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

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1 Animal maintenance prior to experimentation

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

2.2 Acclimation to different environmental salinities

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

Sacramento, CA, USA) to ensure optimal water conditions. Water conditions during experimentation were: temperature, ranging between 19.1 and 19.8 ºC; 5, 15, 40 and 55 ppt salinity (variations <1 ppt for each tank); pH, ranging between 7.82 and 7.88; dissolved oxygen, >5 mg O_2 L^{-1}; nitrites, between 0.05 and 1.69 mg L^{-1}; nitrates, between 4.13 and 36.41 mg L^{-1}; and ammonium, 0.0-0.2 mg L^{-1}. These parameters were checked daily and did not vary significantly for the duration of the experiment. 20 % of the water in circuits was replaced every other day. Fish were maintained in these conditions for 14 days and were fasted for 24 h before sampling. No mortality was observed during the acclimation period.

2.3 Sampling

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

2.4 Water chemistry

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

expressed as mOsm kg^{-1} H_2O. Water chemistry data are shown in Supplementary File 1.

2.5 Cloning of tshb

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

2.6 Phylogenetic analyses

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

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

2.7 Real-time quantitative PCR (qPCR)

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

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

2.8 Outer ring deiodination (ORD) activities

Tracers used for measurements of deiodinase activity were prepared using the chloramine-T method to produce [^{125}I$^-$]rT3, [^{125}I$^-$]T3 and [^{125}I$^-$]T4 from the 3,3´-T2, 3,5-T2 and 3,5,3´-T3, respectively (Visser et al., 1977). All those molecules have been reported as substrates for ORD activity in teleost fish (Klaren et al., 2012).

Iodothyronines were purchased from Sigma Chemical Co. (St. Louis, MO, USA), $Na^{125}I$ was obtained from NEN Life Science Products Inc., Boston, MA, USA. Radiolabelled iodothyronines from the radioiodination reaction were purified using 10 % (w/v) Sephadex LH-20 minicolumns as described previously (Mol and Visser, 1985) followed by high pressure liquid chromatography (HPLC, platinum column EPS C18, 150 mm length, internal diameter 4.6 mm, Alltech Associated Inc., Illinois, USA) with a reverse phase isocratic elution of 35/65 % acetonitrile 0.05 M K_2HPO_4, pH 3.2. Sephadex LH-20 was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were analytical grade and obtained from commercial suppliers.

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

2.9 Plasma parameters

Plasma osmolality was measured with a vapour pressure osmometer and expressed as mOsm kg^{-1} H_2O. Plasma glucose, lactate and triglyceride levels were measured using commercial kits from Spinreact adapted to 96-well microplates. The total plasma protein concentration was determined in diluted plasma samples using a bicinchoninic acid BCA Protein Assay Kit (Pierce, IL, USA) using BSA as a standard. All assays were performed with a Bio Kinetic EL-340i Automated Microplate Reader (BioTek Instruments, Winooski, VT, USA) using Deltasoft3 software for Macintosh (BioMetallics Inc., Princeton Junction, NJ, USA).

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

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

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

2.10 Na^+/K^+-ATPase activity

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

2.11 Statistics

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

3. Results

3.1 Biometrics

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

3.2 Tshb amino acid sequence

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

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

3.3 Pituitary tshb *mRNA expression*

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

3.4 Plasma fTH levels

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

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

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

3.6 ORD activity in gills and kidney

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

3.7 Plasma osmolality, metabolites and cortisol levels

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

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

3.8 Correlations between components of the thyroid system

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

3.9 Na^+/K^+-ATPase activity in gills and kidney

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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637

638 **Figure legends**

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

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

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

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

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

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

Legends to supplementary files

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

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

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

Supplementary file 4. Tshb sequence identity matrix.

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

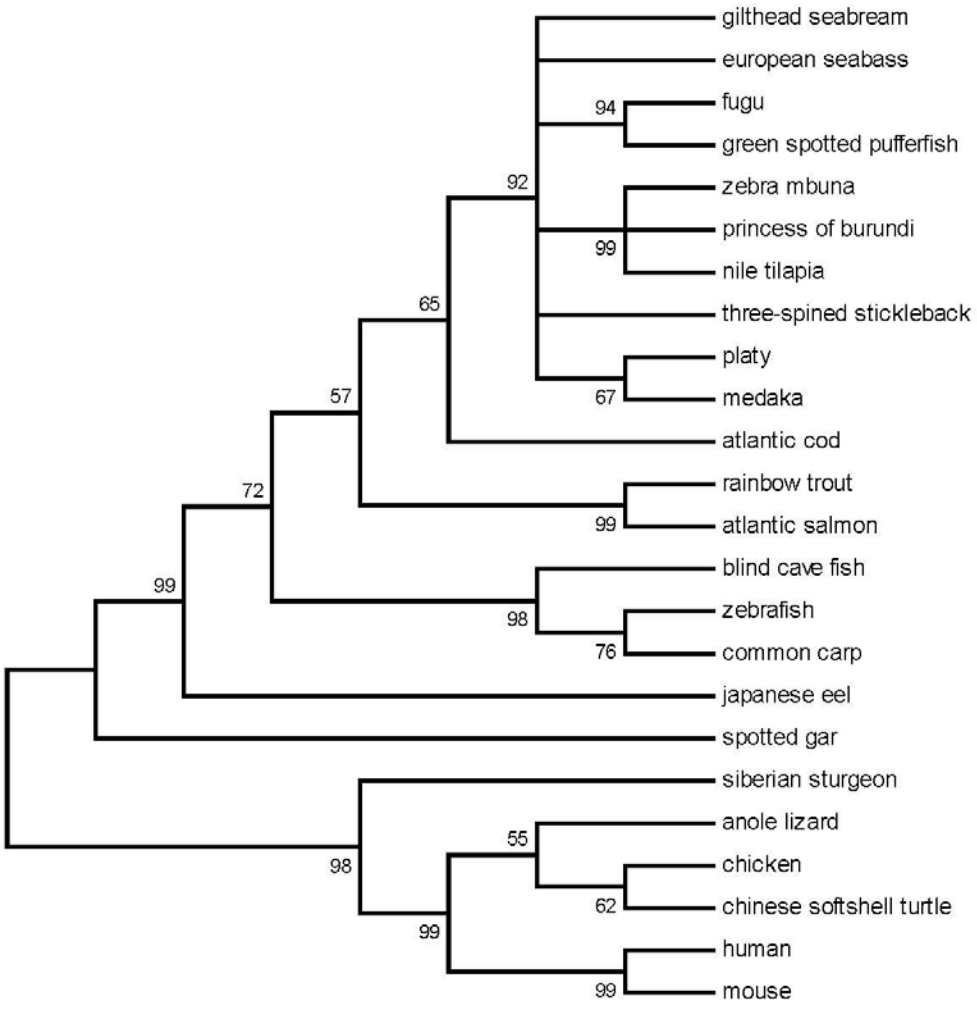
cichlids Nile tilapia (*Oreochromis niloticus*, XP_005478198), princess of Burundi (*Neolamprologus brichardi*, XP_006782879) and zebra mbuna (*Maylandia zebra*, XP_004547638); the tetraodontiformes green spotted pufferfish **(***Tetraodon nigroviridis*, H3DLQ2_TETNG) and fugu (*Takifugu rubripes*, XP_003973164); cyprinodontiformes like the platy (*Xiphophorus maculatus*, XP_005813805); the non Percomorph fish including the Atlantic cod (*Gadus morhua*, GADMO16328) and two salmonids, Atlantic salmon (*Salmo salar*, AAC77908) and rainbow trout (*Oncorhynchus mykiss*, P37240); the cyprinids zebrafish (*Danio rerio*, AAN08914) and common carp (*Cyprinus carpio*, BAA20082); the characiform blind cave fish (*Astyanax mexicanus*, XP_007253483.1); the ancient teleost Japanese eel (*Anguilla japonica*, AAO17791); the holostean spotted gar (*Lepisosteus oculatus*, *XP*_006628446); two reptile species, the Chinese softshell turtle (*Pelodiscus sinensis*, NP_001273864) and the green anole lizard (*Anolis carolinensis*, XP_008108073); one bird, chicken (*Gallus gallus*, AAB88127); and two mammals, mouse (*Mus musculus*, AAA40492) and human (*Homo sapiens*, AAA36782). The presumptive signal peptide sequence is *underlined*, the conserved amino acids residues that share 100 % identity are indicated with bold white letters black boxed. All designated sites and sequences specified are presumptive and based on sequence analysis and comparison. Thus, *hairpin loops*, the *long loop* and the *seatbelt* are underlined. The quoted numbers at the end of each sequence indicate the number of amino acids that each deduced protein contains.

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

Supplementary file 7. Branchial and renal Na^+/K^+-ATPase activities (in μmol ADP mg^{-1} protein h^{-1}) in seabream individuals acclimated to four different environmental

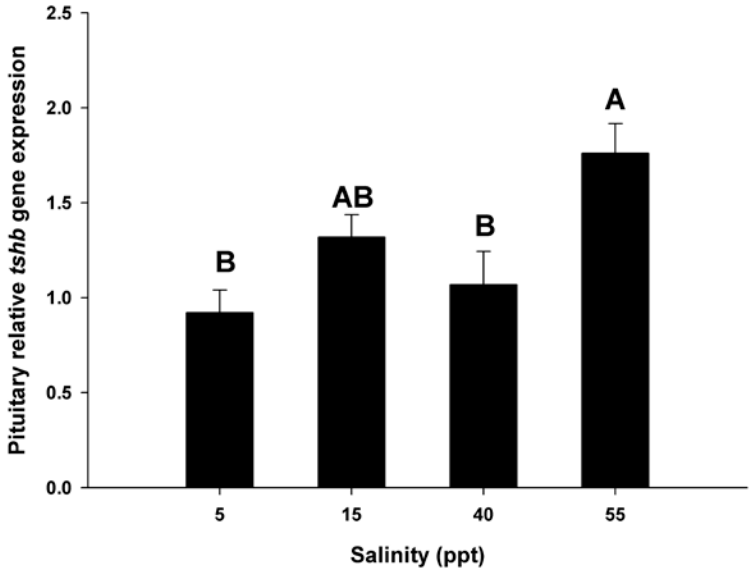
742 salinities for two weeks. Results are expressed as mean ± SEM (N=8). Different
743 letters indicate significant differences among groups (one-way ANOVA followed by a
744 Tukey test, $p<0.05$).
745
746

747 **Figure 1**



748

749 **Figure 2**



750
751

752 **Figure 3**

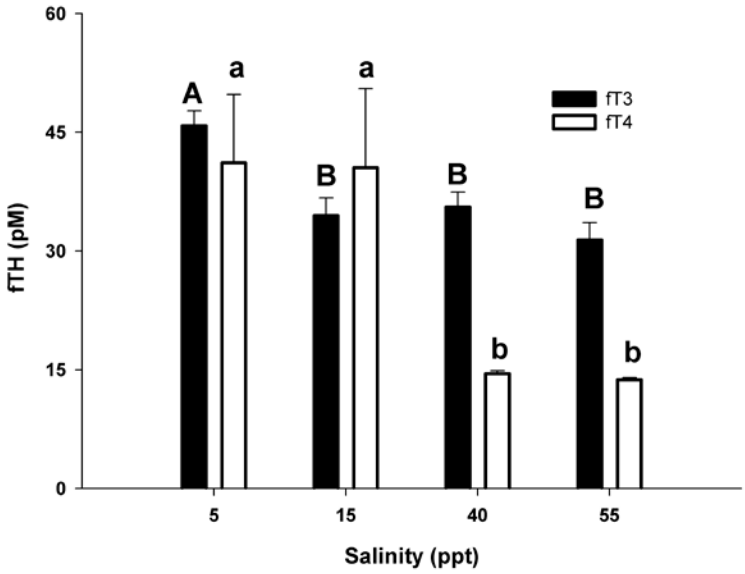


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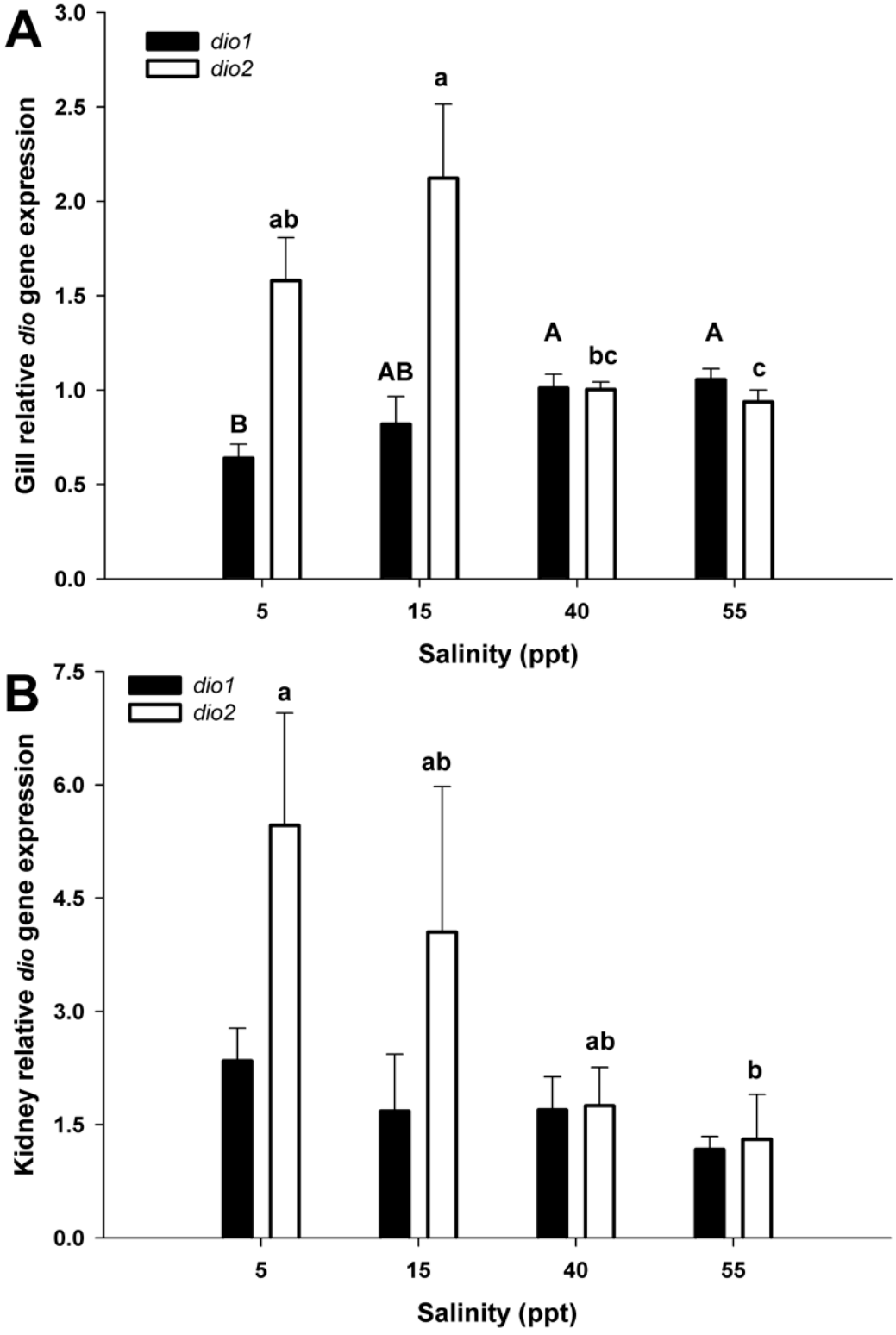
Figure 4


A
dio1
dio2
Gill relative dio gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
A
bc
A
c
AB
B
5
15
40
55
Salinity (ppt)
B
dio1
dio2
Kidney relative dio gene expression
7.5
6.0
4.5
3.0
1.5
0.0
a
ab
ab
b
5
15
40
55
Salinity (ppt)

759 **Figure 5**

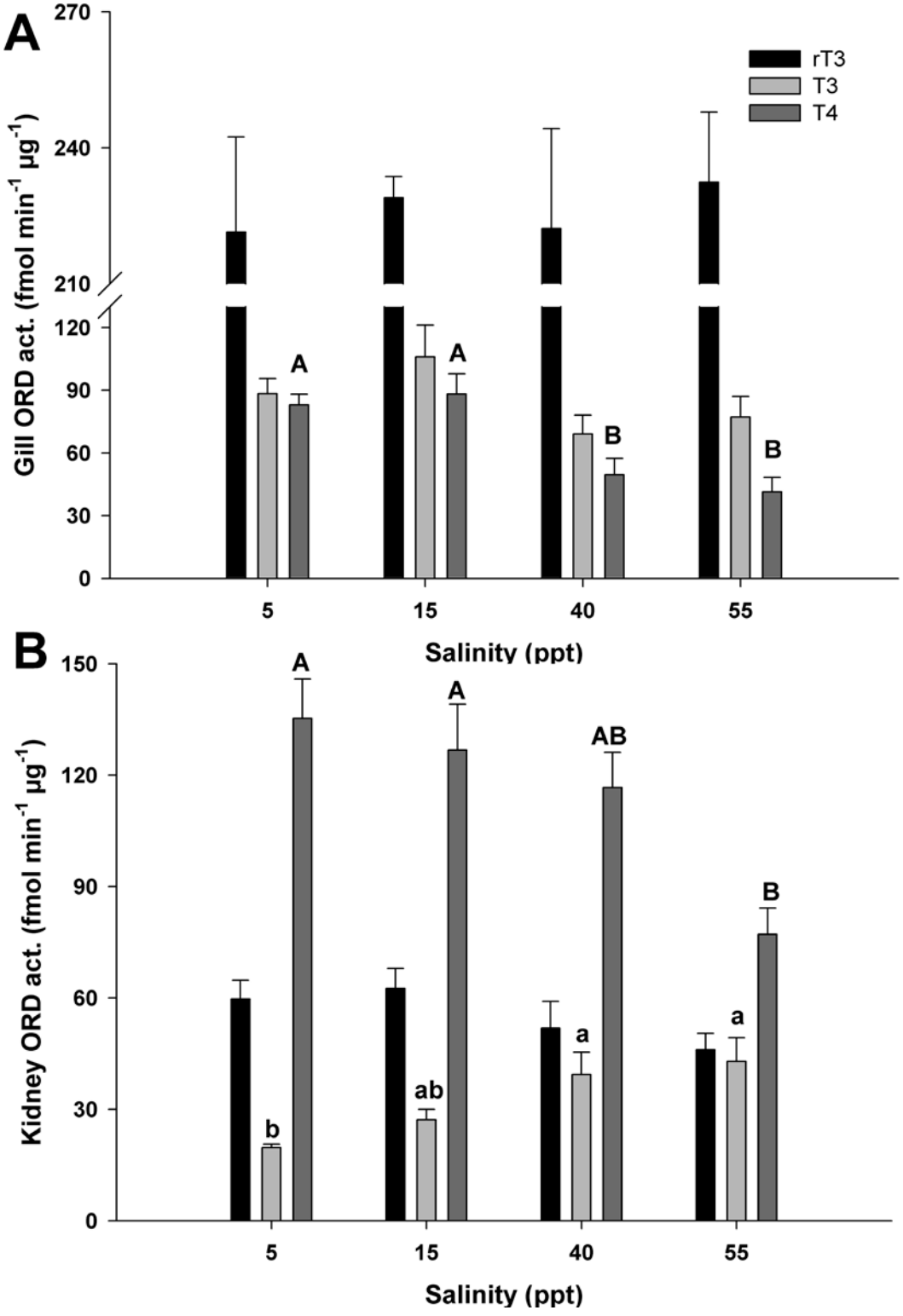


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Table 1. Plasmatic metabolites and osmoregulatory parameters in seabream individuals acclimated to four different environmental salinities for two weeks. Results are expressed as mean ± SEM (N=8). Different letters indicate significant differences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

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

Suppl. 1

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

Suppl. 2

Gene	Primer	Sequence (5´→ 3´)	Conc. (nM)	Amplicon (bp)
tshb	SaTshb_Fw	ACGTCATCCTTCAGCTTGTGAT	200	128
	SaTshb_Rv	CGCTAATGAAAATACCCAGCAG	200	
dio1	SaDio1_Fw	AGGACAAGAGGCTTTTGTGG	400	123
	SaDio1_Rv	CTTCCAAAACTCAGCACCAG	400	
dio2	SaDio2_Fw	GGTTGAGGACTTCAGTGATG	400	103
	SaDio2_Rv	GAAAGAGCAAGAGCCCATAG	400	
actb	Saβactin_Fw	TCTTCCAGCCATCCTTCCTCG	200	108
	Saβactin_Rv	TGTTGGCATACAGGTCCTTACGG	200	

Suppl. 3


5′-ttcagactcagacaggcaccggcatctcctgagcaggtcccaaattgcttggaa 54
aaaaaataacactagctgaacATGGAGACTGCGGTGTTCAGCTGCTGGCTCCTTTTT 111
CTGCTCTTCAGTCCAGCTGTTCCCATGTGTTTACCCACTGACTTCACCCTGTATGTG 168
GACAGGCCAGAGTGTGACTTCTGTGTGGCCATCAACACGACCATCTGCATGGGATTC 225
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TGCAGGACTGACAGCGATGAATGCGCACACAGGGCCGGCGCGAACGGAGCTCGGTGT 453
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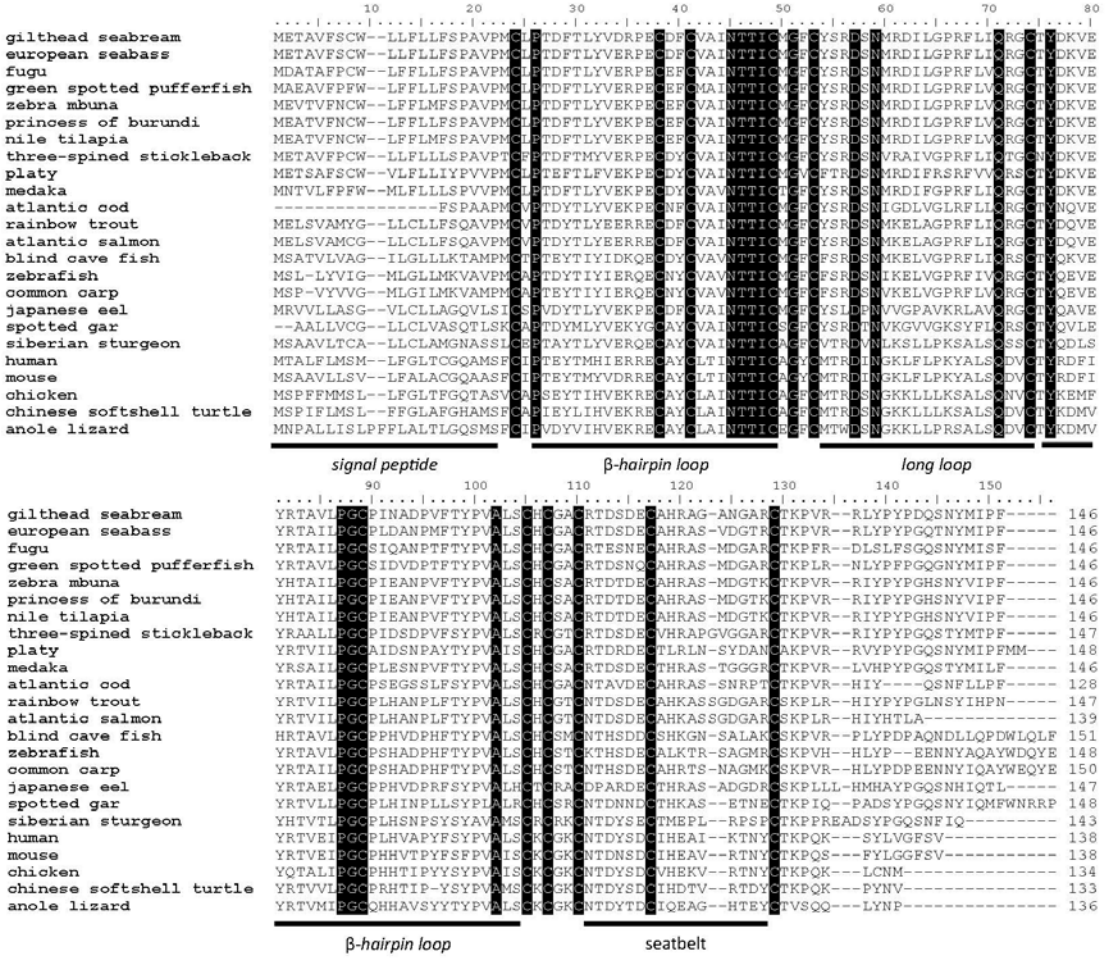
catgtttactgtggaagggatacttggaattcaataaaatgaagaagctctggaagc 852
tggcgtcctcgaaatagc-polyA tail -3′ 860

781 **Suppl. 4**

Suppl. 5


signal peptide
β-hairpin loop
long loop
β-hairpin loop
seatbelt

Suppl. 6

Tissue	Variable	Plasma fT3 r	Plasma fT3 r^2	Plasma fT3 p	Plasma fT4 r	Plasma fT4 r^2	Plasma fT4 p
Plasma	**fT3**				0.633	0.401	0.367
Pituitary	***tshb***	-0.820	0.672	0.180	-0.486	0.236	0.514
Gills	***dio1***	-0.876	0.767	0.124	-0.926	0.857	0.074
	dio2	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	***dio1***	0.967	0.935	0.033	0.713	0.508	0.287
	dio2	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 *dio1* r	Gill *dio1* r^2	Gill *dio1* p	Gill *dio2* r	Gill *dio2* r^2	Gill *dio2* p
Gill	**rT3-ORD**	0.506	0.256	0.494	-0.013	0.000	0.987
	T3-ORD	-0.607	0.368	0.393	0.966	0.933	0.034
	T4-ORD	-0.884	0.781	0.116	0.945	0.894	0.055

		Kidney *dio1* r	Kidney *dio1* r^2	Kidney *dio1* p	Kidney *dio2* r	Kidney *dio2* r^2	Kidney *dio2* p
Kidney	**rT3-ORD**	0.702	0.493	0.298	0.874	0.764	0.126
	T3-ORD	-0.879	0.773	0.121	-0.999	0.998	0.001
	T4-ORD	0.888	0.788	0.112	0.827	0.684	0.173

Suppl. 7

Na^+/K^+-ATPase activity (μmol ADP mg^{-1} prot h^{-1})	5 ppt	15 ppt	40 ppt	55 ppt
Gills	12.6 ± 1.1^{b}	8.8 ± 0.4^{c}	9.7 ± 0.7^{bc}	22.2 ± 2.9^{a}
Kidney	12.2 ± 0.9	11.7 ± 0.4	11.6 ± 1.0	11.7 ± 0.8