**Variations in the expression of vasotocin and isotocin receptor genes in the
gilthead sea bream *Sparus aurata* during different osmotic challenges**

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27 **Abstract**

28

29 The dynamic changes in vasotocin (AVT) and isotocin (IT) receptor gene expression
30 levels were assessed in a time-course response study in immature male specimens of
31 the gilthead sea bream (*Sparus aurata*) submitted to hyper- (55 ‰ salinity) and hypo-
32 osmotic (5 ‰ salinity) challenges. Two different cDNAs for the AVT receptor and
33 one for the IT receptor (V1a2-type and V2-type AVTR, and ITR, respectively) were
34 cloned by screening an *S. aurata* brain cDNA library. Genes for these receptors were
35 expressed differentially and nearly ubiquitously in 26 of the examined tissues. In the
36 gills, both environmental salinity challenges up-regulated AVTR V1a2-type gene
37 expression concomitantly with Na⁺, K⁺-ATPase gene expression and protein activity,
38 whereas the AVTR V2-type and cystic fibrosis transmembrane conductance regulator
39 (CFTR) mRNA levels were associated with environmental salinity, indicating a
40 possible connection between AVTRs and these transporters. In the kidney, AVTR
41 V1a2-type gene expression peaked rapidly and lasted only a short time (12 to 24 h) in
42 response to both osmotic challenges. In contrast, AVTR V2-type mRNA levels were
43 enhanced in specimens exposed to hyperosmotic conditions, whereas they decreased
44 under hypoosmotic environments, suggesting an antidiuretic role related to the
45 vasoconstriction function. In the hypothalamus, only the expression of the AVTR V2-
46 type gene was enhanced at 7 and 14 days under both experimental conditions. In the
47 liver, both AVTRs had increased mRNA levels, with the upregulation of the AVTR
48 V2-type gene increasing faster than the V1a2-type. The ITR gene was not sensitive to
49 variations of external salinity in any of the analyzed tissues. Our results demonstrate
50 the involvement of the vasotocinergic, but not the isotocinergic, pathway as well as
51 the hypothalamic function, in the adjustments of osmoregulatory and metabolic
52 processes after osmotic challenges.

53

54 **Keywords:** *isotocin receptor; metabolism; osmoregulation; Sparus aurata; stress;*
55 *teleost; vasotocin receptors*

56

57 1. Introduction

58

59 Hormones of the vasopressinergic and oxytocinergic families participate in a large
60 variety of physiological processes, such as vascular and osmoregulatory actions,
61 reproduction, behavior, metabolism, stress response, and circadian and seasonal
62 biology (Warne et al., 2002; Balment et al., 2006; Kleszczynska et al., 2006;
63 Kulczykowska, 2007; Rodriguez-Illamola et al., 2011). In teleosts, arginine vasotocin
64 (AVT) and isotocin (IT) are homologous to mammalian vasopressin (AVP) and
65 oxytocin (OXY), respectively (Acher, 1993).

66 Both vasotocinergic and isotocinergic pathways are complex and are activated by
67 several external or internal factors involving different neural processes at the cellular
68 level (gene expression, protein synthesis, maturation, transport, storage, and release)
69 that finally enhance plasma AVT or IT levels. In those systems, some negative
70 feedback relationships that inhibit hormonal synthesis could be present, supporting
71 the survival of specimens in a variable environment and seasonal changes in
72 physiological demands (e.g., nutrition, reproduction, water quality, etc.) within a
73 controlled range (Balment et al., 2006). In both pathways, the presence of vasotocin
74 and/or isotocin receptors (AVTR and ITR, respectively, forming part of the G-protein
75 coupled receptor family) in specific tissues and/or organs indicates a role of both
76 nonapeptides in the physiological processes in which these organs are involved
77 (Mahlmann et al., 1994; Hausmann et al., 1995).

78 Euryhaline teleosts have the capacity of preserving their plasma osmolality within
79 well-delimited values despite the ionic and water imbalances with the surrounding
80 environment. Several osmoregulatory organs (e.g., gills, kidney, gastrointestinal tract,
81 etc.) and hypophyseal and extrahypophyseal hormones are involved in this process
82 (Takei and McCormick, 2013). In addition, a greater energy demand is generated to
83 maintain this homeostasis, and a metabolic organ (i.e., the liver) provides part of the
84 required energy (Hylland et al., 1997; Purdon and Rapoport, 1998; Sangiao-
85 Alvarcellos et al., 2003, 2005). Furthermore, the brain, especially at the hypothalamic
86 level, where nonapeptide synthesis begins, is also involved in the central control of
87 these processes, showing a metabolic activation (Acher, 1993; Soengas and
88 Aldegunde, 2002). Moreover, the presence of AVT and IT receptors or changes in
89 physiological functions in organs/tissues after hormone treatment have been
90 previously described in several species of fish (Henderson and Wales, 1974; Janssens

91 and Lowrey, 1987; Guibbolini et al., 1988; Moon and Mommsen, 1990; Mahlmann et
92 al., 1994; Amer and Brown, 1995; Hausmann et al., 1995; Warne, 2001; Warne et al.,
93 2002; Dantzler, 2003; Sangiao-Alvarellos et al., 2004, 2006; Lema, 2010).
94 Still, changes in AVTR and ITR due to different physiological situations have not
95 been studied extensively. The Amargosa pupfish *Cyprinodon nevadensis amargosae*
96 (Lema, 2010) exposed to environmental salinity changes and the African lungfish
97 *Protopterus annectens* (Konno et al., 2010) under aestivation condition (dehydration)
98 showed alterations in AVTR and ITR, and/or in pro-vasotocin mRNA levels.
99 The gilthead sea bream (*Sparus aurata*) has been proposed by several authors to be a
100 good research model for studying the endocrine control of osmoregulatory processes.
101 In fact, osmoregulatory and metabolic reorganization have been reported in specimens
102 of this euryhaline teleost under hypo- and hyperosmotic acclimations (Mancera et al.,
103 1993; Sangiao-Alvarellos et al., 2003, 2005; Laiz-Carrión et al., 2005; Vargas-
104 Chacoff et al., 2009; Fuentes et al., 2010; Balmaceda-Aguilera et al., 2012; Gregorio
105 et al., 2013; Martos-Sitcha et al., 2013b). The vasotocinergic and, of minor importance
106 (if any), isotocinergic pathways are involved in these processes (Kleszczynska et al.,
107 2006; Sangiao-Alvarellos et al., 2006; Martos-Sitcha et al., 2013a, 2013b). However,
108 to our knowledge, there are no data on the involvement of AVTR and ITR in the
109 osmoregulatory and metabolic processes in *S. aurata*, despite the putative importance
110 of these receptors during osmotic adaptation. We propose that changes in
111 environmental salinity induce modifications in the expression of the AVTR and/or
112 ITR genes in osmoregulatory organs (the gills and kidney). In addition, we considered
113 non-osmoregulatory organs (the liver and hypothalamus/brain) because previous
114 studies with *S. aurata* have shown that osmotic acclimation induces changes in the
115 metabolism and control processes in both organs (Polakof et al., 2006; Sangiao-
116 Alvarellos et al., 2006).
117 To test this hypothesis, cDNA for AVT and IT receptors from *S. aurata* was cloned,
118 and the tissue distribution of each mRNA was studied to identify the putative targets
119 in which both nonapeptides are involved to achieve their final physiological action.
120 Moreover, to obtain information about osmoregulation and metabolism, changes in
121 gene expression levels over time following different environmental salinity challenges
122 were assessed.

123

124 **2. Materials and methods**

125

126 *2.1. Animals and experimental protocol*

127 Immature male specimens of gilthead sea bream (*Sparus aurata* L., 80-100 g body
128 weight, $n = 128$) were provided by Planta de Cultivos Marinos (CASEM, University
129 of Cadiz, Puerto Real, Cadiz, Spain; experimental animal facility registry numbers
130 CA/4/CS and CA/3/U) and randomly distributed into six 400-L tanks in an open
131 system circuit of seawater (SW, 40 ‰ salinity) at a density of $4.5 \text{ kg} \cdot \text{m}^{-3}$ under a
132 natural photoperiod (May, 2009) for our latitude ($36^{\circ} 31' 44'' \text{ N}$) and at constant
133 temperature ($18\text{-}19^{\circ}\text{C}$). After an initial period of acclimation over 10 days, the
134 animals were directly transferred to the following environmental salinities: seawater
135 (SW, control group), low salinity water (LSW, 5 ‰ salinity), and high salinity water
136 (HSW, 55 ‰ salinity). Groups were maintained in duplicate experimental tanks (each
137 400 L in volume; $n = 20$ fish per tank, $4.5 \text{ kg} \cdot \text{m}^{-3}$ initial density) under a closed
138 recirculation system. Water quality was checked twice a day to confirm stability. Fish
139 were fed a daily ration of 1 % of their body weight with commercial pellets
140 (Dibaq-Dibroteg S.A., Segovia, Spain).

141 On day 0, eight fish from the six main tanks containing SW were anaesthetized with
142 2-phenoxyethanol ($1 \text{ mL} \cdot \text{L}^{-1}$) and sampled (control time 0 before transfer), and the
143 remainder of the experimental specimens were transferred directly to the three
144 experimental salinities. Then, eight fish (four per tank) from each experimental
145 salinity (SW, LSW, and HSW) were anesthetized at the specific salinity, weighed, and
146 sampled at 12 hours and 1, 3, 7, and 14 days post-transfer. Biopsies (20-30 mg) from
147 each organ were extracted in duplicate, including the gills (filaments from the second
148 branchial arch), liver, kidney, and the two complete hypothalamic lobules (including
149 all neuronal regions contained in this brain division), and placed in sterile Eppendorf
150 tubes containing 600 μL of RNeasy[®] (Life Technologies). Tubes were kept for 24
151 hours at 4°C and stored at -20°C until processing for total RNA isolation. In all
152 protocols involving commercial kits, the manufacturer's instructions were followed,
153 except where noted. No mortality was observed in any of the groups during
154 experimentation. The experiment was performed following the Guidelines of the
155 European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law
156 32/2007) for the use of laboratory animals.

157

158 *2.2. Molecular cloning of partial AVT and IT receptors*

159 First, a set of degenerate primers was designed according to the most highly
 160 conserved sequences of cDNA between different species for AVTRs, such as for V1-
 161 type (*Platichthys flesus*, GenBank acc. no. AF184966; *Astatotilapia burtoni*, acc. no.
 162 AF517936; *Catostomus commersoni*, acc. no. X76321; *Cyprinodon variegatus* V1a1,
 163 acc. no. GU120189; *Cyprinodon variegatus* V1a2, acc. no. GU120190; *Cyprinodon*
 164 *navadensis amargosae* V1a1, acc. no. GU014233; *Cyprinodon navadensis amargosae*
 165 V1a2, acc. no. GU014234); for V2-type as described by Lema (2010) (acc. no.
 166 GQ981414); and for isotocin receptor (ITR) (*Cyprinodon navadensis amargosae*, acc.
 167 no. GQ981415; *Haplochromis burtoni*, acc. no. GQ288467; *Danio rerio*, NCBI
 168 reference sequence: NM_001199370.1; *Catostomus commersoni*, acc. no. X87783).
 169 Next, degenerate primers were synthesized by IDT[®] (Integrated DNA Technologies,
 170 Leuven, Belgium) and supplied by BIOMOL (Seville, Spain). Nucleotide sequences
 171 are shown in Table 1.

172 Total RNA was prepared from *S. aurata* whole brain (≈ 1 g) from the main stock
 173 maintained in SW using an Ultra-Turrax[®] T25 (IKA[®]-Werke) and the NucleoSpin[®]
 174 RNA II kit (Macherey-Nagel) in a volume of buffer RA1 proportional to their weights
 175 and by loading the column with the maximum allowed volume (600 μ L) and storing
 176 the rest at -80 °C for further use if necessary. Genomic DNA (gDNA) was eliminated
 177 using the on-column RNase-free DNase digestion (included in the kit) at 25 °C for 15
 178 min. RNA quality was checked in a Bioanalyzer 2100 with the RNA 6000 Nano kit
 179 (Agilent Technologies), whereas RNA quantity was measured spectrophotometrically
 180 at 260 nm in a BioPhotometer Plus (Eppendorf). Brain total RNA (2 μ g) was
 181 reverse-transcribed in a 20- μ L reaction using 250 ng random primers (Invitrogen[™],
 182 LifeTechnologies) and 200 U Superscript III reverse transcriptase (Invitrogen[™],
 183 LifeTechnologies) with the manufacturer's first strand buffer (1x final concentration),
 184 DTT (5 mM final concentration), and dNTPs (0.5 mM final concentration) at 25 °C
 185 for 5 min, 50 °C for 60 min, and 70 °C for 15 min. PCR amplifications were carried
 186 out with 1 U Platinum[®] Taq DNA Polymerase (Invitrogen[™], Life Technologies) with
 187 the first strand cDNA (corresponding to 10 ng of input total RNA), manufacturer's
 188 PCR buffer (1x final concentration), 200 nM of each sense and antisense primers, 200
 189 μ M dNTP mixture, and 1.5 mM MgCl₂ in a total volume of 20 μ L. Samples were
 190 cycled at 94 °C for 2 min for initial template denaturation, followed by 35 cycles of
 191 94 °C for 30 s for DNA denaturation, 62-58.5 °C touchdown for 30 s for primer
 192 annealing, and 72 °C for 1 min for primer extension. Next, we performed 10

193 additional three-step cycles at 94 °C for 30 s for DNA denaturation, 60 °C for 30 s for
194 primer annealing, 72 °C for 1 min for primer extension, and a final extension at 72 °C
195 for 10 min in a Mastercycler[®]proS vapo.protect (Eppendorf). PCR products were
196 identified by 1.2 % agarose gel electrophoresis stained with GelRed (Biotium) and
197 ligated with the TOPO TA Cloning[®] Kit for Sequencing (Invitrogen[™], Life
198 Technologies) into the pCR[®]4 TOPO[®] vector. Following sequencing of a single clone
199 for each receptor in both strands using M13 forward (-20) and M13 reverse primers
200 by the dideoxy method at the Bioarray S.L. sequencing services (Elche, Alicante,
201 Spain), the sequence homology of the PCR products to AVTRs and ITR was
202 confirmed using NCBI blastn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

203 For the preparation of the AVTRs and ITR probes, 2 µg of each plasmid DNA
204 containing the corresponding putative partial sequences was digested with 10 U of
205 *Eco*RI (Takara) in a volume of 40 µL. The digestion products were separated on a
206 1.2% agarose gel, and the specific bands of 392 bps (V1a2-type), 490 bps (V2-type),
207 and 260 bps (ITR) were excised and purified with the QIAquick kit (Qiagen). The
208 cDNA fragments were diluted to a final concentration of 25 ng·µL⁻¹ in TE buffer (10
209 mM Tris-HCl, 1 mM EDTANa₂, pH 8.0) and stored at -20 °C.

210

211 *2.3. Cloning of a full-length AVTRs and ITR cDNAs from S. aurata by screening a* 212 *brain cDNA library*

213 The brain cDNA library was prepared from 5 µg of poly(A) RNA using the lambda
214 ZAP-cDNA/Gigapack III Kit (Stratagene, Agilent Technologies; discontinued) with
215 some modifications, as described by Balmaceda-Aguilera et al. (2012).

216 Three 240 x 240 mm NZY agar plates (Nunc) were each plated with approximately
217 250,000 pfu from the amplified *S. aurata* cDNA brain library, and each plate was
218 transferred onto a Hybond-N Nylon membrane (GE LifeSciences). Blots were
219 prehybridized for 1 h at 42 °C in prehybridization solution (50% formamide, 6x
220 SSPE, 0.5% SDS, 5x Denhardt's solution, and 0.1 mg·mL⁻¹ yeast RNA type III). For
221 hybridization, 25 ng of each probe was radiolabeled using the RadPrime DNA
222 Labeling System (Invitrogen[™], LifeTechnologies) and [α^{32} P]dCTP (PerkinElmer),
223 and allowed to hybridize separately with the Nylon membranes overnight at 42 °C.
224 Blots were washed twice in 2x SSC-0.1 % SDS for 30 min each at room temperature,
225 twice in 1x SSC-0.1 % SDS for 30 min at 42 °C, and twice in 0.5x SSC-0.1 % SDS
226 for 30 min at 60 °C. Membranes were exposed to autoradiography film (Amersham,

GE LifeSciences) for two days with intensifying screens at -80 °C. Four positive plaques from approximately 15 positives for ITR and a single positive in each AVTR V1- and V2-type were isolated and subjected to two additional 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). Next, two positive colonies from each clone were picked and plasmid DNA-prepared in a mini-prep column (NucleoSpin® Plasmid Kit; Macherey-Nagel). Excised pBluescript SK(-) containing the clones was double digested by *EcoRI* and *XhoI* (Takara) to release the inserts, 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 (Bioarray S.L., Elche, Alicante, Spain). Following sequencing, sequence homology of all genes was confirmed by NCBI blastn as above. eBiox (v1.5.1) software was used for sequencing fragment assemblage, as well as for translation of the sequences to obtain the open reading frames (ORFs). Homology analysis of putative protein sequences was run with NCBI blastp. Homologous protein sequences were retrieved from the NCBI protein database (accessed in January 2013) and used for phylogenetic analysis conducted with MEGA5 software (Tamura et al., 2011) with the neighbor-joining analysis (Saitou and Nei, 1987) based on amino acid difference (p-distance) and pairwise deletions. The reliability of the tree was assessed with bootstrapping (1,000 replicates) (Felsenstein, 1985).

249

250 2.4. Optimizing qPCR primers

First, specific primer pairs were designed using the software Primer3 v.0.4.0 (available in <http://frodo.wi.mit.edu/>) for relative quantification of AVTR V1a2-type, AVTR V2-type, and ITR gene expression; the primer pairs were synthesized by IDT® (Integrated DNA Technologies, Leuven, Belgium) and supplied by BIOMOL (Seville, Spain) (Table 1). 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 of 5 min at 22 °C, 30 min at 42 °C, and 5 min at 85 °C. cDNA was diluted

10-fold in sterile Milli-Q water to a final estimated concentration of 2.5 ng μL^{-1} . Second, to optimize the qPCR conditions, several primer concentrations (100 nM, 200 nM, 400 nM, and 600 nM) and a temperature gradient (from 54 to 64 °C) were used. Different cDNA template concentrations estimated from total input RNA were applied in triplicate (1 ng $\cdot\mu\text{L}^{-1}$ [10 ng/reaction], 250 pg $\cdot\mu\text{L}^{-1}$, 62.5 pg $\cdot\mu\text{L}^{-1}$, 15.62 pg $\cdot\mu\text{L}^{-1}$, 3.90 pg $\cdot\mu\text{L}^{-1}$, and 976 fg $\cdot\mu\text{L}^{-1}$) to assess the assay linearity and amplification efficiency (AVTR V1a-type (400 nM): $r^2 = 0.998$, efficiency (E) = 1.02; AVTR V2-type (600 nM): $r^2 = 0.996$, E = 1.02; ITR (400 nM): $r^2 = 0.992$, E = 1.03). Primers for the Na^+ , K^+ -ATPase alpha-subunit (NKA α) were designed and optimized as above from the partial sea bream nucleotide sequence available in GenBank (accession no. GU183821). For the cystic fibrosis transmembrane conductance regulator (CFTR) (acc. no. GU183822), primers were used as described by Gregorio et al. (2013). The assay linearity and amplification efficiency for NKA α and CFTR were $r^2 = 0.994$ and 0.991 and E = 1.01 and 0.98, respectively, with 200 nM of primer concentration used in each case. β -actin gene expression (acc. no. X89920) was used to normalize the results and as a positive control in tissue distribution. In this case, the same procedures of testing were carried out as above, using a final concentration of 200 nM of each forward and reverse primer, with $r^2 = 0.999$ and E = 0.99. Finally, although the assay was linear from 1 ng $\cdot\mu\text{L}^{-1}$ to 3.90 pg $\cdot\mu\text{L}^{-1}$ of cDNA per reaction for AVTRs and ITR and from 1 ng $\cdot\mu\text{L}^{-1}$ to 976 fg $\cdot\mu\text{L}^{-1}$ of cDNA per reaction for NKA α , CFTR, and β -actin, 1 ng $\cdot\mu\text{L}^{-1}$ of cDNA was used for all the amplifications. To confirm the correct amplification of each primer pair, the obtained PCR amplicons were cloned and sequenced as above.

284

285 2.5. Distribution of AVTRs and ITR mRNA in Sparus aurata

Total RNA from three different specimens kept at 40 ‰ salinity was isolated using an Ultra-Turrax® T8 (IKA®-Werke) from 30 mg of tissue from the operculum, gills, mouth epithelium, esophagus, stomach, pyloric caeca, anterior intestine, middle intestine, rectum, head kidney, caudal (distal) kidney, heart, spleen, liver, gallbladder, perivisceral fat, ovary, testis, gas bladder, muscle, skin, and bone (vertebral) ($n = 3$) using the NucleoSpin® RNA II kit (Macherey-Nagel) and the on-column RNase-free DNase digestion (included in the kit) at 37 °C for 30 min. Whole brains and eyes were processed similar to the other tissues but in a volume of buffer RA1 proportional to

294 their weights, loading the columns with the maximum allowed volume (600 μL) and
295 storing the rest at $-80\text{ }^{\circ}\text{C}$ for further use if necessary. Pituitaries and urophysis were
296 processed as above but using the NucleoSpin[®] RNA XS kit (Macherey-Nagel). After
297 RNA quality and quantity analyses (see above), reverse transcription on 500 ng of
298 total RNA was performed with the qScript[™] cDNA synthesis kit (Quanta
299 BioSciences). Then, the cDNA was diluted 10-fold to a concentration of $2.5\text{ ng}\cdot\mu\text{L}^{-1}$,
300 and PCR amplifications were carried out with the PerfeCTa SYBR[®] Green FastMix[™]
301 (Quanta BioSciences). Each reaction mixture (10 μL) contained 0.5 μL of each of the
302 specific forward and reverse primers at 600 nM (AVTR V2-type), 400 nM (AVTR
303 V1a2-type and ITR), or 200 nM (β -actin), with 5 μL of PerfeCTa SYBR[®] Green
304 FastMix[™] (Quanta BioSciences), and with 4 μL cDNA (10 ng; estimated from the
305 total RNA reverse transcription reaction) for each sample. Reactions were conducted
306 in semi-skirted 96-well twin.tec real-time PCR plates (Eppendorf) covered with
307 adhesive Masterclear real-time PCR film (Eppendorf). Plates were cycled ($95\text{ }^{\circ}\text{C}$, 10
308 min; [$95\text{ }^{\circ}\text{C}$, 20 s; $60\text{ }^{\circ}\text{C}$, 35 s] x 35 cycles; *melting curve* [$60\text{ }^{\circ}\text{C}$ to $95\text{ }^{\circ}\text{C}$, 20 min];
309 $95\text{ }^{\circ}\text{C}$, 15 s) for AVTRs, ITR and β -actin, which was used as a positive control. The
310 negative control was run using sterile water as template. The melting curve was used
311 to ensure that a single product was amplified and confirm the absence of primer-dimer
312 artifacts. PCR products were separated on a 2% agarose gel stained with GelRed to
313 evaluate the presence or absence of each mRNA in all tissues. The PCR conditions for
314 each primer pair were established in the exponential part of the curve for unique
315 reaction products after establishing a relationship of signal *vs.* number of cycles.

316

317 2.6. Quantification of gene expression levels (qPCR)

318 Total RNA was isolated using an Ultra-Turrax[®] T8 (IKA[®]-Werke) from
319 hypothalamus or 30 mg of tissue from the gills, kidney and liver using the
320 NucleoSpin[®] RNA II kit (Macherey-Nagel). RNA quality and quantity were measured
321 as described above. Only samples with a RNA Integrity Number (RIN) higher than
322 8.5 were used for qPCR.

323 qPCR was carried out with the Fluorescent Quantitative Detection system (Eppendorf
324 Mastercycler ep *realplex* 2S). Each reaction mixture (10 μL) was treated as described
325 above for tissue distribution, except for the PCR profile, which was as follows: $95\text{ }^{\circ}\text{C}$,
326 10 min; [$95\text{ }^{\circ}\text{C}$, 20 s; $60\text{ }^{\circ}\text{C}$, 35 s] x 40 cycles; *melting curve* [$60\text{ }^{\circ}\text{C}$ to $95\text{ }^{\circ}\text{C}$, 20 min];

95 °C, 15 s. Each sample was run in triplicate. The results were normalized to β -actin due to 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) with the cDNA from hypothalamus total RNA of the control group at day 0 as baseline.

Furthermore, gill samples from specimens acclimated to the three environmental salinities (LSW, SW, and HSW) over 14 days (end-point of the experiment) were used for relative quantification of expression levels of NKA α and CFTR genes. qPCR reactions were carried out as described above. In addition, the correlation analysis between AVTR V1a2-type and NKA α , as well as AVTR V2-type and CFTR gene expression, was performed for the three experimental salinities at day 14.

2.7. Statistics

Significant differences were analyzed with a two-way ANOVA with salinity (LSW, SW, and HSW) and time (day 0; 12 hours; days 1, 3, 7, and 14) as main factors, followed by post-hoc comparison with Tukey's test. For single time point data, significant differences were analyzed with a one-way ANOVA (LSW, SW, and HSW), followed by post-hoc comparison with Tukey's test. Linear relationship analyses between AVTR and ion transporter mRNA levels were assessed by a general linear model. All tests were performed using GraphPad Prism[®] (v.5.0b) software. Significance was defined as $P < 0.05$.

3. Results

3.1. Cloning of AVT and IT receptors

The full-length cDNA sequences obtained for the sea bream AVTR V1a2-type (GenBank acc. no. KC195974) and AVTR V2-type (GenBank acc. no. KC960488) receptors were 2,732 bps and 3,479 bps, respectively, whereas the longest full-length sequenced clone for ITR (GenBank acc. no. KC195973) was 1,749 bps. Both AVTR V1a2-type and V2-type receptor nucleotide sequences comprised an ORF of 1,167 bps and 1,521 bps, respectively, encoding predicted amino acid sequences of 388 and 507, respectively (Figures 1 and 2). For ITR, the ORF was 1,188 bps long and encoded a putative 395-amino acid sequence (Figure 3). The ORFs from the three genes contain the typical seven hydrophobic transmembrane domains as highlighted

in Figures 1, 2, and 3, with an extracellular N-terminal segment and intracellular cytoplasmic C-terminal domain for each one. The isolated full-length sequences clustered within three independent branches of the V1a AVTR/AVPR, V2 AVTR/AVPR, and ITR/OXYR families, as shown by phylogenetic analysis (Figure 4) using the translated protein sequences of AVT/AVP and IT/OXY receptors.

3.2. Tissue distribution of AVT and IT receptors

The gene expression patterns for sea bream AVTR V1a2-type (V1a2), AVTR V2-type (V2), and ITR were examined by qPCR in SW-acclimated specimens (Figure 5). V1a2-type receptor mRNA was ubiquitously expressed in all the tissues examined, except in the eye, where it was not detected. The gills, intestine (anterior, middle, and rectum), testis, and gas bladder showed strong gene expression, whereas it was low in the pituitary and ovary. The V2-type receptor was present in all tissues assessed, although different gene expression levels were observed; the gills, anterior intestine, and testis had the highest gene expression, and the heart and ovary had the lowest. Finally, ITR gene expression was not detected in the pituitary, stomach, heart, spleen, ovary, or testis; the gills, mouth epithelium, gall, and gas bladders had the highest gene expression levels, whereas the eye, liver, muscle, skin, and bone had the lowest gene expression.

3.3. Expression of AVT and IT receptor genes after salinity challenges

3.3.1. Gill

Both hyper- and hypoosmotic challenges significantly enhanced AVTR V1a2-type receptor gene expression from day 7 until the end of the experiment (Figure 6a). In contrast, hyperosmotic transfer increased V2-type receptor expression from day 7 until the end of the experiment, whereas hypoosmotic challenge decreased gene expression from 12 h and was significantly different compared with the control group from day 3 until the end of the experiment (Figure 6b). ITR did not show significant modifications in mRNA levels after either osmotic challenge (Figure 6c).

3.3.2. Kidney

AVTR V1a2-type gene expression was significantly increased in both hyper- and hypoosmotic challenges during the first 24 h post-transfer, reaching values close to the control group from day 3 until the end of experiment (Figure 7a). In specimens

submitted to HSW, the V2-type receptor showed enhanced mRNA levels throughout the experimental period, with a significant increase at 12 h and 1 day post-transfer, as well as at the last time-point (14 days). In contrast, transfer to LSW significantly decreased the expression of this receptor gene at day 3, presenting lower values from day 1 to day 14 compared with fish maintained in SW (Figure 7b). ITR gene expression did not change significantly in any of the three salinity challenges (Figure 7c).

3.3.3. Hypothalamus

Hyper- and hypoosmotic challenges did not induce significant changes in AVTR V1a2-type and ITR gene expression compared with the control group (transferred from SW to SW) throughout the 14-day experiment period (Figure 8a and 8c). However, both challenges significantly enhanced AVTR V2-type mRNA levels from day 7 until the end of the experiment (Figure 8b).

3.3.4. Liver

Hepatic expression of the AVTR V1a2- and V2-type genes showed a significant modification following both LSW and HSW. Hypoosmotic challenge significantly increased V1a2-type receptor mRNA levels from day 3 to 14, whereas under the HSW condition, this enhancement was only observed from day 7 onwards (Figure 9a). V2-type gene expression increased under both experimental conditions during the entire duration of the experiment, although significant differences between LSW and HSW mRNA levels from day 3 post-transfer were also observed (Figure 9b). ITR mRNA values did not change significantly in any of the salinity challenges (Figure 9c).

3.4. *NKA α* and *CFTR* expression in gills

Acclimation of *S. aurata* specimens over 14 days induced a significant increase in *NKA α* mRNA levels at both extreme salinities (LSW and HSW) compared with the SW-acclimated group (Figure 10a). In addition, clear salinity dependence in the expression of the *CFTR* gene was observed, with significantly lower mRNA levels in LSW-acclimated specimens compared with those acclimated to HSW (Figure 10b). Furthermore, comparison between ion transporters and AVTRs at the mRNA level

showed a significant ($P < 0.05$) correlation (NKAA and AVTR V1a2-type, $r^2 = 0.5783$; CFTR and AVTR V2-type, $r^2 = 0.6139$) at the final time point. No other associations of ion transporter mRNA levels (NKAA and CFTR) and AVTR or ITR gene expression were observed.

4. Discussion

In this study, three different full-length mRNAs/cDNAs encoding specific AVTR and ITR were cloned and identified in a brain cDNA library from the gilthead sea bream (*Sparus aurata*). Phylogenetic analysis of the predicted proteins revealed that two of the three neuropeptide receptors belong to the family of AVPR/AVTR, clustering independently within the AVT V1a-type (specifically with the V1a2-type receptor) and V2-type receptor clades, while the third cDNA shared greater amino acid structural similarity with ITR.

Analysis of the predicted transmembrane domains (TMDs) revealed amino acid residues previously identified as important counterparts for the interaction between ligands and receptors (Hausmann et al., 1996; Acharjee et al., 2004; Slusarz et al., 2006; Cho et al., 2007; Lema, 2010). Thus, the amino acid residues described in other species as essential for the TMD to form the hydrophilic cleft for hormonal binding or the lipophilic pocket (Konno et al., 2009; Lema, 2010) are also conserved in both sea bream AVTR and ITR. Together with the structural properties of the three receptors cloned in the present study, our data demonstrate that these mRNAs/cDNAs are translated into the corresponding functional proteins.

Recent studies have previously characterized different types of AVT receptors (two V1-types and one V2-type) in *C. nevadensis amargosae* (Lema, 2010) and the sheepshead minnow *Cyprinodon variegatus* (acc. numbers in Figure 4 legend). However, only one V1a-type and one V2-type have been described in *S. aurata* (present results), which is in agreement with that reported in *Protopterus annectens* (Konno et al., 2009). This divergence in the presence of different AVTR subtypes has been highlighted in other teleost species, some in which V1 and V2 types have been characterized, whereas both the V1a1 and V1a2 sub-types but not V2-type were reported in others (acc. numbers in Figure 4 legend). In the isotocinergic pathway, a single ITR was observed in *S. aurata*, which is in agreement with that reported for other species, such as *C. nevadensis amargosae* (Lema, 2010), *Catostomus*

462 *commersoni* (Hausmann et al., 1995), and *Cyprinus carpio* (Flores et al., 2007),
463 although two different mRNAs were described in *Orizyas latipes* (ITR1: acc. no.
464 AB646240; ITR2: acc. no. AB646241) and *Stegastes partitus* (ITR1: acc. no.
465 JX051871; ITR2: acc. no. JX051872). However, no evidence for the possible
466 sub-functionalization of each isoform was reported.

467 Tissue distribution patterns observed for AVTRs and ITR suggest a physiological role
468 of both neuroendocrine pathways related to the processes in which those tissues are
469 involved (Warne, 2002; Balment et al., 2006; Sangiao-Alvarellos et al., 2006;
470 Kulczykowska, 2007; Rodriguez-Illamola et al., 2011; present results); clear
471 differences between the expression of the AVTR V1a-type and V2-type genes have
472 been previously shown (Konno et al., 2009; Lema, 2010; present results).
473 Furthermore, our analysis demonstrates an interesting *S. aurata* tissue distribution
474 pattern. Although there is a ubiquitous expression pattern of V1a2- and V2-types in
475 the intestine, higher mRNA levels of the V2-type receptor in the anterior intestine
476 reflect a putative greater importance of the V2-type than the V1a2-type in this
477 intestinal portion. Moreover, Martos-Sitcha et al. (2013a) also demonstrated higher
478 V1a2-type gene expression in the rectum than the anterior intestine using real-time
479 PCR (qPCR), which is in agreement with the clear functional distinction between both
480 regions based on the effects induced by AVT treatment in the *in vitro* study. In
481 addition, the hypothalamus had the lowest mRNA levels of the three receptors,
482 whereas the gills, kidney and liver showed 7- to 60-fold higher levels for AVTR
483 genes and 3- to 5-fold higher for ITR compared with baseline and depending on the
484 time post-transfer and environmental conditions. These data suggest that
485 osmoregulatory (i.e., gills, kidney) and metabolic (i.e., liver) organs could be more
486 responsive to AVT/IT plasma changes in specimens submitted to osmotic challenge.

487 The vasotocinergic and isotocinergic pathways appear to be involved in branchial
488 sodium and chloride exchange, in which the direction of ion and water movement
489 depends on the relative imbalance compared with the surrounding environment
490 (Maetz et al., 1964; Motaïs and Maetz, 1967). To this end, the Na^+, K^+ -ATPase (NKA)
491 has been described as one of the most important transporters to drive osmoregulatory
492 actions in the gills (Epstein et al., 1980; Marshall, 2002). Furthermore, different
493 expression levels of different NKA isoforms could be associated with several
494 functions, such as ion secretion and uptake and acid-base regulation (Evans et al.,
495 2005; Hiroi and McCormick, 2012). This result has been demonstrated in the gills of

496 tilapia (*Oreochromis mossambicus*) adapted to hyper- or hypoosmotic conditions (Lee
497 et al., 1998). Moreover, differential expression of branchial NKA isoforms in
498 Salmonids has been reported during salinity adjustments (Richards et al., 2003;
499 Bystriansky et al., 2006; Nilsen et al., 2007), suggesting that the switching of isoforms
500 can be an important modulator in gill function after osmotic adaptation (Nilsen et al.,
501 2007). In addition, gill AVTR mRNA levels confirm this tissue as a target for these
502 hormones in teleosts (Mahlmann et al., 1994; Warne, 2001; Lema, 2010) and ITR
503 (Hausmann et al., 1995; Lema, 2010). In SW-acclimated *S. aurata* specimens,
504 long-term treatment with exogenous AVT (6 days) has a stimulatory effect on gill
505 NKA activity. These data suggest a role for this hormone during the regulatory period
506 of acclimation to high salinity environments (Sangiao-Alvarellos et al., 2006).
507 Additionally, during this period, an enhancement of plasma AVT values has also been
508 reported in HSW-acclimated specimens (Kleszczynska et al., 2006), as well as an
509 increase in gill NKA protein activity (Laiz-Carrion et al., 2005). It is interesting to
510 note that the expression of the V1a2-type and Na⁺,K⁺-ATPase alpha-subunit (NKA α)
511 genes (present study, Figure 10a) and gill NKA enzymatic activity were also
512 stimulated in specimens fully acclimated to hyper- and hypoosmotic environments
513 (Laiz-Carrion et al., 2005). These data suggest that AVT available at the plasma level
514 may produce the physiological action *via* AVTR V1a2-type, stimulating by NKA the
515 ion uptake under hypoosmotic conditions and the ion excretion under hyperosmotic
516 conditions. Interestingly, the NKA response is also carried out *via* the PLC and IP3
517 signaling pathways. Therefore, co-regulation between V1a-type receptor and NKA
518 activity in *S. aurata* after hyper- and hypoosmotic challenges could be proposed.
519 For the V2-type receptor, a clear direct linear relationship between its mRNA levels
520 and environmental salinity was detected in both extreme conditions from 3 and/or 7
521 days post-transfer until the end of the experiment. These results also suggest an
522 important role of this receptor in the acclimation to HSW. In this osmotic condition, a
523 relationship between AVTR V2-type and other transporters responsible for ion
524 extrusion could be possible in sea bream gills. Indeed, a relationship between
525 environmental salinity and gill CFTR gene expression has been previously described
526 in the killifish (*Fundulus heteroclitus*), although there were no parallel changes in
527 protein abundance, suggesting post-transcriptional/post-translational regulation of
528 protein levels (Scott et al., 2004). Additionally, CFTR mRNA levels have been shown

529 to be up-regulated in *F. heteroclitus* after transfer from FW to SW (Singer et al.,
530 1998). Our results, which were obtained during the regulatory period (at 14 days post-
531 transfer, the end-point of the experiment), also showed a direct linear relationship
532 between branchial CFTR gene expression and environmental salinity. These results fit
533 with gill V2-type receptor mRNA levels and the hypothesis that both proteins are
534 regulated by the same intracellular signaling pathways (PKA), suggesting that AVTR
535 V2-type could control the trafficking of ion transporters (CFTR, at least) to the apical
536 membranes. In addition, the two positive correlations between the analyses performed
537 (ATPase alpha-subunit *vs.* AVTR V1a2-type; CFTR *vs.* AVTR V2-type) support the
538 hypothesis of a salinity-dependent role of AVTR on gill ion regulation, in which the
539 electrogenic mechanism *via* NKA is highly suggested to be regulated by the V1a2-
540 type receptor, and the secretory mechanism *via* CFTR regulated by the V2-type
541 receptor.

542 In terrestrial vertebrates, AVP function is highly specialized in maintaining water and
543 ion balance at the renal level (Balment et al., 1984; Burgess et al., 1994). However, in
544 teleosts, the contribution of the kidney to osmoregulatory processes is not universal
545 (Beyenbach, 1995; Renfro, 1999; Dantzler, 2003; Nishimura and Fan, 2003). The
546 mechanisms responsible for the antidiuretic effect of AVT are not well understood,
547 but the existence of a decrease in glomerular filtration rate and an increase in tubular
548 water reabsorption has been proposed (Warne, 2002; Warne et al., 2002). In those
549 cases, and according to the mammalian model, diuretic or antidiuretic actions by
550 modifications of the glomerular filtration processes, as well as changes in systemic
551 blood pressure, can be associated with AVT and/or IT depending on the hormone
552 concentration at either pharmacological or physiological ranges (Henderson and
553 Wales, 1974; Amer and Brown, 1995). The V2-type AVT/AVP receptor has been
554 implicated in antidiuretic actions, playing a pivotal role in tubular water preservation
555 in the kidney, where the involvement of specific water channel proteins, namely
556 aquaporin 2 (AQP2), are necessary (Lolait et al., 1992; Konno et al., 2010). Thus, the
557 V2-type receptor allows the accumulation of AQP2 into the apical membrane through
558 the activation of PKA (Lolait et al., 1992; Nielsen et al., 2002). According to the
559 mammalian renal model and in *S. aurata* specimens submitted to hyperosmotic
560 conditions (Figure 7b), co-activation of AQP water channels could be expected, and
561 then, the combination of AVTR V2-type and AQP water channels could produce an

antidiuretic effect by avoiding renal water loss. The lower expression levels of the V2 receptor gene in specimens submitted to LSW compared with those measured in hyperosmotic conditions (SW and HSW) indicate that the physiological actions mediated by the V2 receptor (antidiuretic effect) are not necessary in specimens kept under hypoosmotic environments. However, antidiuretic vascular effects throughout the kidney have been reported in fish for AVTR V1a2-type (Mahlmann et al., 1994; Warne, 2001; Konno et al., 2009; Lema, 2010). The activation of V1a-type receptor expression during the adaptive period to hyperosmotic environments supports this hypothesis, suggesting that AVT affects the renal function *via* the V1a-type receptor to cope with the first effects of this osmotic shock. However, the similar enhancement of AVTR V1a2-type gene expression observed during the adaptive period after hypoosmotic challenge could reflect a role of AVT during the reabsorption processes of monovalent ions (Na^+ , Cl^-) in the tubular epithelium *via* basolateral NKA and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ (NKCC) co-transporters (Dantzler, 2003) to prevent ionic losses due to glomerular water filtration stimulation.

The hypothalamus is the main region of the brain responsible for the central control of neuropeptide synthesis (Acher, 1993). The exclusive presence of AVTR V2-type in the pituitary gland of *S. aurata*, in addition to the unique abundance of V2-type mRNA after salinity transfer, strongly suggest that control of AVT gene expression and mature hormone release is carried out *via* the V2-type receptor, whereas changes in pro-VT mRNA levels, AVT storage, and hormone release have been previously demonstrated after the same salinity transferences (Kleszczynska et al., 2006; Martos-Sitcha et al., 2013b). Furthermore, previous studies demonstrated that AVT treatment produced changes in brain glycogenolytic routes in *S. aurata* (Sangiao-Alvarellos et al., 2004), suggesting that the AVTR V2-type is at least partially responsible for the control of the metabolic processes carried out in this organ. In addition, the possible feedback process of plasmatic AVT on vasotocinergic neurons could be regulated *via* the V2-type receptor at a hypothalamic level. A similar observation has been described in *C. commersoni* (Mahlmann et al., 1994). Interestingly and in contrast with the mammalian model, the V1-type receptor (specifically the V1b-subtype) has been historically described to be present exclusively in the anterior pituitary (Antoni, 1984). Nevertheless, subsequent studies have suggested its presence in other regions of the brain (Barberis and Tribollet, 1996; Vaccari et al., 1998). These observations reinforce the hypothesis of this receptor monitoring the synthesis and release of the

596 AVT hormone. Still, to the best of our knowledge, no evidence of this hypothesis has
597 been reported in other species of teleosts. Thus, future studies on the control of AVT
598 release are necessary to elucidate this paradigm not only in fish but also in mammals.
599 The liver is one of the main reservoirs of energy in vertebrates, whereas the brain is
600 responsible for between 1.5 and 8.5% of the total body energy consumption in
601 vertebrates (Van Ginneken et al., 1996). Previous studies have demonstrated that
602 AVT and IT treatment, as well as osmotic challenges, enhance hepatic glycogenolytic
603 routes and free-glucose production processes in teleosts, including *S. aurata* (Janssens
604 and Lowrey, 1987; Moon and Mommsen, 1990; Sangiao-Alvarellos et al., 2006).
605 Moreover, other studies on this species have demonstrated that plasma AVT levels
606 (Kleszczynska et al., 2006) and the expression of the pro-VT gene (Martos-Sitcha et
607 al., 2013b) are enhanced during acclimation to hyper- and/or hypoosmotic
608 environments. Similar observations have been made after overcrowding or social
609 stress (Sokolowska et al., 2013), suggesting a clear interaction between both
610 vasotocinergic/isotocinergic pathways and cortisol levels, which have been
611 demonstrated to enhance metabolic rates (Mommsen et al., 1999; Laiz-Carrión et al.,
612 2003; Sangiao-Alvarellos et al., 2004, 2006). Moreover, previous studies on *S. aurata*
613 have demonstrated that AVT injection enhances hepatic and brain glycogenolysis, as
614 indicated by glycogen depletion levels and GPase activity enhancement after
615 acclimation to extreme (6 and 55 ‰) salinities (Sangiao-Alvarellos et al., 2006). Our
616 results showed a clear activation of mRNA levels of both AVTRs but not ITR. Thus,
617 similar to the brain, AVTR V2-type function is mediated by PKA activation,
618 suggesting that the metabolic actions of AVT are controlled by this receptor in both
619 extreme salinities due to the higher energy requirement. Furthermore, hyper- and
620 hypoosmotic transfers also increased hepatic AVTR V1a2-type mRNA levels, the
621 effect of which could be associated with the incorporation of inorganic phosphate in
622 phosphatidyl inositol routes, acting as a substrate for phospholipase C, which is the
623 intracellular pathway for this type of AVT receptor (Kirk et al., 1977; 1979). These
624 results suggest that gene activation of V1a2-type and/or V2-type receptors in the liver
625 and brain is related to the control of the energy supply due to the higher requirements
626 produced by the environmental salinity challenge.

627 In conclusion, this study provides evidence of the presence of two different types of
628 AVTR and a single ITR in the marine teleost *S. aurata*. In addition, our results also
629 reveal a wide tissue distribution of these genes, with different expression patterns.

Furthermore, changes in mRNA after osmotic challenges indicate that the abundance of both AVTR V1a2-type and AVTR V2-type mRNA is salinity- and tissue-dependent and that their variations could be associated with different proteins (enzymes, ion transporters, or channels) to produce the necessary osmoregulatory or metabolic effects. Surprisingly, ITR appears to be undisturbed by the osmotic challenge, suggesting a minor role (if any) for isotocin in the sea bream osmoregulatory system during salinity challenge.

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870 *Molecular Biology* 132, 231-7.

871

872 **Table legend**

873

874 **Table 1.** Degenerate primers designed for molecular identification of vasotocin
875 receptors (AVTR V1-type and AVTR V2-type) and isotocin receptor (ITR) partial
876 cDNA sequences, specific primers used for semi-quantitative gene expression by
877 qPCR, and the amplified size by each pair of both degenerate and specific primers.

878

879 **Figure legends**

880

881 **Figure 1.** Nucleotide and predicted amino acid sequences of *S. aurata* vasotocin
882 receptor (AVTR) V1a2-type cDNA. The start and stop codons are underlined and
883 presented in italics and bold text. The predicted amino acid sequence is displayed in
884 bold above the underlined nucleotide sequence, indicating the open reading frame
885 (ORF). The seven transmembrane segments predicted using MEMSTAT
886 (<http://bioinf.cs.ucl.ac.uk/psipred/>) are represented in bold and highlighted in black in
887 the amino acid sequence. GenBank accession number KC195974.

888

889 **Figure 2.** Nucleotide and predicted amino acid sequences of *S. aurata* AVTR V2-type
890 cDNA. The start and stop codons are underlined and presented in italics and bold text.
891 The predicted amino acid sequence is displayed in bold above the underlined
892 nucleotide sequence, indicating the ORF. The seven transmembrane segments
893 predicted using MEMSTAT (<http://bioinf.cs.ucl.ac.uk/psipred/>) are represented in
894 bold and highlighted in black in the amino acid sequence. GenBank accession number
895 KC960488.

896

897 **Figure 3.** Nucleotide and predicted amino acid sequences of *S. aurata* isotocin
898 receptor (ITR) cDNA. The start and stop codons are underlined and presented in
899 italics and bold text. The predicted amino acid sequence is displayed in bold above
900 the underlined nucleotide sequence, indicating the ORF. The seven transmembrane
901 segments predicted using MEMSTAT (<http://bioinf.cs.ucl.ac.uk/psipred/>) are
902 represented in bold and highlighted in black in the amino acid sequence. GenBank
903 accession number KC195973.

904

905 **Figure 4.** Phylogenetic tree of different vasotocin/vasopressin receptor type amino
906 acid sequences from several teleosts, including the sea bream (*Sparus aurata*) and
907 human, using neighbor-joining analysis and based on amino acid differences
908 (p-distance). GenBank and NCBI reference sequence accession numbers are as
909 follows: *Sparus aurata* V1a2 (AGO05923), V2 (AGT02335), and ITR (AGO05922);
910 *Oryzias latipes* V1a1 (BAL45623), V1a2 (BAL45624), V2 (BAJ04637), and ITR
911 (BAL45626); *Thalassoma bifasciatum* V1a1 (AFJ96998) and V1a2 (BAL70406);
912 *Epineohelus adscensionis* V1a1 (AEI54996) and V1a2 (ADO33897); *Cyprinodon*
913 *nevadensis amargosae* V1a1 (ACY07771), V1a2 (ACY07772), V2 (ACX85730), and

914 ITR (ACY07774); *Cyprinodon variegatus* V1a1 (ACY74366), V1a2 (ACY74367),
915 and V2 (ACY74368); *Homo sapiens* V1a (AAD17891), V2 (ACR39021) and OXYR
916 (NP 000907); *Amphiprion ocellaris* V2 (BAL70406) and ITR (BAL70407);
917 *Polypterus senegalus* V2 (BAJ04635); *Catostomus commersonii* ITR (CAA61050);
918 *Haplochromis burtoni* ITR (ACT99617); and *Stegastes partitus* ITR (AFP20280).

919

920 **Figure 5.** Tissue distribution of mRNA/cDNA from AVT receptor V1a2-type, AVT
921 receptor V2-type, and IT receptor in *S. aurata*. qPCR products were analyzed by
922 electrophoresis on a 2 % agarose gel stained with GelRed. PCR product sizes for each
923 transcript are provided. –C: negative control.

924

925 **Figure 6.** Branchial time course changes in the expression of the AVTR V1a2-type
926 (a), AVTR V2-type (b) and ITR (c) genes (relative to β -actin) after transfer from SW
927 to different environmental salinities (LSW, SW, and HSW). Values are presented as
928 the mean $\pm$ S.E.M. ($n = 8$ fish per group). Significant differences between salinity and
929 different time points are identified with different letters; different symbols show
930 differences between groups at the same time point ($P < 0.05$, two-way ANOVA,
931 followed by Tukey's test).

932

933 **Figure 7.** Renal time course changes in the expression of the AVTR V1a2-type (a),
934 AVTR V2-type (b), and ITR (c) genes (relative to β -actin) after transfer from SW to
935 different environmental salinities (LSW, SW, and HSW). Values are presented as the
936 mean $\pm$ S.E.M. ($n = 8$ fish per group). Significant differences between salinity and
937 different time points are identified with different letters; different symbols show
938 differences between groups at the same time point ($P < 0.05$, two-way ANOVA,
939 followed by Tukey's test).

940

941 **Figure 8.** Hypothalamic time course changes in the expression of the AVTR
942 V1a2-type (a), AVTR V2-type (b), and ITR (c) genes (relative to β -actin) after
943 transfer from SW to different environmental salinities (LSW, SW, and HSW). Values
944 are presented as the mean $\pm$ S.E.M. ($n = 8$ fish per group). Significant differences
945 between salinity and different time points are identified with different letters; different
946 symbols show differences between groups at the same time point ($P < 0.05$, two-way
947 ANOVA, followed by Tukey's test).

948

949 **Figure 9.** Hepatic time course changes in the expression of the AVTR V1a2-type (a),
950 AVTR V2-type (b), and ITR (c) genes (relative to β -actin) after transfer from SW to
951 different environmental salinities (LSW, SW, and HSW). Values are presented as the
952 mean $\pm$ S.E.M. ($n = 8$ fish per group). Significant differences within salinity and
953 different time points are identified with different letters; different symbols show
954 differences between groups at the same time point ($P < 0.05$, two-way ANOVA,
955 followed by Tukey's test).

956
957 **Figure 10.** Branchial mRNA levels (relative to β -actin) of NKA α (a) and CFTR (b) in
958 sea breams acclimated to different environmental salinities (LSW, SW, and HSW)
959 over 14 days. Values are presented as the mean $\pm$ S.E.M. ($n = 8$ fish per group).
960 Significant differences between salinities are identified with different letters ($P < 0.05$,
961 one-way ANOVA, followed by Tukey's test). The Na⁺, K⁺-ATPase alpha-subunit was
962 correlated with AVTR V1a2-type (c), whereas CFTR was correlated with AVTR V2-
963 type (d). Correlations were performed on both mRNA levels in the gills of specimens
964 maintained for 14 days at different environmental salinities ($P < 0.05$).

965

Figure 1. *Martos-Sitcha et al.*

5' - tgagagagcaacttgttccctctgtgagcaccaggtagctccatatacgggt 53
 tggagaagcacttcacagcccctctggcgctgtatctgctcgggcgacagaggggc 110
 agtgccacgcacactcccgaactatgcgcttgctctgagcggagggaaaccggtctctg 167

M G T P G N D S V H 10
 gttttcagtcceactgatgacttttacg**atgg**gaacccctggaaacgacagcgtccac 224

Q N G S D P F Q G N E E V A Q I E I M 29
 cagaacggatccgatccttttcaggggaacgagggtagcccaaatcgagatcatg 281

V L S I T F V V A V V G N V S V L L A 48
 gtgctgagcatcactttcgtggtcgctgtggtcgggaaacgtgagcgtcctgctggca 338

M Y N T K K K M S R M H L F I K H L S 67
 atgtacaacacgaagaagaagatgtcgcggtgacaccttttcatcaaacacctcagc 395

L A D L V V A F F Q V L P Q L C W E I 86
 ctggctgacctgggtggtcgcttcttccaggtgctgcccgcagctctgctgggagatc 452

T F R F Y G P D F L C R I V K H L Q V 105
 acctccgcttctacgggtccggactttctctgcaggatagtgaaacacctgcagggtg 509

M G M F A S T Y M M V M M T L D R Y I 124
 atggggatggtcgcggtccacctacatgatgggtgatgatgacctggacctgtacatt 566

A I C H P L K T L Q Q P T Q R S Y I M 143
 gccatctgccccccctgaaaacccctcagcagcccacccagcgtcctacatcatg 623

I I S T W M C S L V L S T P Q Y F I F 162
 attatctcaacgtggatgtgcagcctggtgttaagcaccgccagctacttcatcttc 680

S L S E I K N G S D V Y D C W A Y F I 181
 tcctctgagcgagatcaagaacggctcggtgtttacgactgctggcgctacttcatc 737

E P W G P K A Y I T W I T V G I F L V 200
 gagccatggggccccaaggcgtacatcacctggataaccgtgggcatcttctctgctg 794

P V V I L M M C Y G F I C H S I W K N 219
 cccgtggtcattctcatgatgtgctacgggttcattctgccacagcatatggaaaaat 851

I K Y K K R K T T A G A A S K N A I I 238
 atcaaatacaagaaaaggaaaacgacggccgggtgctgagcaagaacgcgataatc 908

G K N S V S S V T T I S R A K L R T V 257
 ggggaagaattcagtcagcagtggttacaacgatatccagagccaagctgaggactgtc 965

K M T F V I V L A Y I I C W A P F F I 276
 aaaaatgacttttctgtagtatttggcgtagattatctgctggcgccgtttttcatc 1022

V Q M W S V W D E N F Q W A D S E N T 295
 gtgcagatgtggtcggtgtgggatgagaacttccagtgggctgattctgagaacaca 1079

A V T L S A L L A S L N S C C N P W I 314
 gcagtgactctgctgctgctccttgccagttctcaacagctgctgtaaccctggata 1136

Y M I F S G H L L Q D F V H C F S C C 333
 tacatgatcttcagtggtgacaccttctccaggatttctgctgactgcttctgctgtgc 1194

L R M N T E F K K E D S D S S I R R T 352
 ctgaggtgaacactgaattcaagaaggaggactcagacagcagtatccgcaggaca 1250

T L L T K M T N Q S P T G S S A N W R 371
 acgctgctgcaaaagtgaccaatcagagccccacggcgagctctgcaaacgtggaga 1307

E L D N S P K S S I Q P E E N H S 388
 gagctggacaatttctcccaagtctccattcagccggaggaaaatcacagct**tagg**ct 1364
 ttttttaacgggggtgggaatttagaaaaatgtctggcatcaaggtacacagctca 1421
 cagtatcaaccatattgtctttgggcacgagtgagcagtgattgaaatgccccag 1478
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 aaacattgggaaggaaattaaattgcaaagattgactcaaaatccttcatgtttgt 1649
 aaaagcactgcatgctgacagttatatttttcatcattttgttgactttacttgaaa 1706
 attcgacagccgcaagtgatcaagaggatttttttggagtcaaacgtggttcttt 1763
 tccatgttttcgatagaaaagcaacttttctggaatacggatgcttgtactgggtg 1820
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 aaggcgtacgtaaacagccaggggtgtgctggtcgggaaaaacatctcttctgaccgc 1934
 acgtctctaagtaacagaagcagccacctcctcttctgctctgcttgtgtgtt 1991
 ttctcaaaaccagattctatttggtcatgtgttcttctgacccctcacaaaaaaa 2048
 gaaaattaatggctgagtggttaaggctcatgtatcactcatggccttgcatgcat 2105
 ccacatttttgaattatttcagtagcagcagggcagccctctgctggtgacccataa 2162
 agagtggaatttaatttctgttgagcagagggcagatgggaaccccttgtaaatagtag 2219
 gacgctcaccgcgtacacatacacaggcagcgacagtggtggggggcgggcagggtg 2276
 cccaatgaggtgagattggccaaagctgggggagtgctacctaaggacccactctcc 2333
 tccccacgctttcacgaaatgtgtggagaagctcacacattccaccacgctgctg 2390
 caagacgcttctctgatttttttttctgacatcctgcaccgctgagggggggagtc 2447
 tgcttgttttttggctggattttacactccagacaaaaacaattttctgcatataat 2504
 gtgaggcagcgtgaggggtgccagaccactgtgtgaaccccgagtgattgcatcaa 2561
 gacatgagtggtgttatattttgtacataaaatgcagttaaatatctgtaaatagga 2618
 gtaatatatgtaaatatgttcaacatgaattctatttgcagtaaatggcctgtgcac 2675
 attgaaagtaataatgaatattcctataaaatatgttgaatatatacatattgacgcac 2732
 -polyA tail -3'

Figure 2. Martos-Sitcha et al.

5' - agatacagtgagcaggcacacaacggacttgacagaaacccctttggcacactg 53
acagttaaaggagaattaagtggtcatttcttatgctcctgctgtattcattcagca 110
gtcagctgattttactcagagcggaggactcgggttgacagcagtgaggatgtgag 167
tggtttggggcagctctctgcaaccactgaaggggatcacatcaccgagacgggg 224
gcaaaatcaccaagagagagaaaaaggacacgctgagaaagcttaccatgcactga 281
M E S I S L E T D W D G L A L S 16
caacaaggaa**tg**gaagcatcagtttgaaacggactgggatgggttagccctctcc 281
S L A A A G R N N F S S S S L F V S E 35
tctctggctgctgcaggaaggaacaacttctcctcctcgctcctgttgtctctgaa 338
L N S L N S S H G G G S F F G I F P E 54
ctgaactccctcaatagctctcacggcggggatccttcttcgggatcttcctgag 395
N S S T T T P H T L P Q P R V R D L G 73
aatagttcaaccaccacgctctcacaccotaccccagccagggtgagggacctgggc 452
L A R A E I T V L A L V L A I T T L G 92
ctggccggggcagagatcacagtccttgcatgtgtgtgctgctcttaccacctggg 509
N S F V L W V L L R R R K H N A P M H 111
aatagttctgtctgttgggtgtctgtcagagcgaagaaagcacaatgcgccaatgcac 566
V F M V N L C V A D L V V A L F Q V L 130
gtgttcatggtcaactgtgctggtgctgactggtgtgtggcactcttccaggttctt 623
P Q L I W D I T E R F Q G P D L L C R 149
ccccagctaatatgggatatacacagagaggtttcaggggcctgactgctctgtcgg 680
S I K Y L Q I V G M F A S S Y M I V A 168
tccatcaagtacctgcagattgtgggcatgttgttctcctcttaccatgattgttggc 737
M T V D R H H A I C C P L Q A Y R G G 187
atgacagtagaccggcaccatgccatctgctgccattgcaggcttaccgtggggga 794
A M S R W N T P V M V A W G L A L V L 206
gcaatgtccccgtggaacccccctgtcatggttgccctggggttggtgactagtcctc 851
S I P Q V F I F S R T E V N H G E Y E 225
agcataccgaggtgttcatcttctctcgcacagaagtgaatcacggagagtagcag 908
C W G H F A E P W G L K A Y V T W M T 244
tgctggggtcactttgccgagccatgggggtgaaggcctacgtcacctggatgacc 965
V A V F L L P A L I I T I C Q I R I F 263
gtggcagcttctcctcgtcctgccctcatcattaccatctgtcagataagaattctc 1022
R E I H N N I Y L K S E R M V T A E M 282
cgagagattcacacaacatctacctgaagtgcagagaggtgtgacagctgagatg 1079
K K N E I L F R F H G F K K E D E R A 301
aagaagaacgaaatcctgttccgttccacggcttcaagaaggaggagcagcggggcg 1136
R E R G T R A S G G G G R G G Q L L 320
agggagagggggaccgggcatctggagagaggggggggagggagagcagctcta 1194
K G V N N N P H N T N H N S Q V A S G 339
aagggtgtgaataaacacccctcacataccaaccataacagccaagttagctcaggt 1250
I Q Y N S C C G E H D T T A S P T Q Q 358
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Q I L S S S D C H E S Y T S Y E L N S 377
caaatactgagcagctcagattgccacagctcatcacgctcctcagcagctgaactca 1364
G S P R C S L D Y A P P L P P A T P P 396
ggctcaccggctgtcctcctcgactacgacccccctctccctccagccactccacct 1421
P S I T K A M S K T V R M T L V I V L 415
ccaagcatcactaaagccatgtccaaagcagtgaggatgacctgggtcatctgtgtg 1478
V Y T I C W S P F F I V Q L W A A W D 434
gtctatactatctgtctgttcaccttcttcatcgtccagctgtggggggcctgggac 1535
P N P P N Q G V A F T I L M L L A S L 453
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N S C T N P W I Y T A F S S S V S R E 472
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L Q S L L Q C R S R P G R R G S L P D 491
ctgcagagcttgctgcagtgctcggtcgagaccggcgccgggggtcccttctgat 1706
D S T T T H T S T T K D S L Y 507
gactccaccaccgcgcacacctccaccaccaaggacagcctgtactgacagagactg 1763
atgcgacagagaattgacacacagtgacattgttgaagagacatgcaacgggtgcacac 1820
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gagagtgctgactctaccagatgtttcoacaacaaggacagctgtactgtcaac 1934
aggacctctatctgtaatcacctgtcctctccaccogagcagaggagggtgtcat 1991
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cagtgacagactgtgcaattcaaatcgcttgagagtgaaataaaagattttcaacctca 2105
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ttatataagccactgtatagtaatgtataacgttctgtgtgataaaagtaagatta 3473
gaagtt-polyA tail -3' 3479

Figure 3. *Martos-Sitcha et al.*

5'- tgaggggctgagaggtccagaaacaacttcccggaggatcggttttaagacat	53
gggcgcttcttttggattatgcaccgatttaactgcgggcaattcaaaaaacaact	110
ttaatggagtgaagctgtaggaatatctgacaggagacacacgtccaacagctga	167
aagcgacccggactcttgtgtgtgatgtgaccgctggctgcgccgaagtggcgctt	224
M E D F L R E Q N S W A Q N V S	16
ttacgcacg atg gaggactttttacgcgagcagaatagttgggcgcagaacgtttca	281
W S N L S R G N E S H L G N T T V N P	35
tggagcaacttaagccgtggaaatgagagtcatttaggaaacacgacagtgaatcct	338
L K R N E E V A K V E V A V L V L V L	54
ctgaagcgaaacgaagaggtggccaaagtggaagttgccgtcctggtgctggtgctc	395
L L A L T G N L C V L W A I H A T K H	73
ctgctcgctctgaccggcaacctgtgctgctctgtgggctatccacgccaccaagcac	452
S Q S R M Y F F M K H L S I A D L V V	92
agccagctcgcggtatgtatttcttcatgaagcacctgagcatcgccggacctcgctggt	509
A V F Q V L P Q L I W D I T F R F Y G	111
gcagtgctttcaggtcttaccgcagctcatctgggatatacacatttcgcttctatgga	566
P D L L C R L V K Y L Q V V G M F A S	130
ccggattgtgcagagctgggtgaaataacctccaggtcggtgggcattgttcgcatcc	623
T Y M L V L M S I D R C L A I C Q P L	149
acctacatgctgctcctgatgtccatcgacaggtgcctggcgatctgccagcctctc	680
R S V H K R N D R L C V V A S W M L S	168
cgctccgctgcacaagaggaacgatcgcttgtgctggtgcgcctcctggatgctcagc	737
L I F S S P Q A Y I F S L K E V G D G	187
ctgatatttcagctccctcaagcctacataattctccttgaaggaggtgggggacgggt	794
V Y D C W G D F V Q P W G A K A Y I T	206
gtgctatgactgctgggggactttgtgcagccgtggggagccaaagcttacattaca	851
W M S L S I Y I F P V A I L S V C Y G	225
tggatgagcctcagcatttacatcttcccagtggcgattttaagcgtctgctatggc	908
L I W F K K W Q N F N L K T R R E H I	244
ttgatttgggtcaaaaaatggcagaatttcaatttgaaaacaagaagggagcacatc	965
L A L T A R P S K G A L P L T R V S S	263
ctggctctgactgcaaggccctccaaagcgctctccccctcaccctgtgagcagc	1022
V R L I S K A K I R T V K M T F V V V	282
gtgaggctcatttcaaaagcgaagatccgcacagtgaatatgacatttgggtgggtg	1079
V A Y I M C W T P F F F V Q M W S A W	301
gtcgccctacataaatgtgctgggactcccttcttcttcttgcagatgtgggtcgcattg	1136
D P A A P R E D M A F I I A M L L A S	320
gaccctgcagcaccagaagagacatggccttcatcatcgccatgttgctggccagc	1194
L N S C C N P W I Y M F F A G H L F Q	339
ctcaacagctgctgtaacccctggatctacatgttcttggccggtcacctgttccag	1250
D L T R C F F C C C R Q Y L T A S T C	358
gacctgacgcgttgcttcttctgctgctgcagacagtacctgacagcctccacctgc	1307
S C D R P C R H K S S S A T Y V I K N	377
agctgcgatcgaccatgccggcacaagagcagctcagccacttacgtcatcaagaac	1364
E S S Q R S L T H T S S T G G L G H	395
gagagcagccagaggagcctcacacacacatccagcacgggggacttgacac tga	1421
agagccagctgaaagcgcagcaggttctctgtagttgagtaacctggcaatccccca	1478
cttttccaggcattttaatgatctgcagcggcgcccaattgcaacatgttttaaatg	1535
agctaataagacaatgtaaacctcctgtcctgaaaaatgccctgcatcctgtgataac	1592
aaagaccagaacaaaatgtctccaatcgtaatggatgtgtgccctcggttctcttc	1649
ctctgcaccagaatcctgcacatgaaatatttctcatataatgtaggcttcagcaat	1706
tccagatttacccttcttctgtataaacagccatgaaga-polyA tail -3'	1749

Figure 4. *Martos-Sitcha et al.*

Figure 5. *Martos-Sitcha et al.*

Figure 6. *Martos-Sitcha et al.*

Figure 7. *Martos-Sitcha et al.*

Figure 8. *Martos-Sitcha et al.*

Figure 9. *Martos-Sitcha et al.*

Figure 10. *Martos-Sitcha et al.*

Table 1. *Martos-Sitcha et al.*

Primers	Nucleotide sequence	Amplicon size
Degenerate		
AVTR-V1 _{Fw1}	5'-AGCGTSCGTGCTGGCSATG-3'	510 bp
AVTR-V1 _{Rv1}	5'-GCAKATRAASCCGTAGCA-3'	
AVTR-V1 _{nestedFw}	5'-AARCACCTSAGCCTBGCBGA-3'	392 bp
AVTR-V1 _{nestedRv}	5'-GTBATCCAGGTGATGTASGC-3'	
AVTR-V2 _{Fw1}	5'-ATGGTCAAYCTGTGTGTTGCGYA-3'	678 bp
AVTR-V2 _{Rv1}	5'-ACCTGACADAYRGTGATRATRAWG-3'	
AVTR-V2 _{nestedFw}	5'-GTTTGCHTCCTCYTACATGATTGT-3'	490 bp
AVTR-V2 _{nestedRv}	5'-GTCATCCAGGTRACRTAGGC-3'	
ITR _{Fw1}	5'-TCTGBCARCCRCTYCGYTCT-3'	469 bp
ITR _{Rv1}	5'-CAKGCWGACCACATCTGHAC-3'	
ITR _{nestedFw}	5'-TACATYACRTGGATNAGYCT-3'	260 bp
ITR _{nestedRv}	5'-GTCCARCANACKATRTAAGC-3'	
qPCR		
qPCR-AVTR-V1 _F	5'-GACAGCCGCAAGTGATCAAG-3'	203 bp
qPCR-AVTR-V1 _R	5'-CCCAGCCGCACACCCCCTGGCT-3'	
qPCR-AVTR-V2 _F	5'-ATCACAGTCCTTGCATTGGTG-3'	120 bp
qPCR-AVTR-V2 _R	5'-GCACAGGTTGACCATGAACAC-3'	
qPCR-ITR _F	5'-GGAGGATCGTTTTAAAGACATGG-3'	120 bp
qPCR-ITR _R	5'-TGTTGTCTCCCTGTCAGATTTTC-3'	
qPCR- α ATPase _F	5'-TCGTTACAGGAGTGGAAGAAGG-3'	123 bp
qPCR- α ATPase _R	5'-GATGTTGGCGATGATGAAGAG -3'	
qPCR-CFTR _F	5'- GGAGGGCCCCGACCTCGTCAT -3'	175 bp
qPCR-CFTR _R	5'- CTGTTCTGTCCCAGCAGGCCCC -3'	
qPCR β -actin _{Fw}	5'-TCTTCCAGCCATCCTTCCTCG-3'	108 bp
qPCR β -actin _{Rv}	5'-TGTTGGCATAACAGGTCCTTACGG-3'	