenal cells to produce and release the typical stress hormone cortisol (Wendelaar Bonga, 1997; Flik et al., 2006; Bernier et al., 2009).

The mature form of CRH polypeptide consists of 41 amino acids, deriving from a larger peptide of 160-210 amino acids, depending on the species, and signals via specific G-protein coupled receptors of which two forms have been described: CRH-R1 and CRH-R2 (Vale et al., 1981, Huising et al., 2008). CRH is highly conserved and can be found within virtually all vertebrates, which indicates its endocrine importance. Besides CRH's key function in the stress response, this hormone is also involved in other processes, like feeding, digestion and metabolism (Bernier et al., 2009; Yayou et al., 2011). In addition, studies in humans and other mammals have also demonstrated that CRH plays a role in anxiety, arousal and depression (Conti, 2012).

The biological activity of CRH can be regulated by a soluble binding protein, named CRH-BP, since CRH presents a higher affinity for CRH-BP than for its own receptors (Huising et al., 2004). Nevertheless, in mammals exist other ligands for CRH with different affinities for the receptors and CRH-BP, like urocortin I (Ucn I), urocortin II (Ucn II) /stresscopin-related peptide, and urocortin III (Ucn III), whereas fishes and amphibians possess Urotensin I or sauvagine, respectively (Majzoub, 2006).

Like CRH, CRH-BP is mainly expressed in the NPO, and even co-locates with CRH, suggesting a direct and rapid mechanism to regulate the stress response (Huising et al., 2004, Flik et al., 2006). Additionally, physiological studies performed in teleostean species, indeed have shown that CRH-BP can be considered as a strong modulator of the stress response (Huising et al., 2004, Wunderink et al., 2011).

The degree of stress, or allostatic load, depends on the intensity and chronicity of the type of stressor. Chronic exposure to stressors can lead to allostatic overload, which negatively affects in reproduction, growth and immune function leading to diseases and reduced animal welfare (Ellis et al., 2002, Conte, 2004, Ashley, 2007). Chronic stress is diagnosed by long-lasting, moderate changes of stress hormone levels as has been shown in several fish species

(Rotllant et al., 2000, Wunderink et al., 2011, 2012). When a stressor is only exposed shortly/intensively, a differential response is seen, defined by short duration, but more pronounced alterations of stress hormone release (Rotllant and Tort, 1997, Ruane et al., 2002, Huising et al., 2004, Doyon et al., 2005). In aquaculture, fish must cope with exposure to a series of acute stressors such as transport weighing and handling, sorting/grading and sudden environmental changes in, for instance, water temperature or salinity (Rotllant et al., 2001, Arjona et al., 2007, Arjona et al., 2008, Mancera et al., 2008, Herrera et al., 2012), and might become more susceptible when chronically stressed (Wunderink et al., 2011). To that account, mapping the CRH-stress system contributes to a better understanding of the stress response and may lead to improvement of aquaculture settings as well.

In gilthead sea bream (*Sparus aurata*) several studies have assessed changes in HPI axis due to acute or chronic stress situations (Arends et al., 1999; Rotllant et al., 1997, 2000, 2001), but no information exists on the role of CRH and CRH-BP during both stress situations. This species is able to adapt to different environmental salinities adjusting their homeostasis in a range of 5 to 60 ppt of salinity during 3 weeks (Laiz-Carrión et al., 2005; Sanguiao-Alvarellos et al., 2005), being unable to withstand freshwater (Fuentes et al., 2010a). In part, this plasticity is carried out by endocrine regulation, in which several hormones, including cortisol, are involved (Takey and McCormick, 2013). However, a suddenly salinity transfer can be considered as an acute stress situation for this species (Mancera et al., 1993; Laiz-Carrión et al., 2005). On the other hand, and related with feeding status of fish, long-term adaptation to food deprivation has been proposed as a clear stress factor, where cortisol can act as an important player in metabolic processes (Vijayan et al., 1993). Similarly, food deprivation also enhanced plasma cortisol levels in *S. aurata* (Sanguiao-Alvarellos et al., 2005b; Mancera et al., 2008).

In this study, the cDNAs coding for *S. aurata* CRH and CRH-BP peptides were cloned, obtaining thus new molecular tools to study the neuroendocrine stress responses in this species. Furthermore, the physiological roles of these genes in the acute and chronic stress responses were characterized by monitoring their expression levels in *S. aurata* juveniles submitted to: i) an acute stressor, viz. exposure to sudden environmental salinity changes, and ii) a chronic stressor, viz. chronic exposure to food deprivation.

2. Material and Methods

2.1 Animals and experimental design

Juveniles of gilthead sea bream (*Sparus aurata* L., 213.13 ± 4.75 g body mass) were provided by Planta de Cultivos Marinos (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Experimental animal facility registry numbers CA/4/CS and CA/3/U). Fish were fed a daily ration of 1 % of their body weight with commercial pellets (Dibaq-Dibroteg S.A., Segovia, Spain). All the experiments were performed with the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.

2.1.1. Experimental design I: Short-term salinity transfer

Fish ($n = 80$, 192.11 ± 4.23 g body mass) were transferred to the wet laboratories at the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz, Spain), where they were acclimated for 14 days to sea water (SW, 38 ‰ salinity) in 400-L tanks in an open system circuit ($5.6 \text{ kg} \cdot \text{m}^{-3}$ density) under natural photoperiod (May, 2011) and constant temperature (18-19 °C). Afterwards, fish were directly transferred to one of the following environmental salinities: SW (control group), low salinity water (LSW, 5 ‰ salinity, hypoosmotic transfer) and high salinity water (HSW, 55 ‰ salinity, hyperosmotic transfer). These experimental salinities were achieved by either mixing SW with dechlorinated tap water (LSW), or mixing with natural marine salt (Salina de la Tapa, El Puerto de Santa María (Cádiz), Spain) (HSW). Experimental groups were maintained in duplicate tanks (400-L volume each; $n = 12$ fish per tank, $5.6 \text{ kg} \cdot \text{m}^{-3}$ initial density) under a closed recirculating water system. Water quality criteria were checked at the end of the trial to confirm their stability during the 24 hours that experiment lasted. On day 0 (10:00 AM), eight fish from the main tanks containing SW were sampled (control time 0 before transfer). Then, on 4, 8, 12 and 24 hours after salinity transfer, six fish from each experimental salinity (SW, LSW and HSW) were anaesthetized with a lethal dose of 2-phenoxyethanol ($1 \text{ mL} \cdot \text{L}^{-1}$ specific salinity water), weighted, heads separated from trunks and sampled.

Blood samples were collected from the caudal peduncle into 1-mL ammonia-heparinised syringes, and centrifuged (3 min at 10,000 g) to obtain plasma, snap-frozen in liquid nitrogen afterwards and stored at -80 °C until further analysis. Whole brains were put in a 1/10-relation w/v of RNeasyTM stabilization solution (Ambion®) for 24 hours at 4 °C and then stored at -20 °C. No mortality was observed during the time that experiment lasted. Moreover, the stocking density of each tank was restructured after each sampling point, by adjusting the

final water volume in the tanks, to keep it constant throughout the experimental period and between all tanks.

2.1.2. Experimental design II: Starving and re-feeding

Fish ($n = 96$; 235.31 ± 5.65 g body mass) were transferred to the wet laboratories at the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz, Spain), where they acclimated for 28 days to sea water (SW, 38 ‰ salinity) in five 1000-L tanks in an open system circuit ($4.3 \text{ kg} \cdot \text{m}^{-3}$ density) under natural photoperiod (March, 2011) and constant temperature (18-19 °C). After this acclimation period to SW, animals were maintained at the following experimental conditions: 2 tanks fed with a daily ration of 1 % of their body mass with commercial pellets (Dibaq-Dibroteg S.A., Segovia, Spain), and 3 tanks without receiving food ($n = 18$ or 20 fish per tank). Furthermore, from day 14 after the start of the experiment, fish from one tank maintained under food-deprived condition were fed again during 7 days with a daily ration of 1 % of their body mass with the same commercial pellets described above, constituting the re-feeding group. On day 0, eight fish from the main tanks containing SW were sampled (control time 0 before transfer). Then, twelve fish from each experimental group (control, starved and re-fed) on 7, 14 and 21 days after the start of the experiment, were anaesthetized with a lethal dose of 2-phenoxyethanol ($1 \text{ mL} \cdot \text{L}^{-1}$ specific salinity water), weighted, heads separated from trunks and sampled. Blood samples and tissue biopsies were taken as described above. No mortality was observed during the time that experiment lasted. In addition, the stocking density of each tank was restructured as described above.

2.2. Plasma parameters

Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske-VT, USA) and expressed as $\text{mOsm} \cdot \text{kg}^{-1}$. Glucose and lactate concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330) adapted to 96-well microplates.

Plasma cortisol levels were measured by enzyme-linked immunosorbent assay (ELISA) adapted to microtiter plates as previously described for testosterone (Rodríguez et al., 2000). Steroids were extracted from 5 μL plasma in 100 μL RB (PPB (Potassium Phosphate Buffer) 100 mM, NaN_3 1.54 mM, NaCl 400 mM, EDTA 1 mM, BSA (Bovine Serum Albumin) 15 mM) and 1.2 mL methanol (Panreac), and evaporated during 48-72 hours at 37 °C. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol

express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate. The percentage of recovery was determined as 95 %, and evaluated as previously described in others fish species (Barry et al., 1993; Mills et al., 2010). The inter- and intra-assay coefficients of variation (calculated from the sample duplicates) were 3.20 ± 0.67 % and 6.41 ± 0.73 %, respectively for salinity transfer, and 2.71 ± 1.03 % and 5.12 ± 0.48 %, respectively for starving experiment. Cross-reactivity for specific antibody with intermediate products involved in steroids synthesis was given by the supplier (cortexolone (1.6 %), 11-deoxycorticosterone (0.23 %), 17-hydroxyprogesterone (0.23 %), cortisol glucurinoide (0.15 %), corticosterone (0.14 %), cortisone (0.13 %), androstenedione (<0.01 %), 17-hydroxypregnenolone (<0.01 %), testosterone (<0.01 %)).

2.3. Cloning and sequencing

PCR was carried out on *S. aurata* brain cDNA with degenerate primers (Table 1) designed on conserved regions of CRH-BP from *Salmo salar* (NM001173799), *D. rerio* (BC164122), *Haplochromis burtoni* (GQ433718), *Cyprinus carpio* 1 (AJ490880), *Cyprinus carpio* 2 (AJ490881), and *S. senegalensis* (FR745428). For CRH, a specific probe obtained in *Solea senegalensis* as previously described in Wunderink et al. (2011) was used. Both CRH and CRH-BP probes were used for screening a brain cDNA library as described in Balmaceda-Aguilera et al. (2012). *In vivo* excision of 4 single positives of the screening were performed using *Escherichia coli* XL-1-Blue MRF' and SOLR strains (Stratagene, Agilent Technologies Life Sciences). Excised pBluescript SK(-) containing the specific clone was double digested by *EcoRI* and *XhoI* (Takara) and the products were revealed in a 1 % agarose gel stained with GelRed™ (Biotium). Clones were fully sequenced in both strands by the dideoxy method (Bioarray S.L., Alicante, Spain).

2.4. Sequence analysis

Sequencing data were compiled, assembled and analyzed using nucleotide and protein BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). eBiox (v1.5.1) software was used for sequencing fragment assemblage, as well as for translation of the sequences to obtain the open reading frames (ORFs). ClustalW2 software was used for protein alignment (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Homology analysis of putative protein sequences was run with NCBI blastp.

2.5. Phylogenetic and evolutionary analyses

Phylogenetic analysis of the CRH-like and CRH-BP amino acid sequences was conducted with MEGA5 software (Tamura et al., 2011) with the Neighbor-Joining algorithm (Saitou and Nei, 1987) based on amino acid differences (p-distances) and pairwise deletion. Reliability of the tree was assessed by bootstrapping (1000 replications). Amino acid sequences were retrieved from the NCBI protein database (www.ncbi.nlm.nih.gov/pubmed), accessed in June 2014).

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted using the commercial kit NucleoSpin[®] RNA II kit (Macherey-Nagel) according to manufacturer's instructions. Incubation with RNase free DNase (Macherey-Nagel) during 30 min at 37 °C was used to eliminate potential genomic DNA contamination. RNA concentrations were measured by spectrophotometry and RNA quality was assessed using the Agilent RNA 6000 Nano Assay Kits on an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (500 ng) was reverse-transcribed in a 20 µL reaction using the qScript[™] cDNA synthesis kit (Quanta BioSciences). Briefly, the reaction was performed using qScript Reaction Mix (1x final concentration) and qScript Reverse Transcriptase (2.5 x final concentration). The reverse transcription program consisted in 5 min at 22 °C, 30 min at 42° and 5 min at 85 °C. Only samples with a RNA Integrity Number (RIN) higher than 8.5 were used for real time PCR.

2.7. Real-time PCR (qPCR)

Specific primers for use in qPCR were designed by use of Primer 3 software (v. 0.4.0) available at <http://fokker.wi.mit.edu/primer3/input.htm> in February 2011. Primer oligonucleotide sequences are shown in Table 2. Previous to qPCR analysis, optimization of qPCR conditions was made on primers annealing temperature (50 to 60 °C), primers concentration (100 nM, 200 nM and 400 nM) and template concentration (six 1:10 dilution series from 10 ng to 100 fg of input RNA). Moreover, two negative controls, with i) RNA (10 ng/reaction) and ii) sterile water, were performed to detect possible gDNA contamination or primer-dimers artefacts. The resulting curves had amplification efficiencies and r^2 of 0.98 and 0.995 for CRH, 0.99 and 0.998 for CRH-BP, and 0.99 and 0.999 for β -actin, respectively. To perform qPCR reactions, 4 µl cDNA (10 ng assumed from RNA input), specific forward and reverse primers (200 nM each) and 5 µl PerfeCta[™] SYBR[®] Green Fastmix[™] (Quanta

BioSciences) were used. qPCR (10 min at 95 °C; 40 cycles of denaturing for 15 s at 95 °C, annealing and extension for 45 s at 60 °C; and a final melting curve from 60 °C to 95 °C for 20 min) was performed on a Mastercycler[®] epgradient S Realplex² with Realplex software (Eppendorf, version 2.2). The melting curve was used to ensure that a single product was amplified by each primer pair. Results were normalized to *S. aurata* β -actin (acc. no. X89920) owing its low variability (less than 0.5 C_T) under our both experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Data were analysed by two-way ANOVA with salinity (LSW, SW, HSW) and time course (day 0, 4, 8, 12 and 24 hours) as main factors for short-term salinity transfer, or by two-way ANOVA with fed conditions (control and starving) and time course (days 0, 7, 14 and 21) as main factors, and one-way ANOVA at day 21 for each treatment (control, starving and re-feeding) for starving experiment. These analysis were followed by a post-hoc comparison made with the Tukey's test, and using GraphPad Prism[®] (v.5.0b) software. Statistical significance was accepted at $P < 0.05$. Statistical parameters (P -value and F) obtained from two-way ANOVA analysis in both sub-experiments are provided in Table 6.

3. Results

3.1. Cloning and characteristics of *S. aurata* CRH and CRH-BP cDNA sequences

Complete sequences of sea bream CRH (GenBank acc. no. KC195964) and CRH-BP (GenBank acc. no. KC195965) were obtained by screening a *S. aurata* brain cDNA library using labelled probes. Sequencing revealed cDNAs to be 1,063 bp for CRH and 1,516 bp for CRH-BP.

Figure 1 shows the obtained full-length nucleotide and deduced amino acid sequence of the sea bream CRH peptide, which comprises an open reading frame (ORF) of 507 bp encoding a 169 amino acid protein with 56-99 % similarity to other teleosts. ORF includes a conserved signal peptide (M¹ – A²⁴), a cryptic motif (R⁵⁵ – N⁶⁶) and a mature peptide (S¹²⁷ – F¹⁶⁷), based on alignment with other CRH sequences. Figure 2 shows a protein alignment done between fish, amphibian, avian and mammalian CRH. The alignment shows 3 highly conserved regions between these species, and scores between all the species are presented in Table 2A. Moreover, as it has been observed in other species, the N-terminal dibasic cleavage site (R¹²⁵

– R¹²⁶) of the mature peptide and the typical C-terminal amidation site (G¹⁶⁸ – K¹⁶⁹) are also conserved.

On the other hand, the complete coding sequence of *S. aurata* CRH-BP is presented in Figure 3. cDNA sequence comprises an ORF of 969 bps encoding 323 amino acids with 58-90 % sequence similarity to other teleosts, and included a signal peptide between amino acids M¹ – C²⁶, the two conserved amino acids R⁵⁹ and D⁶⁵, and the ten conserved cysteine residues (position numbers 63, 84, 107, 143, 186, 208, 239, 266, 279 and 320) involved in the formation of five C-C disulphide loops. In addition, a protein alignment is shown in Figure 4 between fish, amphibian, avian and mammalian CRH-BP, revealing highly conserved sequences at nucleotide (data not shown) and protein levels (Table 3B).

Phylogenetic analysis of non-mammalian and mammalian CRH-like and CRH-BP amino acid sequences (Figure 5) indicated that *S. aurata* CRH clusters within the fish branch of CRH, and just in the same branch of the CRH-family including CRH, UI, UcnI, UcnII and UcnII of different species of fishes, amphibians, birds, and mammals. In addition, the vertebrate CRH and UI/Ucn clusters together from the same clade, supported by a bootstrap value of 92. Related to CRH-BP, vertebrates and invertebrates (insects) species are evolutionary more distant, showing in vertebrates than amphibians, birds and mammals cluster independently from fish species, supported by a bootstrap value of 100.

3.2. Effects of short-time salinity transfer (acute stress response)

Time courses of osmoregulatory and metabolic response of *S. aurata* to transfer to different environmental salinities are shown in Table 4. These parameters did not show variations in the control group (from SW to SW) along the time that experiment lasted. Plasma osmolality revealed a clear time-course increased in its values in those fish submitted to hyperosmotic transfer (from SW to HSW), showing a statistically increase (~12 %) on this parameter at the end of the trial. In addition, a significant decrease (~12-15 %) was observed in osmolality after hypoosmotic challenge (from SW to LSW) from 8 hours post-transfer compared with the control group. On the other hand, fish transferred to HSW showed a significant increase of around 55 to 65 % in plasma glucose, whereas in hypoosmotic transfer this enhancement was of around 35 to 40 %. In addition, plasma lactate did not show variations in any of the salinities tested in all experimental time.

Plasma cortisol levels rise in all groups tested, being significantly higher 4 hours after hyperosmotic transfer, while for control group and hypoosmotic challenge (from SW to LSW)

a significant enhancement was not produce till 8 hours post-transfer. Later, in control group, it returned to values from time 0 at 12 hours, remaining thus until the end of the experiment. At 24 hours post-transfer, cortisol levels dropped down to almost initial values in HSW group, but not for fish transferred to LSW (Figure 6).

Expression levels of both CRH and CRH-BP after osmotic challenge are shown in Figure 7. CRH mRNA expression presented similar time-course changes after LSW and HSW transfer. Thus, both groups showed an increase of around 50 % in mRNA expression levels respect to control group during all times tested, except at 8 hours where values close to the control group were observed. Regarding CRH-BP mRNA expression, all groups showed a similar pattern change at 4 hour post-transfer, increasing its values in a 50 %. After this time, control group remained unchanged until the end of experimental time. CRH-BP mRNA expression levels of fish submitted to hypoosmotic transfer showed a ~30 % increase in mRNA levels compared to control group at 12 hours, while under hyperosmotic condition enhanced ~60 and ~35 % its expression at 12 and 24 hours post-transfer, respectively.

Statistical values of *P*-value and *F* obtained from the two-way ANOVA analysis for all parameters tested in this sub-assay are shown in Table 5A.

3.3. Effects of starving and re-feeding situation (chronic stress response)

Time courses related to metabolic response of sea breams maintained under different feeding conditions are shown in Table 5. Plasma glucose did not show changes in fish maintained under normal fed conditions. Moreover, fish held under starving conditions significantly enhanced its values respect to the control group, although the highest plasma glucose values were observed in those fish re-fed during one week till day 21 (*P*-value: 0.041; *F*: 3.124). In contrast, plasma lactate only showed statistically higher levels in fish maintained food-deprived during 21 days (*P*-value: 0.047; *F*: 2.963).

Plasma cortisol levels did not change in fish fed with a daily ration of 1 % of their body mass and maintained as control group. However, fish submitted to starving situation significantly increased these values around 7- to 8-fold respect to the control group during the first 14 days of experiment, reaching the highest levels (11-fold) at the end of the trial (*P*-value: <0.001; *F*: 16.009). Moreover, re-feeding group during one week presented higher values respect to fasting group, being 20-fold higher than the control group at the same sampling point (Figure 8). CRH mRNA expression was unchanged in all the groups and time points tested (Figure 9A). On the other hand, only the starved group showed around ~50 % of decreased values in

CRH-BP expression levels after 21 days of food deprivation respect to de control group and the last time point (P -value: 0.031; F : 4.497) (Figure 9B).

Statistical values of P -value and F obtained from the two-way ANOVA analysis for all parameters tested in this sub-assay are shown in Table 5B.

4. Discussion

In this study, the full-length cDNA sequences of CRH and CRH-BP in the teleost species *S. aurata* was characterized, obtaining new tools to study their physiological roles in the acute and chronic stress responses.

4.1. Sea bream CRH and CRH-BP sequences

The cDNA sequence of *S. aurata* CRH involves 1,073 bp that translates into a peptide of 169 amino acids. This is comparable in length with other teleost species like tilapia mossambica (*Oreochromis mossambicus*) (167 amino acids), Senegalese sole (*Solea senegalensis*) (181 amino acids), zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) (both 162 amino acids) (van Enkevort et al., 2000, Huising et al., 2004; Wunderink et al., 2011). The CRH prohormone can be subdivided into 3 regions: the signal peptide, the cryptic motive and the mature peptide. *S. aurata* CRH prohormone appears to be between 42 % and 85 % identical to other vertebrates. However, the mature peptide shows up to 68 % identity, which indicates that the mature peptide is indeed the most important part of the hormone, namely the one involved in receptor-binding. Likewise, *S. aurata* CRH-BP is highly conserved. CRH-BP is known to be conserved throughout vertebrate and even invertebrate species (Huising and Flik, 2005), which underlines that CRH-BP might be as much as important in the stress response as CRH to control all the processes in which it is involved in. In addition, both CRH and CRH-BP are strongly conserved throughout evolution. Both genes can be found in virtually all vertebrates, and these genes can even be traced back as far as the insect lineage. Furthermore, Huising and Flik (2005) found CRH-BP sequence in Honeybee (*Apis mellifera*). This implies that the origin dates back more than 400 million years (Knecht et al., 2011) and underlines the importance of these genes, complemented by the structurally similar molecules involved.

4.2. Effects of salinity challenges

Hypoosmotic and hyperosmotic transfer induced changes in plasma osmolyte levels due to the existing imbalance between the environmental and internal medium of the animal (Laiz-

Carrión et al., 2005; Sangiao-Alvarellos et al. 2005a; Martos-Sitcha et al., 2013). Therefore, during the adaptative period after salinity challenge of *S. aurata* specimens plasma osmolality is disturbed, and an activation of several ion transporters located in different osmoregulatory organs (mainly gills, intestine and kidney) is expected in order to maintain or adjust their plasma osmolality within a certain range (Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al. 2005a; Martos-Sitcha et al., 2013). In addition, our results related to plasma glucose suggest the existence of an energetic reorganization that ensures the proper functioning of the osmoregulatory system, although no variations in lactate values were presumable in a short-time response (24 hours) due to this metabolite has been described as one of the most important metabolites during the chronic osmoregulatory period (Sangiao-Alvarellos et al., 2003, 2005a).

Moreover, this kind of acute stress agent activated HPI axis with early stimulation of CRH and CRH-BP, followed by a plasma cortisol level enhancement as well as a metabolic and osmoregulatory disorder. These data are in agreement with those obtained after acute stress experiment performed on *Cyprinus carpio* (Huisin et al., 2004), or even on *S. aurata* in which this kind of stress can trigger an enhancement in cortisol values (Arends et al., 1999; Sangiao-Alvarellos et al., 2005a). This hormone is also involved in other physiological processes such as osmoregulation and metabolism (Wendelaar Bonga, 1997; Mommsen et al., 1999; McCormick, 2001), which explain the metabolic and osmoregulatory reorganization observed. Fish in this experiment were maintained for 24 hours after transfer to both hypo- and hyper-osmotic environment. Thus, plasma cortisol significantly increased during at least the first 12 hours in both experimental transfers, indicating a primary stress response due to salinity changes, similarly as previously observed after the same salinity transfer in this species (Martos-Sitcha et al., 2013) and in *Solea senegalensis* (Herrera et al., 2012). In this regard, plasma cortisol values as well as brain CRH and CRH-BP mRNA expression levels showed a clear relationship in their values. Moreover, our results indicated a two-phase activation of HPI axis with a good correspondence between plasma cortisol levels and CRH and CRH-BP expression in the first moment after salinity transfer. Thus, just 4 hours post-transfer CRH and CRH-BP enhanced its mRNA levels, together with an increase in the cortisol release into the bloodstream. However, at 8 hours post-transfer, the highest cortisol values induced a clear negative feedback, which controls the down-regulation of both CRH and CRH-BP factors. On the other hand, the subsequent decrease of plasma cortisol levels (12 hours post-transfer) is most likely the result of a drop in CRH expression combined with the up-regulation of CRH-BP expression on the same sample-point in both extreme salinities.

Interestingly, at 24 hours (end point of experiment), fish submitted to hypoosmotic transfer presented the highest plasma cortisol values, while that under hyperosmotic condition returned to basal levels. This could reflect an osmoregulatory role for cortisol during adaptative phase in *S. aurata* transferred to hypoosmotic environments, and it agrees with the previously proposed hyperosmotic role for cortisol in this species increasing gill Na⁺,K⁺-ATPase activity, plasma osmolality, and ions after transfers from seawater to brackish water (Mancera et al., 2002). Both groups showed up-regulation of CRH expression but only hypoosmotic-transferred fish presented down-regulation of CRH-BP expression. These results suggested that a coordination between both hypothalamic factors are thus clearly involved in a fast regulation of plasma cortisol levels, inducing the strongly-pronounced, but short-lived, cortisol response typical in acute stress situations (Huising et al., 2004). The high fluctuation in CRH-BP expression compared to that in CRH expression might suggest that CRH-BP acts stronger as a modulator of the acute stress response than CRH does, as it has been suggested as well for the Senegalese sole (*S. senegalensis*) (Wunderink et al., 2011). Moreover, activation of the hypothalamo-pituitary axis, with CRH as the first player implicated, and the release of ACTH into the circulation by the pituitary is an integral part of the primary stress response of fish (Donaldson, 1981; Sumpter et al., 1986; Balm and Pottinger, 1995). Moreover, in the control group the lack of variations regarding with an expected increase in CRH mRNA at 4 h in agreement with the cortisol enhancement at 8 h could suggest that only handling stress required less amounts of stored protein (CRH), making that any additional gene transcription initiated on top of the constitutive gene expression will remain undetectable, although a contribution of daily rhythms on HPI-axis cannot be ruled out (Montoya et al, 2010).

4.3. Effects of food deprivation

Studies assessing effects of food deprivation on stress axis in adult fish are scarce. Metabolic reorganization after a prolonged stress source is expected due to the need to maintain vital functions in the organisms. In fact, the reorganization observed in those fish maintained food deprived is different compared with those submitted to an acute stress process (see above). Thus, sea breams maintained under starvation revealed an enhancement in their plasma glucose levels during the time that experiment lasted, together with a substantial increase in lactate at the end of the trial. This fact demonstrated that i) food deprivation produced a metabolic imbalance, and ii) re-feeding returned lactate concentration close to the control values, but glucose remained enhanced as a consequence of high cortisol values (see below)

that probably produced higher glycogenolytic activity rates in several important metabolic organs as liver, as has been previously demonstrated after different chronic stress situations, including food deprivation (Sangiao-Alvarellos et al., 2003, 2005b).

Moreover, in this study food-deprived *S. aurata* enhanced plasma cortisol levels. Likewise, elevated whole-body cortisol concentrations were found in zebrafish as a result of crowding and food deprivation (Ramsay et al., 2006), and reduced stress resistance was demonstrated in food-deprived Atlantic cod (*Gadus morhua*) (Olsen et al., 2008). Similarly, food-deprived *S. senegalensis* juveniles significantly enhanced plasma cortisol levels (Costas et al., 2011a). In addition, during early development, food-deprived *S. senegalensis* larvae showed an increase in whole-body cortisol levels, as the result of an up-regulation of CRH expression and a downregulation of CRH-BP expression (Wunderink et al., 2012). However, the lack of variation in CRH mRNA expression as well as the down-regulation of CRH-BP values suggests that, in *S. aurata* exposed to a long period of food deprivation, plasma cortisol level could be regulated by both hypothalamic factors due to the putative lower regulation by the soluble binding protein. Even so, specific changes in CRH-BP mRNA levels could varied in each brain region depending on the stressor applied (Alderman et al., 2008), so a more comprehensive study addressing i) each portion of the brain, deal with ii) different sources of stress would be necessary to clarify the limited changes observed in our results.

In the re-fed group, fish showed the highest values of plasma cortisol and glucose together with a lack of variation in CRH and CRH-BP mRNA expression. Although these results could be a paradigm, the existence of such high values of cortisol could be explained by several situations: i) the existence of a permanent state of alert to a situation of re-feeding after a prolonged starving period (Uchida et al., 2003); ii) the stimulation of food intake by cortisol (Bernier et al., 2004), where this hormone would act on food intake regulation enhancing the stress recovery after food deprivation (Mommsen et al., 1999, Bernier et al., 2004); or iii) the important role of cortisol during the metabolism reorganization (Mommsen et al., 1999).

Moreover, the absence of changes in CRH expression suggests that those processes focused in cortisol production and release could be carried out through a different pathway. In fact, other hormones and factors than just CRH and CRH-BP have been already described as putative players involved in the stress response (Majzoub, 2006; Bernier et al., 2009), and the use of CRH as a regulator of stress during food deprivation is somewhat of a paradox, since CRH also acts as anorexigenic peptide (Uehara et al., 1998). Potential candidates to direct the stress response independently of CRH are TRH through activating α -MSH (Lamers et al., 1991; Rotlland et al., 2000; Van der Salm et al., 2004), and AVT nonapeptide that also stimulates

the release of ACTH (Baker et al., 1996). Thus, α -MSH is a key player in the neuroendocrine stress response, depending on the type and source of the stressor (Wendelaar Bonga et al., 1995). Even so, the corticotrope activity of α -MSH is relatively weak (100 times less potent) compared to that of ACTH (Wendelaar Bonga et al., 1995). Moreover AVT binding sites have been described to be located in the zones occupied by corticotroph cells in *Dicentrarchus labrax* (Moons et al., 1989) and *Catostomus commersoni* (Yulis and Lederis, 1987). In addition, the *in vitro* co-administration of AVT/AVP and CRF stimulate ACTH secretion in preparations *in vitro* (Baker et al., 1996). Furthermore, AVT treatment plus hypo- and hyperosmotic transfer enhanced plasma cortisol levels in *S. aurata*, suggesting a role of AVT on stress axis activation in this species (Sangiao-Alvarellos et al., 2006). Recently, Martos-Sitcha et al. (2013) demonstrated in *S. aurata* that pro-vasotocin mRNA synthesis and pituitary storage of mature hormone is involved in the regulation of stress process after salinity challenges, and also that food deprivation enhanced AVT storage in the pituitary gland, suggesting that this hormone could acts as a paracrine factor on the ACTH cells (Gesto et al., 2014).

5. Conclusions

Both, CRH and CRH-BP cDNA sequences were cloned in *S. aurata*. Their phylogenetic and sequence analysis showed good gene conservation throughout evolution. Moreover, the dynamics of change of osmoregulatory and metabolic parameters after two different sources of stress (osmotic challenge –acute-, or food deprivation –chronic-) conditions confirmed the internal derangement of the animals and its control mediated by the endocrine system.

Thus, the mRNA expression of these hormones, together with these changes reported on plasma cortisol levels, indicated that the cortisol enhancement observed can be controlled by different pathways, in which CRH seems to be regulated by CRH-BP during the acute stress response, whereas during chronic stress (food deprivation) it could be controlled by other factors acting as modulators (AVT or TRH hormones, among others). Even so, the impossibility to discriminate variations in hypothalamic neurons alone could skew these results in a complex endocrine system in which different pathways could regulate its proper operation depending on the stressor. Moreover, the sequence in which stressors (acute or chronic) occurs can produce different responses in this endocrine system as it has been previously reported in *S. senegalensis* (Wunderink et al., 2011).

Acknowledgements

The authors wish to thank *Planta de Cultivos Marinos* (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish. Experiment has been carried out at the *Campus de Excelencia Internacional del Mar* (CEI-MAR) facilities from the University of Cádiz. Study funded by project AGL2010-14876 from Ministerio de Ciencia e Innovación to J.M.M. (Spain). J.A.M-S was supported by a PhD fellowship (FPU, Reference AP2008-01194) from Ministry of Education (Spain).

References

- Alderman SL, Raine JC, Bernier NJ (2008) Distribution and regional stressor-induced regulation of corticotrophin-releasing factor binding protein in rainbow trout (*Oncorhynchus mykiss*). *Journal of Neuroendocrinology* 20:347-358.
- Arends RJ, Mancera JM, Muñoz JL, Wendelaar Bonga SE, Flik G (1999) The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *Journal of Endocrinology* 163:149-157.
- Arjona FJ, Vargas-Chacoff L, Martín del Río MP, Flik G, Mancera JM, Klaren PHM (2008) The involvement of thyroid hormones and cortisol in the osmotic acclimation of *Solea senegalensis*. *Gen Comp Endocrinol* 155:796-803.
- Arjona FJ, Vargas-Chacoff L, Ruiz-Jarabo I, Martín del Río MP, Mancera JM (2007) Osmoregulatory response of Senegalese sole (*Solea senegalensis*) to changes in environmental salinity. *Comp Biochem Physiol A* 148:413-421.
- Ashley PJ (2007) Fish welfare: Current issues in aquaculture. *Appl Anim Behav Sci* 104:199-235.
- Baker BI, Bird DJ, Buckingham JC (1996) In the trout, CRH and AVT synergize to stimulate ACTH release. *Regul Pept* 67:207-210.
- Balm PHM, Pottinger TG (1995) Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* 98:279-88.
- Balmaceda-Aguilera C, Martos-Sitcha JA, Mancera JM, Martinez-Rodriguez G (2012) Cloning and expression pattern of facilitative glucose transporter 1 (GLUT1) in gilthead sea bream *Sparus aurata* in response to salinity acclimation. *Comp Biochem Physiol A Mol Integr Physiol* 163:38-46.
- Barry TP, Lapp AF, Kayes TB, Malison JA (1993). Validation of a microplate ELISA for measuring cortisol in fish and comparison of stress responses of rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*). *Aquaculture* 117:351-363.

554 Bernier NJ, Bedard N, Peter RE (2004) Effects of cortisol on food intake, growth, and forebrain
 555 neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. *Gen Comp*
 556 *Endocrinol* 135:230-240.

557 Bernier NJ, Flik G, Klaren PHM (2009) Regulation and contribution of the corticotropic, melanotropic
 558 and thyrotropic axes to the stress response in fishes. *In: Fish Neuroendocrinology* N.J.
 559 Bernier, G. Van Der Kraak, A.P. Farrel and C.J. Brauner (Eds.). Academic Press, 236-312.

560 Blom S, Andersson TB, Forlin L (2000) Effects of food deprivation and handling stress on head
 561 kidney 17 α -hydroxyprogesterone 21-hydroxylase activity, plasma cortisol and the
 562 activities of liver detoxification enzymes in rainbow trout. *Aquat Toxicol* 48:265-274.

563 Conte FS (2004) Stress and the welfare of cultured fish. *Appl Anim Behav Sci* 86:205-223.

564 Conti LH (2012) Interactions between corticotropin-releasing factor and the serotonin 1A receptor
 565 system on acoustic startle amplitude and prepulse inhibition of the startle response in two rat
 566 strains. *Neuropharmacology* 62:256-263.

567 Costas B, Aragão C, Ruiz-Jarabo I, Vargas-Chacoff L, Arjona FJ, Dinis MT, Mancera JM, Conceição
 568 LEC (2011a) Feed deprivation in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles:
 569 Effects on blood plasma metabolites and free amino acid levels. *Fish Physiol Biochem*
 570 37:495-504.

571 Costas B, Conceição LEC, Aragão C, Martos JA, Ruiz-Jarabo I, Mancera JM, Afonso A (2011b)
 572 Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress
 573 challenge: Effects on non-specific immune parameters, plasma free amino acids and energy
 574 metabolism. *Aquaculture* 316:68-76.

575 Donaldson EM (1981) The pituitary-interrenal axis as an indicator of stress in fish. *In: Pickering AD,*
 576 *editor. Stress and Fish. London: Academic Press, 11–48.*

577 Doyon C, Trudeau VL, Moon TW (2005) Stress elevates corticotropin-releasing factor (CRF) and
 578 CRF-binding protein mRNA levels in rainbow trout (*Oncorhynchus mykiss*). *J Endocrinol*
 579 186:123-130.

580 Ellis T, North B, Scott AP, Bromage NR, Porter M, Gadd D (2002) The relationships between
 581 stocking density and welfare in farmed rainbow trout. *J Fish Biol* 61:493-531.

582 Felsenstein J, (1985) Confidence-limits on phylogenies – an approach using the bootstrap. *Evolution*
 583 39:783–791.

584 Ferlazzo A, Carvalho ES, Gregorio SF, Power DM, Canario AV, Trischitta F, Fuentes J (2012)
 585 Prolactin regulates luminal bicarbonate secretion in the intestine of the sea bream (*Sparus*
 586 *aurata* L.). *J Exp Biol* 215:3836-3844.

587 Flik G, Klaren PHM, van den Burg EH, Metz JR, Huising MO (2006) CRF and stress in fish. *Gen*
 588 *Comp Endocrinol* 146:36-44.

589 Fuentes J, Brinca L, Guerreiro PM, Power DM (2010a) PRL and GH synthesis and release from the
 590 sea bream (*Sparus auratus* L.) pituitary gland in vitro in response to osmotic challenge. *Gen*
 591 *Comp Endocrinol* 168:95-102.

592 Fuentes J, Figueiredo J, Power DM, Canário AVM (2006) Parathyroid hormone-related protein
 593 regulates intestinal calcium transport in sea bream (*Sparus auratus*). *American Journal of*
 594 *Physiology - Regulatory Integrative and Comparative Physiology* 291:R1499-R1506.

595 Fuentes J, Power DM, Canário AVM (2010b) Parathyroid hormone-related protein-stanniocalcin
 596 antagonism in regulation of bicarbonate secretion and calcium precipitation in a marine fish
 597 intestine. *American Journal of Physiology - Regulatory Integrative and Comparative*
 598 *Physiology* 299:R150-R158.

599 Gesto, M., Soengas, J.L., Rodríguez-Illamola, A. and Míguez, J.M. (2014) Arginine vasotocin
 600 treatment induces a stress response and exerts a potent anorexigenic effect in rainbow trout,
 601 *Oncorhynchus mykiss*. *Journal of Neuroendocrinology* 26, 89-99

602 Herrera M, Aragão C, Hachero I, Ruiz-Jarabo I, Vargas-Chacoff L, Mancera JM, Conceição LEC
 603 (2012) Physiological short-term response to sudden salinity change in the Senegalese sole
 604 (*Solea senegalensis*). *Fish Physiology and Biochemistry* 38:1741-1751.

605 Huising MO, Flik G (2005) The remarkable conservation of corticotropin-releasing hormone (CRH)-
 606 binding protein in the honeybee (*Apis mellifera*) dates the CRH system to a common ancestor
 607 of insects and vertebrates. *Endocrinology* 146:2165-2170.

608 Huising MO, Metz JR, van Schooten C, Taverne-Thiele AJ, Hermesen T, Verburg-van Kemenade BM,
 609 Flik G (2004) Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP
 610 and CRH-R1, and the role of these proteins in the acute stress response. *J Mol Endocrinol*
 611 32:627-648.

612 Huising MO, Vaughan JM, Shah SH, Grillot KL, Donaldson CJ, Rivier J, Flik G, Vale WW (2008)
 613 Residues of corticotropin releasing factor-binding protein (CRF-BP) that selectively abrogate
 614 binding to CRF but not to urocortin 1. *J Biol Chem* 283:8902-8912.

615 Knecht RJ, Engel MS, Bennera JS (2011) Late Carboniferous paleoichnology reveals the oldest full-
 616 body impression of a flying insect. *Proceedings of the National Academy of Sciences of the*
 617 *United States of America* 108:6515-6519.

618 Laiz-Carrion R, Guerreiro PM, Fuentes J, Canario AV, Martin Del Rio MP, Mancera JM (2005)
 619 Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. *J Exp*
 620 *Zool A Comp Exp Biol* 303:563-576.

621 Laiz-Carrión R, Sangiao-Alvarellos S, Guzmán JM, Martín del Río MP, Míguez JM, Soengas JL,
 622 Mancera JM (2002) Energy metabolism in fish tissues related to osmoregulation and cortisol
 623 action. *Fish physiology and biochemistry* 27:179-188.

624 Lamers AE, Balm PH, Haenen HE, Jenks BG, Wendelaar Bonga SE (1991) Regulation of differential
625 release of alpha-melanocyte-stimulating hormone forms from the pituitary of a teleost fish,
626 *Oreochromis mossambicus*. J Endocrinol 129:179-187.

627 Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F,
628 Wallace IM, Wilm A, Lopez R, et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics
629 23:2947–2948.

630 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using Real-Time
631 Quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. Methods 25:402-408.

632 Majzoub JA (2006) Corticotropin-releasing hormone physiology. Eur J Endocrinol 155:S71-S76.

633 Mancera JM, Perez-Figares JM, Fernandez-Llebrez P (1993) Osmoregulatory responses to abrupt
634 salinity changes in the euryhaline gilthead sea bream (*Sparus aurata* L.). Comp Biochem
635 Physiol A 106, 245-250.

636 Mancera JM, Laiz-Carrión R, Martín del Río MP (2002) Osmoregulatory action of PRL, GH and
637 cortisol in the gilthead seabream (*Sparus aurata* L.) Gen Comp Endocrinol 129:95-103.

638 Mancera JM, Vargas-Chacoff L, García-López A, Kleszczynska A, Kalamarz H, Martínez-Rodríguez
639 G, Kulczykowska E (2008) High density and food deprivation affect arginine vasotocin,
640 isotocin and melatonin in gilthead sea bream (*Sparus auratus*). Comp Biochem Physiol A
641 149:92-97.

642 Martos-Sitcha JA, Wunderink YS, Gozdowska M, Kulczykowska E, Mancera JM, Martinez-
643 Rodriguez G (2013) Vasotocinergic and isotocinergic systems in the gilthead sea bream
644 (*Sparus aurata*): An osmoregulatory story. Comp Biochem Physiol A Mol Integr Physiol
645 166:571-581.

646 McCormick SD (2001) Endocrine control of osmoregulation in teleost fish. American Zoologist
647 41:781–794

648 Mills SC, Mourier J, Galzin R (2010) Plasma cortisol 11-ketotestosterone enzyme immunoassay (EIA)
649 kit validation for three fish species: the orange clownfish *Amphiprion percula*, the orangefin
650 anemonefish *Amphiprion chrysopterus* and the blacktip reef shark *Carcharhinus melanopterus*.
651 J Fish Biol 77:769-777.

652 Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: Dynamics, mechanisms of action,
653 and metabolic regulation. Rev Fish Biol Fish 9:211-268.

654 Montoya A, López-Olmeda JF, Garayzar ABS, Sánchez-Vázquez FJ. (2010). Synchronization of daily
655 rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in
656 gilthead seabream (*Sparus aurata*) under a light-dark cycle. Physiol Behav 101:101-107.

657 Moons L, Cambre M, Batten TF, Vandesande F (1989) Autoradiographic localization of binding sites
658 for vasotocin in the brain and pituitary of the sea bass (*Dicentrarchus labrax*). Neurosci Lett
659 100:11-16.

660 Olsen RE, Sundell K, Ringø E, Myklebust R, Hemre GI, Hansen T, Karlsen Ø (2008) The acute stress
661 response in fed and food deprived Atlantic cod, *Gadus morhua* L. Aquaculture 280:232-241.

662 Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB (2006) Whole-body cortisol
663 is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture 258:565-574.

664 Rodríguez L, Begtashi I, Zanuy S, Carrillo M (2000) Development and validation of an enzyme
665 immunoassay for testosterone: Effects of photoperiod on plasma testosterone levels and
666 gonadal development in male sea bass (*Dicentrarchus labrax*, L.) at puberty. Fish Physiology
667 and Biochemistry 23:141-150.

668 Rotllant J, Balm PHM, Perez-Sanchez J, Wendelaar-Bonga SE, Tort L (2001) Pituitary and interrenal
669 function in gilthead sea bream (*Sparus aurata* L., Teleostei) after handling and confinement
670 stress. Gen Comp Endocrinol 121:333-342.

671 Rotllant J, Balm PHM, Ruane NM, Perez-Sanchez J, Wendelaar Bonga SE, Tort L (2000) Pituitary
672 proopiomelanocortin-derived peptides and hypothalamus-pituitary-interrenal axis activity in
673 gilthead sea bream (*Sparus aurata*) during prolonged crowding stress: Differential regulation
674 of adrenocorticotropin hormone and alpha-melanocyte-stimulating hormone release by
675 corticotropin-releasing hormone and thyrotropin-releasing hormone. Gen Comp Endocrinol
676 119:152-163.

677 Rotllant J, Tort L (1997) Cortisol and glucose responses after acute stress by net handling in the sparid
678 red porgy previously subjected to crowding stress. J Fish Biol 51:21-28.

679 Ruane NM, Carballo EC, Komen J (2002) Increased stocking density influences the acute
680 physiological stress response of common carp *Cyprinus carpio* (L.). Aquacult Res 33:777-
681 784.

682 Saitou N, Nei M (1987) The neighbor-joining method – a new method for reconstructing phylogenetic
683 trees. Mol. Biol. Evol. 4:406–425.

684 Sangiao-Alvarellos S, Laiz-Carrión R, Guzmán JM, Martín del Río MP, Míguez JM, Mancera JM,
685 Soengas JL (2003) Acclimation of *S. aurata* to various salinities alters energy metabolism of
686 osmoregulatory and nonosmoregulatory organs. Am J Physiol Regul Integr Comp Physiol
687 285:R897-R907.

688 Sangiao-Alvarellos S, Arjona FJ, Martín del Río MP, Míguez JM, Mancera JM, Soengas JL (2005a)
689 Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus*
690 *auratus*. J Exp Biol 208:4291-4304.

691 Sangiao-Alvarellos S, Guzmán JM, Laiz-Carrión R, Míguez JM, Martín del Río MP, Mancera JM,
692 Soengas JL (2005b) Interactive effects of high stocking density and food deprivation on
693 carbohydrate metabolism in several tissues of gilthead sea bream *Sparus auratus*. J Exp Zool
694 303A:761-775

695 Sangiao-Alvarellos S, Polakof S, Arjona FJ, Kleszczynska A, Martín del Río MP, Míguez JM,
696 Soengas JL, Mancera JM (2006) Osmoregulatory and metabolic changes in the gilthead sea

697 bream *Sparus auratus* after arginine vasotocin (AVT) treatment. Gen. Comp. Endocrinol.
698 148:348–358.

699 Sumpter JP, Dye HM, Benfey TJ (1986) The effects of stress on plasma ACTH, a-MSH, and cortisol
700 levels in salmonid fishes. Gen Comp Endocrinol 62:377–85.

701 Sumpter JP, Le Bail PY, Pickering AD, Pottinger TG, Carragher JF (1991) The effect of starvation on
702 growth and plasma growth hormone concentrations of rainbow trout, *Oncorhynchus mykiss*.
703 Gen Comp Endocrinol 83:94-102.

704 Takei Y, McCormick SD (2013) Hormonal control of fish euryhalinity. In: *Euryhaline Fish* S.D.
705 McCormick, A. P. Farrell and C. J. Brauner (Eds.). Academic Press, 69-124.

706 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular
707 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and
708 maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739.

709 Tusnady GE, Simon I (1998) Principles governing amino acid composition of integral membrane
710 proteins: application to topology prediction. J Mol Biol 283:489-506.

711 Tusnady GE, Simon I (2001) The HMMTOP transmembrane topology prediction server.
712 Bioinformatics 17:849-850.

713 Uchida K, Kajimura S, Riley LG, Hirano T, Aida K, Grau EG (2003) Effects of fasting on growth
714 hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis mossambicus*. Comp
715 Biochem Physiol A Mol Integr Physiol 134:429-439.

716 Uehara Y, Shimizu H, Ohtani K, Sato N, Mori M (1998) Hypothalamic corticotropin-releasing
717 hormone is a mediator of the anorexigenic effect of leptin. Diabetes 47:890-893.

718 Vale W, Spiess J, Rivier C, Rivier J (1981) Characterization of a 41-residue ovine hypothalamic
719 peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213:1394-1397.

720 Van der Salm AL, Pavlidis M, Flik G, Wendelaar Bonga SE (2004) Differential release of alpha-
721 melanophore stimulating hormone isoforms by the pituitary gland of red porgy, *Pagrus*
722 *pagrus*. Gen Comp Endocrinol 135, 126-133.

723 van Enkevort FHJ, Pepels PPLM, Leunissen JAM, Martens GJM, Wendelaar Bonga SE, Balm PHM
724 (2000) *Oreochromis mossambicus* (tilapia) corticotropin-releasing hormone: cDNA sequence
725 and bioactivity. J Neuroendocrinol 12:177-186.

726 Vijayan MM, Maule AG, Schreck CB, Moon TW (1993) Hormonal control of hepatic glycogen
727 metabolism in food deprived, continuously swimming coho salmon (*Oncorhynchus kisutch*).
728 Canadian Journal of Fisheries and Aquatic Sciences 50:1676-1682.

729 Wendelaar Bonga SE (1997) The stress response in fish. Physiol Rev 77:591-625.

730 Wendelaar Bonga SE, Balm PHM, Lamers AE (1995) The involment of ACTH and MSH in the
731 stress-response in teleost fish. Neth J Zool 45:103-106.

732 Wunderink YS, Engels S, Halm S, Yúfera M, Martínez-Rodríguez G, Flik G, Klaren PHM, Mancera
 733 JM (2011) Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): The
 734 involvement of cortisol, CRH and CRH-BP. *Gen Comp Endocrinol* 171:203-210.
 735 Wunderink YS, Martinez-Rodriguez G, Yufera M, Martin Montero I, Flik G, Mancera JM, Klaren PH
 736 (2012) Food deprivation induces chronic stress and affects thyroid hormone metabolism in
 737 Senegalese sole (*Solea senegalensis*) post-larvae. *Comp Biochem Physiol A Mol Integr*
 738 *Physiol* 162:317-322.
 739 Yayou K, Kitagawa S, Ito S, Kasuya E, Sutoh M (2011) Effect of oxytocin, prolactin-releasing
 740 peptide, or corticotropin-releasing hormone on feeding behavior in steers. *Gen Comp*
 741 *Endocrinol* 174:287-291.
 742 Yulis CR, Lederis K (1987) Co-localization of the immunoreactivities of corticotropin-releasing factor
 743 and arginine vasotocin in the brain and pituitary system of the teleost *Catostomus*
 744 *commersoni*. *Cell Tissue Res* 247:267-273.
 745
 746

Tables

Degenerate primers	Nucleotide sequence	Amplicon length
<i>CRH-BP_Fw1</i>	5'-CARTTYACMTTCACAGCAGA-3'	718 bp
<i>CRH-BP_Rv1</i>	5'-CARGAGCTRCAGRYGATYAA-3'	
<i>CRH-BP_Fw2</i>	5'-GTRTTYGAYTGGGTGATGAA-3'	501 bp
<i>CRH-BP_Rv2</i>	5'-ATGAARRTYGGYTGTGAYAAC-3'	

Table 1. Nucleotide sequences of degenerate primers designed for molecular identification of CRH-BP partial cDNA sequence, and size amplified by each pair of primers.

Primer	Nucleotide sequence	Amplicon length
<i>qCRH_Fw</i>	5'-ATGGAGAGGGGAAGGAGGT-3'	176 bp
<i>qCRH_Rv</i>	5'-ATCTTTGGCGGACTGGAAA-3'	
<i>qCRH-BP_Fw</i>	5'-GCAGCTTCTCCATCATCTACC-3'	147 bp
<i>qCRH-BP_Rv</i>	5'-ACGTGTCGATACCGCTTCC-3'	
<i>qb-actin_Fw</i>	5'-TCTTCCAGCCATCCTTCCTCG-3'	108 bp
<i>qb-actin_Rv</i>	5'-TGTTGGCATAACAGGTCCTTACGG-3'	

Table 2. Nucleotide sequences of specific primers designed for qPCR analysis and size amplified by each pair of primers.

A) CRH	<i>S. aurata</i>	<i>S. senegalensis</i>	<i>C. carpio</i>	<i>D. rerio</i>	<i>H. sapiens</i>	<i>M. musculus</i>	<i>G. gallus</i>	<i>X. laevis</i>
<i>S. aurata</i>	100							
<i>S. senegalensis</i>	85 (78)	100						
<i>C. carpio</i>	61 (78)	55 (60)	100					
<i>D. rerio</i>	62 (78)	55 (63)	95 (97)	100				
<i>H. sapiens</i>	49 (75)	44 (68)	59 (90)	53 (92)	100			
<i>M. musculus</i>	46 (75)	41 (68)	50 (85)	51 (92)	79 (100)	100		
<i>G. gallus</i>	49 (75)	46 (68)	48 (90)	48 (92)	79 (100)	57 (100)	100	
<i>X. laevis</i>	42 (68)	45 (60)	47 (85)	48 (87)	53 (92)	50 (92)	57 (92)	100

777

B) CRH-BP	<i>S. aurata</i>	<i>S. senegalensis</i>	<i>C. carpio</i>	<i>D. rerio</i>	<i>H. sapiens</i>	<i>M. musculus</i>	<i>G. gallus</i>	<i>X. laevis</i>
<i>S. aurata</i>	100							
<i>S. senegalensis</i>	60	100						
<i>C. carpio</i>	69	59	100					
<i>D. rerio</i>	69	61	97	100				
<i>H. sapiens</i>	57	53	62	63	100			
<i>M. musculus</i>	55	53	60	61	87	100		
<i>G. gallus</i>	58	55	60	61	74	75	100	
<i>X. laevis</i>	56	54	59	61	68	67	74	100

778 **Table 3.** A) Alignments scores of amino acid sequence identity for CRH (A) and CRH-BP (B) sequences of various vertebrate species. For CRH,
779 the identities were given for the complete sequence and for the mature peptide (in parentheses).

780

781

Metabolite	Treatment	0 hours	4 hours	8 hours	12 hours	24 hours
<i>Osmolality</i> (<i>mOsm·kg⁻¹</i>)	SW → LSW		317.3 ± 3.4 ^{ab,*}	289.5 ± 2.4 ^{b,*}	295.3.6 ± 8.6 ^{b,*}	297.1 ± 6.4 ^{b,*}
	SW → SW	336.2 ± 12.5 ^a	340.5 ± 6.1 ^a	335.1 ± 3.9 ^a	336.2 ± 2.1 ^a	335.5 ± 3.2 ^a
	SW → HSW		346.1 ± 5.4 ^{ab}	343.5 ± 10.3 ^{ab,#}	353.2 ± 11.4 ^{ab,#}	373.1 ± 5.6 ^{b,#}
<i>Glucose</i> (<i>mM</i>)	SW → LSW		11.248 ± 0.244 ^{b,*}	11.406 ± 0.680 ^{b,*}	11.693 ± 0.687 ^{b,*}	11.111 ± 0.686 ^{b,*}
	SW → SW	8.143 ± 0.078 ^a	8.122 ± 0.377 ^a	8.445 ± 0.485 ^a	8.319 ± 0.134 ^a	8.325 ± 0.184 ^a
	SW → HSW		12.665 ± 1.054 ^{b,*}	13.639 ± 1.132 ^{b,*}	13.626 ± 1.272 ^{b,*}	13.402 ± 0.586 ^{b,*}
<i>Lactate</i> (<i>mM</i>)	SW → LSW		0.416 ± 0.018 ^a	0.365 ± 0.009 ^a	0.413 ± 0.032 ^a	0.402 ± 0.029 ^a
	SW → SW	0.397 ± 0.016 ^a	0.390 ± 0.026 ^a	0.398 ± 0.040 ^a	0.392 ± 0.027 ^a	0.390 ± 0.011 ^a
	SW → HSW		0.396 ± 0.011 ^a	0.395 ± 0.027 ^a	0.419 ± 0.035 ^a	0.383 ± 0.031 ^a

Table 4. Time course changes in plasma osmolality and metabolite (glucose and lactate) levels after transfer from SW to different environmental salinities (LSW, SW and HSW). Values are represented as mean ± S.E.M. (n = 7-8 fish per group). Significant differences between sampling points at the same salinity are identified with different letters; different symbols show differences between groups at the same time (P<0.05, two-way ANOVA followed by Tukey's test).

Metabolite	Treatment	Day 0	Day 7	Day 14	Day 21
<i>Glucose</i> (mM)	<i>Control</i>	4.844 ± 0.095 ^a	4.838 ± 0.204 ^a	4.872 ± 0.172 ^a	4.759 ± 0.164 ^a
	<i>Starved</i>		5.297 ± 0.173 ^b	5.351 ± 0.212 ^b	5.374 ± 0.204 ^{b*}
	<i>Re-fed</i>				5.535 ± 0.276 [*]
<i>Lactate</i> (mM)	<i>Control</i>	2.908 ± 0.199 ^a	3.038 ± 0.154 ^a	2.775 ± 0.110 ^a	2.833 ± 0.261 ^a
	<i>Starved</i>		2.982 ± 0.183 ^a	2.826 ± 0.224 ^a	3.637 ± 0.467 ^{b*}
	<i>Re-fed</i>				2.589 ± 0.213 ^a

Table 5. Time course changes in plasma metabolite (glucose and lactate) levels in fish maintained under feeding, food deprivation and re-feeding situations. Values are represented as mean ± S.E.M. (*n* = 10-12 fish per group). Significant differences among sampling points at the same condition are identified with different letters; different symbols show differences between groups at the same time (*P*<0.05, one-way ANOVA followed by Tukey's test or Student t-test, in each case).

A	Time		Salinity		Interaction	
Parameter	P-value	F	P-value	F	P-value	F
Osmolality	0.015	3.315	<0.001	66.670	<0.001	6.989
Glucose	<0.001	13.309	<0.001	43.28	0.002	12.410
Lactate	0.871	0.309	0.940	0.061	0.971	0.278
Cortisol	<0.001	32.131	<0.001	17.362	<0.001	8.544
CRH	0.005	5.660	0.001	7.487	0.594	0.812
CRH-BP	<0.001	14.481	0.039	3.356	0.017	2.512
B	Time		Fed condition		Interaction	
Parameter	P-value	F	P-value	F	P-value	F
Glucose	0.032	3.937	0.008	7.397	0.021	6.278
Lactate	0.002	4.988	0.032	3.937	0.465	0.872
Cortisol	0.002	5.222	<0.001	31.932	0.003	5.143
CRH	0.315	1.213	0.116	2.557	0.575	0.668
CRH-BP	0.011	4.233	0.743	0.109	0.724	0.443

792 **Table 6.** Statistical parameters (*P*-value and *F*) obtained from two-way ANOVA analysis in
793 fish transferred to different environmental salinities in a short-time response (A) or in fish
794 maintained under different feeding situations (B).

Legends to Figures

Figure 1. Nucleotide and deduced amino acid sequences of the sea bream (*S. aurata*) CRH cDNA. The start and stop codon are presented in bold, underlined and italic. ORF is highlighted in italic and underlined. The deduced amino acid sequence is displayed above the nucleotide sequence. The predicted signal peptide M¹-A²⁴ and the conserved cryptic motif R⁵⁵-N⁶⁶ are indicated in bold capitals. Predicted mature peptide S¹²⁷-F¹⁶⁷ is presented in bold and underlined. The cleavage site and C-terminal amidation site are both underlined. Accession number: KC195964.

Figure 2. Comparison of CRH amino acid sequences of four fish species [*Sparus aurata* (AGO05917), *Solea senegalensis* (CBY78066), *Cyprinus carpio* (CAC84859) and *Danio rerio* (ABS86029)], two of mammals [*Homo sapiens* (AAH11031) and *Mus musculus* (AAI19037)], one of birds [*Gallus gallus* (CAF18561)] and one of amphibians [*Xenopus laevis* (P49188)]. Alignment was carried out by ClustalW2 software (Larkin et al., 2007). Gaps marked by hyphens have been inserted to optimize homology. Identical amino acid residues are indicated in black. Signal peptide, cryptic motif and mature hormone structures are noted behind the amino acid residues alignment.

Figure 3. Nucleotide and deduced amino acid sequences of the sea bream (*S. aurata*) CRH-BP cDNA. The start and stop codon are presented in bold, underlined and italic. ORF is marked in italic and underlined. The deduced amino acid sequence is displayed above the nucleotide sequence. The predicted signal peptide M¹-C²⁶ is indicated in bold capitals. The ten cysteines involved in the formation of five C-C disulfide bonds are boxed, underlined and in bold. R⁵⁹ and D⁶⁵, probably implicated in ligand-binding with CRH are underlined and indicated in bold capitals. Accession number: KC195965.

Figure 4. Comparison of CRH-BP amino acid sequences of four fish species [*Sparus aurata* (AGO05918), *Solea senegalensis* (CBY78067), *Cyprinus carpio* (CAD35748) and *Danio rerio* (NP_001003459)], two of mammals [*Homo sapiens* (NP_001873) and *Mus musculus* (AAH61247)], one of birds [*Gallus gallus* (XP_003643006)] and one of amphibians [*Xenopus laevis* (NP_001079273)]. Alignment was carried out by ClustalW2 software (Larkin et al., 2007). Gaps marked by hyphens have been inserted to optimize homology. Conserved cysteine residues (essential for protein folding) are presented underlined, in bold, italics, and

highlighted in grey. Curved lines behind cysteine residues represent the formation of disulphide bonds. R⁵⁹ and D⁶⁵, probably implicated in ligand-binding with CRH, are in italics and double underlined. Identical amino acid residues are indicated in black.

Figure 5. Phylogenetic tree of CRH-like and CRH-BP amino acid sequences from several fish species, including the sea bream (*Sparus aurata*), as well as amphibians, birds, mammals and insects using Neighbor-Joining analysis and based on amino acid difference (p-distance). Reliability of the tree was assessed by bootstrapping (1,000 replicates). GenBank and NCBI Reference Sequences accession numbers are as follows: *Sparus aurata* CRH (AGO05917) and CRH-BP (AGO05918); *Oreochromis mossambicus* CRH (CAB77056); *Solea senegalensis* CRH (CBY78066) and CRH-BP (CBY78067); *Danio rerio* CRH (ABS86029), UI (NP_001025351), UII (NP_998013) and CRH-BP (NP_001003459); *Cyprinus carpio* CRH (CAC84859), UI (AAA49214) and CRH-BP (CAD35748); *Oryzias latipes* UI (BAG16734), UcnII (BAG16730) and UcnIII (BAG16732); *Platichthys flesus* UI (CAD56906) and UII (CAD56908); *Takifugu rubripes* CRH-BP (CAF18402); *Salmo salar* CRH-BP (ACN11242); *Osmerus mordax* CRH-BP (ACO09096); *Xenopus laevis* CRH (P49188), UcnI (NP_001086429), UcnIII (AAT70727), UII (NP_001267509) and CRH-BP (NP_001079273); *Spea hammondi* CRH (AAP20883); *Rana sylvatica* CRH (AEQ37345); *Gallus gallus* CRH (CAF18561); UcnIII (AGC65587), UII (NP_996873) and CRH-BP (XP_003643006); *Bos taurus* CRH (AAI47873); *Mus musculus* CRH (AAI19037), UcnI (NP_067265), UcnII (Q99ML8), Ucn III (Q924A4), UII (AAD55767) and CRH-BP (AAH61247); *Tupaia belangeri* CRH (AFJ95881); *Homo sapiens* CRH (AAH11031), UcnI (NP_003344), UcnII (Q96RP3), Ucn III (Q969E3), UII (AAD13070) and CRH-BP (NP_001873); *Rattus norvegicus* UcnI (NP_062023), UcnII (Q91WW1), UII (EDL81198) and CRH-BP (NP_631922); *Ovis aries* CRH-BP (NP_001009339); *Apis mellifera* CRH-BP (NP_001012633); and *Apis cerana cerana* CRH-BP (ADG21869).

Figure 6. Plasma cortisol values in fish transferred from 38 ‰ to 38 ‰ (SW→SW), from 38 ‰ to 55 ‰ (SW→HSW) or from 38 ‰ to 5 ‰ (SW→LSW). Values are represented as mean ± S.E.M. (n = 7-8 fish per group). Significant differences among sampling points at the same salinity are identified with different letters; different symbols show differences between groups at the same time (P<0.05, two-way ANOVA followed by Tukey's test).

Figure 7. Expression levels of CRH (A) and CRH-BP (B) in fish transferred from 38 ‰ to 38 ‰ (SW→SW), from 38 ‰ to 55 ‰ (SW→HSW) or from 38 ‰ to 5 ‰ (SW→LSW). Further details as described in the legend of Figure 6.

Figure 8. Plasma cortisol values in fish maintained under feeding, food deprivation and re-feeding situations. Values are represented as mean \pm S.E.M. ($n = 10-12$ fish per group). Significant differences among sampling points at the same condition are identified with different letters; different symbols show differences between groups at the same time ($P < 0.05$, one-way ANOVA or two-way ANOVA followed by Tukey's test, in each case).

Figure 9. Hypothalamic expression levels of CRH (A) and CRH-BP (B) in fish maintained under feeding, food deprivation and re-feeding situations. Values are represented as mean \pm S.E.M. ($n = 6-7$ fish per group). Further details as described in the legend of Figure 8.

877 **Figure 1. Martos-Sitcha et al.**

5'-atacttgtttctcctaagaagtgaaggagggcgccatctcgccaacta	48
ccttgcaaactgcacggctgttcttgacctcctctaagactgaagattcc	99
M K L N L L G T T V I L	12
tgctgatatacctgac <u>atgaagctcaatttacttggcaccaccgtgattctg</u>	150
L V A F L P R Y E C R A I E S P G	29
<u>ctagttgccttcttaccctcgctacgaatgtcgggctattgagagccctggc</u>	201
G A L R V P A P Q T Q N S Q Q Q Q	46
<u>ggtgccctgcgcgtcccagctccccaaccccaaaactcccagcagcagcaa</u>	252
Q Q S G P I L E R L G E E Y F I R	63
<u>cagcagtctggtcccatcctggagcggcttggagaggagtatttcatccga</u>	303
L G N G D S N S F P S S S M Y P G	80
<u>ctgggcaacggggactctaactctttcccatcttctgtccatgtatcccggc</u>	354
G S P A I Y N R A L Q L Q L T R R	97
<u>ggatcacctgcgatctacaacagagcggttgcaactccagctgacgcggcgt</u>	405
L L Q G K V G N I R A L I S G F G	114
<u>cttttacaaggaaaaagttgggaacatcagggcgctcataagcggcttcgga</u>	456
D R G D D S M E R G R R S E D P P	131
<u>gaccgcggggacgactcgatggagaggggaaggaggtccgaggacccgccg</u>	507
I S L D L T F H L L R E M M E M S	148
<u>atctccctggatctgaccttccacctgctccgggagatgatggagatgtcc</u>	558
R A E Q L A Q Q A Q N N R R M M E	165
<u>agggcggaacagctggcccagcaagcgcaaaaataacagaagaatgatggag</u>	609
L F G K	169
<u>ctcttcgggaaatga</u> agacctctttccagtcgcccaaagatctccctttcc	660
tttcattttcttttcttcttcttcttttttttgttgcatttttaccatca	711
gcacaaaacatgctctgtacaatatagtgtgcttcttactctattatctt	762
atagctttaacctcaaactatggagcttaaacgggcttgacttataatgat	813
ccgattgtaccttgccatttttaatgttggtgtcaaactctgtagaattaagc	864
cgttcttcatgtttgagatgaaatactttgggttgacatgaaatactgcat	915
taacaaaactggcatactttgttttagatttgcgaatcactgtattttatgat	966
atztatgtttgttaataaaacttatgtgcaaccagtcattttctgttggtgca	1017
agagaacgtcttatatctatatatttttaataaaaaaattaaaagcaaaaaa	1068
aaaaaaaaaaa -3'	1079

878

879

	Signal peptide	
<i>Sparus aurata</i>	MKLNLLGTTVILLVAFLLPRYECRAIESPPGGALRVPAPOTONSQQQQQQQ-----	48
<i>Solea senegalensis</i>	MKLNLFEGTTVILLVAFLLPRHECRAVDSRGGALRVLPAPOTPNQQQQQQQ-----	54
<i>Cyprinus carpio</i>	MKLNFLVTTVALLVAFPPPYECRAIESS-----SNQPAADPDGERQ-----	41
<i>Danio rerio</i>	MKLNFLVTTVALLVAFPPPYECRAIESS-----SNQPAADPDGERQ-----	41
<i>Homo sapiens</i>	MRLPLLVSAGVLLVALLCPPCRALLSRGPVPGARQAPQHPQPLDFFQPPQSEQPQQPQ	60
<i>Mus musculus</i>	MRLRLLVSAGMLLVALSSCLPCRALLSRGSVP---RAPRAPQPLNPLQP---EQPQQPQ	53
<i>Gallus gallus</i>	MKLQPLVCAGILLALLLCEHCRALESK---SPG---AARGALQQPDFFPQ---QQQQQQQ	52
<i>Xenopus laevis</i>	MKFQLWVSTGILLVSLLECEHCRAFIK---SPA---SSPGALLP-----ALSNSQ	44
	*:: : *::: . ***.	
	Cryptic motif	
<i>Sparus aurata</i>	SGPILERLGEEYFIRLNGDSNSFPSSS-----MYPGGSXAIYNRALQLQLT	95
<i>Solea senegalensis</i>	SAPILERLGEEYFVRLNGEDSNLPPSSSSS-----SSSMYPGGAPATYNRALQLQLT	107
<i>Cyprinus carpio</i>	SPPVLARLGEEYFIRLGNRNONSPPSPADS-----FPETS-QYSKRALQLQLT	88
<i>Danio rerio</i>	SPPVLARLGEEYFIRLGNRNPTSPSPADS-----FPETS-QYPKRALQLQLT	88
<i>Homo sapiens</i>	AREVLLRMGEEYFIRLGNLNKSPAAPLSPASSLLAGGSGSRPSPSQATANFRRVLLQQLL	120
<i>Mus musculus</i>	--PVLIRMGEEYFIRLGNLNKSPAARLSPNSTPLTAGRSGSRPSHDQAAANFRRVLLQQLQ	111
<i>Gallus gallus</i>	TLFVLLRMGEEYFIRLGLTKRPAGPFSASS-----GGHLRP---EASAEELLRAAAQQLQ	104
<i>Xenopus laevis</i>	--PFLIRMGEEYFIRLGNLHKHSPGSFPEAS-----AGNFVRAVQQLQA	86
	. *:*****:***: . *	
	Mature hormone	
<i>Sparus aurata</i>	RRLLOGKVGNIIRALISGFGDRG--DDSMERGRSEDPPIISLDLTFHLLREMEMSRAEQ	153
<i>Solea senegalensis</i>	RRLLOGKVGNIIRALISGFGDRG--DDSMERGRSEDPPIISLDLTFHLLRGMMMSRAEQ	165
<i>Cyprinus carpio</i>	QRLLEGKVGNIIRLDGNYALRA--LDSMERERRSEEPPIISLDLTFHLLREVLEMAEAEQ	146
<i>Danio rerio</i>	QRLLEGKVGNIIRLDGNYALRA--LDSMERERRSEEPPIISLDLTFHLLREVLEMAEAEQ	146
<i>Homo sapiens</i>	LPRRSLDSPAALAEERGARNALGHQEAPEERERRSEEPPIISLDLTFHLLREVLEMAEAEQ	180
<i>Mus musculus</i>	MPQRSLSRAEPAERGAEDALGHQGALEERRSEEPPIISLDLTFHLLREVLEMAEAEQ	171
<i>Gallus gallus</i>	G-----SGSPEGDEGAG-----EAVEREKRSEEPPIISLDLTFHLLREVLEMAEAEQ	151
<i>Xenopus laevis</i>	QQWSSQPGMRAASLDGADSPYSAQEDPTEKAKRAEPPPIISLDLTFHLLREVLEMAEAEQ	146
	. *: :*: :*.***** :*:*****:	
<i>Sparus aurata</i>	AQQAQNNRRMMELFGK	169
<i>Solea senegalensis</i>	AQQAQNNRRMMELFGK	181
<i>Cyprinus carpio</i>	AQQAHSNRKMMELFGK	162
<i>Danio rerio</i>	AQQAHSNRKMMELFGK	162
<i>Homo sapiens</i>	AQQAHSNRKMMELFGK	196
<i>Mus musculus</i>	AQQAHSNRKMMELFGK	187
<i>Gallus gallus</i>	AQQAHSNRKMMELFGK	167
<i>Xenopus laevis</i>	AQQAHSNRKMMELFGK	162
	:::.. :*: **	

881

882

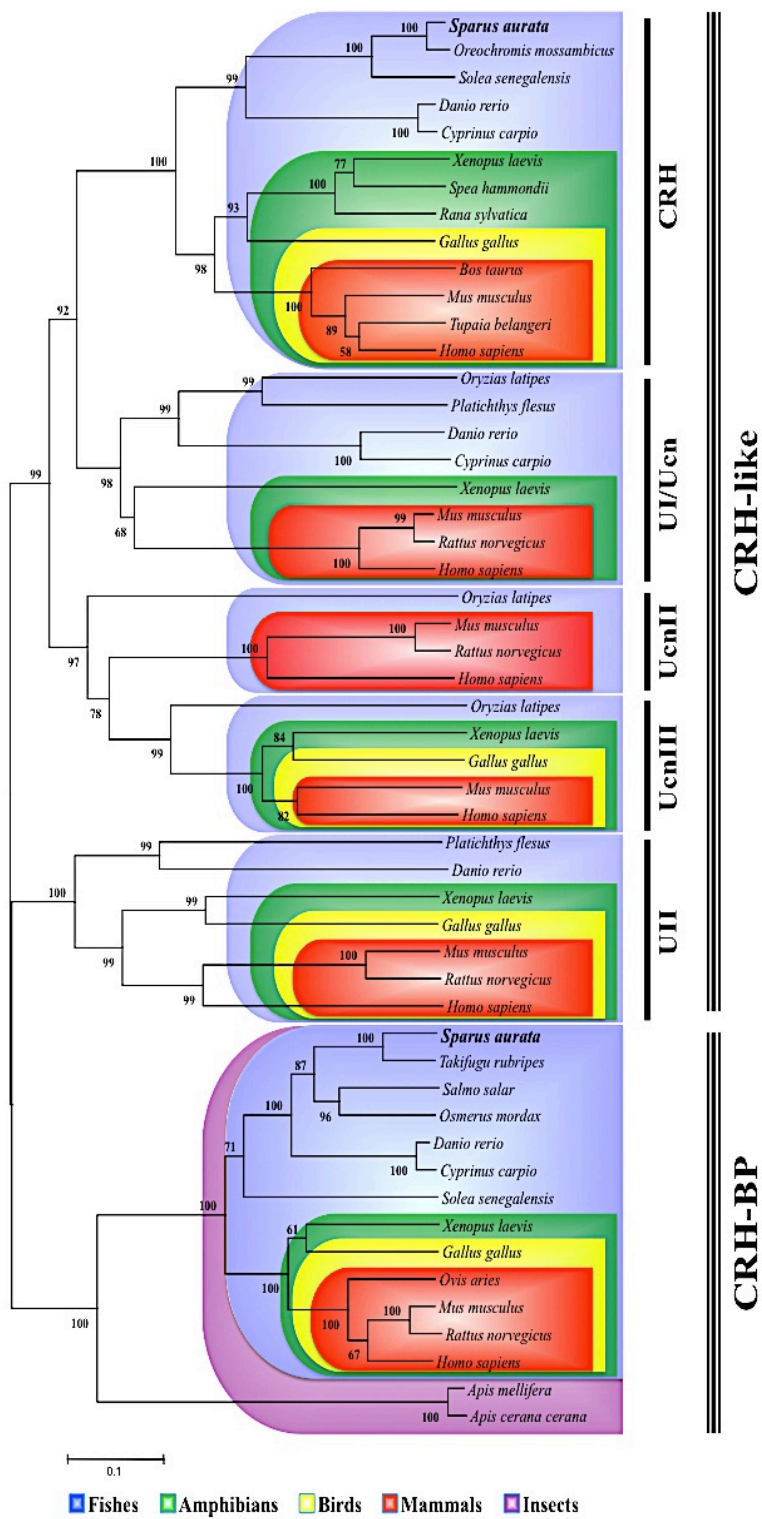
Figure 3. *Martos-Sitcha et al.*

M R V M E R T F R E Q L	12
5'-ctgcagacagag <u>atg</u> cgcgatgatggagcgcacgttccgcgagcagctg	48
F F L L L C A S V L K G D C R Y I	29
ttcttcctgctgttgtgctgcgctcggtgctgaagggagactgcaggtacatc	99
E N N E I S K D E L Y S F F N S E	46
gagaacaacgagatctccaaagatgagttatatattctttcttcaactcggag	150
L K R E T T E E L M Y R R P L R C	63
ctgaagagagaaacaacggaggagttaatgtaccgtcgcacctctacgctgt	201
L D M V A V E G Q F T F T A E R P	80
ctggacatggtggctgtggagggtcagttcaccttcacggccgagcgtcct	252
Q L S C A A F F M A E P N E V I T	97
cagctcagctgcgccgcttttcttcatggccgagcccaacgaggtgatcacg	303
V E Y D N V D I D C R G G D F I T	114
gtggagtacgacaacgtcgcacatcgactgcaggggaggagacttcatcacg	354
V F D G W V M K G E K F P S S Q D	131
gtgtttgacggctgggtgatgaaaggagagaagtccccagctcccaggat	405
H P L P L Y E R Y V D Y C D S G A	148
cacccgctgcctctgtacgagcgatatgtggattactgcgactcgggagcg	456
L R R S V R S S Q N V A M I F F R	165
ctgaggagaagcgtgcgctcctctcagaacgtcgccatgatcttcttttcgg	507
I H N A G S T F T L T V R K H I N	182
attcacaacgccggcagcaccttcacgctgaccgtcaggaaacacatcaat	558
P F P C N V I S Q S P E G S Y T M	199
ccgttcccctgtaatgtcatctcccagtcaccagagggcagttacacgatg	609
V I P Q Q H R K C S F S I I Y P V	216
gtgatcccgcagcagcacaggaaatgcagcttctccatcatctacccggtg	660
E I D V S E F S L G H F N N F P Q	233
gagatcgacgtctctgagttcagcctcggacacttcaacaactttcccca	711
R S M P G C A E S G D F V Q L L G	250
aggatccatgcccggttgtgcagaatcaggagatttcgtgcagctgttggga	762
G S G I D T S K L L P I T D L C I	267
ggaagcggtatcgacacgtcgaagctgctgcccacacggacactctgcatc	813
S L L D P T H M K I G C D N T V V	284
tccttactggacccccaccacatgaagatcgggtgcgacaacacgggtggtg	864
R M V S S G K F V S R V S F S Y R	301
aggatggtgtccagcgggaagtgtgtgagccgagtgctcgttcagctacagg	915
L L D S Q E L Q T I K L N N V E D	318
ctactggacagccaggagctgcagaccatcaaactcaacaacgtggaggat	966
F C F N N	323
ttctgtttcaacaactgaacccgacaggatcctccagtgacacacgcatca	1017
tctgactgcaaacatttttttaattctttgaagagccacagatccacccgg	1068
tcgctccatctgattaggtgaaacgtcttaaatccgaagacgtaaacataa	1119
aagaaaaataagatgaattacgatccacgctttttgttttcggtccatttt	1170
tccatcttattttcagtcggtccggtgtcgtgaataaagctcgatgaagtg	1221
tccttttgtgtttggggaactgctattgttttatttttgtgtattttattaa	1272
gacttactgatgatgtttgttattttgttacgctgtatgagttgtggtcaaca	1323
ttcttgcaaagggacgggctaaaaaagttaccttctgtttatgttgctgaa	1374
cgacacgcgatgtgccgattcatttcctgcagcaggtgaccaggaggggac	1425
ggtgaagtgttccatgtaataaatacagtggttttcttaatgcggttcaatt	1476
tgtataaaacctttttgttaactcatcgcatgacaaagcaaaaaaaaaaaaa	1527
aaaa -3'	1531

Sparus aurata MKIGDNTVVRMVSSGKGFVSRVSFSYRLLDSQELQTIKLNNVEDFCFNN- 323
Solea senegalensis MKVGCDNSVVRLVSSGNYNRVSEFYRLLGRNELPKNRENSLENFCLE- 321
Cyprinus carpio MKVGCDNTVVRMVSSGKGFVNRVSFYRLLGHQELQOMKGNSVEDVCLRA- 321
Danio rerio MKVGCDNTVVRMVSSGKGFVNRVSFYRLLGHQELQOMKGNSVEDVCLRA- 321
Homo sapiens MKVGCDNTVVRMVSSGKHINRVTFEYROLEPYELENPNGNSIEGFCLSGL 322
Mus musculus MKTSCDNADVVRMVSSGKHINRVTFEYROLEPFLELTSTGNSIPEYCLSSL 322
Gallus gallus MKTIGCDNTVVRMVSSGKHINRVTFEYYQLDLQEIENRKENSIEEFQPPGI 345
Xenopus laevis MKIGCDNTVVRMVSSGNFINRVTFEYNYOLD-ROLEKKQGNSVEEAQFPSD 321
.**.*****.***.*.***.*.***.*.***.*.

888 **Figure 5. Martos-Sitcha et al.**

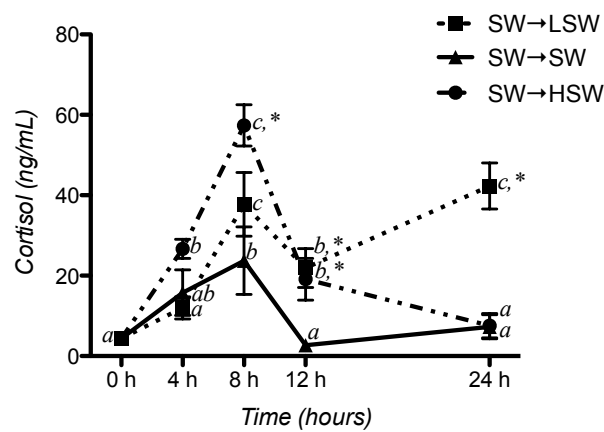
889



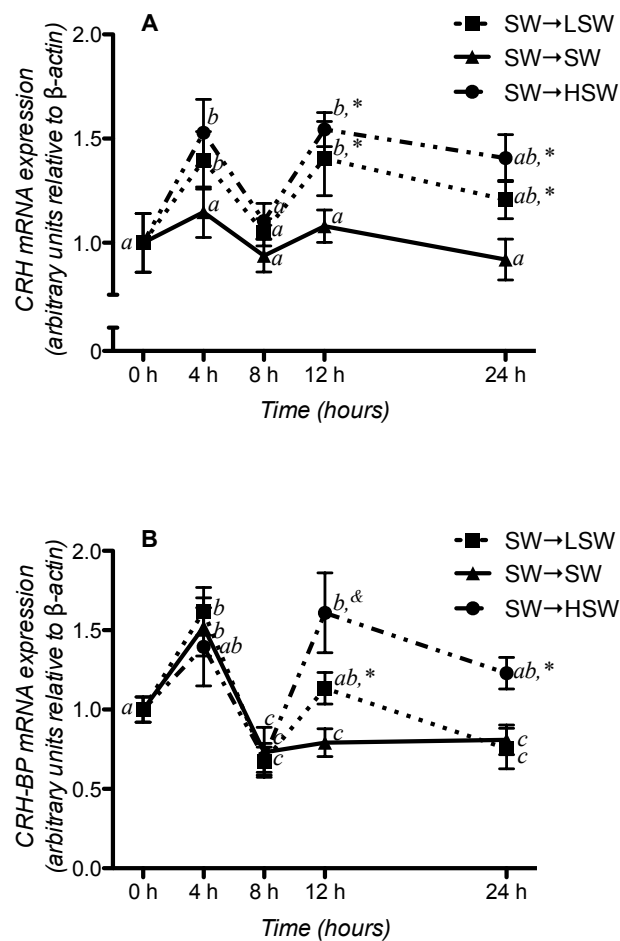
917

918

919 **Figure 6.** *Martos-Sitcha et al.*



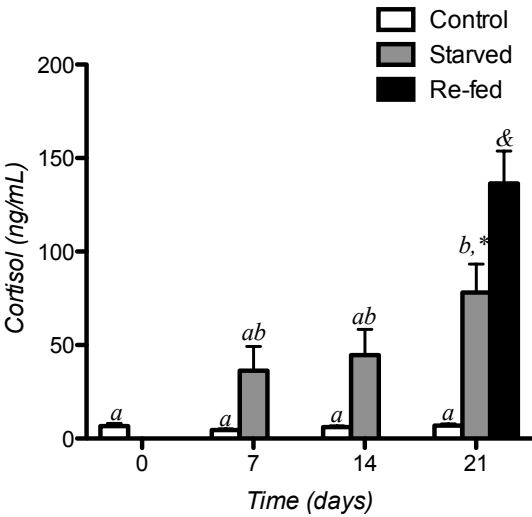
920
921
922



924

925

926 **Figure 8.** *Martos-Sitcha et al.*



927
928
929

