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

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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* (Sangiao-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

115 *2.1 Animals and experimental design*

116 Juveniles of gilthead sea bream (*Sparus aurata* L., 213.13 ± 4.75 g body mass) were provided
117 by Planta de Cultivos Marinos (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain;
118 Experimental animal facility registry numbers CA/4/CS and CA/3/U). Fish were fed a daily
119 ration of 1 % of their body weight with commercial pellets (Dibaq-Dibroteg S.A., Segovia,
120 Spain). All the experiments were performed with the Guidelines of the European Union
121 (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of
122 laboratory animals.

123

124 *2.1.1. Experimental design I: Short-term salinity transfer*

125 Fish (n = 80, 192.11 ± 4.23 g body mass) were transferred to the wet laboratories at the
126 Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz, Spain), where they were
127 acclimated for 14 days to sea water (SW, 38 ‰ salinity) in 400-L tanks in an open system
128 circuit (5.6 $kg \cdot m^{-3}$ density) under natural photoperiod (May, 2011) and constant temperature
129 (18-19 ºC). Afterwards, fish were directly transferred to one of the following environmental
130 salinities: SW (control group), low salinity water (LSW, 5 ‰ salinity, hypoosmotic transfer)
131 and high salinity water (HSW, 55 ‰ salinity, hyperosmotic transfer). These experimental
132 salinities were achieved by either mixing SW with dechlorinated tap water (LSW), or mixing
133 with natural marine salt (Salina de la Tapa, El Puerto de Santa María (Cádiz), Spain) (HSW).
134 Experimental groups were maintained in duplicate tanks (400-L volume each; n = 12 fish per
135 tank, 5.6 $kg \cdot m^{-3}$ initial density) under a closed recirculating water system. Water quality
136 criteria were checked at the end of the trial to confirm their stability during the 24 hours that
137 experiment lasted. On day 0 (10:00 AM), eight fish from the main tanks containing SW were
138 sampled (control time 0 before transfer). Then, on 4, 8, 12 and 24 hours after salinity transfer,
139 six fish from each experimental salinity (SW, LSW and HSW) were anaesthetized with a
140 lethal dose of 2-phenoxyethanol (1 $mL \cdot L^{-1}$ specific salinity water), weighted, heads separated
141 from trunks and sampled.

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

148 final water volume in the tanks, to keep it constant throughout the experimental period and
149 between all tanks.
150
151 *2.1.2. Experimental design II: Starving and re-feeding*
152 Fish (*n* = 96; 235.31 ± 5.65 g body mass) were transferred to the wet laboratories at the
153 Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz, Spain), where they
154 acclimated for 28 days to sea water (SW, 38 ‰ salinity) in five 1000-L tanks in an open
155 system circuit (4.3 $kg \cdot m^{-3}$ density) under natural photoperiod (March, 2011) and constant
156 temperature (18-19 ºC). After this acclimation period to SW, animals were maintained at the
157 following experimental conditions: 2 tanks fed with a daily ration of 1 % of their body mass
158 with commercial pellets (Dibaq-Dibroteg S.A., Segovia, Spain), and 3 tanks without receiving
159 food (*n* = 18 or 20 fish per tank). Furthermore, from day 14 after the start of the experiment,
160 fish from one tank maintained under food-deprived condition were fed again during 7 days
161 with a daily ration of 1 % of their body mass with the same commercial pellets described
162 above, constituting the re-feeding group. On day 0, eight fish from the main tanks containing
163 SW were sampled (control time 0 before transfer). Then, twelve fish from each experimental
164 group (control, starved and re-fed) on 7, 14 and 21 days after the start of the experiment, were
165 anaesthetized with a lethal dose of 2-phenoxyethanol (1 $mL \cdot L^{-1}$ specific salinity water),
166 weighted, heads separated from trunks and sampled. Blood samples and tissue biopsies were
167 taken as described above. No mortality was observed during the time that experiment lasted.
168 In addition, the stocking density of each tank was restructured as described above.
169
170 *2.2. Plasma parameters*
171 Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten
172 Osmometer, Fiske-VT, USA) and expressed as $mOsm \cdot kg^{-1}$. Glucose and lactate
173 concentrations were measured using commercial kits from Spinreact (Barcelona, Spain)
174 (Glucose-HK Ref. 1001200; Lactate Ref. 1001330) adapted to 96-well microplates.
175 Plasma cortisol levels were measured by enzyme-linked immunosorbent assay (ELISA)
176 adapted to microtiter plates as previously described for testosterone (Rodríguez et al., 2000).
177 Steroids were extracted from 5 μL plasma in 100 μL RB (PPB (Potassium Phosphate Buffer)
178 100 mM, NaN_3 1.54 mM, NaCl 400 mM, EDTA 1 mM, BSA (Bovine Serum Albumin) 15
179 mM) and 1.2 mL methanol (Panreac), and evaporated during 48-72 hours at 37 ºC. Cortisol
180 EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002),
181 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 *Eco*RI and *Xho*I (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.

215

216 *2.5. Phylogenetic and evolutionary analyses*

217 Phylogenetic analysis of the CRH-like and CRH-BP amino acid sequences was conducted
218 with MEGA5 software (Tamura et al., 2011) with the Neighbor-Joining algorithm (Saitou and
219 Nei, 1987) based on amino acid differences (p-distances) and pairwise deletion. Reliability of
220 the tree was assessed by bootstrapping (1000 replications). Amino acid sequences were
221 retrieved from the NCBI protein database (www.ncbi.nlm.nih.gov/pubmed), accessed in June
222 2014).

223

224 *2.6. RNA extraction and cDNA synthesis*

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

236

237 *2.7. Real-time PCR (qPCR)*

238 Specific primers for use in qPCR were designed by use of Primer 3 software (v. 0.4.0)
239 available at http://fokker.wi.mit.edu/primer3/input.htm in February 2011. Primer
240 oligonucleotide sequences are shown in Table 2. Previous to qPCR analysis, optimization of
241 qPCR conditions was made on primers annealing temperature (50 to 60 ºC), primers
242 concentration (100 nM, 200 nM and 400 nM) and template concentration (six 1:10 dilution
243 series from 10 ng to 100 fg of input RNA). Moreover, two negative controls, with i) RNA (10
244 ng/reaction) and ii) sterile water, were performed to detect possible gDNA contamination or
245 primer-dimers artefacts. The resulting curves had amplification efficiencies and r^2 of 0.98 and
246 0.995 for CRH, 0.99 and 0.998 for CRH-BP, and 0.99 and 0,999 for β-actin, respectively. To
247 perform qPCR reactions, 4 µl cDNA (10 ng assumed from RNA input), specific forward and
248 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® ep*gradient* S Realplex^2 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 whit 56-99 % similarity to other teleosts. ORF includes a conserved signal peptide ($M^1 – A^{24}$), a cryptic motif ($R^{55} – N^{66}$) and a mature peptide ($S^{127} – F^{167}$), 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 (R^{125}

– R^{126}) of the mature peptide and the typical C-terminal amidation site (G^{168} – K^{169}) 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 M^1 – C^{26}, the two conserved amino acids R^{59} and D^{65}, 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)

316 a significant enhancement was not produce till 8 hours post-transfer. Later, in control group, it
317 returned to values from time 0 at 12 hours, remaining thus until the end of the experiment. At
318 24 hours post-transfer, cortisol levels dropped down to almost initial values in HSW group,
319 but not for fish transferred to LSW (Figure 6).
320 Expression levels of both CRH and CRH-BP after osmotic challenge are shown in Figure 7.
321 CRH mRNA expression presented similar time-course changes after LSW and HSW transfer.
322 Thus, both groups showed an increase of around 50 % in mRNA expression levels respect to
323 control group during all times tested, except at 8 hours where values close to the control
324 group were observed. Regarding CRH-BP mRNA expression, all groups showed a similar
325 pattern change at 4 hour post-transfer, increasing its values in a 50 %. After this time, control
326 group remained unchanged until the end of experimental time. CRH-BP mRNA expression
327 levels of fish submitted to hypoosmotic transfer showed a ~30 % increase in mRNA levels
328 compared to control group at 12 hours, while under hyperosmotic condition enhanced ~60
329 and ~35 % its expression at 12 and 24 hours post-transfer, respectively.
330 Statistical values of *P*-value and F obtained from the two-way ANOVA analysis for all
331 parameters tested in this sub-assay are shown in Table 5A.
332
333 *3.3. Effects of starving and re-feeding situation (chronic stress response)*
334 Time courses related to metabolic response of sea breams maintained under different feeding
335 conditions are shown in Table 5. Plasma glucose did not show changes in fish maintained
336 under normal fed conditions. Moreover, fish held under starving conditions significantly
337 enhanced its values respect to the control group, although the highest plasma glucose values
338 were observed in those fish re-fed during one week till day 21 (*P*-value: 0.041; F: 3.124). In
339 contrast, plasma lactate only showed statistically higher levels in fish maintained food-
340 deprived during 21 days (*P*-value: 0.047; F: 2.963).
341 Plasma cortisol levels did not change in fish fed with a daily ration of 1 % of their body mass
342 and maintained as control group. However, fish submitted to starving situation significantly
343 increased these values around 7- to 8-fold respect to the control group during the first 14 days
344 of experiment, reaching the highest levels (11-fold) at the end of the trial (*P*-value: <0.001; F:
345 16.009). Moreover, re-feeding group during one week presented higher values respect to
346 fasting group, being 20-fold higher than the control group at the same sampling point (Figure
347 8). CRH mRNA expression was unchanged in all the groups and time points tested (Figure
348 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 Enckevort 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 linage. 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* (Huising 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.

417 Interestingly, at 24 hours (end point of experiment), fish submitted to hypoosmotic transfer
418 presented the highest plasma cortisol values, while that under hyperosmotic condition
419 returned to basal levels. This could reflect an osmoregulatory role for cortisol during
420 adaptative phase in *S. aurata* transferred to hypoosmotic environments, and it agrees with the
421 previously proposed hyperosmotic role for cortisol in this species increasing gill Na^+,K^+-
422 ATPase activity, plasma osmolality, and ions after transfers from seawater to brackish water
423 (Mancera et al., 2002). Both groups showed up-regulation of CRH expression but only
424 hypoosmotic-transferred fish presented down-regulation of CRH-BP expression. These results
425 suggested that a coordination between both hypothalamic factors are thus clearly involved in
426 a fast regulation of plasma cortisol levels, inducing the strongly-pronounced, but short-lived,
427 cortisol response typical in acute stress situations (Huising et al., 2004). The high fluctuation
428 in CRH-BP expression compared to that in CRH expression might suggest that CRH-BP acts
429 stronger as a modulator of the acute stress response than CRH does, as it has been suggested
430 as well for the Senegalese sole (*S. senegalensis*) (Wunderink et al., 2011). Moreover,
431 activation of the hypothalamo-pituitary axis, with CRH as the first player implicated, and the
432 release of ACTH into the circulation by the pituitary is an integral part of the primary stress
433 response of fish (Donaldson, 1981; Sumpter et al., 1986; Balm and Pottinger, 1995).
434 Moreover, in the control group the lack of variations regarding with an expected increase in
435 CRH mRNA at 4 h in agreement with the cortisol enhancement at 8 h could suggest that only
436 handling stress required less amounts of stored protein (CRH), making that any additional
437 gene transcription initiated on top of the constitutive gene expression will remain
438 undetectable, although a contribution of daily rhythms on HPI-axis cannot be ruled out
439 (Montoya et al, 2010).
440
441 *4.3. Effects of food deprivation*
442 Studies assessing effects of food deprivation on stress axis in adult fish are scarce. Metabolic
443 reorganization after a prolonged stress source is expected due to the need to maintain vital
444 functions in the organisms. In fact, the reorganization observed in those fish maintained food
445 deprived is different compared with those submitted to an acute stress process (see above).
446 Thus, sea breams maintained under starvation revealed an enhancement in their plasma
447 glucose levels during the time that experiment lasted, together with a substantial increase in
448 lactate at the end of the trial. This fact demonstrated that i) food deprivation produced a
449 metabolic imbalance, and ii) re-feeding returned lactate concentration close to the control
450 values, but glucose remained enhanced as a consequence of high cortisol values (see below)

451 that probably produced higher glycogenolitic activity rates in several important metabolic
452 organs as liver, as has been previously demonstrated after different chronic stress situations,
453 including food deprivation (Sangiao-Alvarellos et al., 2003, 2005b).
454 Moreover, in this study food-deprived *S. aurata* enhanced plasma cortisol levels. Likewise,
455 elevated whole-body cortisol concentrations were found in zebrafish as a result of crowding
456 and food deprivation (Ramsay et al., 2006), and reduced stress resistance was demonstrated in
457 food-deprived Atlantic cod (*Gadus morhua*) (Olsen et al., 2008). Similarly, food-deprived *S.*
458 *senegalensis* juveniles significantly enhanced plasma cortisol levels (Costas et al., 2011a). In
459 addition, during early development, food-deprived *S. senegalensis* larvae showed an increase
460 in whole-body cortisol levels, as the result of an up-regulation of CRH expression and a
461 downregulation of CRH-BP expression (Wunderink et al., 2012). However, the lack of
462 variation in CRH mRNA expression as well as the down-regulation of CRH-BP values
463 suggests that, in *S. aurata* exposed to a long period of food deprivation, plasma cortisol level
464 could be regulated by both hypothalamic factors due to the putative lower regulation by the
465 soluble binding protein. Even so, specific changes in CRH-BP mRNA levels could varied in
466 each brain region depending on the stressor applied (Alderman et al., 2008), so a more
467 comprehensive study addressing i) each portion of the brain, deal with ii) different sources of
468 stress would be necessary to clarify the limited changes observed in our results.
469 In the re-fed group, fish showed the highest values of plasma cortisol and glucose together
470 with a lack of variation in CRH and CRH-BP mRNA expression. Although these results could
471 be a paradigm, the existence of such high values of cortisol could be explained by several
472 situations: i) the existence of a permanent state of alert to a situation of re-feeding after a
473 prolonged starving period (Uchida et al., 2003); ii) the stimulation of food intake by cortisol
474 (Bernier et al., 2004), where this hormone would act on food intake regulation enhancing the
475 stress recovery after food deprivation (Mommsen et al., 1999, Bernier et al., 2004); or iii) the
476 important role of cortisol during the metabolism reorganization (Mommsen et al., 1999).
477 Moreover, the absence of changes in CRH expression suggests that those processes focused in
478 cortisol production and release could be carried out through a different pathway. In fact, other
479 hormones and factors than just CRH and CRH-BP have been already described as putative
480 players involved in the stress response (Majzoub, 2006; Bernier et al., 2009), and the use of
481 CRH as a regulator of stress during food deprivation is somewhat of a paradox, since CRH
482 also acts as anorexigenic peptide (Uehara et al., 1998). Potential candidates to direct the stress
483 response independently of CRH are TRH through activating α-MSH (Lamers et al., 1991;
484 Rotlland et al., 2000; Van der Salm et al., 2004), and AVT nonapeptide that also stimulates

485 the release of ACTH (Baker et al., 1996). Thus, α-MSH is a key player in the neuroendocrine
486 stress response, depending on the type and source of the stressor (Wendelaar Bonga et al.,
487 1995). Even so, the corticotrope activity of α-MSH is relatively weak (100 times less potent)
488 compared to that of ACTH (Wendelaar Bonga et al., 1995). Moreover AVT binding sites
489 have been described to be located in the zones occupied by corticotroph cells in
490 *Dicentrarchus labrax* (Moons et al., 1989) and *Catostomus commersoni* (Yulis and Lederis,
491 1987). In addition, the *in vitro* co-administration of AVT/AVP and CRF stimulate ACTH
492 secretion in preparations *in vitro* (Baker et al., 1996). Furthermore, AVT treatment plus hypo-
493 and hyperosmotic transfer enhanced plasma cortisol levels in *S. aurata*, suggesting a role of
494 AVT on stress axis activation in this species (Sangiao-Alvarellos et al., 2006). Recently,
495 Martos-Sitcha et al. (2013) demonstrated in *S. aurata* that pro-vasotocin mRNA synthesis and
496 pituitary storage of mature hormone is involved in the regulation of stress process after
497 salinity challenges, and also that food deprivation enhanced AVT storage in the pituitary
498 gland, suggesting that this hormone could acts as a paracrine factor on the ACTH cells (Gesto
499 et al., 2014).
500
501
502 **5. Conclusions**
503 Both, CRH and CRH-BP cDNA sequences were cloned in *S. aurata*. Their phylogenetic and
504 sequence analysis showed good gene conservation throughout evolution. Moreover, the
505 dynamics of change of osmoregulatory and metabolic parameters after two different sources
506 of stress (osmotic challenge –acute-, or food deprivation –chronic-) conditions confirmed the
507 internal derangement of the animals and its control mediated by the endocrine system.
508 Thus, the mRNA expression of these hormones, together with these changes reported on
509 plasma cortisol levels, indicated that the cortisol enhancement observed can be controlled by
510 different pathways, in which CRH seems to be regulated by CRH-BP during the acute stress
511 response, whereas during chronic stress (food deprivation) it could be controlled by other
512 factors acting as modulators (AVT or TRH hormones, among others). Even so, the
513 impossibility to discriminate variations in hypothalamic neurons alone could skew these
514 results in a complex endocrine system in which different pathways could regulate its proper
515 operation depending on the stressor. Moreover, the sequence in which stressors (acute or
516 chronic) occurs can produce different responses in this endocrine system as it has been
517 previously reported in *S. senegalensis* (Wunderink et al., 2011).
518

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Tables

Degenerate primers	Nucleotide sequence	Amplicon length
CRH-BP_Fw1	5'-CARTTYACMTTCACAGCAGA-3'	718 bp
CRH-BP_Rv1	5'-CARGAGCTRCAGRYGATYAA-3'	
CRH-BP_Fw2	5'-GTRTTYGAYTGGGTGATGAA-3'	501 bp
CRH-BP_Rv2	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
qCRH_Fw	5'-ATGGAGAGGGGAAGGAGGT-3'	176 bp
qCRH_Rv	5'-ATCTTTGGCGGACTGGAAA-3'	
qCRH-BP_Fw	5'-GCAGCTTCTCCATCATCTACC-3'	147 bp
qCRH-BP_Rv	5'-ACGTGTCGATACCGCTTCC-3'	
qb-actin_Fw	5'-TCTTCCAGCCATCCTTCCTCG-3'	108 bp
qb-actin_Rv	5'-TGTTGGCATACAGGTCCTTACGG-3'	

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

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

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

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

Metabolite	Treatment	0 hours	4 hours	8 hours	12 hours	24 hours
Osmolality (mOsm·Kg^{-1})	SW → LSW	336.2 ± 12.5^{a}	317.3 ± 3.4^{ab,*}	289.5 ± 2.4^{b,*}	295.3.6 ± 8.6^{b,*}	297.1 ± 6.4^{b,*}
	SW → SW		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$^{b,\#}$
Glucose (mM)	SW → LSW	8.143 ± 0.078^{a}	11.248 ± 0.244^{b,*}	11.406 ± 0.680^{b,*}	11.693 ± 0.687^{b,*}	11.111 ± 0.686^{b,*}
	SW → SW		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,*}
Lactate (mM)	SW → LSW	0.397 ± 0.016^{a}	0.416 ± 0.018^{a}	0.365 ± 0.009^{a}	0.413 ± 0.032^{a}	0.402 ± 0.029^{a}
	SW → SW		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
Glucose (mM)	*Control*	4.844 ± 0.095^{a}	4.838 ± 0.204^{a}	4.872 ± 0.172^{a}	4.759 ± 0.164^{a}
	Starved		5.297 ± 0.173^{b}	5.351 ± 0.212^{b}	5.374 ± 0.204^{b}*
	Re-fed				5.535 ± 0.276*
Lactate (mM)	*Control*	2.908 ± 0.199^{a}	3.038 ± 0.154^{a}	2.775 ± 0.110^{a}	2.833 ± 0.261^{a}
	Starved		2.982 ± 0.183^{a}	2.826 ± 0.224^{a}	3.637 ± 0.467^{b}*
	Re-fed				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

Table 6. Statistical parameters (*P*-value and F) obtained from two-way ANOVA analysis in fish transferred to different environmental salinities in a short-time response (A) or in fish 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 M^{1}-A^{24} and the conserved cryptic motif R^{55}-N^{66} are indicated in bold capitals. Predicted mature peptide S^{127}-F^{167} 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 M^{1}-C^{26} is indicated in bold capitals. The ten cysteines involved in the formation of five C-C disulfide bonds are boxed, underlined and in bold. R^{59} and D^{65}, 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. R^{59} and D^{65}, 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 hammondii* 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).

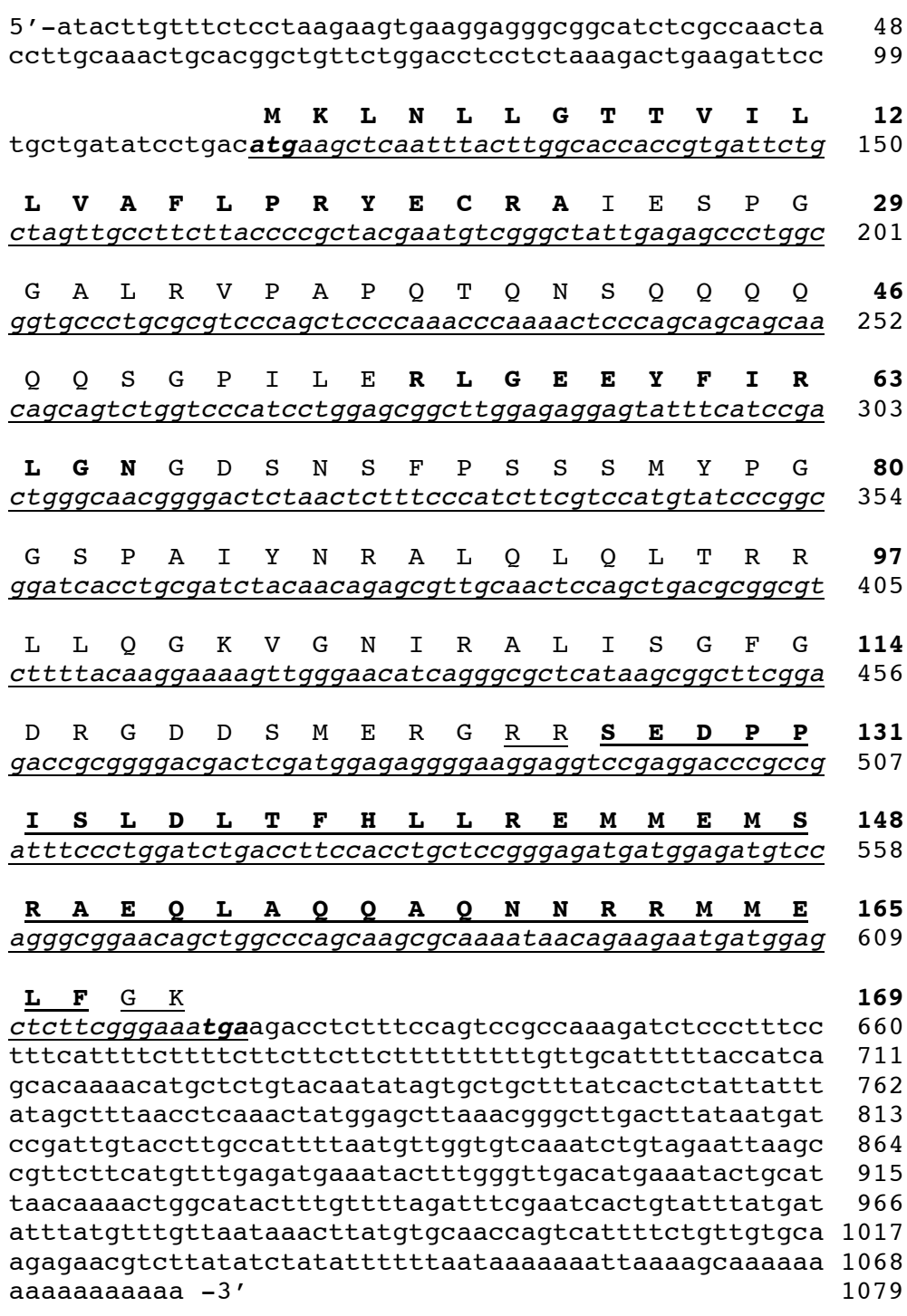
Figure 6. Plasma cortisol values in fish transferred from 38 ‰ to 38 ‰ (SW→SW), from 38 ‰ to 55 ‰ (SW→HSW) or from 38 ‰ to 5 ‰ (SW→LSW). Values are represented as mean ± S.E.M. (n = 7-8 fish per group). Significant differences among sampling points at the same salinity are identified with different letters; 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 ± 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 ± S.E.M. (n = 6-7 fish per group). Further details as described in the legend of Figure 8.

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



880 **Figure 2.** ***Martos-Sitcha et al.***

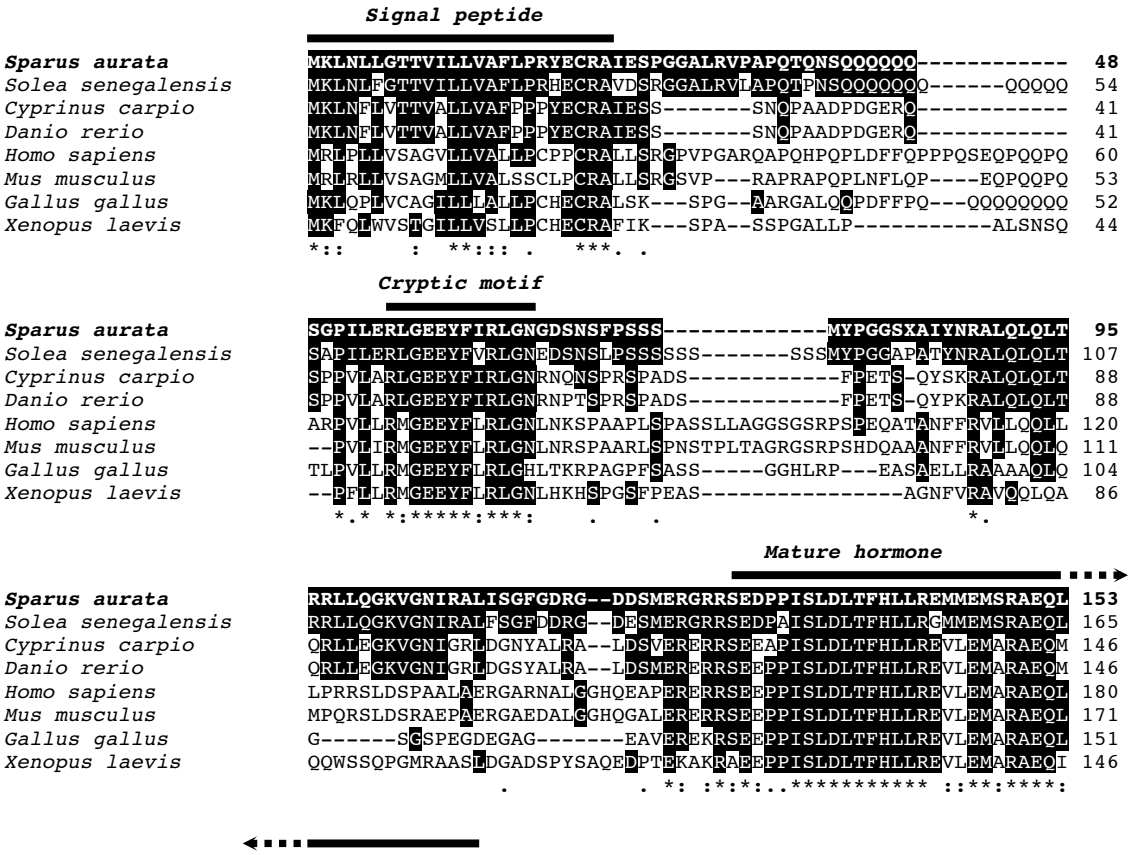


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Figure 3. ***Martos-Sitcha et al.***

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                     M  R  V  M  E  R  T  F  R  E  Q  L     12
5′-ctgcagacagag***atg***cgcgtgatggagcgcacgttccgcgagcagctg    48

 F  F  L  L  L  C  A  S  V  L  K  G  D  C  R  Y  I     29
ttcttcctgctgttgtgcgcgtcggtgctgaagggagactgcaggtacatc    99

 E  N  N  E  I  S  K  D  E  L  Y  S  F  F  N  S  E     46
gagaacaacgagatctccaaagatgagttatattctttcttcaactcggag   150

 L  K  R  E  T  T  E  E  L  M  Y  R  R  P  L  R  C     63
ctgaagagagaaacaacggaggagttaatgtaccgtcgacctctacgctgt   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
cagctcagctgcgccgctttcttcatggccgagcccaacgaggtgatcacg   303

 V  E  Y  D  N  V  D  I  D  C  R  G  G  D  F  I  T    114
gtggagtacgacaacgtcgacatcgactgcaggggaggagacttcatcacg   354

 V  F  D  G  W  V  M  K  G  E  K  F  P  S  S  Q  D    131
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 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
ctgaggagaagcgtgcgctcctctcagaacgtcgccatgatcttctttcgg   507

 I  H  N  A  G  S  T  F  T  L  T  V  R  K  H  I  N    182
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 P  F  P  C  N  V  I  S  Q  S  P  E  G  S  Y  T  M    199
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 G  S  G  I  D  T  S  K  L  L  P  I  T  D  L  C  I    267
ggaagcggtatcgacacgtcgaagctgctgcccatcacggacctctgcatc   813

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 R  M  V  S  S  G  K  F  V  S  R  V  S  F  S  Y  R    301
aggatggtgtccagcgggaagtttgtgagccgagtgtcgttcagctacagg   915

 L  L  D  S  Q  E  L  Q  T  I  K  L  N  N  V  E  D    318
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 F  C  F  N  N                                         323
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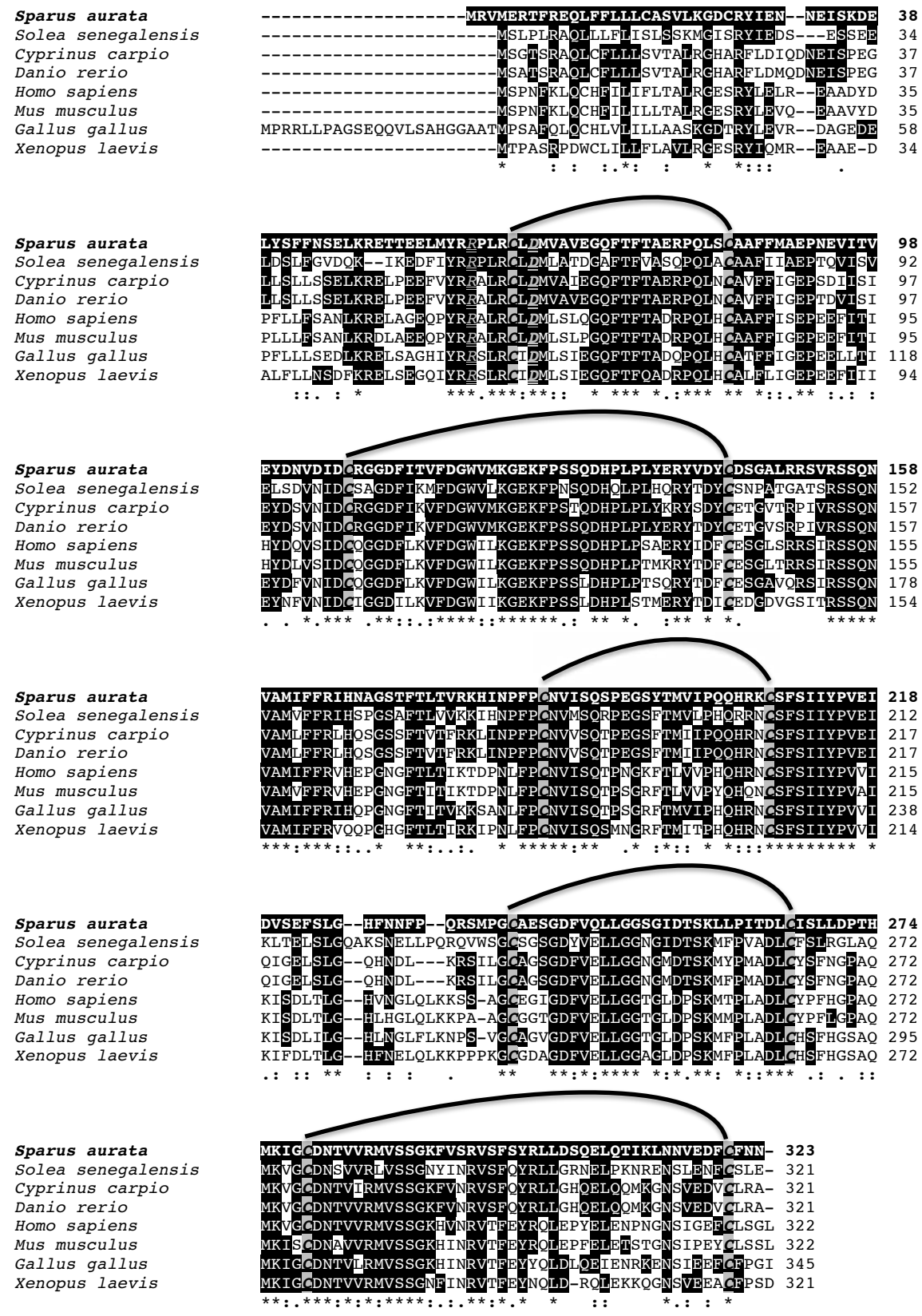
Figure 4. ***Martos-Sitcha et al.***



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

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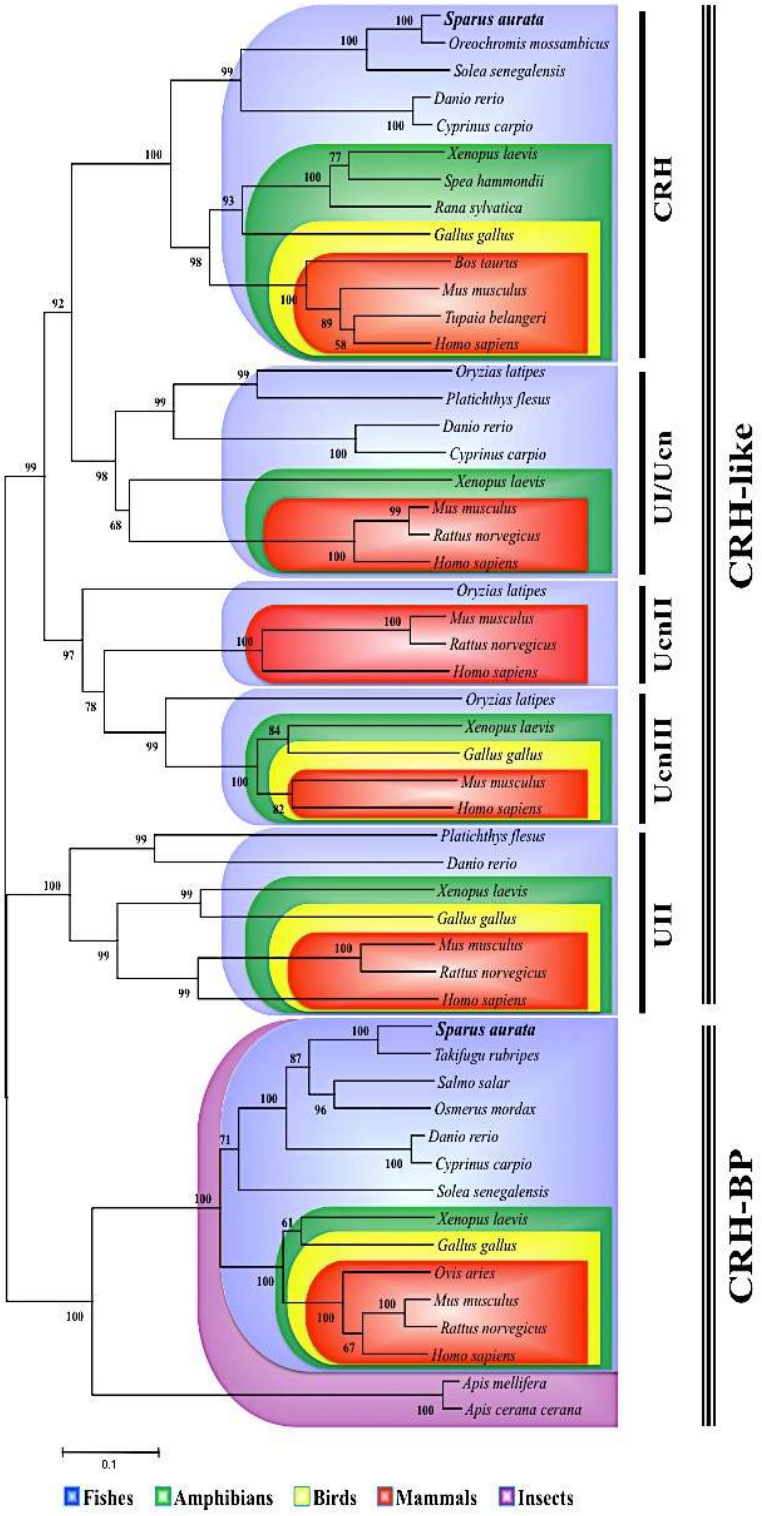

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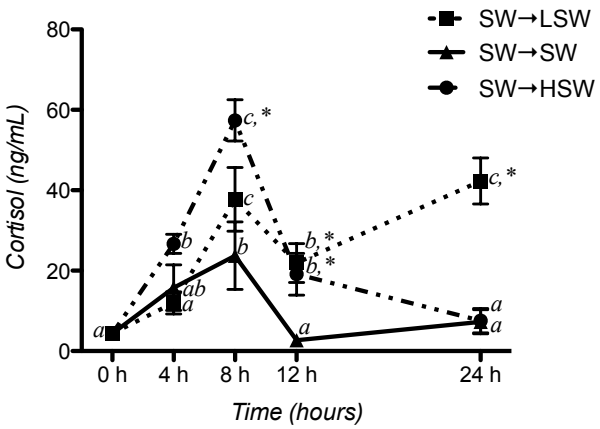
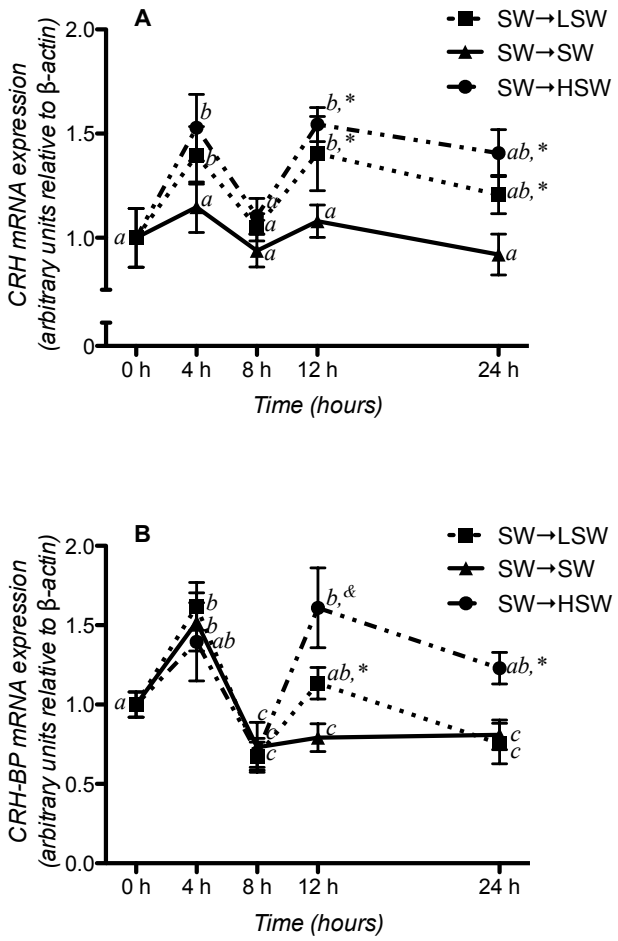
Figure 6. ***Martos-Sitcha et al.***



923 **Figure 7.** ***Martos-Sitcha et al.***



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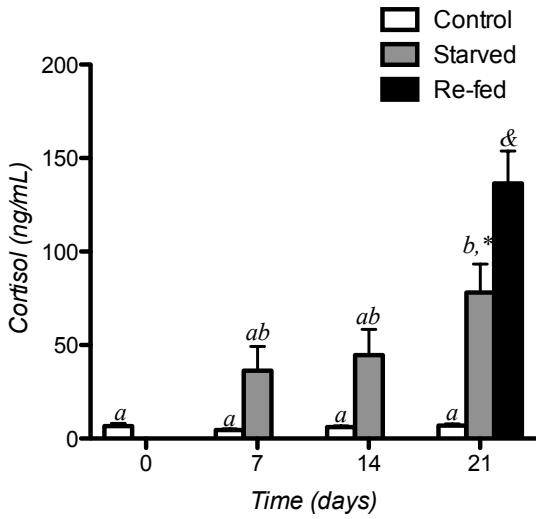
926 **Figure 8.** ***Martos-Sitcha et al.***



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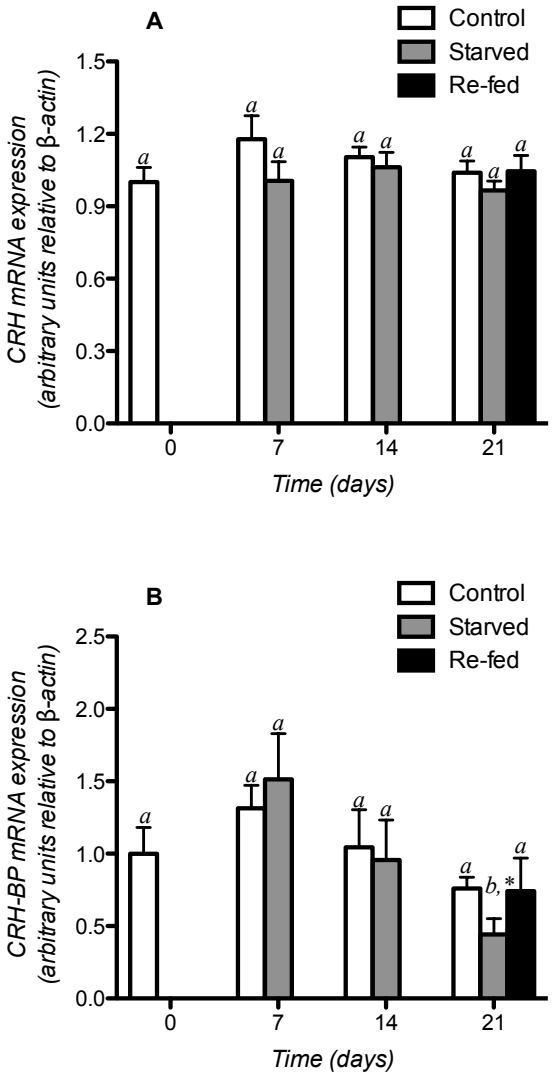
930 **Figure 9.** ***Martos-Sitcha et al.***



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