

1 **Characterization of the peripheral thyroid system of gilthead seabream**  
2 **acclimated to different ambient salinities**

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28 **Running title:** Salinity and the thyroid system in a euryhaline fish.

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41 **Abstract**

42 Thyroid hormones are involved in many developmental and physiological processes,  
43 including osmoregulation. The regulation of the thyroid system by environmental  
44 salinity in the euryhaline gilthead seabream (*Sparus aurata*) is still poorly  
45 characterized. To this end seabreams were exposed to four different environmental  
46 salinities (5, 15, 40 and 55 ppt) for 14 days, and plasma free thyroid hormones (fT3,  
47 fT4), outer ring deiodination and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities in gills and kidney, as  
48 well as other osmoregulatory and metabolic parameters were measured. Low salinity  
49 conditions (5 ppt) elicited a significant increase in fT3 (29 %) and fT4 (184 %)  
50 plasma concentrations compared to control animals (acclimated to 40 ppt, natural  
51 salinity conditions in the Bay of Cádiz, Spain), while the amount of pituitary thyroid  
52 stimulating hormone subunit  $\beta$  (*tshb*) transcript abundance remained unchanged. In  
53 addition, plasma fT4 levels were positively correlated to renal and branchial  
54 *deiodinase type 2 (dio2)* mRNA expression. Gill and kidney T4-outer ring  
55 deiodination activities correlated positively with *dio2* mRNA expression and the  
56 highest values were observed in fish acclimated to low salinities (5 and 15 ppt). The  
57 high salinity (55 ppt) exposure caused a significant increase in *tshb* expression (65  
58 %), but *deiodinase* gene expression (*dio1* and *dio2*) and activity did not change and  
59 were similar to controls (40 ppt). In conclusion, acclimation to different salinities led  
60 to changes in the peripheral regulation of thyroid hormone metabolism in seabream.  
61 Therefore, thyroid hormones are involved in the regulation of ion transport and  
62 osmoregulatory physiology in this species. The conclusions derived from this study  
63 may also allow aquaculturists to modulate thyroid metabolism in seabream by  
64 adjusting culture salinity.

65

66 **Keywords:** deiodinases, osmoregulation, outer ring deiodination, *Sparus aurata*,  
67 thyroid hormones.

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69

## 70 **1. Introduction**

71

72 Thyroid hormones (THs) are truly pleiotropic in fish, affecting metabolism,  
73 reproduction, growth and osmoregulation, relevant physiological processes for  
74 aquaculture (Blanton and Specker, 2007). Thus, understanding how this system is  
75 regulated by the environment in cultured species, is key for the optimization of their  
76 culture. In the aquaculture ponds of the South of Spain, where culture of gilthead  
77 seabream (*Sparus aurata*) is carried out, salinity is highly variable and may well  
78 influence the thyroid system. In general, the fish thyroid system responds to stimuli  
79 by regulating the release of thyroid stimulating hormone (Tsh) that in turn stimulates  
80 the thyroid follicle to secrete thyroxine (T4) into the blood stream (Eales and Brown,  
81 1993). Within the plethora of stimuli regulating the release of Tsh in fish, different  
82 salinity concentrations are postulated (Leatherland and Farbridge, 1992). Pituitary  
83 *thyroid stimulating hormone subunit  $\beta$*  (*tshb*) gene expression is under negative  
84 feedback control by plasma (free) thyroid hormones (Cohn et al., 2010; Manchado et  
85 al., 2008).

86 The pro-hormone T4 is deiodinated into bioactive triiodothyronine (T3) in the  
87 peripheral tissues (Bernier et al., 2009; Klaren et al., 2008). The regulation of  
88 deiodination in peripheral tissues is therefore a determining factor for the  
89 physiological effects of thyroid hormones.

90 Two iodothyronine deiodinases (Dio1 and Dio2) have outer ring deiodination (ORD)  
91 activities and in peripheral organs such as the gills and the kidney produce T3 from  
92 T4 that are directly involved in ion transport and osmoregulation (Arjona et al., 2008).  
93 The inactivation pathways of THs are catalysed also by Dio1 and by a third  
94 iodothyronine deiodinase, Dio3. Both Dio1 and Dio2 ORD activities have distinct  
95 substrate and co-substrate preferences (Klaren et al., 2012; Orozco et al., 2000).  
96 Reverse T3 (rT3) is usually the preferred substrate for Dio1 in mammals (Orozco et  
97 al., 1997) while T4 is the preferred substrate of Dio2 (Garcia-G et al., 2004).

98 One consequence of increased TH activity is the stimulation of the basal metabolic  
99 rate, which seems to result, at least in part, in increased oxygen consumption and ATP  
100 hydrolysis. Several studies have reported species-specific changes in plasma TH  
101 levels, ORD activity (Arjona et al., 2008) or deiodinase gene expression (Lorgen et  
102 al., 2015) when fish are submitted to an osmotic challenge. Osmotic acclimation in  
103 fish is also associated with variations in plasma THs and in gilthead seabream plasma

104 free T4 and gill ORD activity respond to a change in environmental salinity from 35  
105 ppt to 1 ppt (Klaren et al., 2007).

106 Other authors have studied the thyroid system in *S. aurata* in hypo-saline conditions  
107 (Klaren et al., 2007; Power et al., 2001). To our knowledge, there are no previous  
108 studies characterizing the effects of acclimation to iso- or hypersaline conditions on  
109 the thyroid system in this species. We therefore set out to compare the effects of  
110 environmental hypo- and hyper-salinity on the thyroid system of the euryhaline  
111 gilthead seabream, an important aquaculture species.

112

## 113 **2. Materials and methods**

114

### 115 *2.1 Animal maintenance prior to experimentation*

116 Immature juvenile gilthead seabream juveniles (N=32; 200 ± 44 g body mass, mean ±  
117 SD) were provided by *Servicios Centrales de Investigación en Cultivos Marinos*  
118 (SCI-CM, CASEM, University of Cádiz, Spain; Operational Code REGA  
119 ES11028000312), and maintained in the fish husbandry facility of the Faculty of  
120 Marine and Environmental Sciences (Puerto Real, Cadiz, Spain). Fish were  
121 acclimated for 35 days in 400-L tanks to seawater (40 ppt, natural salinity condition in  
122 the Bay of Cadiz, Spain) in a flow-through system under natural photoperiod (month  
123 of May in Cadiz, 14 h light:10 h dark) and temperature (environmental temperature of  
124 approximately 19.5°C). Fish were fed commercial pellets (1% body mass) once a day  
125 (9:00) (Dibaq-Diproteg, Segovia, Spain). The experimental procedures complied with  
126 the guidelines of the University of Cadiz (Spain) and the European Union  
127 (86/609/EU) for the use of animals in research.

128

### 129 *2.2 Acclimation to different environmental salinities*

130 Fish were lightly anaesthetized in 0.05 % (v/v) 2-phenoxyethanol, netted and  
131 randomly allocated to 400-L cubic tanks with different salinities (5, 15, 40 and 55 ppt  
132 with 140, 364, 1090 and 1546 mOsm kg<sup>-1</sup> osmolality, respectively) (N=8 per group).  
133 During transfer to the experimental tanks, the mass and length of the animals were  
134 recorded. Experimental salinities were achieved by mixing full-strength seawater with  
135 dechlorinated tap water (Puerto Real, Spain) or by mixing seawater with natural  
136 marine salt (Salina La Tapa, Puerto de Santa María, Cádiz, Spain). Each tank had a  
137 water recirculation system, which consisted of an external filter (Hydor Prime 30,

138 Sacramento, CA, USA) to ensure optimal water conditions. Water conditions during  
139 experimentation were: temperature, ranging between 19.1 and 19.8 °C; 5, 15, 40 and  
140 55 ppt salinity (variations <1 ppt for each tank); pH, ranging between 7.82 and 7.88;  
141 dissolved oxygen, >5 mg O<sub>2</sub> L<sup>-1</sup>; nitrites, between 0.05 and 1.69 mg L<sup>-1</sup>; nitrates,  
142 between 4.13 and 36.41 mg L<sup>-1</sup>; and ammonium, 0.0-0.2 mg L<sup>-1</sup>. These parameters  
143 were checked daily and did not vary significantly for the duration of the experiment.  
144 20 % of the water in circuits was replaced every other day. Fish were maintained in  
145 these conditions for 14 days and were fasted for 24 h before sampling. No mortality  
146 was observed during the acclimation period.

147

### 148 *2.3 Sampling*

149 Fish were netted, anaesthetized in 0.1 % (v/v) 2-phenoxyethanol, weighed and  
150 sampled. Blood was collected with ammonium-heparinized syringes from the caudal  
151 vessels and placed into heparinized tubes. Plasma was separated from cells by  
152 centrifugation of whole blood (3 min, 10,000 x g, 4°C). Fish were then euthanized by  
153 spinal transection and the pituitary gland was collected from each fish. The first gill  
154 arch on the left side of fish was excised. Adherent blood was removed by blotting  
155 with absorbent paper and a smaller subsample consisting of a few branchial filaments  
156 was collected using fine-point scissors. A small portion of the caudal part of the  
157 kidney was also collected. Gill filaments and kidney were placed in 100 µL of ice-  
158 cold sucrose-EDTA-imidazole (SEI) buffer (150 mM sucrose, 10 mM EDTA, 50 mM  
159 imidazole, pH 7.3) for the analysis of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. The remaining gill  
160 tissue and kidney were snap frozen in liquid nitrogen and stored at -80°C until  
161 measurement of outer ring deiodination activities or mRNA extraction. Liver was also  
162 collected and weighed to determine the hepatosomatic index (HSI).

163

### 164 *2.4 Water chemistry*

165 Water samples were filtered (0.22 µm pore size) prior to analysis. Na<sup>+</sup>, K<sup>+</sup> and Mg<sup>2+</sup>  
166 levels were measured using a flame atomic absorption spectrophotometer (UNICAM  
167 939, Servicios Centrales, University of Cadiz). Cl<sup>-</sup> and Ca<sup>2+</sup> levels were measured  
168 with commercially available kits following the manufacturers protocol (Spinreact  
169 S.A, Sant Esteve d'en Bas, Girona, Spain). Osmolality was measured using a vapour  
170 pressure osmometer (Fiske One-Ten osmometer, Fiske, Massachusetts, USA) and

171 expressed as mOsm kg<sup>-1</sup> H<sub>2</sub>O. Water chemistry data are shown in Supplementary File  
172 1.

173

### 174 2.5 Cloning of *tshb*

175 The sequence of the beta subunit of *tsh* was originated using a cDNA cloned from a  
176 seabream pituitary cDNA library (Louro et al., 2005). Plasmid DNA was extracted  
177 using the alkaline lysis procedure (Birnboim and Doly, 1979) and sequenced using the  
178 Sanger sequencing method. Sequence identity was determined using the tblastx and  
179 blastn algorithms (Altschul et al., 1994) against the non-redundant nucleotide (nr db)  
180 and GenBank EST databases. Homologues were defined as those with an E-value  
181 <1e<sup>-5</sup> and a score of >40. Several cDNA clones corresponding to *tshb* were identified;  
182 one cDNA clone (281 EP10C7 Sa) was selected as reference and fully sequenced in  
183 order to obtain 3-fold coverage.

184

### 185 2.6 Phylogenetic analyses

186 Clustal Omega (SeaView v4 software, Gouy et al., 2010) with default parameters was  
187 used to generate a multiple sequence alignment of *tshb* sequences from  
188 representatives of the main vertebrate taxa.

189 Model Generator v0.85 (Keane et al., 2006) was used to test which substitution model  
190 best fitted the amino acid (aa) sequence alignment data. The Maximum Likelihood  
191 (ML) method, based on the selected optimal matrix-based model (JTT) (Jones et al.,  
192 1992), was used for the evolutionary analyses conducted in MEGA6 (Tamura et al.,  
193 2013). The bootstrap consensus tree was inferred from 1,000 replicates (Felsenstein,  
194 1985), and only branches corresponding to partitions reproduced in more than 50 %  
195 bootstrap replicates were presented. Initial tree(s) for the heuristic search were  
196 obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix  
197 of pairwise distances estimated using a JTT model, and then selecting the topology  
198 with a superior log likelihood value. A discrete gamma distribution was used to model  
199 evolutionary rate differences among sites [5 categories (+G, parameter = 1.7029)]. All  
200 positions containing gaps and missing data were eliminated.

201

### 202 2.7 Real-time quantitative PCR (qPCR)

203 Total RNA was extracted from the pituitary, gills and kidney using Mini or Midi  
204 RNeasy kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. The

205 concentration of RNA was determined at 260 nm using BioPhotometer Plus  
206 Spectrophotometer (Eppendorf, Hamburg, Germany) and its quality was determined  
207 in a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies, Santa  
208 Clara, CA, USA). Only samples with an RNA Integrity Number (RIN) higher than 9.0  
209 were used for qPCR. Synthesis of cDNA was carried out in a final reaction volume of  
210 20  $\mu$ L using qSCRIPT™ cDNA synthesis kit (Quanta BioSciences, Gaithersburg,  
211 MD, USA). Primers used for the analysis were designed using Primer3 software (v.  
212 0.4.0.) (<http://frodo.wi.mit.edu/primer3>) and seabream cDNA sequences available in  
213 GenBank: *deiodinase type 1 (dio1, DQ888894)*; *deiodinase type 2 (dio2, DQ888895)*;  
214 *tshb* (KM014688); and  *$\beta$ -actin (actb, X89920)*. qPCR assay linearity and  
215 amplification efficiencies (Supplementary File 2) were checked using dilution curves  
216 (six serial 1/4 dilutions, in triplicate, starting from 10 ng of cDNA, calculated from  
217 total input of RNA per reaction). All optimized qPCR assay were linear through 6  
218 serial dilutions (*dio1*:  $r^2 = 0.982$ , efficiency (E) = 0.90; *dio2*:  $r^2 = 0.982$ , E = 0.90;  
219 *tshb*:  $r^2 = 0.998$ , E = 0.90;  *$\beta$ -actin*:  $r^2 = 0.999$ , E = 1.01). To confirm the correct  
220 amplification of these primer pairs, the obtained PCR amplicons were cloned and  
221 sequenced (CloneJET PCR Cloning Kit, ThermoFisher Scientific, Waltham, MA,  
222 USA). qPCR was carried out with a Fluorescent Quantitative Detection System  
223 (Mastercycler ep *realplex*<sup>2</sup> S, Eppendorf, Hamburg, Germany). Each reaction was  
224 carried out in triplicate and contained 10 ng cDNA/total input of RNA, 0.5  $\mu$ L of each  
225 specific forward and reverse primer, and 5  $\mu$ L of PerfeCTa SYBR® Green FastMix™  
226 (Quanta BioSciences) in a final reaction volume of 10  $\mu$ L. The thermal cycle utilized  
227 was 10 min at 95°C; 40 cycles of 20 s at 95°C followed by 30 s at 60°C; melting curve  
228 (60°C to 95°C, 20 min); 95°C, 15 s. A final melt curve showed single  
229 product/dissociation curves in all reactions. The results for each gene were normalized  
230 to *actb*, which was stable between all samples analysed (< 0.35 C<sub>T</sub> variation).  
231 Relative gene quantification was performed using the  $\Delta\Delta C_T$  method (Livak and  
232 Schmittgen, 2001).

233

### 234 2.8 Outer ring deiodination (ORD) activities

235 Tracers used for measurements of deiodinase activity were prepared using the  
236 chloramine-T method to produce [<sup>125</sup>I]rT3, [<sup>125</sup>I]T3 and [<sup>125</sup>I]T4 from the 3,3'-T2,  
237 3,5-T2 and 3,5,3'-T3, respectively (Visser et al., 1977). All those molecules have  
238 been reported as substrates for ORD activity in teleost fish (Klaren et al., 2012).



239 Iodothyronines were purchased from Sigma Chemical Co. (St. Louis, MO, USA),  
240 Na<sup>125</sup>I was obtained from NEN Life Science Products Inc., Boston, MA, USA.  
241 Radiolabelled iodothyronines from the radioiodination reaction were purified using 10  
242 % (w/v) Sephadex LH-20 minicolumns as described previously (Mol and Visser,  
243 1985) followed by high pressure liquid chromatography (HPLC, platinum column  
244 EPS C18, 150 mm length, internal diameter 4.6 mm, Alltech Associated Inc., Illinois,  
245 USA) with a reverse phase isocratic elution of 35/65 % acetonitrile 0.05 M K<sub>2</sub>HPO<sub>4</sub>,  
246 pH 3.2. Sephadex LH-20 was obtained from Amersham Pharmacia Biotech (Uppsala,  
247 Sweden). All other chemicals were analytical grade and obtained from commercial  
248 suppliers.

249 Gills and kidneys were homogenized in 1 mL and 3 mL of phosphate buffer (100 mM  
250 Na-phosphate, 2 mM EDTA, pH 7.2), respectively. To determine ORD activities,  
251 homogenates were incubated with [<sup>125</sup>I]rT3, [<sup>125</sup>I]T3 or [<sup>125</sup>I]T4 without DTT as  
252 previously described (Klaren et al., 2005). Protein concentrations in the homogenates  
253 were measured with a Coomassie Brilliant Blue reagent kit (Bio-Rad, München,  
254 Germany) using bovine serum albumin (BSA) as the standard. Deiodination rates  
255 were normalized using the total homogenate protein in the reaction and were  
256 corrected for non-enzymatic deiodination.

257

### 258 *2.9 Plasma parameters*

259 Plasma osmolality was measured with a vapour pressure osmometer and expressed as  
260 mOsm kg<sup>-1</sup> H<sub>2</sub>O. Plasma glucose, lactate and triglyceride levels were measured using  
261 commercial kits from Spinreact adapted to 96-well microplates. The total plasma  
262 protein concentration was determined in diluted plasma samples using a bicinchoninic  
263 acid BCA Protein Assay Kit (Pierce, IL, USA) using BSA as a standard. All assays  
264 were performed with a Bio Kinetic EL-340i Automated Microplate Reader (BioTek  
265 Instruments, Winooski, VT, USA) using Deltasoft3 software for Macintosh  
266 (BioMetallics Inc., Princeton Junction, NJ, USA).

267 Plasma cortisol was measured by radioimmunoassay (RIA) (Arends et al., 1999).  
268 Plasma free thyroxine (fT4) concentrations were determined using a commercially  
269 available kit (DELFI<sup>®</sup> fT4, PerkinElmer Life and Analytical Sciences, Turku,  
270 Finland), which consists of a solid phase time-resolved fluoroimmunoassay reaction  
271 and measurements were performed using a Wallac Victor<sup>2</sup> 1420 multilabel counter.

272 Serially diluted *S. aurata* charcoal-stripped plasma produced binding curves that were  
273 parallel to the standard curve (results not shown).

274 Plasma free triiodothyronine (fT3) levels were measured with a solid phase  
275 competitive ELISA (Human Diagnostics, Wiesbaden, Germany) according to the  
276 manufacturer's instructions as previously described for this species (Vargas-Chacoff et  
277 al., 2016). Absorbance was measured in a Bio-Rad Model-680 microplate reader (Bio-  
278 Rad, Veenendaal, The Netherlands). Samples were diluted with *S. aurata* charcoal-  
279 stripped plasma when the measured concentrations of fT3 were above the maximum  
280 standard concentration.

281

### 282 2.10 $\text{Na}^+/\text{K}^+$ -ATPase activity

283  $\text{Na}^+/\text{K}^+$ -ATPase activities in gill and kidney homogenates were determined in  
284 microplates using McCormick's method (McCormick, 1993) with modifications  
285 (Mancera et al., 2002).

286

### 287 2.11 Statistics

288 Differences between groups were tested using a one-way ANOVA with  
289 environmental salinity as the factor of variance. When necessary, data were  
290 logarithmically transformed to fulfil the requirements for parametric ANOVA.  
291 Normality was analysed using the Kolmogorov-Smirnov's test. The homogeneity of  
292 variances was analysed using Levene's test. When ANOVA yielded significant  
293 differences, Tukey's post-hoc test was used to identify significantly different groups.  
294 When data did not comply with the premises of the parametric ANOVA, data were  
295 analysed using a Kruskal–Wallis ANOVA by ranks. Correlations between free THs,  
296 relative to mRNA expression of *tsh $\beta$* , *dio1* and *dio2*, and ORD activities in gill and  
297 kidney were analysed using linear regression on mean values of parameters measured  
298 in the experimental groups, as previously described (Speers-Roesch et al., 2015).  
299 Statistical significance was accepted at  $p < 0.05$ . All the results are given as mean  $\pm$   
300 standard error of the mean (SEM).

301

## 302 3. Results

303

### 304 3.1 Biometrics

305 None of the groups differed in length or body mass at the start of the 14-days  
306 acclimation period (data not shown). No mortality was recorded during the  
307 experimental period. At the end of the acclimation period HSI decreased significantly  
308 in animals exposed to 55 ppt (HSI  $0.67 \pm 0.05$  %) compared to animals acclimated to  
309 15 ppt (HSI  $0.94 \pm 0.06$  %) or 40 ppt (HSI  $0.96 \pm 0.06$  %)

310

### 311 *3.2 Tshb amino acid sequence*

312 The full-length sequence of *S. aurata tshb* consisted of 870 bp (accession number  
313 KM014688) and had an open reading frame (ORF) of 438 nucleotides that encoded a  
314 146 aa protein (Supplementary File 3). Multiple sequence alignment of Tshb  
315 (Supplementary File 4) from seabream and a wide selection of vertebrates revealed  
316 they shared from 39 % aa sequence conservation with mammals and reptiles (anole  
317 lizard) up to 92 % aa sequence conservation with Perciformes (European sea bass)  
318 (Supplementary File 5). In common with other jawed vertebrates *S. aurata*, Tshb  
319 possessed a signal peptide of 20 aa and contained 12 conserved cysteine residues and  
320 a putative site for asparagine-linked glycosylation (Supplementary File 5).

321 Evolutionary analysis of *S. aurata* Tshb using the maximum likelihood method  
322 confirmed its identity and revealed that the branching of the consensus phylogenetic  
323 tree was consistent with established evolutionary relationships (Figure 1). The  
324 exception was the chondrosteian Siberian sturgeon that grouped with the tetrapods.  
325 Percomorphs grouped into one clade, with tetraodontidae (82-84 % aa sequence  
326 conservation), cichlids (84 % aa sequence conservation) and ovalentaria (medaka and  
327 platy fish, 71-78 % aa sequence conservation) in subclades. The Perciformes grouped  
328 into a consistent clade with the exception of the ovalentaria, a newly established fish  
329 clade (Wainwright et al., 2012). Seabream Tshb shared 70 % aa sequence identity  
330 with Salmoniformes and 60 % with Cypriniformes. The aa sequence identity between  
331 seabream Tshb and tetrapod TSHB was approximately 40 %.

332

### 333 *3.3 Pituitary tshb mRNA expression*

334 Pituitary *tshb* gene expression was significantly ( $p < 0.05$ ) higher (65 %) in animals  
335 acclimated to 55 ppt salinity compared to groups acclimated to 5 or 40 ppt salinity  
336 (Figure 2). No significant differences were detected between animals acclimated to 15  
337 ppt and any of the other groups.

338

339 *3.4 Plasma fTH levels*

340 Free T4 (fT4) concentrations in plasma were significantly higher ( $p=0.0002$ , one-way  
341 ANOVA followed by a Tukey post hoc test; salinity effect  $p=0.014$ ;  $N=4$  per group)  
342 in animals acclimated to 5 and 15 ppt compared to fish acclimated to 40 and 55 ppt  
343 salinities. Plasma free T3 was also significantly higher ( $p$  between 0.019 and 0.010,  
344 one-way ANOVA followed by a Tukey post hoc test; salinity effect  $p=0.0019$ ;  $N=4$   
345 per group) in fish acclimated to 5 ppt compared to those maintained at 15, 40 and 55  
346 ppt (Figure 3).

347

348 *3.5 deiodinases type 1 and 2 mRNA expression in gills and kidney*

349 Branchial *dio1* transcript abundance was significantly lower in seabream acclimated  
350 to 5 ppt salinity, with 37 % lower mRNA expression in the 5 ppt-acclimated fish  
351 relative to those maintained at 40 ppt (control) and 55 ppt salinity (salinity effect  
352  $p=0.025$ ;  $N=5$  per group). Conversely, *deiodinase type 2 (dio2)* gene expression was  
353 significantly higher (salinity effect  $p=0.007$ ;  $N=5$  per group) in fish acclimated to 15  
354 ppt compared to fish at 40 and 55 ppt salinity (Figure 4A). In contrast, transcript  
355 abundance of *deiodinase type 1 (dio1)* in kidney did not vary between groups.  
356 However, *dio2* expression was significantly higher in the 5 ppt group (210 % higher  
357 than the control group) compared to the 55 ppt salinity group (25 % less expression  
358 than the control group (Figure 4B).

359

360 *3.6 ORD activity in gills and kidney*

361 Branchial and renal T4-ORD activities were higher in animals acclimated to salinities  
362 of 5 and 15 ppt compared to animals acclimated to 40 and 55 ppt salinity (Figure 5).  
363 However, when incubated with rT3, gill and renal ORD activities were not  
364 significantly different between groups. Kidney T3-ORD activity increased with  
365 increased salinity with T3-ORD rates measured at 40 and 55 ppt twice as high as  
366 those measured at 5 ppt.

367

368 *3.7 Plasma osmolality, metabolites and cortisol levels*

369 Plasma parameters significantly differed between the experimental groups (Table 1).  
370 Plasma osmolality increased with increasing salinity. In general, all plasma metabolite  
371 concentrations were significantly higher in fish at 55 ppt and lower in fish at 5 ppt

372 compared with fish at 15 ppt and 40 ppt salinities. Plasma cortisol concentrations  
373 were similar between all the experimental groups.

374

### 375 *3.8 Correlations between components of the thyroid system*

376 Correlations between elements of the thyroid system in fish exposed to different  
377 salinities are indicated in Supplementary File 6. Correlation analysis revealed the  
378 highest plasma fT3 levels inhibited pituitary *tshb* mRNA expression (Pearson r  
379 coefficient,  $r=-0.820$ ). Hence, 67.2 % of the variance of *tshb* expression was  
380 explained by plasma fT3 concentrations ( $r^2=0.672$ ,  $p=0.180$ ). A positive correlation  
381 was found between plasma fT4 and higher *dio2* expression in gills and kidney  
382 ( $r^2=0.827$  and  $r^2=0.917$ , respectively). Branchial *dio2* expression correlated positively  
383 with T3- and T4-ORD branchial activities ( $r^2=0.933$  and  $r^2=0.894$ , respectively). In  
384 this sense, renal *dio2* expression correlated positively with rT3- and T4-ORD renal  
385 activities ( $r^2=0.764$  and  $r^2=0.684$ , respectively), but was negatively correlated with  
386 T3-ORD activity in this tissue ( $r^2=0.998$ ). Finally, plasma fT3 displayed a positive  
387 and strong correlation with renal *dio1* expression ( $r^2=0.935$ ).

388

### 389 *3.9 Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in gills and kidney*

390 Branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase activity as a function of ambient salinity was significantly  
391 higher in fish acclimated to 55 and 5 ppt salinities compared to fish at 15 ppt salinity.  
392 Renal Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was not significantly different between experimental  
393 fish groups (Supplementary File 7).

394

## 395 **4. Discussion**

396

397 The present study substantiates the notion that the thyroid system is regulated by  
398 changes in salinity in gilthead seabream. In this sense, a range covering 5 to 55 ppt  
399 salinity modified *tshb* gene expression, fTHs concentrations in plasma, ORD activities  
400 and relative mRNA levels of deiodinases in osmoregulatory organs (gills and kidney).  
401 The change in the thyroid system in response to a salinity challenge suggests it is  
402 involved and/or affected during the acclimation of seabream to changing osmolality  
403 conditions. Physiological processes regulated by the thyroid system such as growth or  
404 reproduction (Nugegoda and Kibria, 2016), of paramount relevance for the

405 aquaculture, will consequently be modified when seabream culture occurs at different  
406 salinities.

407 Although pituitary *tshb* is differentially expressed in response to environmental  
408 salinity, only 67.2 % of its variance is explained by changes in plasma fT3, and less  
409 by plasma fT4 (23.6 %) (Supplementary Figure 6). Our findings reveal that although  
410 fT3 levels partially modulate pituitary expression of *tshb*, its fine regulation is  
411 dependent on the total amount of T3 and/or T4 in plasma. Thus, the classical feedback  
412 mechanism in which plasma T3 regulates TSH secretion was evident only in the  
413 extreme-salinity groups (5 and 55 ppt). On the other hand, the absence of a clear  
414 correlation between plasma fTHs and pituitary expression of *tshb* may suggest that the  
415 thyroid system is not fully controlled by the pituitary, but may be fine-tuned at the  
416 peripheral tissue level.

417 Our findings indicate that gilthead seabream maintains thyroidal homeostasis (*viz.*  
418 stable fT3 concentrations in plasma) in a wide range of environmental salinities (from  
419 15 to 55 ppt) by changing plasma fT4 levels (higher levels at hypo- and isosmotic  
420 environments), in common with what occurs in *Solea senegalensis* (Arjona et al.,  
421 2008) and *Acipenser stellatus* (Krayushkina et al., 2015). The differences in plasma  
422 fT4 levels in our study are probably due to changes in T4 production/secretion by the  
423 thyroid gland, and/or changes in peripheral thyroid hormone metabolism.

424 Deiodination of T4 towards the formation of the active T3 is carried out by Dio1 and  
425 Dio2 enzymes (Klaren et al., 2008). The substrate specificity of gilthead seabream  
426 Dio1 and Dio2 is not well established. In the present study, incubations with different  
427 substrates for the Dio1 and Dio2 deiodinases (T3, rT3 and T4), reveal that T4-ORD  
428 activity in gills and kidney decreases with environmental salinity while no changes in  
429 rT3-ORD activity occurred in any of the groups tested. Despite these similarities,  
430 there are some differences between both tissues that should be mentioned. In this  
431 sense, gill ORD activity is maximal when rT3 is the substrate (Figure 5A), while the  
432 highest renal activity occurs with T4 as the substrate (Figure 5B). These differences in  
433 deiodinase activity between the gills and the kidney may be explained by differing  
434 ratios of Dio1 and Dio2 enzymes in these tissues. Thus, the apparent substrate  
435 preference of mammalian and fish Dio1 for rT3 rather than T4 (Klaren et al., 2005;  
436 Kohrle, 1999) could indicate that the main deiodinase in gilthead seabream gills is  
437 Dio1. Herein, the expression of *dio2* in both tissues positively correlates with T4-  
438 ORD activity (Supplementary Figure 6), pointing to T4 as the preferential substrate

439 for gilthead seabream Dio2. Thus, the high T4-ORD activity revealed for the  
440 seabream kidney in the present study may indicate that the predominant deiodinase in  
441 this tissue is Dio2 rather than Dio1, even though Dio1 is also expressed.

442 The presence of Dio2 in gills seems to depend on the fish species studied (Lorgen et  
443 al., 2015; Orozco et al., 2000), although recent studies in *S. aurata* have illustrated  
444 that the thyroid metabolism canonical pathway is clearly regulated by salinity changes  
445 in this osmoregulatory tissue (Martos-Sitcha et al., 2016). In *S. aurata* *dio2* mRNA  
446 expression occurs not only in gills, but also in kidney indicating that Dio2 is relevant  
447 in osmoregulatory organs. We provide some correlations between plasma fT4  
448 concentrations and expression of *dio2* in both gills and kidney, indicating an  
449 enhancement in peripheral ORD activity when circulating T4 levels increase. The  
450 results of the present study in seabream coincide with those of previous studies in  
451 fish, as the expression of Dio2 is described to increase in hyposmotic salinities  
452 (López-Bojórquez et al., 2007) due to the presence of osmotic response elements in  
453 the *dio2* promoter region (Lorgen et al., 2015). Moreover, gill *dio1* expression  
454 increases with environmental salinity in *S. aurata* (Figure 4A), and this may suggest  
455 that TH inactivation pathways (as this enzyme also presents inner ring deiodinase  
456 activity) are involved in acclimation to hyperosmotic conditions. The results obtained  
457 *ex vivo* when T3 was used as the substrate for kidney were negatively correlated with  
458 renal *dio2* expression (Supplementary Figure 6), and this may indicate that high levels  
459 of T3 inhibit *dio2* expression in this tissue. ORD and IRD (inner ring deiodination)  
460 processes could then be modulated jointly, as renal *dio1* expression was upregulated  
461 by plasma fT3 concentrations (Supplementary Figure 6), sustaining an increased IRD  
462 activity in the kidney when T3 levels were high. However, the enhanced T3-ORD  
463 activity in kidney at higher salinities (40 and 55 ppt) was not accompanied by  
464 differences in *dio1* transcript abundance suggesting that regulation of deiodinase  
465 transcription and translation diverge. Overall, our results of ORD activity and mRNA  
466 expression support the idea of Dio2 as the main “osmoregulatory deiodinase” in  
467 seabream with Dio1 taking a secondary role.

468 The elevated fTH levels measured in hyposmotic conditions (5 ppt) can be interpreted  
469 as an acclimation response that may increase the activity of ion transporters in  
470 osmoregulatory organs (Laiz-Carrion et al., 2005a). In this sense it was postulated that  
471 THs interact with other hormones such as cortisol and GH/IGF-I in order to increase  
472 the osmoregulatory capacity of fish (McCormick, 2011). As the highest fT3 levels

473 shown in this study (fish acclimated to 5 ppt) are related to reduced growth rates  
474 (Laiz-Carrion et al., 2005b), it could be suggested that T3 reallocates metabolic  
475 energy from growth processes to ion transport and osmoregulation so that seabream  
476 can cope with the ionoregulatory demands dictated by low salinity (5 ppt)  
477 environments (higher ion transport and water retention). In agreement with this, gill  
478  $\text{Na}^+/\text{K}^+$ -ATPase activity was maximal at 5 ppt. The metabolic actions of THs have  
479 been associated with increased plasma levels of energy metabolites (Vargas-Chacoff  
480 et al., 2016). However, the results of the present study are not in total concordance  
481 with this idea as seabream acclimated to 55 ppt had low levels of fTHs but a high  
482 concentration of metabolites in plasma and highest branchial NKA activity.  
483 Regarding to this, previous works reported in this species that extreme salinities are  
484 associated with higher gill NKA (Laiz-Carrión et al., 2005a) and thus metabolic  
485 activities. This may suggest that metabolite turnover is not only regulated by the  
486 thyroid system at this salinity (55 ppt).

487 In conclusion, the thyroid system of gilthead seabream (*Sparus aurata*) is regulated  
488 by salinity. Hypo- and isosmotic environments cause an increase in plasma fTH levels  
489 evoking a hyperthyroid condition. Environmental salinity modulated ORD activity in  
490 osmoregulatory tissues such as the gills and kidney supporting the idea that the  
491 thyroid system is involved in osmoregulation in fish. Gills seem to have  
492 predominantly Dio1 activity as indicated by high rT3-ORD activity, while kidney has  
493 mainly Dio2 activity, as indicated by the high T4-ORD activity. Dio2 seems to be  
494 more responsive to osmoregulatory changes than Dio1, and we propose Dio2 should  
495 be considered as the main “osmoregulatory deiodinase” in the seabream. Our results  
496 indicate that the peripheral tissue plays an important role in TH regulation during  
497 osmoregulation in seabream.

498

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506



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637

638 **Figure legends**

639

640 **Figure 1. Phylogenetic analysis of vertebrate *Tshb* amino acid sequences using**  
641 **the maximum likelihood method based and a JTT matrix-based model.** A  
642 bootstrap test of phylogeny was performed with 1,000 replications. Branches with less  
643 than 50 % bootstrap support are collapsed into a single clade. The consensus tree is  
644 drawn to represent the evolutionary history of the taxa analysed. Partial proteins were  
645 not used for phylogenetic analysis. Species included in the analysis were gilthead  
646 seabream (*S. aurata*), European seabass (*Dicentrarchus labrax*), fugu (*Takifugu*  
647 *rubripes*), green spotted pufferfish (*Tetraodon nigroviridis*), zebra mbuna (*Maylandia*  
648 *zebra*), princess of Burundi (*Neolamprologus brichardi*), Nile tilapia (*Oreochromis*  
649 *niloticus*), three-spined stickleback (*Gasterosteus aculeatus*), platy (*Xiphophorus*  
650 *maculatus*), medaka (*Oryzias latipes*), Atlantic cod (*Gadus morhua*), rainbow trout  
651 (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), blind cave fish (*Astyanax*  
652 *mexicanus*), zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*), Japanese eel  
653 (*Anguilla japonica*), spotted gar (*Lepisosteus oculatus*), Siberian sturgeon (*Acipenser*  
654 *baerii*), anole lizard (*Anolis carolinensis*), chicken (*Gallus gallus*), Chinese softshell  
655 turtle (*Pelodiscus sinensis*), human (*Homo sapiens*), and mouse (*Mus musculus*).

656

657 **Figure 2. Expression of *tshb* in pituitary of seabream.** Quantitative real-time PCR  
658 analysis of *tshb* transcript abundance in the pituitary gland of seabream after  
659 acclimation to 5, 15, 40 and 55 ppt salinity for two weeks. Data were normalized by  
660 dividing transcript number by the absolute value of  $\beta$ -actin in every sample. Results  
661 are expressed as mean  $\pm$  SEM (N=8). Different letters indicate significant differences  
662 among groups (one-way ANOVA followed by a Tukey test,  $p < 0.05$ ).

663

664 **Figure 3. Plasma concentrations for free thyroid hormones (fT3 and fT4) in**  
665 seabreams acclimated to four different environmental salinities for two weeks (black  
666 bars, fT3; white bars, fT4). Results are expressed as mean  $\pm$  SEM (N=4). Different  
667 letters indicate significant differences among groups (capital and lowercase letters  
668 represent fT3 and fT4, respectively). Further details as in legend of Figure 2.

669

670 **Figure 4. Expression of *dio1* and *dio2* in osmoregulatory tissues of seabream.**  
671 Branchial (A) and renal (B) quantitative real-time PCR analysis of *deiodinases 1* and  
672 *2* (*dio1*, black bars; and *dio2*, white bars) relative transcript abundance in seabream  
673 individuals acclimated to four different environmental salinities for two weeks.

674 Different letters indicate significant differences among groups (capital and lowercase  
675 letters represent *dio1* and *dio2* mRNA expression, respectively). Further details as in  
676 legend of Figure 2.

677

678 **Figure 5. Outer ring deiodination activity in osmoregulatory tissues of seabream.**

679 Branchial (A) and renal (B) outer ring deiodination (ORD) activities when incubating  
680 with reverse T3 (black bars), 3,5,5'-T3 (light grey bars) and T4 (dark grey bars) in  
681 seabream animals acclimated to four different environmental salinities for two weeks.  
682 Results are expressed as mean  $\pm$  SEM (N=5). Different letters indicate significant  
683 differences among groups (capital and lowercase letters represent T4- and T3-ORD  
684 activity, respectively). Further details as in legend of Figure 2.

685

686 **Legends to supplementary files**

687

688 **Supplementary file 1.** Water parameters at different environmental salinities in the  
689 experiment.

690

691 **Supplementary file 2.** Primers, concentrations (in nM) and amplicon sizes used for  
692 qPCR analysis of seabream *tshb*, *dio1*, *dio2* and *actb*. Sa denotes *Sparus aurata*; Fw  
693 and Rv indicate forward and reverse primers, respectively.

694

695 **Supplementary file 3.** Nucleotide sequence for *tshb* (GenBank acc. no. KM014688)  
696 cDNA cloned from seabream. Nucleotides shown in lower case at the beginning and  
697 the end designate 5' and 3' untranslated regions. Nucleotides for ORF are indicated in  
698 upper case, bold and italics. Start and stop codons are indicated in black boxes.  
699 Putative adenylation signal aataaa sequence is lower case letter, bold and underlined.

700

701 **Supplementary file 4.** Tshb sequence identity matrix.

702

703 **Supplementary file 5.** Multiple sequence alignments for twenty four complete  
704 Tshb/TSHB proteins, including the deduced seabream protein sequence. Common and  
705 scientific names of the species used and their respective GenBank or NCBI Reference  
706 Sequence numbers are as follows: the Percomorph fish gilthead seabream (*Sparus*  
707 *aurata*, KM014688) and European seabass (*Dicentrarchus labrax*, CBN80754); the

708 cichlids Nile tilapia (*Oreochromis niloticus*, XP\_005478198), princess of Burundi  
709 (*Neolamprologus brichardi*, XP\_006782879) and zebra mbuna (*Maylandia zebra*,  
710 XP\_004547638); the tetraodontiformes green spotted pufferfish (*Tetraodon*  
711 *nigroviridis*, H3DLQ2\_TETNG) and fugu (*Takifugu rubripes*, XP\_003973164);  
712 cyprinodontiformes like the platy (*Xiphophorus maculatus*, XP\_005813805); the non  
713 Percomorph fish including the Atlantic cod (*Gadus morhua*, GADMO16328) and two  
714 salmonids, Atlantic salmon (*Salmo salar*, AAC77908) and rainbow trout  
715 (*Oncorhynchus mykiss*, P37240); the cyprinids zebrafish (*Danio rerio*, AAN08914)  
716 and common carp (*Cyprinus carpio*, BAA20082); the characiform blind cave fish  
717 (*Astyanax mexicanus*, XP\_007253483.1); the ancient teleost Japanese eel (*Anguilla*  
718 *japonica*, AAO17791); the holostean spotted gar (*Lepisosteus oculatus*,  
719 XP\_006628446); two reptile species, the Chinese softshell turtle (*Pelodiscus sinensis*,  
720 NP\_001273864) and the green anole lizard (*Anolis carolinensis*, XP\_008108073); one  
721 bird, chicken (*Gallus gallus*, AAB88127); and two mammals, mouse (*Mus musculus*,  
722 AAA40492) and human (*Homo sapiens*, AAA36782). The presumptive signal peptide  
723 sequence is underlined, the conserved amino acids residues that share 100 % identity  
724 are indicated with bold white letters black boxed. All designated sites and sequences  
725 specified are presumptive and based on sequence analysis and comparison. Thus,  
726 *hairpin loops*, the *long loop* and the *seatbelt* are underlined. The quoted numbers at  
727 the end of each sequence indicate the number of amino acids that each deduced  
728 protein contains.

729

730 **Supplementary file 6.** Product-moment correlation analysis between plasma free  
731 thyroid hormones (fT3 and fT4), pituitary *tshb* and gills and kidney *dio1* and *dio2*  
732 mRNA expression, and branchial and renal rT3-, T3- and T4-ORD activities in  
733 seabream individuals acclimated to four different environmental salinities for two  
734 weeks. Correlation matrixes were performed using the means for each group  
735 (environmental salinity of 5, 15, 40 or 55 ppt). Linear regression results are displayed  
736 as the Pearson's coefficient (r), coefficient of determination (r<sup>2</sup>) and p value (p).  
737 Variables from the second column on the left are linearly correlated with those  
738 variables shown in bold in the top rows.

739

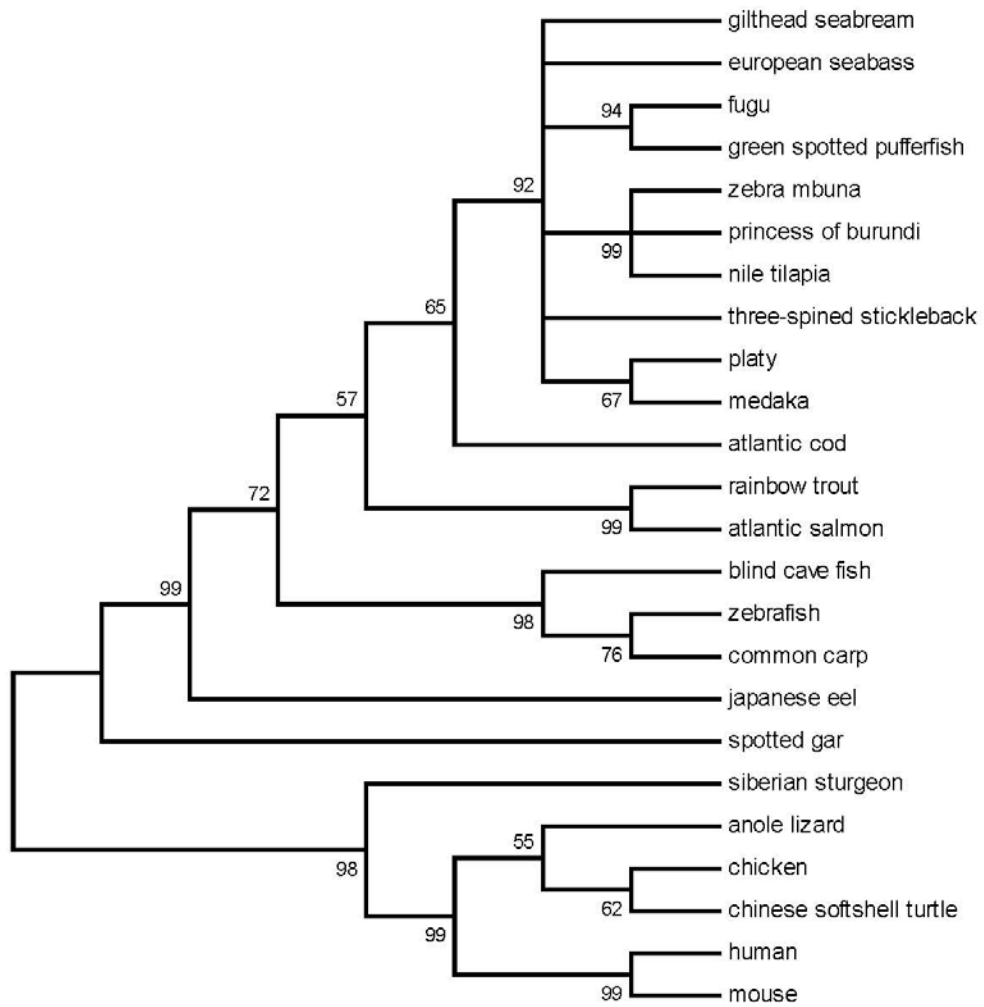
740 **Supplementary file 7.** Branchial and renal Na<sup>+</sup>/K<sup>+</sup>-ATPase activities (in μmol ADP  
741 mg<sup>-1</sup> protein h<sup>-1</sup>) in seabream individuals acclimated to four different environmental

742 salinities for two weeks. Results are expressed as mean  $\pm$  SEM (N=8). Different  
743 letters indicate significant differences among groups (one-way ANOVA followed by a  
744 Tukey test,  $p < 0.05$ ).

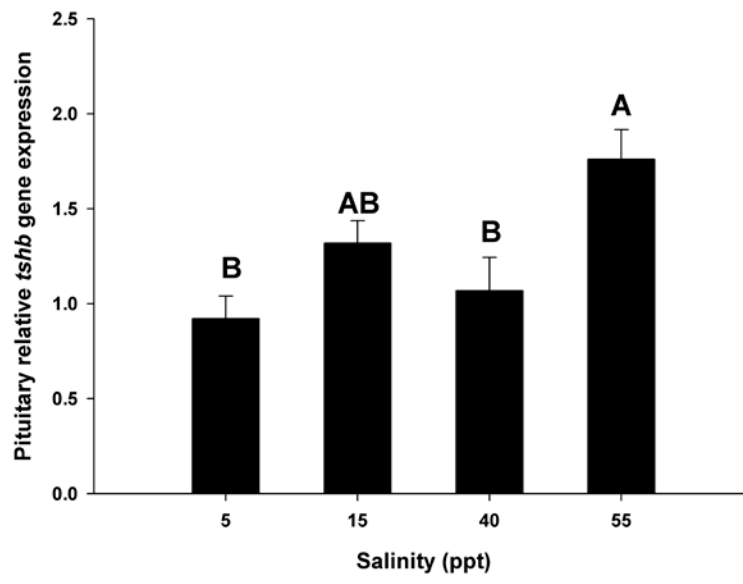
745

746



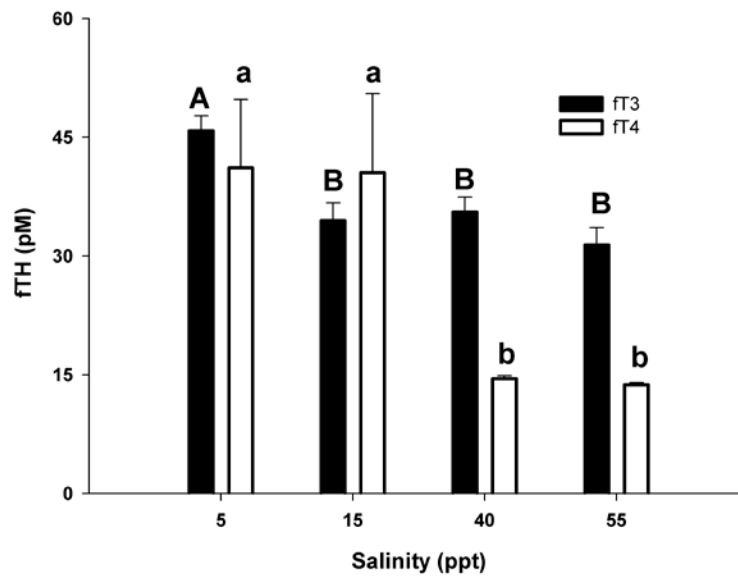


749 **Figure 2**



750  
751

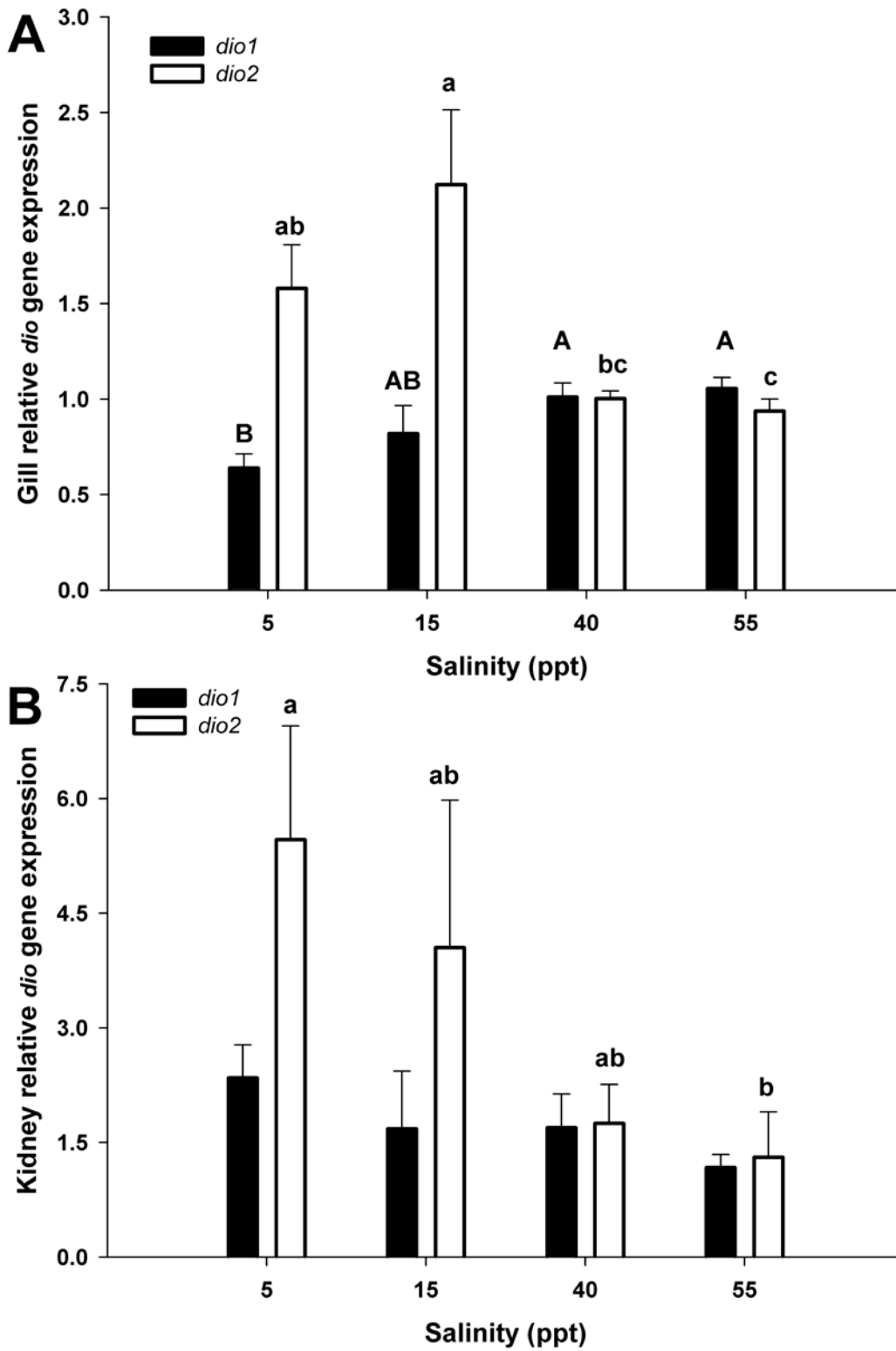
752 **Figure 3**



753

754

755 **Figure 4**

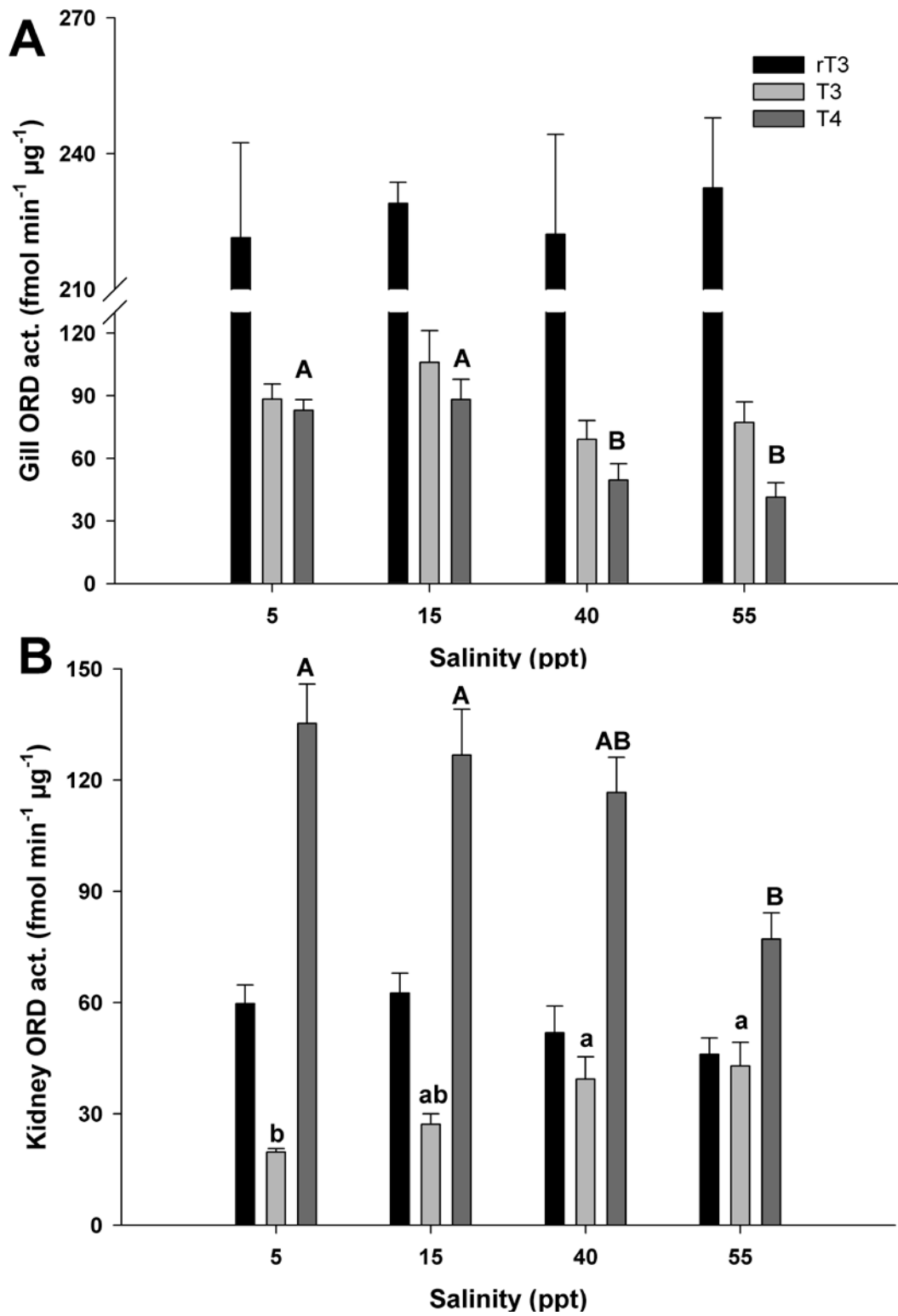


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759 **Figure 5**



760

761

762 **Table 1.** Plasmatic metabolites and osmoregulatory parameters in seabream  
 763 individuals acclimated to four different environmental salinities for two weeks.  
 764 Results are expressed as mean  $\pm$  SEM (N=8). Different letters indicate significant  
 765 differences among groups (one-way ANOVA followed by a Tukey test,  $p < 0.05$ ).  
 766

<b>Parameter</b>	<b>5 ppt</b>	<b>15 ppt</b>	<b>40 ppt</b>	<b>55 ppt</b>
Glucose (mM)	$3.4 \pm 0.3^b$	$4.3 \pm 0.3^{ab}$	$3.3 \pm 0.2^b$	$4.5 \pm 0.2^a$
Lactate (mM)	$1.7 \pm 0.1^b$	$2.7 \pm 0.3^a$	$2.2 \pm 0.2^{ab}$	$3.5 \pm 0.1^a$
TAG (mM)	$1.1 \pm 0.1^b$	$1.8 \pm 0.1^a$	$1.8 \pm 0.1^a$	$1.5 \pm 0.1^{ab}$
Proteins (g L <sup>-1</sup> )	$37.8 \pm 1.2^b$	$37.1 \pm 1.4^b$	$38.6 \pm 0.7^b$	$44.5 \pm 1.8^a$
Osmolality (mOsm kg <sup>-1</sup> )	$358 \pm 2^c$	$384 \pm 5^b$	$381 \pm 5^b$	$444 \pm 9^a$
Cortisol (ng mL <sup>-1</sup> )	$19.4 \pm 4.7$	$18.8 \pm 5.2$	$20.8 \pm 5.7$	$7.2 \pm 1.6$

767  
 768

769 **Suppl. 1**

770

<b>Water parameter</b>	<b>5 ppt</b>	<b>15 ppt</b>	<b>40 ppt</b>	<b>55 ppt</b>
Na <sup>+</sup> (mM)	63	169	570	780
Cl <sup>-</sup> (mM)	77	194	588	957
Ca <sup>2+</sup> (mM)	2.67	5.19	13.00	17.72
K <sup>+</sup> (mM)	1.28	3.48	11.28	15.36
Mg <sup>2+</sup> (mM)	6.95	19.46	57.11	88.65

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773 **Suppl. 2**

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<b>Gene</b>	<b>Primer</b>	<b>Sequence (5' → 3')</b>	<b>Conc. (nM)</b>	<b>Amplicon (bp)</b>
<i>tshb</i>	SaTshb_Fw	ACGTCATCCTTCAGCTTGTGAT	200	128
	SaTshb_Rv	CGCTAATGAAAATACCCAGCAG	200	
<i>dio1</i>	SaDio1_Fw	AGGACAAGAGGCTTTTGTGG	400	123
	SaDio1_Rv	CTTCCAAAACCTCAGCACCAG	400	
<i>dio2</i>	SaDio2_Fw	GGTTGAGGACTTCAGTGATG	400	103
	SaDio2_Rv	GAAAGAGCAAGAGCCCATAG	400	
<i>actb</i>	Sa $\beta$ actin_Fw	TCTTCCAGCCATCCTTCCTCG	200	108
	Sa $\beta$ actin_Rv	TGTTGGCATAACAGGTCCTTACGG	200	

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775

776



777 **Suppl. 3**

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5' -ttcagactcagacagggcaccggcatctcctgagcaggtcccaaattgcttgaa 54  
aaaaataaacactagctgaac**ATGGAGACTGCGGTGTT**CAGCTGCTGGCTCCTTTTT 111  
**CTGCTCTTCAGTCCAGCTGTTCCCATGTGTTTACCCACTGACTTCACCCTGTATGTG** 168  
**GACAGGCCAGAGTGTGACTTCTGTGTGGCCATCAACACGACCATCTGCATGGGATTC** 225  
**TGCTACTCGAGGGACAGCAACATGAGGGACATACTCGGCCCCCGCTTCCTTATCCAG** 282  
**AGAGGCTGTACTTATGACAAAGTGAATACCGCACAGCCGTGCTGCCCGGCTGTCCC** 339  
**ATCAACGCCGACCCTGTCTTACCTACCCCGTGGCCCTCAGCTGCCACTGTGGGGCC** 396  
**TGCAGGACTGACAGCGATGAATGCGCACACAGGGCCGGCGGAACGGAGCTCGGTGT** 453  
**ACCAAACCAGTCAGACGTCTCTACCCGTATCCCGACCAGAGCAACTACATGATCCCG** 511  
**TTC**TGA****tcttcctggtgtagogcttttatcttggccttccttttctttttttt 567  
cccccttaaattaccaggtggaaactgcatgattcatcaatgttttgggagcagaca 624  
tacgtcatccttcagcttggatggagacactgatgtctgtccagctctgtgttatct 681  
cttgtaccagcctgttttattgtgcctttgttgcccaactcaaggtgatcctgctgg 738  
gtattttcattagcgtcattattaatcccactgtacactcatgtgtgtgtgactaat 795  
catgtttactgtggaagggatacttggaattcaataaaatgaagaagctctggaagc 852  
tggcgtcctcgaaatagc-polyA tail -3' 860

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780



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      10      20      30      40      50      60      70      80
gilthead seabream METAVFSCW--LFLFLSPAVPMCFPTDFTLYVDRPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
european seabass METAVFSCW--LFLFLSPAVPMCFPTDFTLYVERPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
fugu MDATAFPWC--LFLFLSPAVPMCFPTDFTLYVERPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
green spotted pufferfish MAEAVFPFW--LFLFLSPAVPMCFPTDFTLYVERPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
zebra mbuna MEVTVFNCW--LFLFLSPAVPMCFPTDFTLYVEKPECFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
princess of burundi MEATVFNWC--LFLFLSPAVPMCFPTDFTLYVEKPECFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
nile tilapia MEATVFNWC--LFLFLSPAVPMCFPTDFTLYVEKPECFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
three-spined stickleback METAVFPWC--LFLFLSPAVPMCFPTDFTLYVERPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
platy METAFSCW--VLFLLIYVPMCFPTDFTLYVEKPECDFGVAINNTTICMGEFYSRDSNMRRDIFRSRFLVQRGCTNDKVE
medaka MNTVLFPPW--MLFLLSPVPMCFPTDFTLYVEKPECDFGVAINNTTICMGEFYSRDSNMRRDIFGPRFLIQRGCTNDKVE
atlantic cod -----FSPAAPMCFPTDFTLYVEKPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
rainbow trout MELSVAMYG--LLCLLFSQAVPMCFPTDFTLYEERPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
atlantic salmon MELSVAMCG--LLCLLFSQAVPMCFPTDFTLYEERPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
blind cave fish MSATVLVAG--ILGLLLKTVAMPMPCTEYTYIYERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
zebrafish MSL-LYVIG--MLGLLKVAVPMCFPTDFTLYEERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
common carp MSP-VYVVG--MLGILMKVAMPMPCTEYTYIYERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
japanese eel MRVVLLASG--VLCLLAGQVLSICSPVDYTYLVEKPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
spotted gar --AALLVCG--LLCLVASQTLKSCAPTDYMLYVEKPECDFGVAINNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
siberian sturgeon MSAAVLTCA--LLCLAMGNASSLCSPTDFTLYEERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
human MTALFLMSM--LFLGLTGGQAMSFCTEYTYMHIERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
mouse MSAAVLLSV--LFLALCGQAASFCTEYTYMVDRECAVCLTINTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
chicken MSPFFMMSL--LFLGLTGGQAMSFCTEYTYMHIERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
chinese softshell turtle MSPIFMSL--LFLGLARGHAMSFCTEYTYMHIERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE
anole lizard MNPALLISLPFFLALTLGQSMSCPTDFTLYEERQECNYGVAVNTTICMGEFYSRDSNMRRDILGPRFLIQRGCTNDKVE

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      signal peptide      β-hairpin loop      long loop
      90      100      110      120      130      140      150
gilthead seabream YRTAVLPGCPINADPVFTYFVVALSCHCGACRTDSDDEAHRAG-ANGARCTKPVV--RLYPYDQSNYMIPIF----- 146
european seabass YRTAILPGCPLDANPMFTYFVVALSCHCGACRTDSDDEAHRAS-VDGTRCTKPVV--RLYPYDQSNYMIPIF----- 146
fugu YRTAILPGCSIQANPFTYFVVALSCHCGACRTDSDDEAHRAS-MDGARCTKPVV--DLSLFSGQSNYMIPIF----- 146
green spotted pufferfish YRTAVLPGCSIDVDPTFTYFVVALSCHCGACRTDSDDEAHRAS-MDGARCTKPLR--NLYPFPGQSNYMIPIF----- 146
zebra mbuna YHTAILPGCPIEANPVFTYFVVALSCHCGACRTDDEAHRAS-MDGTCTKPVV--RIYPYGHNSYVPIF----- 146
princess of burundi YHTAILPGCPIEANPVFTYFVVALSCHCGACRTDDEAHRAS-MDGTCTKPVV--RIYPYGHNSYVPIF----- 146
nile tilapia YHTAILPGCPIEANPVFTYFVVALSCHCGACRTDDEAHRAS-MDGTCTKPVV--RIYPYGHNSYVPIF----- 146
three-spined stickleback YRAALLPGCPIDSDPVFTYFVVALSCHCGACRTDSDDEAHRAS-VHRAPVGGARCTKPVV--RIYPYDQSNYMIPIF----- 147
platy YRTVILPGCAIDSNPAFTYFVVALSCHCGACRTDDEAHRAS-SYDANCAKPVV--RVYPYDQSNYMIPIF----- 148
medaka YRSAILPGCPLESNPVFTYFVVALSCHCGACRTDSDDEAHRAS-TGGGRTKPVV--LVHPYDQSNYMIPIF----- 146
atlantic cod YRTAILPGCPSEGSFLFTYFVVALSCHCGACRTAVDEAHRAS-SNRPTCTKPVV--HIY---QSNFLLPF----- 128
rainbow trout YRTVILPGCPHMANPLFTYFVVALSCHCGACRTDSDDEAHRAS-SGDRGASKPLR--HIYPTGLNSYIHPN----- 147
atlantic salmon YRTVILPGCPHMANPLFTYFVVALSCHCGACRTDSDDEAHRAS-SGDRGASKPLR--HIYPTGLNSYIHPN----- 139
blind cave fish HRTAVLPGCPHVDPHFTYFVVALSCHCGACRTDSDDEAHRAS-SALAKSAPVH--FLYDPAQNDLQPDWLQLF 151
zebrafish YRTAVLPGCPSHADPHFTYFVVALSCHCGACRTDSDDEAHRAS-SAGMRSKPVH--HLYP--EENNYAQAYWDQYE 148
common carp YRTAILPGCPSHADPHFTYFVVALSCHCGACRTDSDDEAHRAS-NAGMRSKPVV--HLYPDPBENNYIQAYWQYE 150
japanese eel YRTAELPGCPHVDPRFTYFVVALSCHCGACRTDSDDEAHRAS-ADGDRSAPVH--HMAYPQSNYMIPIF----- 147
spotted gar YRTVILPGCPHINPLLSYPLALRCHCGACRTDSDDEAHRAS--ETNECTKPIQ--PADSYDQSNYMIPIF----- 148
siberian sturgeon YHTVTLPGCPHNSNPSYSYAVAMSCRCRCKNTDYSDEIHEAL--RPSCTKPPREADSYDQSNYMIPIF----- 143
human YRTVEIPGCPHVAHYFYSYFVVALSCKCGKNTDYSDEIHEAL--RTNYCTKPKQ---SYLVGFSV----- 138
mouse YRTVEIPGCPHHTVPIYYSYFVVALSCKCGKNTDYSDEIHEAV--RTNYCTKPKQ---FYLGGFSV----- 138
chicken YQTALIPGCPHHTVPIYYSYFVVALSCKCGKNTDYSDEIHEAV--RTNYCTKPKQ---LCNM----- 134
chinese softshell turtle YRTVVLPGCPRHTIP-YYSYFVVALSCKCGKNTDYSDEIHDTV--RTDYCTKPKQ---PYNV----- 133
anole lizard YRTVMIPGCPHHAHVSYTYFVVALSCKCGKNTDYSDEIHEAV--HTEYCTVSSQ---LYNP----- 136

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β-hairpin loop                      seatbelt

Tissue	Variable	Plasma fT3			Plasma fT4		
		r	r <sup>2</sup>	p	r	r <sup>2</sup>	p
Plasma	fT3				0.633	0.401	0.367
Pituitary	<i>tshb</i>	-0.820	0.672	0.180	-0.486	0.236	0.514
Gills	<i>dio1</i>	-0.876	0.767	0.124	-0.926	0.857	0.074
	<i>dio2</i>	0.281	0.079	0.719	0.910	0.827	0.090
	rT3-ORD	-0.781	0.611	0.218	-0.252	0.063	0.748
	T3-ORD	0.148	0.022	0.852	0.858	0.737	0.142
	T4-ORD	0.579	0.335	0.421	0.987	0.974	0.013
Kidney	<i>dio1</i>	0.967	0.935	0.033	0.713	0.508	0.287
	<i>dio2</i>	0.828	0.686	0.172	0.958	0.917	0.042
	rT3-ORD	0.546	0.298	0.454	0.939	0.882	0.061
	T3-ORD	-0.830	0.688	0.170	0.957	0.916	0.043
	T4-ORD	0.742	0.551	0.258	0.781	0.609	0.219
Gill	rT3-ORD T3-ORD T4-ORD	Gill <i>dio1</i>			Gill <i>dio2</i>		
		r	r <sup>2</sup>	p	r	r <sup>2</sup>	p
		0.506	0.256	0.494	-0.013	0.000	0.987
		-0.607	0.368	0.393	0.966	0.933	0.034
		-0.884	0.781	0.116	0.945	0.894	0.055
Kidney	rT3-ORD T3-ORD T4-ORD	Kidney <i>dio1</i>			Kidney <i>dio2</i>		
		r	r <sup>2</sup>	p	r	r <sup>2</sup>	p
		0.702	0.493	0.298	0.874	0.764	0.126
		-0.879	0.773	0.121	-0.999	0.998	0.001
		0.888	0.788	0.112	0.827	0.684	0.173

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789 **Suppl. 7**  
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<b>Na<sup>+</sup>/K<sup>+</sup>-ATPase activity</b>				
<b>(<math>\mu\text{mol ADP mg}^{-1} \text{prot h}^{-1}</math>)</b>	<b>5 ppt</b>	<b>15 ppt</b>	<b>40 ppt</b>	<b>55 ppt</b>
Gills	$12.6 \pm 1.1^b$	$8.8 \pm 0.4^c$	$9.7 \pm 0.7^{bc}$	$22.2 \pm 2.9^a$
Kidney	$12.2 \pm 0.9$	$11.7 \pm 0.4$	$11.6 \pm 1.0$	$11.7 \pm 0.8$

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792