J Comp Physiol B (2015) 185:87–101
DOI 10.1007/s00360-014-0871-7

ORIGINAL PAPER

Acclimation to different environmental salinities induces molecular endocrine changes in the GH/IGF-I axis of juvenile gilthead sea bream (*Sparus aurata* L.)

Khaled Mohammed-Geba · J. M. Mancera · G. Martínez-Rodríguez

Received: 13 April 2014 / Revised: 15 October 2014 / Accepted: 26 October 2014 / Published online: 14 November 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract To assess the role of the GH/IGF-I axis in osmotic acclimation of the gilthead seabream *Sparus aurata*, juvenile specimens were acclimated to four environmental salinities: hyposmotic (5 ‰), isosmotic (12 ‰) and hyperosmotic (40 and 55 ‰). The full-length cDNAs for both pituitary adenylate cyclase-activating peptide (PACAP) and prepro-somatostatin-I (PSS-I), the precursor for mature somatostatin-I (SS-I), were cloned. Hypothalamic PACAP and PSS-I, hypophyseal growth hormone (GH) and prolactin (PRL), and hepatic insulin-like growth factor-I (IGF-I) mRNA expression levels were analyzed in the four rearing salinities tested. PACAP and IGF-I mRNA values increased significantly in response to both 5 and 55 ‰ salinities, showing a U-shaped curve relationship with the basal level in the 40 ‰ group. Hypothalamic PSS-I expression increased strongly in the 55 ‰ environment. GH mRNA levels did not change in any of the tested environmental salinities. PRL mRNA maximum levels were encountered in the 5 and 12 ‰ environments, but significantly down-regulated in the 40 ‰. Plasma cortisol levels significantly increased in the 40 ‰ environment. These results are discussed in relation to the well-known high adaptability of *Sparus aurata* to different environmental salinities and the role of the GH/IGF-I axis in this process.

Keywords GH · IGF-I · Osmoregulation · PACAP · PSS-I · *Sparus aurata*

Communicated by H.V. Carey.

K. Mohammed-Geba (✉) · G. Martínez-Rodríguez
Departamento de Biología Marina y Acuicultura, Instituto de Ciencias Marinas de Andalucía, Consejo Superior Investigaciones Científicas (CSIC), 11510 Puerto Real, Cádiz, Spain
e-mail: khaled.mohammed@icman.csic.es

K. Mohammed-Geba · J. M. Mancera
Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Campus de Excelencia Internacional del Mar (CEIMAR), Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain

K. Mohammed-Geba
Molecular Biology and Genetic Engineering Division, Department of Zoology, Faculty of Sciences, Menoufia University, Shebin El-Kom, Menoufia, Egypt

Introduction

Hormones and neuropeptides play a key role in maintaining the physiological balance of euryhaline fishes in a stable state during changes in the environmental salinity. Neuroendocrine axes sense the osmotic and ionic changes and coordinate the tissue-specific response for internal adjustment. This adjustment can be rapid, as through the actions of angiotensins, or slow, as through prolactin (PRL) and growth hormone/insulin-like growth factor-I (GH/IGF-I) actions. The rapid-acting hormones cope with immediate challenges by controlling drinking rate and the activity of ion transporters in the gill, gut and kidney. The slow-acting hormones alter the abundance of ion transporters and trigger cell proliferation and differentiation of ionocytes and other osmoregulatory cells, reorganizing then the body for long-term acclimation (Takei and McCormick 2012).

GH and IGF-I exhibited plasma-hypoosmoregulatory actions in salmonid fishes (Sakamoto et al. 1993), increasing opercular chloride cells number, gill Na^+, K^+-ATPase activity and mRNA expression of Na^+, K^+-ATPase subunits, as well as salinity tolerance when administered (McCormick 1995, 2001). However, in non-salmonid fishes, the osmoregulatory roles of this axis are

controversial due to the contradictory results obtained among different teleost species (Mancera and McCormick 1998). For this reason, more research on both euryhaline and stenohaline fish species to determine the widespread osmoregulatory actions of the GH/IGF-I axis is required (Sakamoto and McCormick 2006; Mancera and McCormick 2007; Link et al. 2010).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a hypothalamic neuropeptide which belongs to glucagon/secretin/growth hormone-releasing hormone (GHRH) superfamily. PACAP regulates cell proliferation, differentiation, development of the nervous system, regeneration following nerve injuries, apoptosis, and metabolism (Somogyvári-Vigh and Reglodi 2004). PACAP analogs are effective in stimulating GH secretion in vitro (Wong et al. 2000). Somatostatin-I (SS-I) is a tetradecapeptide secreted by the hypothalamus and it acts as an inhibitor of pituitary GH secretion (Peterson et al. 2003; Xu and Volkoff 2009). Prepro-somatostatin (PSS), the precursor of mature SS peptide, is present in all vertebrate classes, especially the precursor for the highly conserved SS-14 form (also named SS-I), presenting many effects on energy allocation, digestion, and metabolism. Both PRL and GH belong to the GH/PRL family of pituitary polypeptide hormones sharing a common structure (Forsyth and Wallis 2002). The major action of PRL in teleost fishes is the maintenance of hydromineral balance in euryhaline species under freshwater condition (Manzon 2002; Sakamoto and McCormick 2006).

Gilthead sea bream (*Sparus aurata*) is a euryhaline species that is being used in our research group as a model organism for osmoregulatory studies. This species tolerates a wide range of environmental salinities (from 5 to 60 ‰), with the best growth rates obtained under isosmotic environments. Acclimation to different environmental salinities induced several osmoregulatory, endocrine and metabolic adjustments (Mancera et al. 1993a, b; Laiz-Carrión et al. 2005a, b; Sangiao-Alvarellos et al. 2005; Vargas-Chacoff et al. 2009b). In addition, this species is extensively cultured in the Mediterranean area, providing considerable economic significance in aquaculture there.

The aim of this study was to investigate the roles of growth-related neuropeptides (PACAP and PSS-I) in controlling modifications in the GH/IGF-I axis in juvenile specimens of *S. aurata* acclimated to different environmental salinity regimens (5, 12, 40 and 55 ‰). Some of these changes were previously tested in response to different osmotic challenges in *S. aurata* (Laiz-Carrión et al. 2009; Vargas-Chacoff et al. 2009b). The cDNAs from two hypothalamic factors (PACAP and PSS-I) involved in GH production were cloned and sequenced. Expression modification in genes related to the GH/IGF-I axis (PACAP, SS-I, GH and IGF-I), alongside with another adenohypophyseal hormone directly involved in osmoregulatory processes (PRL) was determined. Also, the phylogeny of the obtained neuropeptide precursors in *S. aurata* was studied in relation to other teleost species using neighbor-joining phylogenetic trees.

Materials and methods

Animals and experimental protocol

Juvenile specimens of *S. aurata* ($n = 32$, 150–180 g body mass) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) where they were kept in 40 ‰, salinity (1,090 mOsm kg^{-1} H_2O osmolality), and temperature between 21 and 22 °C). Fish were transferred to the wet laboratories of the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz), where they were acclimated during 7 days to 40 ‰ salinity, under the same water conditions mentioned before, in 400-L tanks with a flow through water system. The experimental salinities were achieved by mixing SW with dechlorinated tap water (until reaching 5 and 12 ‰ salinities) or with natural marine salt (Salina de la Tapa, El Puerto de Santa María, Cádiz, Spain) until reaching 55 ‰ salinity. Experimental animals were divided into four groups ($n = 8$) and transferred directly to 400-L tanks (3 kg m^{-3} density) with the specific salinity, where they were maintained under environmental photoperiod and temperature (October 2010) for 14 days. After this experimental time, the osmoregulatory system of *S. aurata* specimens is known to reach a chronic regulatory period (Laiz-Carrión et al. 2005a; Sangiao-Alvarellos et al. 2005). Groups were maintained in a closed recirculating water system with a Teflon physical filter and a biological filter of rock beads. Water quality criteria were checked periodically to affirm their stability. 20 % of tank water was changed daily. Fish were fed a daily ration of 1 % of their body mass with commercial pellets (Dibaq-Dibroteg S.A., Segovia, Spain). Every morning before feeding, rearing tanks were checked and no food was left. No mortality was observed during experimental time in all salinities used.

After 14 days, fish were netted, anesthetized with 2 mL L^{-1} of 2-phenoxyethanol (Sigma-Aldrich), weighed and sampled. Blood was immediately collected from the caudal peduncle into 1-mL syringes that were rinsed with a solution containing 25,000 units of ammonium heparin per 3 mL of 0.6 % NaCl. Plasma was separated from cells by whole blood centrifugation (3 min, 10,000×*g*, 4 °C), snap frozen in liquid nitrogen and stored at −80 °C until analysis. For quantitative reverse transcription polymerase chain reaction (qPCR) analysis, the entire pituitary gland, both hypothalami lobes, and biopsies from the liver of each animal were immediately preserved in 5–10× volumes (w/v) of RNAlater® (Ambion, LifeTechnologies), kept

Table 1 Sequences for primers used for obtaining the 5′, 3′, intermediate and full-length cDNA fragments for PACAP and PSS-I

Primer	Gene	Orientation	Sequence 5′ → 3′	Location in *S. aurata*
Intermediate cDNA fragment	PACAP	Sense	CAT TAC AGC GTC TAC TGC T	322–340
		Antisense	ATG TGT GTC CTC AAG TG	1,007–1,024
	PSS-I	Sense	ACT CCA AAC TCC GCC TG	121–140
		Antisense	ATA GTT TCA TCA GTT TAC ATT C	504–525
5′-, 3′-RACE	PSS-I	3′-RACE 1	CCG GCT GCA AGA ACT TCT T	349–366
		3′-RACE 2	ACC TTC ACC TCC TGC TGA GA	375–394
	PSS-I	cDNA-synthesizer and 5′-RACE 1	AGA ATC AGC TCA GAG AAG TCT AA	499–477
		5′-RACE 2	CTC CAG ATC CAC ATG GAT GT	283–302
Re-Clon	PSS-I	Sense	AGA TCC GCC GAC CCG TCA GC	1–20
		Antisense	TTT GAC TCA AAC GGA TTT TAT TCA	541–564

overnight at 4 °C and then transferred to −20 °C until further analysis.

All experimental procedures complied 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.

Cloning of sea bream PACAP and PSS-I partial cDNAs

Gene-specific primers for PACAP and PSS-I were designed from published partial cDNA sequences in *S. aurata* (Acc. No.: DQ659328.1) and from very conserved regions in the full-length cDNA sequences from other teleost fishes (PSS-I: *Astatotilapia burtoni*, Acc. No.: AY585720.1; *Epinephelus coioides*, Acc. No.: AY677120.1; *Lophius americanus*, Acc. No.: V00640.1). All primers used for cloning (Table 1) were purified by desalting and purchased from biomers.net (Germany). All kits were used according to manufacturers´ instructions; otherwise any modification will be mentioned. Total RNA was extracted with the NucleoSpin® RNA II kit (Macherey-Nagel), using single hypothalamus, homogenized by an IKA® Ultra-Turrax® T25 with the dispersing tool S25N-8G (IKA-Werke), and including the on-column DNA digestion using the RNase-free DNase provided with the kit. RNA quality was checked in the Bioanalyzer 2100 system (Agilent Technologies, Life Sciences), using Agilent RNA 6000 Nano kit (Agilent Technologies, Life Sciences). RNA quantity was measured spectrophotometrically at 260 nm with a BioPhotometer Plus (Eppendorf). For cDNA synthesis, the purest (RIN >8) and the most abundant RNA samples (>200 ng μL^{-1}) were selected.

cDNA was synthesized from the hypothalamic RNA using the SuperScript™ III Reverse Transcriptase (Invitrogen, LifeTechnologies), using ~3 µg of total RNA. 1 U of BIOTAQ™ DNA polymerase (Bioline) was used in each PCR reaction applied for the amplification of the target genes in a total volume of 25 µL. The PCRs were accomplished in a Mastercycler®pro (Eppendorf). The PCR program is shown in Table 2. Fresh, adequate-size PCR products were cloned into the pCR®4-TOPO cloning vector (Invitrogen, Life Technologies) and sequenced in the Unidad de Genómica of the University of Córdoba. For all putative clones, forward and reverse sequencing was carried out using the dideoxynucleotide chain-termination method with T3 and T7 universal primers. The obtained 703 bp PACAP cDNA fragment shared 100 % nucleotide sequence similarity with the published partial GHRH/PACAP precursor in the gilthead sea bream (GenBank Accession No. DQ659328.1). PSS-I cDNA clone was 408 bp-long and showed 95 % identity with *Astatotilapia burtoni* PSS-I (GenBank Accession No. AY585720.1), and 85 % with PSS-I from *Epinephelus coioides* (GenBank Accession No. AY677120.1) and from *Siniperca chuatsi* (GenBank Accession No. JN034584.1).

Table 2 PCR programs applied for (1) cloning of PACAP and PSS-I intermediate fragments and (2) qPCR amplifications

Step	Temperature	Time
(1)		
Initial denaturation	95 °C	10 min
40 cycles		
Denaturation	95 °C	30 s
Annealing	55 °C	30 s
Extension	72 °C	60 s
Final extension	72 °C	10 min
(2)		
Initial denaturation	95 °C	10 min
40 cycles		
Denaturation	95 °C	15 s
Annealing and extension	60 °C	30 s

Screening of a brain cDNA library

To isolate the full-length PACAP cDNA, a gilthead sea bream brain cDNA library constructed in lambda ZAP

(Stratagene, Agilent Technologies Life Sciences) (Martos-Sitcha et al. 2013) was screened. Approximately, 250,000 plaques were plated in a NZY 240 × 240 mm plate (Nunc), and transferred onto a Hybond-N Nylon membrane (GE LifeSciences). Membrane was prehybridized for 1 h at 42 °C in prehybridization buffer (50 % formamide, 6× SSPE, 0.5 % SDS, 5× Denhardt's solution, 0.1 mg mL^{-1} yeast RNA type III). For hybridization, 25 ng of the putative cDNA fragment coding for gilthead sea bream PACAP, previously cloned by PCR, was radiolabeled using the RadPrime DNA Labeling System (Invitrogen, LifeTechnologies) and (α-^{32}P)dCTP (PerkinElmer), and allowed to hybridize with the Nylon membrane overnight at 42 °C. Then membrane was washed twice in 2× SSC-0.1 % SDS for 30 min each at room temperature, twice in 1× SSC-0.1 % SDS for 30 min at 42 °C, and twice in 0.5× SSC-0.1 % SDS for 30 min at 60 °C, till background was very low. Membranes were exposed to autoradiography film (Amersham, GE LifeSciences) for 2 days with intensifying screens at −80 °C. Positive plaques were isolated and subjected to further two rounds of hybridization/isolation. After the third round of the screening, several putative pBluescript SK(−) phagemid clones from the brain library were excised in vivo using *E. coli* XL1-Blue MRF' and SOLR strains (Stratagene, Agilent Technologies Life Sciences). The phagemids were double digested by *Eco*RI and *Xho*I (Takara) and the products were revealed in a 1 % agarose gel stained with GelRed™ (Biotium). Positive clones with adequate sizes were sequenced in a sequencing service (Unidad de Genómica, University of Córdoba, Spain).

Although the same protocol for screening the brain mRNA library was applied for obtaining the full PSS-I cDNA, no positive clones were obtained, and therefore a RACE approach was followed to get the full-length cDNA.

3′ and 5′ RACE

All primers used for RACE are shown in Table 1. For cloning the 3′-end of the PSS-I cDNA, two rounds of PCR were performed with two forward primers designed from the 3′-end of the obtained intermediate cDNA fragment of PSS-I against a poly-T(V) as the reverse primer. All gene-specific primers used were purified by desalting and purchased from biomers.net (Germany). PCR conditions were the same as used before for obtaining the intermediate cDNA fragment and are shown in Table 2. To clone the 5′-end of PSS-I, the 5′ RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, LifeTechnologies) was used. In brief, hypothalamic cDNA was synthesized from 1 µg of total RNA with the gene-specific primer (cDNA-synthesizer primer in Table 1). Two rounds of PCR, with the same conditions for cloning of the intermediate cDNA fragments, were run with two reverse primers designed from the 5′-end of the obtained intermediate cDNA fragment of PSS-I. Forward primers were poly G adapter primers provided with the 5′-RACE kit. Fresh PCR product was purified using Microcon YM-100 model (Centrifugal filter devices, Millipore) as follows. The PCR reaction was adjusted to 500 µL with 10 mM Tris–HCl (pH 8). The solution was applied to Microcon filters and centrifuged at 500×*g* for 10 min. The volume was re-adjusted to 500 µL with 10 mM Tris–HCl pH 8 and re-centrifuged. The filter was placed upside-down and centrifuged for 5 min at 1,000×*g* for recovering of the PCR product purified from primers, free nucleotides, and other contaminants. After running a 1 % agarose gel stained with GelRed™ (Biotium) in a horizontal electrophoresis for checking the product integrity, the purified PCR product was cloned into the pCR®4-TOPO vector (Invitrogen, LifeTechnologies) for sequencing.

Finally, the three cDNA sequences (3′-, intermediate, and 5′-) of the PSS-I were assembled according to the 100 % identities between their overlapping ends using the algorithm merger (http://pro.genomics.purdue.edu/emboss/). A re-cloning primer pair was designed from the outer-most 5′ and 3′ extremities (Table 1) of the obtained hypothalamic cDNA fragments of the PSS-I. These were used to re-clone the full-length cDNA of the PSS-I by PCR to assure the effectiveness of the RACE strategy.

Phylogenetic analyses

PACAP and PSS-I sequences obtained from various vertebrate classes were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/protein/) and aligned using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html). The results were uploaded to MEGA 5.0 (Tamura et al. 2011). Neighbor-joining algorithm, based on open reading frame nucleotide sequences and complete deletion, was applied for constructing the phylogenetic trees. One thousand (1,000) bootstraps were applied for enhancing the reliability of the test.

Total RNA extraction and quantitative reverse transcription polymerase chain reaction (qPCR)

Total RNA from hypothalamus and liver was extracted using the NucleoSpin® RNA II kit (Macherey-Nagel), whereas total RNA from pituitary was extracted using the NucleoSpin® RNA XS kit (Macherey-Nagel), following manufacturer's protocols (for all kits in this study only originally supplied components and protocols were used). RNA concentration and quality were assessed using the Biophotometer plus (Eppendorf) and Bioanalyzer 2100 system (Agilent Technologies, Life Sciences), using Agilent RNA 6000 Nano kit (Agilent Technologies, Life Sciences). All samples had RNA integrity number (RIN)

Table 3 Sequences, positions, amplicon sizes, amplification efficiencies and regression coefficients of primers used for qPCR study

Primer	Orientation	Sequence 5′ → 3′	Position	Acc. no.	Amplicon size (bp)	*E*	r^2
PACAP	Sense	AGA CAG CTA CAG CCG CTA CC	678	JN585761	135	1	0.99
	Antisense	AAG GGA GGA GGA GGA TGC TA	812				
PSS-I	Sense	CTG GGC TCC AAA CAG GAC	159	JN585762	142	0.99	0.99
	Antisense	CCA GAT CCA CAT GGA TGT CTT	300				
GH	Sense	CGT CTC TTC TCA GCC GAT	90	U01301.1	131	0.99	0.99
	Antisense	GCT GGT CCT CCG TCT GC	220				
PRL	Sense	TGA CAT CGG CGA GGA CAA CAT T	540	AF060541.1	111	1	0.99
	Antisense	CGG CAG CGG AGG ACT TTC AG	650				
IGF-I	Sense	GCC ACA CCC TCT CAC TAC TG	184	AY996779.2	196	1.01	0.998
	Antisense	AAG CAG CAC TCG TCC ACA	379				
β-actin	Sense	TCT TCC AGC CAT CCT TCC TCG	782	X89920.1	108	1	0.99
	Antisense	TGT TGG CAT ACA GGT CCT TAC GG	889				

values ≥8.00. A total of 500 ng (for hypothalamus and liver) or 50 ng (for pituitary) of total RNA from each tissue was used for cDNA synthesis using qScript™ cDNA Synthesis Kit (Quanta BioSciences). Generated cDNAs were stored at −20 °C for a period never exceeding 1 month.

All qPCR steps were performed using PerfeCTa™ SYBR®Green FastMix™ (Quanta BioSciences), with cycling conditions detailed in Table 2. The qPCR primers (Table 3) were designed using the software primer3 (http://frodo.wi.mit.edu/primer3/) based on the full cDNA sequences published in GenBank (http://www.ncbi.nlm.nih.gov/nuccore) for gilthead sea bream PACAP (Acc. No.: JN585761), preproSS-I (Acc. No.: JN585762), IGF-I (Acc. No.: AY996779), GH (Acc. No.: U48221) and β-actin (Acc. No.: X89920). PRL primers were designed and used in previous studies with *S. aurata* (Vargas-Chacoff et al. 2009a, b). All qPCR primers were purified by HPLC and purchased from biomers.net (Germany).

To optimize the qPCR conditions, several primer concentrations (100, 200, 400 and 500 nM) and temperature gradient (from 50 to 60 °C) were used. Different hypothalamus and liver cDNA template quantities were applied in triplicate (10 ng, 1 ng, 100 pg, 10 pg and 1 pg of input RNA) to check the assay linearity and the amplification efficiency. For pituitary cDNAs, the same range was applied, but excluding the first point (10 ng). Finally, although the assay was linear between 10 and 1 pg of cDNA per reaction (amplification efficiencies and regression coefficients are shown in Table 3), a final in-well cDNA quantity of 100 pg of cDNA was used for all the amplifications.

Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). qPCR reactions (10 µL), composed of 4 µL cDNA template, 5 µL PerfeCTa™ SYBR®Green FastMix (2× concentrated, Life Technologies), and 0.5 µL from each primer, were performed with the Mastercycler®ep Realplex^2 (Eppendorf) operated with Realplex 2.2 software (Eppendorf). Reactions, ran in triplicate, were incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Non-template controls (NTCs) were used as negative controls in every experiment. A single-peak melting curve was used to check for the absence of primer–dimer artifacts and non-specific amplifications. Beta actin (β-actin) was used as the internal reference gene for normalizing mRNA expression data, owing its low C_T variability in agreement to the results reported before for *S. aurata* (Hang et al. 2005; Vargas-Chacoff et al. 2009a, b) and what we found during the qPCR runs (not exceeding 0.5 C_T differences among different salinities).

Plasma cortisol levels

To determine the state of stress of the fishes under their diverse environments, plasma cortisol levels were determined. Plasma levels of free cortisol were quantified in each experimental group ($n = 8$) by indirect enzyme assay (EIA) following a method described before for testosterone (Rodríguez et al. 2000). Extraction and measurement of gilthead sea bream plasma cortisol followed the procedure used by Martos-Sitcha et al. (2013). The lower limit of cortisol detection (93 % of binding, ED93) was 3.91 pg mL^{-1}. The intra-assay coefficient of variation (calculated from the sample duplicates) was <4 %. The inter-assay coefficient of variation was not calculated since all samples were run in the same plate.

Statistics

Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey-HSD post hoc test. Significant values were considered when $P < 0.01$.

Fig. 1 Nucleotide and predicted amino acid sequence of sbPRP/PACAP. *White letters* over *black background*: signal peptide. *White letters* over *dark gray background*: PACAP-related peptide (PRP). *Black letters* over *light gray background*: mature PACAP precursor. Putative polyadenylation signals are shown as *white letters* over *light gray background*. *Boxes* mark dibasic cutting sites Lys^{126}-Arg^{127} within the PRP and Lys^{155}-Arg^{156} within the PACAP sequences. Stop codon motif TAG was referred to by the *at symbol*

Results

Cloning of sbPACAP and sbSS-I

Gilthead sea bream PACAP cDNA was successfully cloned, with the nucleotide and predicted amino acid sequences shown in Fig. 1. The oligonucleotide primers used for cloning PACAP were successfully located in the cDNA sequences. Their positions are shown in Table 1. *S. aurata* full-length PACAP cDNA consisted of 2,051 bp including an open reading frame (ORF) of 522 bp, a 5′-untranslated region (UTR) of 273 bp and a 3′-UTR of 1,256 bp. The ORF shared 91 % identity with *Paralichthys olivaceus* PACAP (JX152586.1), 87 % with *Oncorhynchus mykiss* long PACAP transcript (NM_001124297.1), 86 % with *Salmo salar* long PACAP transcript (NM_001139927.1), 74 % with *Oreochromis niloticus* PACAP (JQ246949.1) and 72 % with *Clarias gariepinus* PACAP (EF524513.1). The cDNA encoded a protein with 173 amino acids in its open reading frame. Sea bream PACAP primary protein structure shared 88 % identity with PACAP precursor of *Paralichthys olivaceus* (AGB14621.1), 86 % with *Oncorhynchus mykiss* long PACAP transcript (CDQ56897.1), 86 % with *Salmo salar* long PACAP transcript (NP_001133399.1), 89 % with *Oreochromis niloticus* PACAP (XP_003439403.1) and 72 % with *Clarias gariepinus* PACAP (65 %). The protein consisted of a 23 amino acid signal peptide at the beginning, followed by the 106 amino acid-long PACAP-related peptide (PRP) that showed high sequence identity with PRP in other teleost fishes, but very low similarity to mammalian GHRH (22–37 %) proteins, according to BLAST comparisons. A dibasic cutting site with lysine–arginine is found in the end of PRP, through which a proteolytic processing to the preproprotein will cause the release of the 45 amino acid-long fish mature PACAP, corresponding to the α-amidated PACAP38 in mammals. Furthermore, the tripeptide Gly^{154}-Lys^{155}-Arg^{156} can be cleaved to generate the α-amidated PACAP27 isoform according to the model reviewed before (Vaudry et al. 2000).

Gilthead sea bream preproSS-I cDNA consisted of 560 bp including an open reading frame (ORF) of 365 bp, a 5′-untranslated region (UTR) of 26 bp and a 3′-UTR of 169 bp (Fig. 2). The locations of the oligonucleotide primers used for cloning PSS-I were detected in the full-length cDNA sequence (Table 1). The ORF shared 87 % identity with *Paralichthys olivaceus* PSS-I (AB693833.1), 74 % with *Salmo salar* PSS-I (NM_001141101.1), 57 % with *Acipenser transmontanus* PSS-I (AF395849.1) and 59 % with *Ictalurus punctatus* PSS-I (M25903.1), and encoded a 121 amino acid protein. This predicted primary amino acid sequence shared 87 % identity with *Paralichthys olivaceus* PSS-I (BAL46409.1), 53 % with *Salmo salar* PSS-I

Fig. 2 Nucleotide and predicted protein sequence of sbPreproSS-I (PSS-I) precursor. *White letters* over *black background*: signal peptide. *Black letters* over *light gray background*: mature SS-I precursor. Putative polyadenylation signals are shown as *white letters* over *light gray background*. *Boxes* mark dibasic cutting site Arg^{106}-Lys^{107} within the PSS-I sequences. Stop codon motif TGA was referred to by the *ampersand symbol*

(NP_001134573.1), 49 % with *Acipenser transmontanus* PSS-I (AAL13248.1) and 49 % with *Ictalurus punctatus* PSS-I (AAA49339.1). Most of the variation produced from the portion of the PSS-I precursor other than the SS-I itself. Mature SS-I deduced amino acid sequence was almost 100 % identical to its analogs in all vertebrate classes, from fishes to mammals. In *S. aurata*, the protein encompassed a putative signal peptide of 28 amino acids with a region of hydrophobic residues that is a common feature of other signal peptides (Nielsen et al. 1997). The deduced PSS-I amino acid sequence comprised possible recognition sites for prohormone convertases. Cleavage at the R–K (Arg^{106}-Lys^{107}) dibasic recognition site would yield the SS-14 peptide and processing at the R (Arg^{93}) monobasic site may generate a large form of SS-I (SS 30). The 3′-UTR of the cDNA contained a polyadenylation signal motif AATAAA for the poly-A^+ tail (Beaudoing et al. 2000; Xu and Volkoff 2009).

Phylogeny of sbPACAP and sbSS-I

For the neighbor-joining (NJ) phylogenetic trees, Kimura 2-parameter was the best model describing the substitution patterns in both PACAP and PSS-I. For PACAP, the phylogenetic tree showed that all fish PACAPs actually belong to one monophyletic group, while the amphibians and the other tetrapods belong to the other one (Fig. 3). Interestingly, both short and long PACAP mRNA precursors of the spotted African lungfish *Protopterus dolloi* were located within the tetrapod clade. Within fish clade, two sub-clades could be distinguished, the first including species whose PACAP precursors are catfish-like; and the second containing species with salmon-like PACAP precursors. Despite having some degree of independence, *S. aurata* belonged to the salmon-like PACAP sub-clade.

For the PSS-I precursors phylogeny, the NJ tree exhibited two monophyletic groups. The first group or clade is divided into two sub-clades: to the first belongs most of siluriformes, cypriniformes and osteoglossiformes, and into the second lies most of perciformes and pleuronectiformes, including that of *S. aurata.* Interestingly, the second monophyletic group not only contains all tetrapod vertebrates' PSS-I precursors, but also PSS-I precursors of sturgeons and sharks. (Fig. 4).

Endocrine and neuroendocrine measurements

Acclimation during 14 days to different environmental salinities increased hypothalamic PACAP mRNA levels in all tested salinities in comparison to the 40 ‰-acclimated group (Fig. 5A). However, hypothalamic PSS-I expression increased significantly in the 55 ‰-acclimated group only (Fig. 5B). Pituitary GH expression levels did not change significantly among different salinity groups (Fig. 5C), except for a tendency to increase in specimens acclimated to the highest salinity ($P = 0.09$). PRL expression levels (Fig. 5D) increased significantly in 5- and 12 ‰-acclimated groups versus specimens acclimated to hyperosmotic environments (40 and 55 ‰). This expression was significantly down-regulated in 55 ‰-acclimated specimens in comparison to 40 ‰-acclimated group. Hepatic IGF-I expression values showed, after 14 days of acclimation, a U-shaped curve pattern in relation to different environmental salinity, with the highest expression in extreme salinities (5 and 55 ‰) (Fig. 5E). This pattern was very similar to that of the PACAP expression in the hypothalamus.

Finally, plasma cortisol levels exhibited the minimum values in 12 ‰ conditions while significantly increased in 55 ‰ environment (Fig. 6).

Discussion

In this study, we aimed to identify the influence of environmental salinity on the GH/IGF-I axis of *S. aurata*. Gilthead sea bream GH and IGF-I were previously cloned (Martínez-Barberá et al. 1994; Tiago et al. 2008). Also, expression

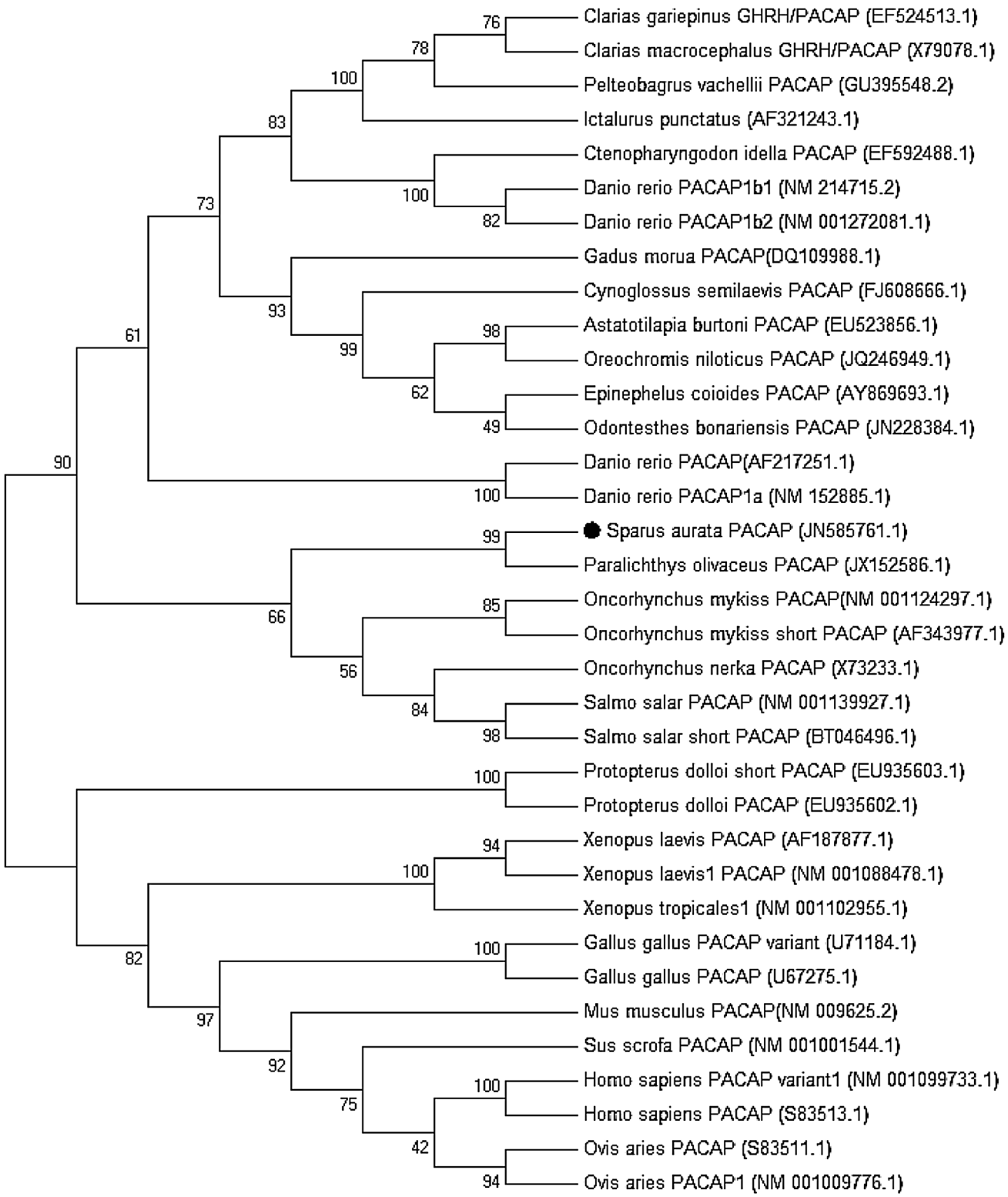

Fig. 3 Neighbor-joining phylogenetic tree of vertebrate prepro-PACAP open reading frame sequences. 1,000 bootstraps were used to assess the efficacy of the test. Only bootstrap values higher than 50 % are shown on each branch. Species and accession numbers are shown in the tree. Location of *S. aurata* sequence is marked by a *black circle*

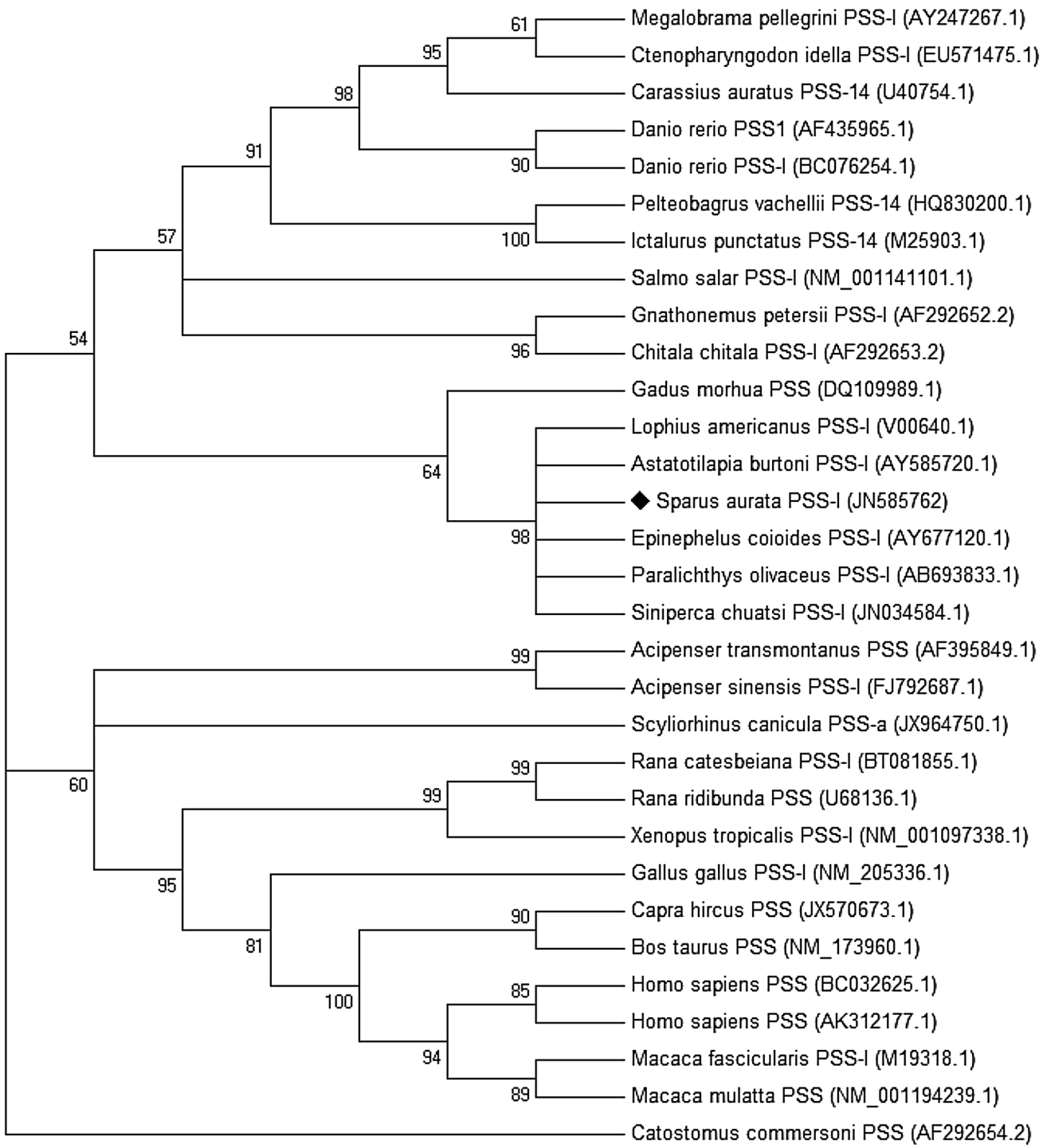

Fig. 4 Neighbor-joining phylogenetic tree of PSS-I open reading frame sequences. 1,000 bootstraps were used to assess the efficacy of the test. Only bootstrap values higher than 50 % are shown on each branch. Species and accession numbers are shown in the tree. Location of *S. aurata* sequence is marked by a *black rhomboid*

analyses assessing the influence of different experimental conditions, including osmoregulatory approaches, were performed (Laiz-Carrión et al. 2009; Vargas-Chacoff et al. 2009a, b; Fuentes et al. 2010). However, molecular tools for hypothalamic factors involved in the control of GH cells were not available until now. In this study, we cloned the full-length cDNAs for PACAP, as a stimulatory factor, and PSS-I, as an inhibitory factor, both for the pituitary GH cells.

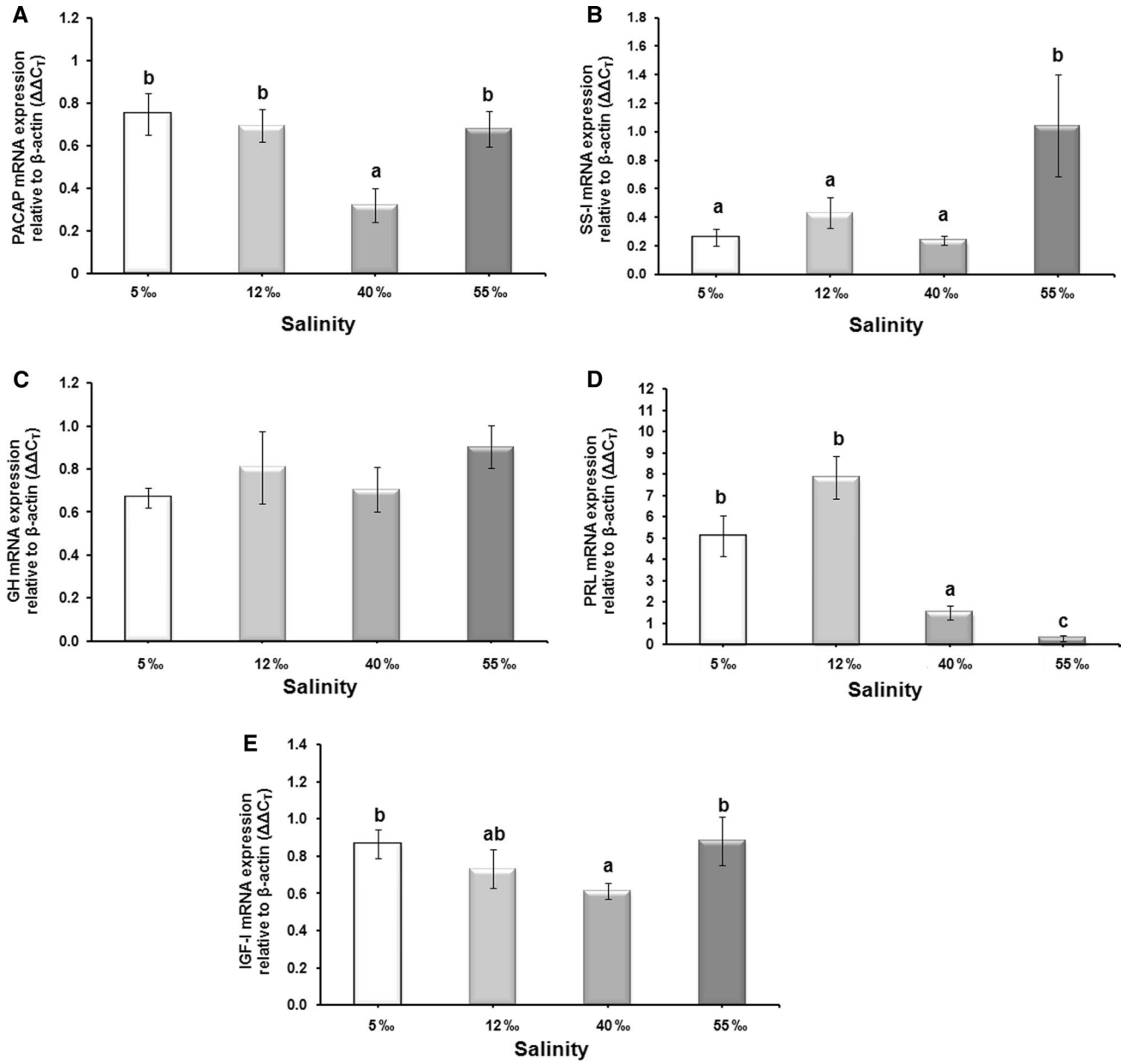

Fig. 5 mRNA expression patterns for **A** PACAP, **B** SS-I, **C** GH, **D** PRL and **E** IGF-I in *Sparus aurata* juveniles acclimated to different environmental salinities (5 ‰; 12 ‰; 40 ‰; 55 ‰) during 14 days. Data ($n = 8$) are represented as mean ± SEM. *Different letters* indicate significant differences among experimental groups (one-way ANOVA, $P < 0.01$)

PACAP is the newest member discovered from the family of brain–gut peptides that also includes, PACAP-related peptide (PRP), growth hormone-releasing hormone (GHRH), and glucagon (GCG). It was discovered in all levels of chordates from tunicates to humans. Its sequence is well conserved in these distant taxa, indicating that it performs important regulatory functions. In teleosts, two transcripts were discovered in several species, such as *Danio rerio*, *Takifugu rubripes*, *Carassius auratus*, *Gadus morhua* and in salmonids—all of which whom both transcripts result from a PRP—exon skipping (Parker et al. 1997; Cardoso et al. 2007; Xu and Volkoff 2009). In our case, we aligned many PACAP cDNA sequences available in the GenBank database, some of which belong to both "short" and "long" PACAP transcripts in the same fish species. We designed our PCR primers from the regions in the PACAP cDNA flanking the skipped exon, aiming to obtain two different-sized PACAP fragments by a single PCR. However, we could not found more than a single PCR product that was a part of the cDNA of the "long" PACAP transcript.

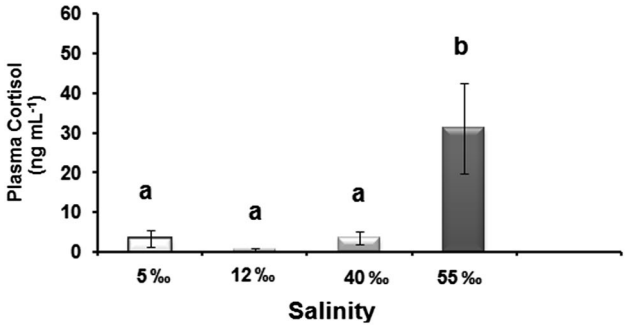

Fig. 6 Plasma cortisol levels in *Sparus aurata* juveniles acclimated to different environmental salinities. Further details are described in the legend of Fig. 5

After labeling this PCR fragment to be used as a probe for screening the brain gene library for the full PACAP cDNA sequence, the resulting clones contained only the long transcript. Therefore, presence of a "short" PACAP transcript in *S. caurata* cannot be clearly judged.

PSS-I is also very well conserved among all vertebrate classes, from agnathans to mammals. The obtained *S. aurata* PSS-I sequence clustered with other fish PSS-I rather than tetrapods, with a clear separation between both groups. Mature SS-I contained the same features of mature SS-I of *Epinephelus coioides*, including the dibasic cutting Arg-Lys site at the beginning that releases it, and the monobasic cutting site Arg^{93} that can give rise to SS-30.

GH/IGF-I axis

In our study, hypothalamic PACAP expression increased in environmental salinities different to 40 ‰. Furthermore, PACAP mRNA expression was completely different of GH and quite similar to that of IGF-I, possibly suggesting that the effect on GH mRNA occurred early during the course of the experiment to transmit the signal from PACAP to the hepatic IGF-I precursor.

The apparent similarity between PACAP and IGF-I expression patterns, but their difference with GH further complicates the dilemma of the direct GH role in osmoregulation in *S. aurata*. In salmonids, GH is considered as a proper seawater-adapting hormone (Sakamoto et al. 1993; Björnsson 1997) although in non-salmonids such function is less evident (Mancera and McCormick 1998, 2007; McCormick 2001; Laiz-Carrión et al. 2005b). In *S. aurata*, GH does not seem to be a key player in comparison to IGF-I. Many studies were carried out, concerning the osmoregulation in *S. aurata*, with special reference to the patterns of GH regulation (Mancera et al. 1995, 2002; Laiz-Carrión et al. 2009; Vargas-Chacoff et al. 2009b; Fuentes et al. 2010). GH injection did not affect any of the osmoregulatory parameters (including gill Na^+, K^+-ATPase activity, plasma osmolality, and plasma ions) in *S. aurata* upon acclimation to different salinities (Mancera et al. 2002). This issue of loss of GH-direct osmoregulatory roles extends far beyond *S. aurata*, to include fish species from proper marine origin, like *Sparus sarba* and eels, in contraposition to tilapia or killifish, for example (Sakamoto and McCormick 2006). Moreover, the contradictory results found in other previous studies with *S. aurata* for GH mRNA patterns under acclimation to different salinities may further confirm the minimum role of GH in *S. aurata* osmoregulation. *S. aurata* GH mRNA levels varied between the increase, the decrease, or even the complete loss of responsiveness (Laiz-Carrión et al. 2009; Vargas-Chacoff et al. 2009b). PACAP, however, stimulates the in vitro and in vivo GH release from the pituitary gland of some fish species, such as the goldfish *Carassius auratus*, the stargazer *Uranoscopus japonicus*, the common carp *Cyprinus carpio*, the turbot *Scophthalmus maximus* and the sockeye salmon *Oncorhynchus nerka* (Parker et al. 1997; Wong et al. 1998; Rousseau et al. 2001; Xiao et al. 2002; Matsuda et al. 2005). However, GH mRNA did not change under PACAP treatment in the olive flounder *Paralichthys olivaceus* (Nam et al. 2013). Also, dietary lipid changes influenced more PACAP, GH receptors and IGF-I than GH itself (Gómez-Requeni et al. 2012).

Therefore, and from all these studies, it is possible to conclude that the absence of a clear GH response to different environmental salinities in our experiment can be attributed to one of two factors: (1) that the GH for itself, at least in *S. aurata*, can be just a part of a more complex system of control, where it is switched on or off by other regulators, like cortisol or IGF-I for example (Sakamoto et al. 1993); or (2) a temporary and early GH response occurred during the course of the experiment, that lasted for 14 days, to affect the IGF-I production and some other osmoregulatory actions then disappeared before the end of the experiment.

PSS-I mRNA expression increased significantly in specimens maintained under the highest environmental salinity (55 ‰). Again, GH mRNA did not show any corresponding changes, which may reflect some osmosensitivity of growth-related neuropeptides, at least under the hyperosmotic condition. The inhibitory role of SS-I on GH cells has been demonstrated in some teleosts (Cook and Peter 1984; Cameron et al. 2005; Very et al. 2008). However, SS-I belongs to a gene family with several active forms that arise from the same or different precursors. SS forms are not equipotent in inhibiting GH production. For instance, three PSS genes have been cloned in the grouper *Epinephelus coioides*, sharing low amino acid identity with each other (Xing et al. 2005). In *Carassius auratus*, hypothalamic SS-II is more potent in inhibiting GH secretion than SS-I, while, in the catfish *Ictalurus punctatus*, the forms SS-22 and SS-14 have no effect on GH release (Klein and

Sheridan 2008). In our study, minimal changes observed in GH expression in relation to SS-I (SS-14) precursor expression may suggest the existence of several SS forms in *S. aurata* that showed different effects on GH cells.

In our study, the hepatic IGF-I mRNA up-regulated upon 14 days of acclimation to extreme salinities (5 and 55 ‰), alongside a non-significant ($P = 0.07$) tendency for increase in the 12 ‰ group. IGF-I is generally considered as a key player for mitochondria-rich cell (MRC) development as well as Na^+, K^+-ATPase increased activity (Sakamoto et al. 1993; McCormick 1995; Reinecke et al. 2005; Link et al. 2010). Therefore, its observed increase in the highest and lowest salinities could aid the proliferation and differentiation of MRC cells as well as the enhancement in Na^+, K^+-ATPase activity related to the acclimation of *S. aurata* specimens to extreme salinities (5 and 55 ‰), where higher number and sizes of MRCs as well as gill Na^+, K^+-ATPase activity have been observed (Laiz-Carrión et al. 2005a). In addition, this IGF-I enhancement could stimulate energy metabolism to support high energetic demand of osmoregulatory organs under extreme environmental salinity conditions, as previously reported in *S. aurata* (Sangiao-Alvarellos et al. 2005; Soengas et al. 2007). Moreover, another issue emerged in our results in what concerns the differences of IGF-I mRNA expression patterns in 12 ‰ salinity in comparison to other very similar teleosts, *Sparus sarba* and *Mylio macrocephalus* (Deane and Woo 2004, 2005). In these two species, the enhancement in IGF-I mRNA was more prominent in brackish water environments. However, taking into consideration the varying durations of acclimation between these experiments and ours, the IGF-I expression in response to different environmental salinities may then be time and species specific. Species-specific osmoregulatory response of IGF-I mRNA expression is well-known (McCormick 2001; Mancera and McCormick 2007). For example, in salmonids, parr-smolt transformation is associated with IGF-I mRNA increase (McCormick 2001; Reinecke et al. 2005; Jørgensen et al. 2007). Also, the transference of the black-chinned tilapia *O. melanotheron heudelotii* to seawater then to freshwater resulted in a transient IGF-I mRNA down-regulation (Link et al. 2010). Ultimately, in the Southern flounder *Paralichthys lethostigma*, transfer from freshwater to seawater increased hepatic IGF-I expression (Tipsmark et al. 2008).

PRL

PRL expression was significantly up-regulated in low-salinity environments, but down-regulated in hypersaline environments. Our results confirmed the hyperosmotic role of PRL in *S. aurata*, in agreement with the reported for this hormone in the same species and in other teleost species (Manzon 2002; Sakamoto and McCormick 2006; Laiz-Carrión et al. 2009; Vargas-Chacoff et al. 2009b; Fuentes et al. 2010; Link et al. 2010). PRL cells increased in volume, occupying larger hypophyseal area, and showed bigger nuclear sizes and/or cytoplasmic features upon low osmolality challenge in both *S. aurata* and *S. sarba*, indicating an osmosensing capability of sea bream PRL cells (Mancera et al. 1993b; Kwong et al. 2009). In addition, ovine PRL treatment significantly decreased gill Na^+, K^+-ATPase activity while increased plasma osmolality and ions in juveniles of *S. aurata* acclimated for 24 h to seawater and brackish water (Mancera et al. 2002). These latter findings refer to a role for PRL in the acute acclimation period to adjust plasma osmolality by reducing Na^+, K^+-ATPase activity and ion loss in a low-salinity environment, consequently. As mentioned elsewhere, after 14 days of acclimation, the fishes are found in a state of chronic adaptation. This, as found by Laiz-Carrión et al. (2005a), is associated with the increase in Na^+, K^+-ATPase activity and a decrease in plasma osmolality. Hence, and especially upon prolonged survival in a low-salinity environment, it seems that the regulatory role of PRL on Na^+, K^+-ATPase activity decreases, while IGF-I plays more crucial role.

Cortisol

Despite being a euryhaline species, *S. aurata* exposed to extreme environmental salinity in our experiment mounted a cortisol stress response, as indicated by the elevated plasma cortisol levels. A similar situation was noted before in a similar trial with *S. aurata* (Sangiao-Alvarellos et al. 2005). Several studies were carried out to investigate the effects of cortisol in relation to different environmental salinities in teleosts, including *S. aurata*. Cortisol increase is beneficial for enhancing gill Na^+, K^+-ATPase activity in response to excessively high and low environmental salinities (Mancera et al. 1993a, b, 2002). In *S. aurata*, stress elevates plasma cortisol which is accompanied by a decrease in GH levels (Pérez-Sánchez and Le Bail 1999).

The cortisol/IGF-I interaction seems to be complicated, multifactorial, and even species specific. Both cortisol and IGF-I may be involved in mediating the action of GH in seawater adaptation (Sakamoto et al. 1993). The direct physiological roles of IGF-I and cortisol seem to be paradoxical, with the cortisol acting as a potent growth inhibitor and IGF-I as a major growth promoter. Yet, some studies in teleosts, even in perciformes, indicated that cortisol does not necessarily reduce the capacity of hepatocytes to produce IGF-I in vitro. Even a co-incubation of hepatocytes with GH and cortisol could increase the hepatocyte IGF-I mRNA, despite the enhancement was less than that when incubating the hepatocytes with GH only (Pierce et al. 2011). On the other hand, another type of stress, the confinement, could increase plasma cortisol levels in *S. aurata,*

but decreased at the same time hepatic IGF-I mRNA expression, so cortisol/IGF-I interaction seems to be dependent also upon the stress situation (Saera-Vila et al. 2009). Finally, the interaction may be also species dependent since the transfer of the Southern flounder *Paralichthys lethostigma* to seawater elevated plasma cortisol levels, but decreased hepatic IGF-I mRNA expression significantly (Tipsmark et al. 2008).

Conclusions

Our results suggested that the increases in both PACAP and IGF-I gene expressions in response to hypo- and hyperosmotic salinity acclimation were greater than those for GH in *Sparus aurata* juveniles. PACAP and IGF-I patterns may then contribute to the successful acclimation and survival of *S. aurata* in response to environmental salinity fluctuations. The loss of GH expression sensitivity to the different tested environmental salinities may further question its direct roles in *S. aurata* osmoregulation, at least upon chronic acclimation to extreme environmental salinities. Somatostatin and cortisol had more prominent roles in osmoregulation in hyperosmotic environments. Finally, prolactin osmosensing and hyperosmoregulatory capabilities are prominent in *S. aurata* regardless of the salinity regime applied or length of acclimation period, as inferred from our study and other similar studies.

Acknowledgments This study was partly supported by Grants AGL2007-61211/ACU (Ministerio de Educación y Ciencia and FEDER, Spain) and Proyecto de Excelencia PO7-RNM-02843 (Junta de Andalucía) to JMM. Authors would like to appreciate Mrs. Asmaa Galal-Khallaf´s precious help during the conduction of the experiment. Also, authors would like to thank Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish. KMG would like to appreciate the role of the Egyptian Bureau in Madrid and the Egyptian Government in facilitating the stay period in Spain. Experimentation has been carried out at the *Campus de Excelencia Internacional del Mar* (CEI-MAR) facilities from the Universidad de Cádiz.

References

Beaudoing E, Freier S, Wyatt JR, Claverie JM, Gautheret D (2000) Patterns of variant polyadenylation signal usage in human genes. Genome Res 10:1001–1010

Björnsson BT (1997) The biology of salmon growth hormone: from daylight to dominance. Fish Physiol Biochem 17:9–24

Cameron C, Moccia RD, Leatherland JF (2005) Growth hormone secretion from the Arctic charr (*Salvelinus alpinus*) pituitary gland in vitro: effects of somatostatin-14, insulin-like growth factor-I, and nutritional status. Gen Comp Endocrinol 141:93–100

Cardoso JC, Vieira FA, Gomes AS, Power DM (2007) PACAP, VIP and their receptors in the metazoa: insights about the origin and evolution of the ligand-receptor pair. Peptides 28:1902–1919

Cook AF, Peter RE (1984) The effects of somatostatin on serum growth hormone levels in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 54:109–113

Deane EE, Woo NY (2004) Differential gene expression associated with euryhalinity in sea bream (*Sparus sarba*). Am J Physiol Regul Integr Comp Physiol 287:R1054–R1063

Deane EE, Woo NY (2005) Upregulation of the somatotropic axis is correlated with increased G6PDH expression in Black Sea bream adapted to iso-osmotic salinity. Ann NY Acad Sci 1040:293–296

Forsyth IA, Wallis M (2002) Growth hormone and prolactin–molecular and functional evolution. J Mammary Gland Biol Neoplasia 7:291–312

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

Gómez-Requeni P, Kraemer MN, Canosa LF (2012) Regulation of somatic growth and gene expression of the GH-IGF system and PRP-PACAP by dietary lipid level in early juveniles of a teleost fish, the pejerrey (*Odontesthes bonariensis*). J Comp Physiol 182B:517–530

Hang XM, Power D, Flik G, Balment RJ (2005) Measurement of PTHrP, PTHR1, and CaSR expression levels in tissues of sea bream (*Sparus aurata*) using quantitative PCR. Ann NY Acad Sci 1040:340–344

Jørgensen EH, Aas-Hansen Ø, Moriyama S, Iwata M, Strand JET (2007) The parr-smolt transformation of Arctic charr is comparable to that of Atlantic salmon. Aquaculture 273:227–234

Klein SE, Sheridan MA (2008) Somatostatin signalling and the regulation of growth and metabolism in fish. Mol Cell Endocrinol 286:148–154

Kwong AKY, Ng AHY, Leung LY, Man AKY, Woo NYS (2009) Effect of extracellular osmolality and ionic levels on pituitary prolactin release in euryhaline silver sea bream (*Sparus sarba*). Gen Comp Endocrinol 160:67–75

Laiz-Carrión R, Guerreiro PM, Fuentes J, Canario AV, Martín Del Río MP, Mancera JM (2005a) Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. J Exp Zool 303A:563–576

Laiz-Carrión R, Sangiao-Alvarellos S, Guzmán JM, Martín del Río MP, Soengas JL, Mancera JM (2005b) Growth performance of gilthead sea bream *Sparus aurata* in different osmotic conditions: implications for osmoregulation and energy metabolism. Aquaculture 250:849–861

Laiz-Carrión R, Fuentes J, Redruello B, Guzmán JM, Martín del Río MP, Power D, Mancera JM (2009) Expression of pituitary prolactin, growth hormone and somatolactin is modified in response to different stressors (salinity, crowding and food-deprivation) in gilthead sea bream *Sparus auratus*. Gen Comp Endocrinol 162:293–300

Link K, Berishvili G, Shved N, D'Cotta H, Baroiller JF, Reinecke M, Eppler E (2010) Seawater and freshwater challenges affect the insulin-like growth factors IGF-I and IGF-II in liver and osmoregulatory organs of the tilapia. Mol Cell Endocrinol 327:40–46

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods 25:402–408

Mancera JM, McCormick SD (1998) Osmoregulatory actions of the GH/IGF-I axis in non-salmonid teleosts. Comp Biochem Physiol 121B:43–48

Mancera JM, McCormick SD (2007) Role of prolactin, growth hormone, insulin-like growth factor I and cortisol in teleost osmoregulation. In: Kapoor BG, Baldisserotto B, Mancera Romero JM (eds) Fish osmoregulation. Science Publishers, Enfield, pp 497–515

Mancera JM, Fernández-Llebrez P, Grondona JM, Pérez-Fígares JM (1993a) Influence of environmental salinity on prolactin and corticotropic cells in the gilthead sea bream (*Sparus aurata* L.). Gen Comp Endocrinol 90:220–231

Mancera JM, Fernández-Llebrez P, Pérez-Fígares JM (1993b) Osmoregulatory responses during abrupt salinity changes in the euryhaline gilthead sea bream (*Sparus aurata* L.). Comp Biochem Physiol 106A:245–250

Mancera JM, Pérez-Fígares JM, Fernández-Llébrez P (1995) Effect of decreased environmental salinity on growth hormone cells in the euryhaline gilthead sea bream (*Sparus aurata* L.). J Fish Biol 46:494–500

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

Manzon LA (2002) The role of prolactin in fish osmoregulation: a review. Gen Comp Endocrinol 125:291–310

Martínez-Barberá JP, Pendón C, Rodríguez RB, Pérez-Sánchez J, Valdivia MM (1994) Cloning, expression, and characterization of a recombinant gilthead seabream growth hormone. Gen Comp Endocrinol 96:179–188

Martos-Sitcha JA, Wunderink YS, Gozdowska M, Kulczykowska E, Mancera JM, Martínez-Rodríguez G (2013) Vasotocinergic and isotocinergic systems in the gilthead sea bream (*Sparus aurata*): an osmoregulatory story. Comp Biochem Physiol 166A:571–581

Matsuda K, Nagano Y, Uchiyama M, Onoue S, Takahashi A, Kawauchi H, Shioda S (2005) Pituitary adenylate cyclase-activating polypeptide (PACAP)-like immunoreactivity in the brain of a teleost, *Uranoscopus japonicus*: immunohistochemical relationship between PACAP and adenohypophysial hormones. Regul Pept 126:129–136

McCormick SD (1995) Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. In: Wood CM, Shuttleworth TJ (eds) Fish physiology, vol 14. Academic Press, New York, pp 285–315

McCormick SD (2001) Endocrine control of osmoregulation in teleost fish. Am Zool 41:781–794

Nam BH, Moon JY, Kim YO, Kong HJ, Kim WJ, Kim DG, Jee YJ, Lee SJ (2013) Structural and functional characterization of pituitary adenylyl cyclase-activating polypeptide (PACAP)/PACAP-related peptide (PRP) and its receptor in olive flounder (*Paralichthys olivaceus*). Comp Biochem Physiol 164B:18–28

Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng 10:1–6

Parker DB, Power ME, Swanson P, Rivier J, Sherwood NM (1997) Exon skipping in the gene encoding pituitary adenylate cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. Endocrinology 138:414–423

Pérez-Sánchez J, Le Bail P-Y (1999) Growth hormone axis as marker of nutritional status and growth performance in fish. Aquaculture 177:117–128

Peterson BC, Simpson PR, Cain KD, Hardy RH, Schelling GT, Ott TL (2003) Effects of administration of somatostatin-14 and immunoneutralization of somatostatin on endocrine and growth responses in rainbow trout. J Fish Biol 63:506–522

Pierce AL, Breves JP, Moriyama S, Hirano T, Grau EG (2011) Differential regulation of Igf1 and Igf2 mRNA levels in tilapia hepatocytes: effects of insulin and cortisol on GH sensitivity. J Endocrinol 211:201–210

Reinecke M, Björnsson BT, Dickhoff WW, McCormick SD, Navarro I, Power DM, Gutiérrez J (2005) Growth hormone and insulin-like growth factors in fish: where we are and where to go. Gen Comp Endocrinol 142:20–24

Rodríguez L, Begtashi I, Zanuy S, Carrillo M (2000) Development and validation of an enzyme inmunoassay for testosterone: effects of photoperiod on plasma testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax*, L.) at puberty. Fish Physiol Biochem 23:141–150

Rousseau K, Le Belle N, Pichavant K, Marchelidon J, Chow BKC, Boeuf G, Dufour S (2001) Pituitary growth hormone secretion in the turbot, a phylogenetically recent teleost, is regulated by a species-specific pattern of neuropeptides. Neuroendocrinology 74:375–385

Saera-Vila A, Calduch-Giner JA, Prunet P, Pérez-Sánchez J (2009) Dynamics of liver GH/IGF axis and selected stress markers in juvenile gilthead sea bream (*Sparus aurata*) exposed to acute confinement: differential stress response of growth hormone receptors. Comp Biochem Physiol 154A:197–203

Sakamoto T, McCormick SD (2006) Prolactin and growth hormone in fish osmoregulation. Gen Comp Endocrinol 147:24–30

Sakamoto T, McCormick SD, Hirano T (1993) Osmoregulatory actions of growth hormone and its mode of action in salmonids: a review. Fish Physiol Biochem 11:155–164

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

Soengas JL, Sangiao-Alvarellos S, Laiz-Carrión R, Mancera JM (2007) Energy metabolism and osmotic acclimation in teleost fish. In: Kapoor BG, Baldisserotto B, Mancera Romero JM (eds) Fish osmoregulation. Science Publishers, Enfield, pp 278–307

Somogyvári-Vigh A, Reglodi D (2004) Pituitary adenylate cyclase activating polypeptide: a potential neuroprotective peptide. Curr Pharm Des 10:2861–2889

Takei Y, McCormick SD (2012) Hormonal control of fish euryhalinity. In: McCormick SD, Farrell AP, Brauner CJ (eds) Fish physiology, vol 32. Academic Press, USA, pp 69–123

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

Tiago DM, Laizé V, Cancela ML (2008) Alternatively spliced transcripts of *Sparus aurata* insulin-like growth factor 1 are differentially expressed in adult tissues and during early development. Gen Comp Endocrinol 157:107–115

Tipsmark CK, Luckenbach JA, Madsen SS, Kiilerich P, Borski RJ (2008) Osmoregulation and expression of ion transport proteins and putative claudins in the gill of Southern flounder (*Paralichthys lethostigma*). Comp Biochem Physiol 150A:265–273

Vargas-Chacoff L, Astola A, Arjona FJ, Martín del Río MP, García-Cózar F, Mancera JM, Martínez-Rodríguez G (2009a) Gene and protein expression for prolactin, growth hormone and somatolactin in *Sparus aurata*: seasonal variations. Comp Biochem Physiol 153B:130–135

Vargas-Chacoff L, Astola A, Arjona FJ, Martín del Río MP, García-Cózar F, Mancera JM, Martínez-Rodríguez G (2009b) Pituitary gene and protein expression under experimental variation on salinity and temperature in gilthead sea bream *Sparus aurata*. Comp Biochem Physiol 154B:303–308

Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, Vaudry H (2000) Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. Pharmacol Rev 52:269–324

Very NM, Kittilson JD, Klein SE, Sheridan MA (2008) Somatostatin inhibits basal and growth hormone-stimulated hepatic insulin-like growth factor-I production. Mol Cell Endocrinol 281:19–26

Wong AO, Leung MY, Shea WL, Tse LY, Chang JP, Chow BK (1998) Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and

molecular cloning of pituitary type I PACAP receptor. Endocrinology 139:3465–3479

Wong AO, Li WS, Lee EK, Leung MY, Tse LY, Chow BK, Lin HR, Chang JP (2000) Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. Biochem Cell Biol 78:329–343

Xiao D, Chu MMS, Lee EKY, Lin H-R, Wong AOL (2002) Regulation of growth hormone release in common carp pituitary cells by pituitary adenylate cyclase-activating polypeptide: signal transduction involves cAMP- and calcium-dependent mechanisms. Neuroendocrinol 76:325–338

Xing Y, Wensheng L, Haoran L (2005) Polygenic expression of somatostatin in orange-spotted grouper (*Epinephelus coioides*): molecular cloning and distribution of the mRNAs encoding three somatostatin precursors. Mol Cell Endocrinol 241:62–72

Xu M, Volkoff H (2009) Cloning, tissue distribution and effects of food deprivation on pituitary adenylate cyclase activating polypeptide (PACAP)/PACAP-related peptide (PRP) and preprosomatostatin 1 (PPSS 1) in Atlantic cod (*Gadus morhua*). Peptides 30:766–776