CBP manuscript 22454 – Part A

# VASOTOCINERGIC AND ISOTOCINERGIC SYSTEMS IN THE GILTHEAD SEA BREAM (*Sparus aurata*): AN OSMOREGULATORY STORY

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**Abstract**

To investigate the physiological roles of arginine vasotocin (AVT) and isotocin (IT) in osmoregulatory process in gilthead sea bream (*Sparus aurata*), a time course study (0, 12 hours, and 1, 3, 7 and 14 days) has been performed in specimens submitted to hypoosmotic (from 40 ‰ salinity to 5 ‰ salinity) or hyperosmotic (from 40 ‰ salinity to 55 ‰ salinity) challenges. Plasma and liver osmoregulatory and metabolic parameters, as well as AVT and IT pituitary contents were determined concomitantly with hypothalamic pro-vasotocin (pro-

VT) and pro-isotocin (pro-IT) mRNA expression levels. Previously, sequences coding for pro-VT and pro-IT cDNAs were cloned. Two osmoregulatory periods related to plasma osmolality and metabolic parameters variations could be distinguished: i) an adaptative period, from 12 hours to 3 days after transfer, and ii) a chronic regulatory period, starting at day 3 after transfer. Higher values in hypothalamic pro-VT and pro-IT mRNA expression as well as in pituitary AVT and IT storage levels in both hypo- and/or hyper-osmotic transfers have been distinguished. These increase correlated with changes in plasma cortisol levels, suggesting an interaction between this hormone and pro-VT expression. Furthermore, pro-IT expression enhancement also suggests a role of the isotocinergic system as a modulator in the acute stress response induced by hyper-osmotic challenge in *S. aurata*.

**Keywords:** *osmoregulation; pro-isotocin; pro-vasotocin; stress; teleost fish.*

## 1. Introduction

Several studies in teleost fish have pointed out that synthesis of neurohypophyseal nonapeptides (arginine vasotocin –AVT- and isotocin –IT-) and their secretion into the circulatory system change in response to environmental salinity, suggesting an hypoosmotic role (at least for AVT) related to its antidiuretic effect (Warne et al., 2002; Balment et al., 2006; Kulczykowska, 2007). Both AVT and IT, homologous to mammalian arginine vasopressin (AVP) and oxytocin (OXY), respectively, are teleostean neuropeptides synthesized in neurons located in the preoptic and lateral tuberal nuclei, from where they are transported to the neurohypophysis for storage and release to systemic bloodstream (Goossens et al., 1977; Peter, 1977; Schreibman and Halpern, 1980; Van den Dungen et al., 1982). AVT and IT, as well as AVP and OXY in mammals, act as neurotransmitters and/or neuromodulators in the central nervous system, or as hormones in the periphery binding to their specific receptors (Goossens et al., 1977; Van den Dungen et al., 1982; Acher, 1993; Acher and Chauvet, 1995; Goodson and Bass, 2000).

The differences in AVT/IT distribution and function have been associated with sex, social tactics (e.g. parental care, courtship) and stage of development (Van den Dungen et al., 1982; Batten et al., 1999; Goodson and Bass, 2000; Ohya and Hayashi, 2006). Furthermore, AVT is related to osmoregulatory processes (mainly to adaptation to hyperosmotic environments due

to its anti-diuretic role), control of blood pressure and cardiovascular activity, metabolism and stress, reproductive behaviour, brain neurotransmission and pituitary endocrine activity (Warne et al., 2002; Balment et al., 2006; Kulczykowska, 2007). Although no clear role for IT has been described, recent studies have shown brain variations in IT levels related to reproductive stage, social status and behaviour in fish (Almeida et al., 2012; Kleszczynska et al., 2012).

The nucleotide cDNA sequences coding for pro-vasotocin (pro-VT) and pro-isotocin (pro-IT) precursors have been described in different teleosts, e.g. *Catostomus commersoni*, *Oncorhynchus keta*, *O. masou* or *Platichthys flesus* (Heierhorst et al., 1989; Hyodo et al., 1991; Suzuki et al., 1992; Warne et al., 2000). However, to date there are no molecular tools to study the functional role of the vasotocinergic and isotocinergic systems in gilthead sea bream (*Sparus aurata*). This species is a marine teleost of high commercial value, widely cultured in the South Atlantic region of Spain mainly in salt marshes, areas of wide salinity and temperature fluctuations due to weather conditions (Arias, 1976). *S. aurata* is an euryhaline species, with the ability to adapt to a wide range of environmental salinities, that involves osmoregulatory, endocrine and metabolic processes (Mancera et al., 1993; Sangiao-Alvarellos et al., 2003; Laiz-Carrion et al., 2005; Sangiao-Alvarellos et al., 2005; Laiz-Carrion et al., 2009; Vargas-Chacoff et al., 2009a; Vargas-Chacoff et al., 2009b). This physiological plasticity is controlled by several pituitary hormones, including AVT and IT, as well as extrapituitary hormones, making sea bream a good model for osmoregulatory studies (Mancera et al., 2002; Kleszczynska et al., 2006; Mancera and Fuentes, 2006). Previous research by our group has demonstrated that acclimation of *S. aurata* specimens to different environmental salinities induced changes in pituitary AVT and/or IT storage, and in their plasma levels, suggesting an osmoregulatory role, as well as a control function, of these hormones in the acclimation to hyperosmotic environments (Kleszczynska et al., 2006). In addition, treatment of *S. aurata* with AVT and their subsequent transfer to different salinity conditions enhanced plasma cortisol level, gill and kidney $Na^+$,$K^+$-ATPase activities and metabolism rate at plasma and tissue levels, reinforcing the idea of a hypoosmoregulatory role for AVT in this species (Sangiao-Alvarellos et al., 2006). Moreover, the effects of those hormones on several osmoregulatory epitheliums, such as gills, kidney or gastrointestinal tract, have been already reported in other species by their actions on ion absorption/secretion,

97 urine production or glomerular filtration (Maetz et al., 1964; Motais and Maetz, 1967;
98 Henderson and Wales, 1974; Amer and Brown, 1995; Martos-Sitcha et al., 2013).
99
100 In order to examine the role of the vasotocinergic and isotocinergic systems in the
101 osmoregulatory processes in *S. aurata*, the nucleotide sequences for pro-VT and pro-IT
102 precursors were cloned. Changes in both systems, at the hypothalamic mRNA expression and
103 pituitary peptide levels, together with osmoregulatory and metabolic parameters, were
104 measured in this species, while submitted to an acute transfer from seawater (SW, 40 ‰
105 salinity) to low salinity water (LSW, 5 ‰ salinity) or high salinity water (HSW, 55 ‰
106 salinity). The osmoregulatory role of AVT and IT in *S. aurata* and their possible interaction
107 with the stress system are also discussed.
108
109 **2. Material and methods**
110 *2.1. Animals and experimental protocol*
111 Immature males of gilthead sea bream (*Sparus aurata* L., 80-100 g body weight, $n$ = 128)
112 were provided by Planta de Cultivos Marinos (CASEM, University of Cadiz, Puerto Real,
113 Cadiz, Spain). Fish were transferred to the wet laboratories at the Faculty of Marine and
114 Environmental Sciences (Puerto Real, Cadiz, Spain), where they were acclimated for 10 days
115 to sea water (SW, 40 ‰ salinity, 1,149 mOsm·kg$^{-1}$ $H_2O$ osmolality), randomly distributed in
116 six 400-L tanks in an open system circuit (4.5 kg·m$^{-3}$ density), under natural photoperiod
117 (May, 2009) and constant temperature (18-19 ºC). After this acclimation period, animals were
118 directly transferred to the following environmental salinities: seawater (SW, 40 ‰ salinity,
119 control group), low salinity water (LSW, 5 ‰ salinity, 139 mOsm·kg$^{-1}$ $H_2O$ osmolality,
120 hypoosmotic environment), and high salinity water (HSW, 55 ‰ salinity, 1,439 mOsm·kg$^{-1}$
121 $H_2O$ osmolality, hyperosmotic environment). The experimental salinities were achieved either
122 by mixing SW with dechlorinated tap water for LSW, or with natural marine salt (Salina de la
123 Tapa, El Puerto de Santa Maria, Cadiz, Spain) for HSW. Groups were maintained in duplicate
124 experimental tanks (each 400-L volume; $n$ = 20 fish per tank, 4.5 kg·m$^{-3}$ initial density) under
125 a closed recirculation system, and at least 10 % of the water of each tank was replaced every
126 two days with water from a reservoir previously adjusted to the experimental salinity
127 required. Water quality was checked twice a day to affirm their stability. Fish were fed a daily

ration of 1 % of their body weight with commercial pellets (Dibaq-Dibroteg S.A., Segovia, Spain).

On day 0, eight fish from the main tanks containing SW were sampled (control time 0 before transfer). Then, at 12 hours, and at 1, 3, 7 and 14 days after salinity transfer, 8 fish (4 per tank) from each experimental salinity (SW, LSW and HSW) were anaesthetized with 2-phenoxyethanol (1 $mL \cdot L^{-1}$ at the specific salinity water), weighted and sampled. Blood samples were collected from the caudal peduncle into 1-mL ammonium-heparinised (Sigma) syringes, and centrifuged (3 min at 10,000 g) to obtain plasma. Plasma and pituitary samples were snap-frozen in liquid nitrogen and stored at -80 ºC until further analysis. After brain extraction, hypothalami were dissected and placed in eppendorf tubes containing 600 µL of RNA*later*© (Life Technologies). Tubes were incubated for 24 hours at 4 ºC and stored at -20 ºC afterwards. In all protocols involving commercial kits, the manufacturer's instructions were followed, except where noted. No mortality was observed in any of the groups during experimentation. The experiment was performed following the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.

*2.2. Plasma and liver parameters*

Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske-VT, USA) and expressed as $mOsm \cdot kg^{-1}$. Glucose and triglycerides (in plasma and liver), lactate (in plasma) and glycogen (in liver) concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Triglycerides ref. 1001311; Lactate Ref. 1001330) adapted to 96-well microplates. Plasma protein concentrations were measured on a 50-fold plasma dilution using the bicinchoninic acid method with the BCA protein kit (Pierce P.O., Rockford, USA), with bovine serum albumin serving as standard. All the assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, USA) controlled by KCjunior™ software. Standards and samples were measured in duplicate.

Plasma cortisol levels were measured by Enzyme Immune-Assay (EIA) using microtiter plates (MaxiSorp™, Nunc, Roskilde, Denmark) as previously described for testosterone

(Rodríguez et al., 2000). Steroids were extracted from 5 µL plasma in 100 µL RB (10 % v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01 % w/v $NaN_3$, 2.34 % w/v NaCl, 0.037 % w/v EDTA, 0.1 % w/v BSA (Bovine Serum Albumin)) and 1.2 mL methanol (Panreac), and evaporated during 48-72 hours at 37 ºC. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate. Standard curve was run from 2.5 $ng \cdot mL^{-1}$ to 9.77 $pg \cdot mL^{-1}$ ($R^2$= 0.993). The lower limit of detection (92.87 % of binding, ED92.87) was 1.51 pg/mL. The percentage of recovery was 95 %. The inter- and intra-assay coefficients of variation (calculated from the sample duplicates) were 2.88 ± 0.33 % and 3.82 ± 0.35 %, respectively. 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. Molecular cloning of partial pro-vasotocin and pro-isotocin sequences*

First, a set of degenerate primers was designed according to the most highly conserved sequences of cDNA between different species for pro-vasotocin (*Thalassoma bifasciatum*, GenBank acc. no. **AY167033**; *Parajulis poecilepterus*, GenBank acc. no. **DQ073094**; *Halichoeres tenuispinis*, GenBank acc. no. **DQ073098**; and *Astatotilapia burtoni*, GenBank acc. no. **AF517935**) and pro-isotocin (*Parajulis poecilepterus*, GenBank acc. no. **DQ073095**; *Halichoeres tenuispinis*, GenBank acc. no. **DQ073099**; *Danio rerio*, GenBank acc. no. **AY069956**; *Catostomus commersoni*, GenBank acc. no. **X16621**; *Salmo salar*, NCBI reference sequence acc. no. **NM_001123652**; *Oncorhynchus nerka*, GenBank acc. no. **D31841**: *Oncorhynchus masou*, GenBank acc. no. **D10944**; and *Oncorhynchus keta*, GenBank acc. no. **D10940**). Later, degenerate primers were synthesized and purified by HPLC (Invitrogen™, Life Technologies), and nucleotide sequences are shown in Table 1.

Total RNA was prepared from *S. aurata* single hypothalamus lobes (≈ 15 mg), using the RNeasy Plus Mini Kit (Qiagen). Briefly, the sample was homogenized in Buffer RLT Plus

(600 µL) with 2-mercaptoethanol 1 % v/v (Sigma) using an Ultra-Turrax® T8 (IKA®-Werke). From this point and further, RNA quality was checked in a Bioanalyzer 2100 with the RNA 6000 Nano kit (Agilent Technologies), whereas RNA quantity was measured spectrophotometrically at 260 nm in a BioPhotometer Plus (Eppendorf). Hypothalamus total RNA (2 µg) was reverse-transcribed in a 20 µL reaction volume using 250 ng random primers (Invitrogen™, LifeTechnologies) and 200 U Superscript III reverse transcriptase (Invitrogen™, LifeTechnologies), with the manufacturer's first strand buffer (1x final concentration), DTT (5 mM final concentration) and dNTPs (0.5 mM final concentration) at 25 ºC for 5 min, 50 °C for 60 min, and 70 ºC for 15 min. PCR amplifications were carried out with 1 U Platinum® Taq DNA Polymerase (Invitrogen™, Life Technologies) with the first strand cDNA (corresponding to 100 ng of input total RNA), manufacturer's PCR buffer (1x final concentration), 200 nM each sense and antisense primers, 200 µM dNTPs mixture, and 1.5 mM $MgCl_2$ in a total volume of 20 µL. Samples were cycled at 94 ºC for 1 min, followed by 35 cycles at [94 ºC for 30 s, 50-60 ºC gradient for 30 s, and 72 ºC for 1 min], and a final step at 72 ºC for 10 min, in a Mastercycler®proS vapo.protect (Eppendorf). PCR products were identified in a 1.5 % agarose gel electrophoresis and ligated with the TOPO TA Cloning® Kit for Sequencing (Invitrogen™, Life Technologies) into the pCR®4 TOPO® vector. Following sequencing of a single clone in both strands using M13 Forward (-20) and M13 Reverse primers by the dideoxy method at the University of Cordoba sequencing services, we confirmed sequence homology of the PCR products to pro-VT and pro-IT.

For the preparation of the pro-VT and pro-IT probes, around 2 µg of each plasmid DNA containing the partial pro-VT or pro-IT sequence were digested with 10 U of *Eco*RI (Takara) in a volume of 40 µL, the digestion products were separate on an 1.5 % agarose gel and the bands of about 300 bp in each case were excised and purified with the QIAquick kit (Qiagen). The cDNA fragments were diluted till a final concentration of 25 $ng \cdot \mu L^{-1}$ in TE buffer (10 mM Tris-HCl, 1 mM $EDTANa_2$, pH 8.0) and stored at -20 ºC afterwards.

*2.4. Construction and screening of a brain cDNA library, and cloning of a full-length pro-VT and pro-IT cDNAs from S. aurata*

The brain cDNA library was prepared from 5 µg of Poly(A) RNA, using the lambda ZAP-cDNA/Gigapack III Kit (Stratagene, Agilent Technologies), with few modifications, as described in Balmaceda-Aguilera et al. (2012).

Two NZY agar 240 x 240 mm plates (Nunc) were plated each with approximately 250,000 pfu from the amplified *S. aurata* cDNA brain library, and subsequent steps were carried out as described in Balmaceda-Aguilera et al. (2012). Four positives from the around 40 positive plaques of the first round of the screening in each case were isolated and subjected to further two rounds of hybridization/isolation. After the third round of the screening, these positives were isolated and excised to pBluescript SK(-) (Stratagene, Agilent Technologies Life Sciences) by *in vivo* excision using *Escherichia coli* XL1-Blue MRF´ and SOLR strains (Stratagene, Agilent Technologies Life Sciences). After that, 2 positive colonies from each clone were picked up and plasmid DNA prepared in a mini-prep column (GenElute™ Five-Minute Miniprep Kit; SIGMA®). Excised pBluescript SK(-), containing the clones, were double digested by *Eco*RI and *Xho*I (Takara) and the products were revealed in a 1 % agarose gel stained with GelRed™ (Biotium). The clones were fully sequenced in both strands by the dideoxy method in a biotechnology company (Newbiotechnique S.A., Sevilla, Spain).

*2.5. Quantification of pro-vasotocin and pro-isotocin mRNA levels (QPCR)*

Each hypothalamus soaked in RNA*later* was hemi-sectioned in both lobes. Total RNA was extracted from one of the lobes using RNeasy® Plus Mini Kit (Qiagen). gDNA was eliminated with gDNA Eliminator spin column (Qiagen). Only samples with a RNA Integrity Number (RIN) higher than 8.5 were used for QPCR.

Firstly, total RNA (500 ng) was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences). Briefly, the reaction was performed using qScript Reaction Mix (1x final concentration) and qScript Reverse Transcriptase (2.5x final concentration). The reverse transcription program consisted in 5 min at 22 °C, 30 min at 42 °C and 5 min at 85 °C. Second, to optimize the QPCR conditions several primers concentrations (100 nM, 200 nM, 400 nM and 500 nM) and temperature gradient (from 50 to 60 ºC) were used. Different cDNA template concentrations were applied in triplicate (1 $ng \cdot \mu L^{-1}$, 100 $pg \cdot \mu L^{-1}$, 10 $pg \cdot \mu L^{-1}$, 1 $pg \cdot \mu L^{-1}$, 100 $fg \cdot \mu L^{-1}$ and 10 $fg \cdot \mu L^{-1}$ of input RNA) to check the assay linearity and the amplification efficiency (pro-VT: $r^2 = 0.998$, efficiency =

1.02; pro-IT: $r^2$ = 0.999, efficiency = 1.04; β-actin: $r^2$ = 0.999, efficiency = 1.01). Finally, although the assay was linear between 1 ng·μL$^{-1}$ to 1 pg·μL$^{-1}$ of cDNA per reaction, 100 pg·μL$^{-1}$ (2 ng) of cDNA was used for all the amplifications. The primer sequences used for QPCR were designed with Primer3 software v. 0.4.0 (available in http://frodo.wi.mit.edu/) and were synthesized by Invitrogen™ Life Technologies (Table 1) and HPLC purified. To confirm the correct amplification of pro-VT and pro-IT primer pairs, the obtained PCR amplicons were cloned and sequenced. QPCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep *realplex* $^2$ S). Each reaction mixture (10 μL) contained 0.5 μL at 200 nM of each specific forward and reverse primers, and 5 μL of PerfeCTa SYBR® Green FastMix™ (Quanta BioSciences). Reactions were conducted in semi-skirted twin.tec real-time PCR plates 96 (Eppendorf) covered with adhesive Masterclear real-time PCR Film (Eppendorf). The PCR profile was as follows: 95 ºC, 10 min; [95 ºC, 30 s; 60 ºC, 45 s] x 40 cycles; *melting curve* [60 ºC to 95 ºC, 20 min], 95 ºC, 15 s. The melting curve was used to ensure that a single product was amplified and to check for the absence of primer-dimer artefacts. Results were normalized to β-actin (acc. no. X89920), owing its low variability (less than 0.3 $C_T$) under our experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

*2.6. AVT and IT pituitary storage*

AVT and IT content in pituitary gland was determined by high-performance liquid chromatography (HPLC) with fluorescence detection preceded by solid-phase extraction (SPE) based on the method previously described by (Gozdowska et al., 2006) with modifications. In short, frozen pituitaries were weighed (452.75 ± 17.59 μg) and sonicated in 0.5 mL Milli-Q water (Microson™XL, Misonix, USA). Then glacial acetic acid (1.25 μL) was added and samples were placed in a boiling water bath for 3.5 minutes. Extracts were cooled down and centrifuged at 6,000 x g for 15 min at 4 °C. Then the supernatants were decanted and loaded onto previously conditioned (1 mL MeOH, 1 mL water) SPE columns (30 mg/1 mL, STRATA-X, Phenomenex). Water (600 μL), then 0.1 % TFA (trifluoroacetic acid) in 5 % acetonitrile (600 μL) was passed through the columns to wash away impurities. The peptides were eluted by 2 x 600 μL of 80 % acetonitrile. The eluate was evaporated to dryness using Turbo Vap LV Evaporator (Caliper Life Scence, USA). Then samples were frozen and stored at -80 ºC until HPLC analysis. Before quantitative analysis the samples

were re-dissolved in 40 µL of 0.1 % TFA and divided into two for repetition. Pre-column derivatization of IT and AVT in each of 20 µL samples was performed using 3 µL NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) solution (30 mg NBD-F in 1 mL of acetonitrile) in mixture of 20 µL phosphoric buffer (0.2 M, pH 9.0) and 20 µL acetonitrile. The solution was heated at 60 ºC for 3 min in a dry heating block and cooled down on ice. Next, 4 µL of 1 M HCl was added. Derivatized samples were measured with Agilent 1200 Series Quaternary HPLC System (Agilent Technologies, USA). Chromatographic separation was achieved on Agilent ZORBAX Eclipse XDB-C18 column (150 mm x 4.6 mm I.D., 5 µm particle size). Gradient elution system was applied for separation of derivatized peptides. The mobile phase consisted of solvent A (0.1 % TFA in $H_2O$) and solvent B (0.1 % TFA in acetonitrile: $H_2O$ (3:1)). A linear gradient was 45-70 % of eluent B in 20 min. Flow rate was set at 1 mL/min and the column temperature at 20 ºC. Injection volume was 67 µL. Fluorescence detection was carried out at 530 nm with excitation at 470 nm.

### *2.7. Statistics*

Statistical differences were analyzed by two-way ANOVA with salinity (LSW, SW, HSW) and time course (day 0, 12 hours, days 1, 3, 7 and 14) as main factors, followed by post-hoc comparison made with the Tukey's test where appropriate, using GraphPad Prism® (v.5.0b) software. Significance was taken at $P < 0.05$. Moreover, Student t-test analysis was carried out between duplicate tanks of each experimental salinities to discard any variation caused by tank factor.

## 3. Results

### *3.1. Cloning and characteristics of* S. aurata *pro-VT and pro-IT sequences*

Sequencing revealed the longest clones to be 1,056 bp for pro-VT and 698 bp for pro-IT cDNAs (GenBank accession numbers **FR851924** for pro-VT, Figure 1, and **FR851925** for pro-IT, Figure 2). Pro-VT nucleotide sequence comprises an open reading frame (ORF) of 459 bp encoding a 153 amino acid pro-peptide with 88-100 % similarity to other teleosts. On the other hand, pro-IT presented an ORF of 468 bp, encoding a 156 amino acid protein with 86-100 % similarity to other fish species. These sequences are composed of three segments: a signal peptide, the hormone and the neurophysin, preceded and followed by 5' and 3' untranslated regions of 60 bp and 534 bp, respectively, for the vasotocin precursor, and 60 bp

and 168 bp, respectively, for the isotocin precursor. Comparisons of the amino acid sequences with their mammalian homologous (pro-vasopressin and pro-oxytocin) are shown in Figure 3.

*3.2. Plasma and hepatic parameters*

Time course of osmoregulatory and metabolic response of *S. aurata* to transfer from SW to LSW or to HSW are shown, respectively, in Tables 2 and 3. In both osmotic challenges specimens enhanced plasma glucose, triglycerides and protein levels from 12 hours to day 1, whereas plasma lactate maintained higher values from 12 hours till the end of experiment. On the other hand, hepatic glycogen decreased its level from 12 h to 1 d in both LSW and HSW groups, while hepatic glucose presented an inverse relationship respect to environmental salinity.

Plasma osmolality was slightly higher in HSW-transferred specimens after 12 hours, staying above the control levels (SW) until the end of the experiment. However, in fish submitted to LSW, plasma osmolality significantly decreased at 12 hours after transfer, returning to the near control levels after day 1 (Figure 4). Plasma cortisol levels did not change in specimens transferred from SW to SW (control group), but increased in fish submitted to hypoosmotic (from SW to LSW) and hyperosmotic (from SW to HSW) transfer after 12 hours, being their levels restored at both salinities after day 1 (Figure 5).

*3.3. Pro-VT and pro-IT mRNA expression*

Acute challenge to hyper- and hypo-osmotic conditions altered pro-VT and pro-IT expression compared to that in control group (transferred from SW to SW) (Figure 6). In specimens submitted to hyperosmotic transfer, both pro-VT and pro-IT mRNA levels were significantly enhanced. Pro-IT expression was significantly lower than that in control fish at day 7 of the experiment. Hypoosmotic condition significantly increased pro-VT expression at 12 hours after transfer. After day 1 the expression significantly decreased. From day 7 till the end of the experiment values were close to those in control group. In the isotocinergic system, only a decrease at day 7 was significant.

*3.4. Nonapeptides content in the pituitary gland*

Pituitary AVT and IT changes are presented in Figure 7. In animals transferred from SW to HSW, AVT and IT levels were increased until day 3, whereas hypoosmotic transfer produced the same response until day 1. Then both AVT and IT nonapeptides decreased to values close to those of the control group.

## 4. Discussion

### *4.1. Cloning of vasotocin and isotocin precursors*

In the present work, changes in the vasotocinergic and isotocinergic systems following transfer to hypo- and hyper-osmotic environments were examined in *S. aurata*. For this purpose, vasotocin and isotocin cDNA precursors were cloned in order to get a new tool for assessing modifications in their hypothalamic expression due to variations in environmental salinity. Our results in *S. aurata* agreed with those previously described for pro-VT and pro-IT in other teleosts, e.g. *Catostomus commersoni*, *Oncorhynchus keta*, *O. masou* and *Platichthys flesus* (Heierhorst et al., 1989; Hyodo et al., 1991; Suzuki et al., 1992; Warne et al., 2000). The 14 Cys residues and the leucine-rich core segment described in other species are conserved along the specific neurophysin. Comparison between pro-VT and pro-IT sequences revealed high homology between them at both amino acid (64 %) and nucleotide (74 %) levels, being higher in the central zone of the neurophysin (between residues 17 and 82) than in the N-terminal and C-terminal ends, as has been described previously for other teleosts (Hyodo et al., 1991; Warne et al., 2000). Moreover, carboxy-terminal portions lack the amino terminal Arg (residue 121) in both sea bream pro-VT and pro-IT, which seems to be important for releasing the copeptin structure coupled to the mammalian orthologue AVP neurophysin, which has been recently proposed to be a relevant prolactin-releasing factor in *Cyprinus carpio* by pituitary explants culture (Flores et al., 2007).

### *4.2. Plasma and hepatic parameters*

Time course modifications in osmoregulatory and metabolic parameters are in agreement with those previously reported for *S. aurata* submitted to similar hypo- and hyperosmotic transfers (Sangiao-Alvarellos et al., 2003; Laiz-Carrion et al., 2005; Sangiao-Alvarellos et al., 2005). These changes revealed two stages during hypo- and hyperosmotic acclimation: (i) an *adaptative period* during the first days of acclimation (12 hours – 3 days), with important

changes in these parameters, and (ii) a *chronic regulatory period* (after day 3 post-transfer) where parameters reached homeostasis.

Changes in plasma osmolality levels indicated the existence of an osmotic imbalance associated with a stress signified by enhanced plasma cortisol values and a new or recovered steady state in both tested parameters at the end of experiment. Moreover, unchanged cortisol value in control group (fish transferred from SW to SW) indicated that handling alone did not provoke any stress as shown by the absence of changes in this hormone as well as in plasmatic and hepatic metabolites, although modifications in other parameters as plasmatic catecholamines could be produced, but probably during the first hours post-handling. Consequently, osmotic mechanisms and stress pathways are involved in short-term modifications of epithelia permeability as has been shown by (Wendelaar Bonga, 1997). The pattern of changes in metabolic parameters at both plasma and hepatic levels also agrees with the previously reported for *S. aurata* submitted to similar salinity transfers (Sangiao-Alvarellos et al., 2005). Thus, the enhancement of plasma metabolites (glucose, triglycerides and protein) levels concomitantly with plasma cortisol values as well as a consumption of energy reserves identified by the lower liver glycogen storage, suggested the existence of a clear energetic reorganization in both LSW and HSW groups in order to ensure the proper operation of the osmoregulatory system (Soengas et al., 2007). However, only lactate reached a new steady state in both hypo- and hyperosmotic transfers, becoming one of the most important metabolites during the chronic osmoregulatory period (Sangiao-Alvarellos et al., 2003, 2005). Thus, higher levels of plasma lactate could reflect the greater supply required by some important tissues (i.e.. gills, kidney or brain), where the use of this metabolite in those organs have been demonstrated (Mommsen, 1984; Mommsen et al., 1985; Soengas et al., 1998).

*4.3. Changes in vasotocinergic and isotocinergic systems*

Vasotocin and isotocin precursors are synthesized in the neural soma and processed in the secretory granules during their axonal transport before being released in the axonal terminal (Acher, 1993). Both synthesis and release of AVT and IT are stimulated by osmotic challenge in euryhaline teleosts, and seem to be different depending on species and environmental salinity, which secretion is sensitive to the osmotic status of the animal (mainly related to

412 dehydration) (Maetz and Lahlou, 1974; Haruta et al., 1991; Hyodo and Urano, 1991; Perrott
413 et al., 1991; Pierson et al., 1995; Kulczykowska, 1997). As it has been described above, our
414 experimental design enabled to differentiate an adaptative response from a chronic regulatory
415 response in the acclimation to new environmental salinities. During the adaptative period,
416 pro-VT cDNA increased in both hypo- and hyperosmotic challenges suggesting a relation
417 between pro-VT expression and the initial stress.
418
419 In *Dicentrarchus labrax*, AVT binding sites are located in the zones occupied by corticotroph
420 (ACTH) cells that controlled cortisol secretion in the interrenal tissue (Moons et al., 1989).
421 Moreover, in *Catostomus commersoni*, both AVT and corticotropin-releasing factor (CRF)
422 neurons of nucleus preopticus (NPO) innervate corticotroph cells, suggesting a control of
423 these cells by AVT (Yulis and Lederis, 1987). However in other species, as in *Anguilla*
424 *anguilla* or *Poecilia latipinna,* this fact has not been evidenced or is inconsistent (Olivereau
425 and Olivereau, 1988; Batten et al., 1990). In addition, the *in vitro* co-administration of AVT
426 and CRF to pituitary cultures of *Oncorhynchus mykiss*, similarly to that observed in mammals
427 (Rivier and Vale, 1983), enhances ACTH secretion and indicates the existence of a synergism
428 between both peptides (Baker et al., 1996).
429
430 In fact, pituitary AVT and IT content changes have been described in several teleostean
431 species (*O. mykiss, Oryzia latipes, P. flesus*), where transfers from SW to hyperosmotic
432 environments deplete AVT pituitary content and enhance its plasma levels (Carlson and
433 Holmes, 1962; Haruta et al., 1991; Perrott et al., 1991). However, the stimulated pro-VT
434 expression observed in hyperosmotic environment (present results), together with increased
435 plasma AVT values (Kleszczynska et al., 2006), strongly suggests an intense synthesis and
436 release of the hormone into the blood circulation in fish under HSW environment, which
437 taken together with the lack of variation in the pituitary storage at the same sampling point,
438 could provide an increase in the bloodstream to perform the physiological action through its
439 receptors. After that, the return of the expression levels to those measured in the control group
440 could be explained by a negative feedback. This process produces the higher storage of the
441 hormone at pituitary level pending on the new osmotic stimulus/stressor or even to be
442 degraded in the pituitary as has been shown from day 7 post-transfer.
443

In SW-acclimated *S. aurata* treated with AVT (Sangiao-Alvarellos et al., 2006) or submitted to similar hypo- and hyperosmotic challenges for 24 h, as in this experiment, plasma cortisol levels were enhanced, suggesting a possible synergy between exogenous AVT and endogenous CRF stimulated by salinity transfer. Our results in *S. aurata* also suggest the relation between the vasotocinergic and the stress response systems during the initial adaptative period of salinity transfer to HSW, where a synergy between both can be expected (Laiz-Carrion et al., 2005; Sangiao-Alvarellos et al., 2005), present results). Moreover, a role for AVT (and IT) has been also suggested in this species during the chronic stress response in specimens submitted to high density (Mancera et al., 2008), which reinforces the vasotocinergic (and the isotocinergic) function during the stress. Pituitary AVT content enhancement observed in *S. aurata* during the adaptative period in both salinity transfers (present results) could result from an increase in pro-VT mRNA expression. The absence of changes in AVT pituitary content in *S. aurata* acclimated to different environmental salinities (LSW, SW and HSW) during the regulatory period is in agreement with the previous results in this species (Kleszczynska et al., 2006).

At the first sampling point post-transfer (12 h) from SW to LSW an enhancement in the pro-VT expression with respect to no transferred specimens was observed. This hypoosmotic transfer induced high plasma cortisol values and could be considered as stressful for fish. According to the proposed role of AVT during the stress response, an increase of pro-VT expression could be expected, as it is observed in *S. aurata* specimens submitted to hypoosmotic challenge. However, the hypoosmotic transfer decreased plasma osmolality values (present results) and, due to the antidiuretic role of AVT described by other authors (Henderson and Wales, 1974; Amer and Brown, 1995) an enhancement in its plasma values to participate in the osmoregulatory action, by an increase in the mRNA expression and pituitary release, will not be necessary. Thus, two antagonistic situations could take place in the specimen after hypoosmotic challenges: i) an enhanced pro-VT expression due to stress situation, and ii) an inhibitory effect on this expression induced by plasma hypoosmolality.

The changes in the vasotocinergic system during the chronic regulatory period are different depending on species and environmental salinity transfer, and are linked to the osmoregulatory role of this neuropeptide (Hyodo and Urano, 1991; Pierson et al., 1995;

476 Warne et al., 2005; Kleszczynska et al., 2006). In *Platichthys flesus*, another euryhaline
477 species, the osmotic challenge from SW to freshwater (FW) induced a non-significant
478 tendency to decrease the hypothalamic pro-VT mRNA levels followed by a fall in AVT
479 secretion from pituitary, in contrast to the lack of changes in these parameters three days after
480 transfer from FW to SW (Warne et al., 2000). In *Oncorhynchus keta*, the upstream migration
481 between coast and river waters originated a fall in pro-VT and pro-IT expression in the
482 preoptic nucleus (Ota et al., 1996). However, in *O. mykiss*, a clear decrease in pro-VT
483 expression two weeks after transfer from FW to 80 % SW, and subsequent restoration to the
484 initial FW levels three days after return to FW, has been previously reported (Hyodo and
485 Urano, 1991). In our studies, pro-VT expression enhanced in HSW-acclimated fish just 12 h
486 after transfer, suggesting a rapid activation of the antidiuretic role for this hormone
487 (Henderson and Wales, 1974; Amer and Brown, 1995). On the other hand, the increase of
488 AVT storage, together with pro-VT expression enhancement, at 12 h post-transfer in
489 specimens submitted to LSW respect to the HSW group, suggest that the vasotocinergic
490 system has been activated only through the stress pathways. These evidences are according to
491 the high values of the hormone in the pituitary on day 1 as well as by the decreasing in the
492 hypothalamic mRNA expression and the observed cortisol values. Also, AVT-treatment of
493 SW-acclimated *S. aurata* specimens enhanced gill $Na^+$,$K^+$-ATPase activity, a key enzyme for
494 extruding the excess of ions (Laiz-Carrion et al., 2005), suggesting a role of AVT for
495 acclimation to hyperosmotic environments in this species (Sangiao-Alvarellos et al., 2006).
496 The differences between all the species tested after osmotic challenge could be explained by
497 the capacity of each one to cope the interaction between both osmoregulatory and stress
498 processes.
499
500 To our knowledge, only few data are available concerning the osmoregulatoy role of IT
501 (Hiraoka et al., 1996; Kulczykowska, 1997; Warne et al., 2000; Kleszczynska et al., 2006;
502 Motohashi et al., 2009), where changes in plasma level, and its control and release, appear
503 differentially sensitive to changes in plasma osmolality. In fact, sea bream AVT/IT secretory
504 system appears to be involved in the mechanism of short-term and/or long-term acclimation
505 to different salinities. Our results indicate, similarly to the pro-VT profile, two periods in
506 pro-IT expression changes: an initial phase associated with the adaptative period, and a later
507 one related to the chronic regulatory period. In the first period only transfer to HSW enhanced

pro-IT expression. These results suggest a role of the isotocinergic system in the stress response that is linked to hyperosmotic, but not to hypoosmotic, transfer during the adaptative period. Moreover, present results shown an enhancement in pituitary IT contents during the adaptative period after hyperosmotic transfer, just in the same direction and time post-transfer respect to the AVT storage values. This fact could be associated with stimulation of pro-IT expression, where IT could be act throughout the vasotocinergic system as an alternative pathway in the control of osmoregulation and/or stress mediated by the paracrine regulation in the AVT cells. Moreover, in *S. aurata* acclimated to hyper- and hypoosmotic waters the differences in the pituitary IT storage may suggest no secretion of the peptide into blood circulation (Kleszczynska et al., 2006).

However, during the chronic regulatory period after transfer from SW to hypoosmotic environments, pro-IT mRNA levels decreased on day 7, as it has been previously described in other species (Hiraoka et al., 1996; Motohashi et al., 2009). The absence of significant differences in hypothalamic pro-IT expression, pituitary IT storage (present results) and plasma IT values (Kleszczynska et al., 2006) in specimens acclimated to different environmental salinities (LSW, SW, and HSW) even during 14 days did not support an osmoregulatory role for IT in *S. aurata* in the chronic regulatory period. Also, pro-IT expression in *P. flesus* submitted to hyper- and hypoosmotic challenges did not show any differences in mRNA level after 3 days of exposure (Warne et al., 2000). Moreover, in *O. mykiss*, no differences were found in pro-IT mRNA expression during hypotonic transfers (Hyodo and Urano, 1991). Nevertheless, some studies have implicated this hormone in the hypo-osmoregulatory process, i.e. enhancement of prolactin (PRL) expression in *Cyprinus carpio* by the putative copeptine present in the neurophysin of the pro-IT structure (Flores et al., 2007). A similar situation produced by OXY (the IT homolog) has been demonstrated in mammals (Nagy et al., 1988), which contain the real copeptin and the glycosylation site, although more information and *in vitro* experiments will be necessary to demonstrate this possible stimulation of IT on PRL secretion in teleostean species, including in *S. aurata* endocrine system.

Due to the small size of animals, it was not possible to measure plasma AVT and IT levels in a time course response, which could have provided more information about the role of these

nonapeptides in both osmoregulatory and stress pathways and could have been compared with the plasma levels with increasing water salinity previously reported (Kleszczynska et al., 2006).

## 5. Conclusions

In summary, this study reports the dynamics of changes in pro-VT and pro-IT hypothalamic expression and pituitary storage after different hyperosmotic and hypoosmotic challenges. These results, together with those reported on osmolality and metabolism, confirmed a hypoosmoregulatory role for AVT, but not for IT in *S. aurata*. Our results corroborate an osmoregulatory role of these nonapeptides in the sea bream, and also suggest a role of the vasotocinergic and/or isotocinergic systems in the regulation of the stress response. In order to clarify this influence of stress on both systems in *S. aurata*, a new experimental protocol involving an acute stress (e.g. air exposure; see (Arends et al., 1999) should be used. Moreover, the possibility to establish the expression pattern of different types of AVT and IT receptors in different osmoregulatory and metabolic organs after salinity change could provide new information on the relative contribution of AVT and IT to osmoregulatory and stress processes.

**Acknowledgements**

The authors wish to thank *Planta de Cultivos Marinos* (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish. Experiment has been carried out at the *Campus de Excelencia Internacional del Mar* (CEI-MAR) facilities from the University of Cádiz. Study funded by projects AGL2010-14876 from Ministerio de Ciencia e Innovación to J.M. Mancera (Spain) and 498/N- HISZP-JPR/2009/0 to E. Kulczykowska (Poland), as well as by project Consolider-Ingenio 2010 (CSD2007-00002). J.A. Martos-Sitcha is supported by a PhD fellowship (FPU, Reference AP2008-01194) from Ministerio de Educación (Spain).

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## FIGURE LEGENDS

**Figure 1.** Nucleotide and deduced amino acid sequences from *S. aurata* pro-vasotocin cDNA. The start and stop codons are represented in italics and bold. The deduced amino acid sequence is displayed above the underlined nucleotide sequence. The predicted nonapeptide (AVT, $C^{20}$-$G^{28}$) is presented in bold capital letters. GenBank accession number **FR851924**.

**Figure 2.** Nucleotide and deduced amino acid sequences from *S. aurata* pro-isotocin cDNA. The start and stop codons are represented in italics and bold. The deduced amino acid sequence is displayed above the underlined nucleotide sequence. The predicted nonapeptide (IT, $C^{20}$-$G^{28}$) is presented in bold capital letters. GenBank accession number **FR851925**.

**Figure 3.** Comparison of amino acid sequences of four species of mammals [*Mus musculus* (mouse) VP (**AAC42027**) and OXY (**AAI17031**), *Bos taurus* (cow) VP (**AAA30806**) and OXY (**BAK09303**), *Ovis aries* (sheep) VP (**NP_001119813**) and OXY (**CAA38924**), and *Homo sapiens* (human) VP (**AAA61291**) and OXY (**AAA59977**)], two teleost fishes [*Platichthys flesus* (European flounder) VT (**BAA98140**) and IT (**BAA98141**), and *Parajulis poecilepterus* (multicolorfin rainbowfish) VT (**ABB90892**) and IT (**ABB90893**)] and *S. aurata* (gilthead sea bream) VT (amino acid sequence deduced from **FR851924** nucleotide sequence) and IT (amino acid sequence deduced from **FR851925** nucleotide sequence) hormone precursors. Alignment was carried out by ClustalW2 software (Larkin et al., 2007).

803 Gaps marked by hyphens have been inserted to optimize homology. Asterisks denote identical
804 amino acid residues between *S. aurata* pro-VT and pro-IT. N-glycosilation site (NXT) and
805 Leucine-rich core are boxed in grey. Identical amino acid residues are indicated in black.
806
807 **Figure 4.** Time course changes in plasma osmolality levels after transfer from SW to different
808 environmental salinities (LSW, SW and HSW). Values are represented as mean ± S.E.M. (*n* =
809 8 fish per group). Significant differences among sampling points at the same salinity are
810 identified with different letters; different symbols show differences between groups at the
811 same time ($P<0.05$, two-way ANOVA followed by Tukey's test).
812
813 **Figure 5.** Time course changes in plasma cortisol levels after transfer from SW to different
814 environmental salinities (LSW, SW and HSW). Values are represented as mean ± S.E.M. (*n* =
815 8 fish per group). Further details as described in the legend of Figure 4.
816
817 **Figure 6.** Time course changes in hypothalamic pro-VT (A) and pro-IT (B) mRNA
818 expression levels (relative to β-actin) after transfer from SW to different environmental
819 salinities (LSW, SW and HSW). Values are represented as mean ± S.E.M. (*n* = 8 fish per
820 group). Further details as described in the legend of Figure 4.
821
822 **Figure 7.** Time course changes in AVT (A) and IT (B) pituitary storage levels after transfer
823 from SW to different environmental salinities (LSW, SW and HSW). Values are represented
824 as mean ± S.E.M. (*n* = 8 fish per group). Further details as described in the legend of Figure 4.
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826 **Figure 1.** ***Martos-Sitcha et al.***
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**Figure 2.** ***Martos-Sitcha et al.***

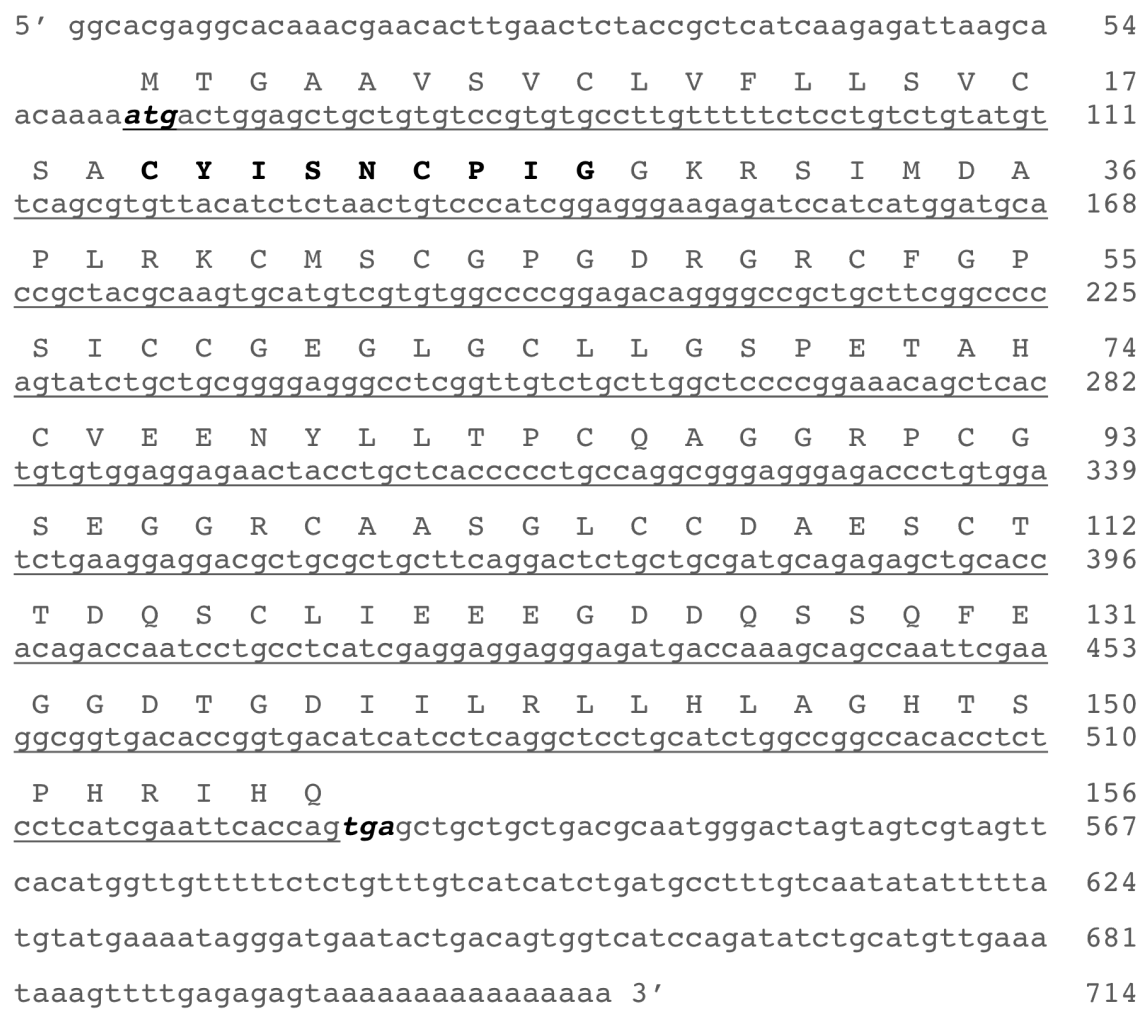
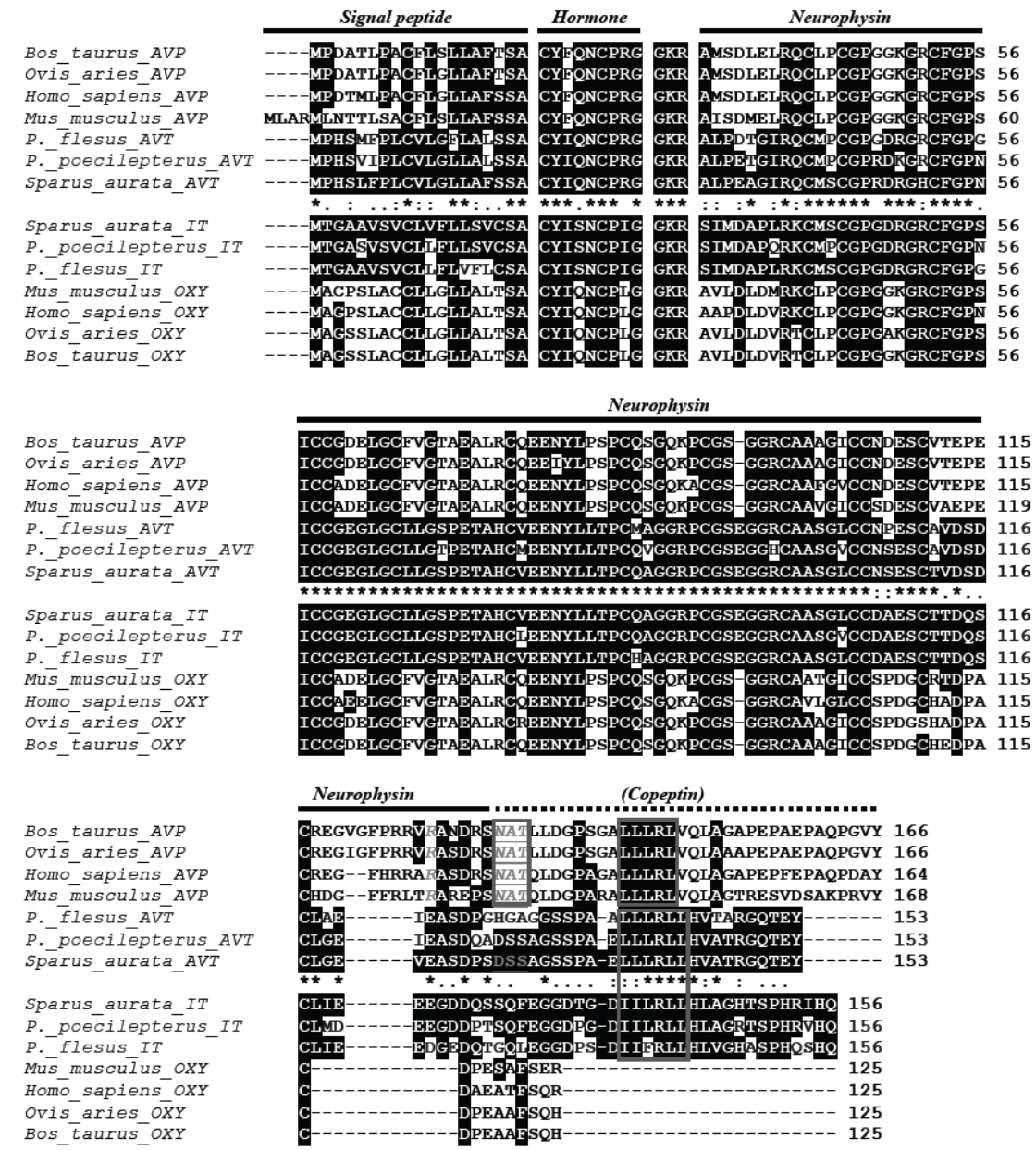


**Figure 3.** ***Martos-Sitcha et al.***



840 **Figure 4.** ***Martos-Sitcha et al.***
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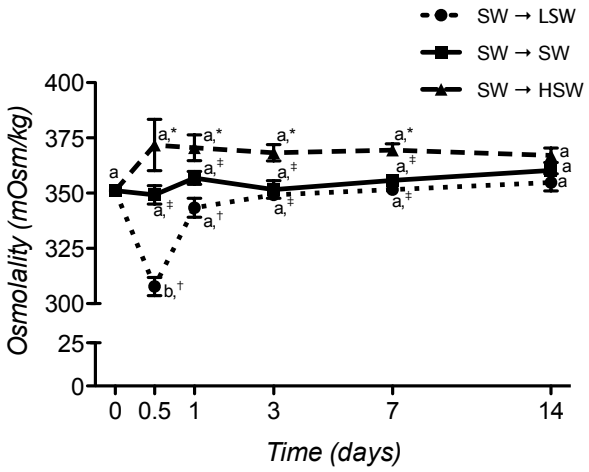


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844 **Figure 5**. ***Martos-Sitcha et al.***
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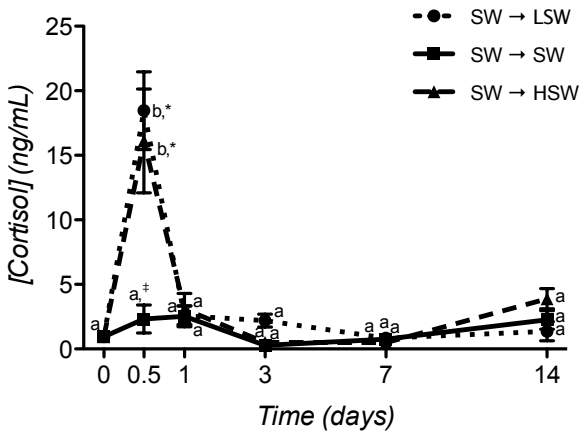


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848 **Figure 6.** ***Martos-Sitcha et al.***
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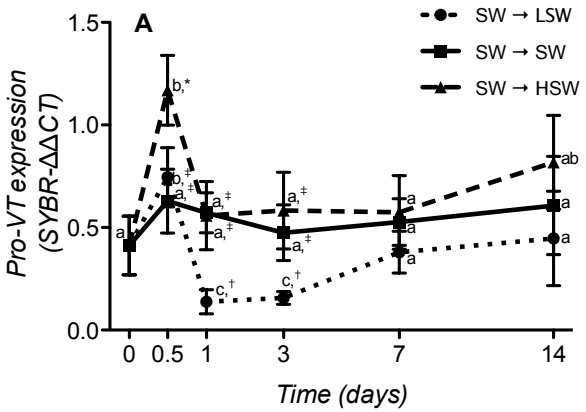


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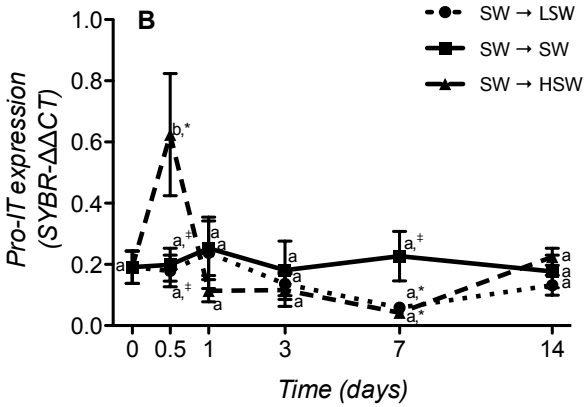


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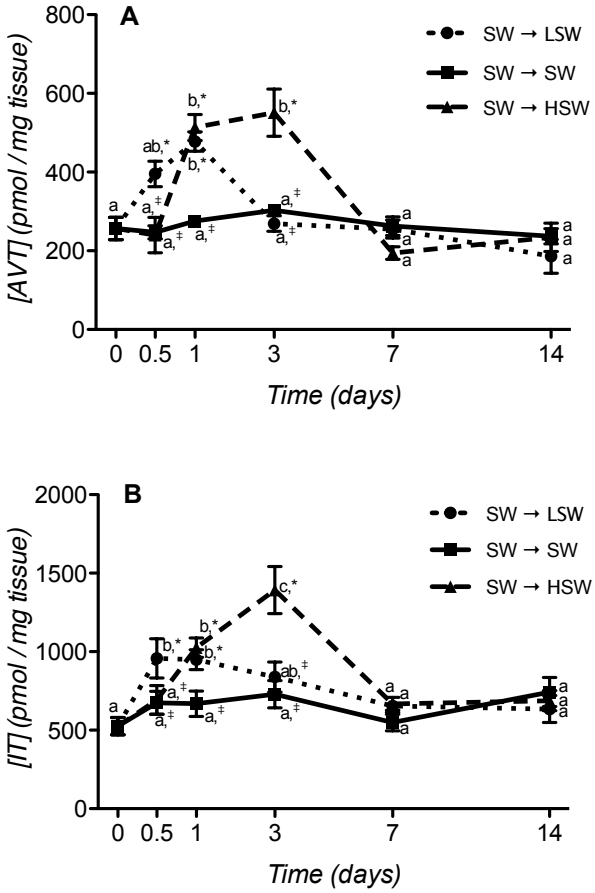
**Figure 7.** ***Martos-Sitcha et al.***



871 **Tables**
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| *Primers* | *Nucleotide sequence* | *Amplicon size* |
|---|---|---|
| *Degenerated* | | |
| **pro-VT$_{Fw1}$** | 5'-ATGCCTCACTCCTTGTTCCC-3' | 436 bp |
| **pro-VT$_{Rv1}$** | 5'-GCMACATGKAGMAGMCGCA-3' | |
| **pro-VT$_{nestedFw}$** | 5'-GCCTGCTACATCCAGAATTG-3' | 299 bp |
| **pro-VT$_{nestedRv}$** | 5'-AGGCAGTCAGAGTCCACC-3' | |
| **pro-IT$_{Fw1}$** | 5'-TTCAGCRTGTTACATCTC-3' | 491 bp |
| **pro-IT$_{Rv1}$** | 5'-GAGGTRAAGACAAACAGAGAA-3' | |
| **pro-IT$_{nestedFw}$** | 5'-CGYAAGTGCATGYCCTGTGG-3' | 379 bp |
| **pro-IT$_{nestedRv}$** | 5'-GYGACCRGCCAGATGCAGCAG-3' | |
| *QPCR* | | |
| **qPCRpro-VT$_{Fw}$** | 5'-AGAGGCTGGGATCAGACAGTGC-3' | 129 bp |
| **qPCRpro-VT$_{Rv}$** | 5'-TCCACACAGTGAGCTGTTTCCG-3' | |
| **qPCRpro-IT$_{Fw}$** | 5'-GGAGATGACCAAAGCAGCCA-3' | 151 bp |
| **qPCRpro-IT$_{Rv}$** | 5'-CAACCATGTGAACTACGACT-3' | |
| **qPCRβ-actin$_{Fw}$** | 5'-TCTTCCAGCCATCCTTCCTCG-3' | 108 bp |
| **qPCRβ-actin$_{Rv}$** | 5'-TGTTGGCATACAGGTCCTTACGG-3' | |

873
874 **Table 1.** Degenerate primers designed for molecular identification of pro-VT and pro-IT
875 partial cDNA sequences, specific primers used for semi-quantitative expression by QPCR,
876 and the amplified size by each pair of both degenerate and specific primers.
877
878