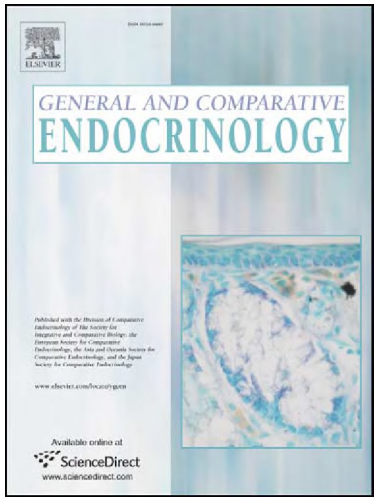

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Low salinity acclimation and thyroid hormone metabolizing enzymes in gilthead seabream (*Sparus auratus*)

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Abstract

We investigated the effect of acclimation to low salinity water of gilthead seabream (*Sparus auratus*), a euryhaline seawater teleost, on the activities of thyroid hormone-metabolizing enzymes in gills, kidney and liver. Following acclimation to low salinity water, the plasma free thyroxine (T_4) concentration increases 2.5-fold, and outer ring deiodination activities towards T_4, 3,5,3'-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (reverse T_3, rT_3) in the gills are reduced by 20 to 32%. Conjugation (catalyzed by sulfotransferase and UDP-glucuronyltransferase) and deconjugation pathways (arylsulfatase, ß-glucuronidase) play a role in the biological activity of native and conjugated thyroid hormones. Branchial, renal and hepatic activities of the enzymes involved in these metabolic pathways respond differentially to low salinity conditions. The results substantiate that thyroid hormones are involved in *Sparus auratus* osmoregulation, and that the gills are well equipped to play an important role in the modulation of plasma hormone titers.

1. Introduction

The vertebrate thyroid gland mainly secretes thyroxine (T_4), which is enzymatically deiodinated in the periphery to yield the biologically active hormone 3,5,3'-triiodothyronine (T_3). Besides deiodination, conjugation reactions are important pathways in the peripheral metabolism of thyroid hormones. Enzymatically catalyzed sulfation and glucuronidation reactions yield iodothyronine sulfates and glucuronides, respectively, in mammals (Visser, 1996; Wu et al., 2005) and teleosts (Finnson and Eales, 1998; Sinclair and Eales, 1972). Sulfations are catalyzed by sulfotransferases that transfer a sulfate group to the phenolic hydroxyl group of the iodothyronine molecule. Glucuronidation reactions, catalyzed by uridine 5'-diphosphate-glucuronyltransferases (UGTs), transfer a glucuronosyl group to the same acceptor group. Conjugated iodothyronines, mainly from hepatic origin, are considered to be biologically inactive, and the increased water so lubility to facilitate urinary and biliary excretion.

The presence of glucuronidated and sulfated iodothyronines in bile of a number of teleost species (Finnson and Eales, 1996; Sinclair and Eales, 1972) is commensurate to the role of hepatic conjugation as a clearance pathway. However, in healthy, fasted tilapia (*Oreochromis mossambicus*), a substantial fraction, *i.e.* 8%, of the total plasma T_3 pool was found to be glucuronidated (DiStefano et al., 1998). Extrahepatic sources of UGT isozymes are well known in mammals, and, indeed, synthesis and secretion of glucuronidated iodothyronines by fibroblasts obtained from neonatal rat heart have been observed (van der Heide et al., 2002). As are UGTs, sulfotransferases are not only involved in the detoxification of exogenous compounds in mammals, but also in the biotransformation of endogenous compounds such as steroids and iodothyronines in anatomical locations other than liver (Esfandiari et al., 1994; Falany et al., 1998). These observations strongly hint at a role of conjugating mechanisms other than merely the facilitation of plasma clearance through biliary excretion.

The extensive peripheral metabolism of thyroid hormones can be regarded as analogous to the posttranslational modifications that determine the unique biological actions of many mature

peptide hormones. Conjugation affects the physico-chemical properties of substrates; compared to native iodothyronines, conjugated thyroid hormones possess highly different biological activities and reactivities towards enzymes, transporters, biological membranes, receptors, and binding proteins (Hays and Hsu, 1987; Visser, 1994). Although unequivocal evidence is lacking, concerted thyroid hormone conjugations and deconjugations could well represent a mechanism for the local or systemic regulation of thyroid hormone bioavailability.

Na^+/K^+-ATPase is a pivotal enzyme involved in ion transport and osmoregulation, and its activity in branchial and renal epithelia of the euryhaline seawater teleost gilthead seabream (*Sparus auratus*) is regulated upon acclimation to different salinities (Sangiao-Alvarellos et al., 2005). In mammals, the expression of both the α- and β-subunits of the Na^+/K^+-ATPase holoprotein are sensitive to T_3 (Horowitz et al., 1990). Several studies pointed to the regulatory actions of T_3 on in-vitro and in-vivo teleost branchial, renal and hepatic Na^+/K^+-ATPase activities (De et al., 1987; Peter et al., 2000). This would make thyroid hormones *a priori* important determinants of osmoregulatory capacity in teleo sts.

We here investigated the hypothesis that altered activities of enzymes involved in deiodination, conjugation and deconjugation reactions reflect the involvement of thyroid hormone and its metabolites in the acclimation of gilthead sea bream (*Sparus auratus*) to low salinity conditions. Since fish gills comprise a major compartment in the circulation, and receive the entire cardiac output following systole, they are a site where modulation of plasma hormone titers can occur (Olson, 1998), and we have accordingly placed an emphasis on the putative role of the gills in thyroid hormone metabolism.

2. Material and Methods

2.1. Animals and animal procedures

Juvenile gilthead seabream (*Sparus auratus* L. 1758, hereafter called seabream) were from laboratory stock. Seabream were kept in artificial seawater of 35 parts per thousand (ppt) salinity

at 20°C and a photoperiod of 12 h light alternating with 12 h darkness. Artificial seawater was prepared by dissolving natural sea salt (Aqua Medic, Bissendorf, Germany) in demineralised water and allowing it to stabilize for at least 72 h. Fish were fed a commercially available feed (Pro-Aqua, Trouvit, The Netherlands) once daily at a ration of 1% of the estimated body weight. Two groups (n = 5 per group) of juvenile seabream (body weight 20-25 g), reared in artificial seawater were used. Seawater of the experimental group was diluted with tap water to reach a final salinity of 1 ppt. The salinity of the experimental tank fell gradually and exponentially with a first order rate constant of 0.16 day^{-1} from the initial salinity of 35 ppt on day 1 to 1 ppt after 18 days, which salinity was maintained for another 5 days after which the animals were euthanised. The half-value time, i.e. the time needed for the salinity in the experimental tank to fall to 50% of the initial value, was calculated to be 4.3 days. Control animals were kept in full-strength seawater at 35 ppt salinity throughout all experiments. No mortality was observed during the experiments. Fish were anesthetised in 0.1% 2-phenoxyethanol, and mixed arterial and venous blood was sampled by puncture from the caudal vessels. Blood plasma was obtained by centrifugation at 1000 × g for 5 min at 4°C, and stored at -20°C until further analyses. Animals were killed by spinal transection, and gills, liver and kidney were collected, homogenised in ice-cold 100-mM Na-phosphate buffer (pH 7.2) using a Potter-Elvehjem device, and stored at -80 °C until further analysis. All animal procedures were approved by the local ethical review committee.

2.2. Materials

Thyroxine (T_4), 3,5,3'-triiodo-L-thyronine (T_3), 3,3',5'-triiodo-L-thyronine (reverse T_3, rT_3), uridine 5'-diphosphate glucuronic acid (UDPGA), 3'-phospho-adenosine-5'-phosphosulfate (PAPS), *p*-nitrophenol (*p*NP), *p*NP sulfate, and phenolphthalein glucuronide were from Sigma Chemical Co. (St. Louis, MO). 3,5-Diiodo-L-thyronine (3,5-T_2) was from ICN Biochemicals, Inc. (Aurora, OH). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech Benelux (Roosendaal, The Netherlands). Outer ring radiolabeled [^{125}I]rT_3 (23.3 TBq/mmol) was obtained

from Perkin Elmer Life Science, Inc. (Boston, MA). All other chemicals were analytical grade and obtained from commercial suppliers. Outer ring labeled [^{125}I]T_4 and [^{125}I]T_3 were prepared by radioiodination of T_3 and 3,5-T_2, respectively, with carrier-free Na^{125}I using chloramine-T. Radiolabeled iodothyronines were purified shortly before use by Sephadex LH-20 column chromatography. Radioactivities were measured in an 1272-Clinigamma gamma counter (LKB/Wallac Oy, Turku, Finland). Protein concentrations were determined using a Coomassie Brilliant Blue G-250 kit (Bio-Rad, München, Germany) and bovine serum albumin as a standard.

2.3. RNA isolation and cDNA synthesis

RNA was isolated using the TRIzol reagent (Gibco BRL, Gaithersburg, US) according to the manufacturer's instructions. Total RNA was treated with 1 U DNase I (amplification grade; Gibco BRL) for 15 min at room temperature to remove DNA traces prior to reverse transcription. One µl 25 mM EDTA was then added and the sample was incubated for 10 min at 65°C to inactivate DNase and to linearise RNA. Complementary DNA was synthesised from 1 µg RNA with 300 ng random primers, 1 µl 10 mM dNTPs, 2 µl 0.1 M DTT, 10 U RNase inhibitor (Gibco BRL), and 200 U Superscript II RT reverse transcriptase (Gibco BRL) for 50 min at 37°C.

2.4. Real-time quantitative PCR

Primer sequences are listed in Table 1. The design of primers saD1.fw and saD1.rv for real-time quantitative PCR (RQ-PCR) of seabream deiodinase type 1 (saD1) was based on the published sequence for saD1 cDNA (Klaren et al., 2005). Primer sequences for the amplification of β-actin (ACT.fw and ACT.rv) were obtained from Pinto et al. (2003). RQ-PCR was performed by the addition of 5 µl cDNA, diluted to a cycle-threshold value of 21, and forward (fw) and reverse (rv) primers (at a final concentration of 300 nM each) to 12.5 µl Quantitect SYBR Green Master Mix (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands), and distilled

water to a final volume of 25 µl. A GeneAmp 5700 Sequence Detection System (Applied Biosystems) was used. The reaction mixture was incubated for 10 min at 95°C, followed by 40 two-step cycles that consisted of a 15-s denaturation step at 95°C, and 1 min annealing and extension at 60°C. Primers saD1.fw and saD1.rv were predicted to yield a 282-bp product, that was resolved by agarose gel electrophoresis. A melting curve was constructed, and the results confirmed the identity and specificity of the PCR product (results not shown).

2.5. Plasma thyroid hormone determinations

Plasma free T_4 (fT_4) and free T_3 (fT_3) concentrations were measured with a solid phase competitive ELISA (Research Diagnostics, Inc., Flanders, NJ) according to the manufacturer's instructions. The intra-assay and inter assay coefficients of variation for the fT_4 and fT_3 ELISA reported by the manufacturer are 4.5 and 3.7%, and 4.1 and 5.2%, respectively. The reported cross reactivity of the anti-T_4 monoclonal antibody to D-T_4 (at 10 µg/dl) is 98% (the reactivity to L-T_4 is set at 100% as a reference), and to L-T_3 and D-T_3 (both at 100 µg/dl) is 3 and 1.5%, respectively. The reported cross reactivity of the anti-T_3 monoclonal to L-T_4 (at 10 µg/ml) is < 0.02% (setting the reactivity to L-T_3 at 100%). Cross reactivities of both monoclonals to diiodothyronine, diiodotyrosine and iodotyrosine (all at 10 µg/ml) are = 0.01%. Quantitative recoveries of the fT_4 and fT_3 ELISA are 97 and 96%, respectively.

2.6. Enzyme assays

Na^+/K^+-ATPase. Na^+/K^+-ATPase activities in homogenates are here defined as the ouabain-sensitive and K^+-dependent liberation of inorganic phosphate from ATP in the presence of 100 mM Na^+, using a colorimetric method described by Flik et al. (1983) that was adapted for 96-wells microplates. Briefly, approximately 10 µg homogenate protein was incubated in triplicate in 100 µl buffer (100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 30 mM imidazole/ HEPES at pH 7.4) to which 3 mM Na_2ATP, and either 1 mg/ml KCl or 1 mg/ml ouabain were added. After a 10-

160 min incubation at 37°C, the reaction was terminated with 100 µl ice-cold 10% trichloroacetic acid (TCA). One hundred µl of an acid molybdate reagent was added, and the amount of inorganic phosphate was measured at 700 nm against a KH_2PO_4-standard.

Sulfotransferase. Sulfotransferase activities were measured in duplicate with *p*NP, T_4 or T_3 as conjugate group acceptors. PAPS was used as the sulfate group donor. Sulfotransferase 165 activity towards *p*NP was measured according to Ohkimoto et al. (2003). Briefly, approximately 50 µg homogenate protein was incubated for 30 min at 37°C in 200 µl 50 mM Na-phosphate buffer (pH 7.0), containing 50 µm PAPS and 50 µm *p*NP. The specific sulfotransferase activity was calculated from the amount of unconjugated *p*NP remaining after the enzyme reaction was terminated by the addition of 100 µl ice-cold 5% trichloroacetic acid (TCA). The incubate was 170 alkalinized by the addition of 500 µl glycine/NaOH buffer at pH 12.9, and unconjugated *p*NP was quantified spectrophotometrically at 405 nm against a *p*NP standard. Similarly, the sulfation of T_4 and T_3 was measured by the incubation of approximately 200 µg homogenate protein at 37°C for 30 min in 200 µl buffer composed of 100 mM Na-phosphate buffer and 2 mM EDTA (pH 7.2), 1 µM ^{125}I-labeled T_4 or T_3 and 50 µM PAPS. The reaction was terminated with 800 µl 175 ice-cold 0.1 M HCl, and the quenched incubate was applied to Sephadex LH-20 minicolumns (2 ml of a 10% w/v suspension) to resolve liberated iodide, water-soluble conjugates and native iodothyronines, respectively, as described in detail previously (van der Heide et al., 2002). Radioiodide activities in the water-soluble fractions were interpreted to have originated from the presence of sulfated iodothyronines. Control incubations in these assays were in the absence of 180 PAPS. Net sulfotransferase activities are expressed as a percentage of the total sum of all fractions of the Sephadex-LH20 chromatograms.

UDP glucuronyltransferase (UGT). UGT activities were measured in duplicate with *p*NP, T_4 or T_3 as conjugate group acceptors. UDPGA was used as the glucuronosyl group donor. The glucuronidation of *p*NP by organ homogenates was measured in duplicate as described in detail 185 previously (van der Heide et al., 2004). Briefly, 50 µg homogenate protein was incubated for 15

min at 37°C in 200 µl buffer containing 100 mM Tris/HCl (pH 7.4), 5 mM $MgCl_2$, and 0.05% Brij56, to which 1 mM *p*NP and 5 mM UDPGA were added. The reaction was quenched with 3.8 ml ice-cold 0.1 M NaOH, and unconjugated *p*NP was quantified spectrophotometrically at 405 nm against a *p*NP standard. The glucuronidation of T_4 and T_3 was measured by the incubation of 10 µg homogenate protein at 37°C for 60 min in 200 µl of the same Tris/HCl buffer, supplemented with 1 µM ^{125}I-labeled T_4 or T_3 and 5 mM UDPGA. The reaction was quenched with 200 µl ice-cold methanol, and the incubate centrifuged for 10 min at 1500 × *g*. To 300 µl of the supernatant thus obtained 700 µl 0.1 M HCl was added, and the mixture was subjected to Sephadex LH-20 column chromatography as described above. Radioiodide activities in the water-soluble fractions were here interpreted to have originated from the presence of glucuronidated iodothyronines. Control incubations were in the absence of UDPGA.

Arylsulfatase. Arylsulfatase activities were measured spectrophotometrically as described by Daniel et al. (1987). Briefly, 50 µg homogenate protein was incubated in duplicate for 15 min at 37°C in 1 ml 100 mM acetate buffer (pH 5.9) containing 1.5 mM *p*NP sulfate as the substrate for deconjugation. The reaction was terminated by the addition of 500 µl ice-cold 5% TCA, and alkalinized with 2 ml 0.5 M NaOH. *Para*-nitrophenol, derived from the deconjugation of *p*NP sulfate, was quantified spectro photometrically at 405 nm against a *p*NP standard.

ß-Glucuronidase. ß-Glucuronidase activities were measured spectrophotometrically as described by Fishman (1974). Briefly, 100 µg homogenate protein was incubated in duplicate for 60 min at 37°C in 250 µl 100 mM acetate buffer (pH 4.5) containing 1 mM phenolphthalein glucuronide. The reaction was terminated with 250 µl ice-cold 5% TCA, and 400 µl glycine/NaOH buffer (pH 12.9) was added to adjust the pH of the incubate to a value of 10.5 (phenolphthalein's colour change is complete at pH 10.0). Phenolphthalein, derived from the deconjugation of phenolphthalein glucuronide, was quantified spectrophotometrically at 540 nm against a phenolphthalein standard.

5'-Deiodinase. 5'-Deiodinase activities were measured in duplicate as the outer ring deiodination (ORD) of rT_3 as described in detail elsewhere (Klaren et al., 2005). Briefly, 50 µg homogenate protein was incubated under saturating substrate conditions of 20 µM rT_3 in 200 µl 100 mM Na-phosphate buffer (pH 7.2). Outer ring labeled [^{125}I]rT_3 was used as a tracer, and was purified on a 10% (w/v) Sephadex LH-20 mini-column shortly before use. The reaction was quenched by the addition of 100 µl ice-cold 5% BSA, followed by 500 µl ice-cold 10% TCA, and centrifuged at 1400 × *g* (15 min, 4°C). To 500 µl of the deproteinized supernatant thus obtained an equal volume of 1.0 M HCl was added, and liberated iodide was separated from the native iodothyronine using Sephadex LH-20 column chromatography. Non-enzymatic outer ring deiodination was determined in the absence of a preparation.

2.7. Calculations and statistics

Mean values ± standard deviation (SD) are presented, unless indicated otherwise. The number of observations (*n*) is in parentheses. Calibration curves were constructed, and plasma hormone concentrations were calculated from non-transformed spectrophotometer readings, using a cubic polynomial interpolation routine that is available in the XNUMBERS multi-precision floating point computation utility add-in (version 3.1) for Microsoft® Excel. Statistical significance was evaluated with Student's *t*-test or Welch's alternate *t*-test, where appropriate, and was accepted at $P < 0.05$ (two-tailed).

3. Results

Plasma fT_4 concentrations in seabream had increased 2.5-fold from 51 ± 22 to 127 ± 41 pM ($P < 0.01$, $n = 5$) following acclimation to 1-ppt low salinity water. Free T_3 levels remained unchanged following the same treatment, and amounted to 46 ± 0.7 and 45 ± 1.9 pM ($P = 0.32$, $n = 5$) in control and low-salinity water acclimated fish, respectively.

Branchial, renal and hepatic Na^+/K^+-ATPase activities were similar in the group acclimated to low-salinity water and the control group (Fig. 1). Conjugating and deconjugating enzyme activities in liver, kidney and gills responded differentially to the salinity change (Fig. 2). In low salinity water the hepatic sulfotransferase activity had reduced to 4% of its control value measured in seawater fishes, and arylsulfatase activities had increased 2- to 3.6-fold in kidney, liver and gills. A reciprocal relationship between conjugating and deconjugating enzymes was less obvious when UGT and ß-glucuronidase activities were analysed. Per enzyme, the specific activities measured in control fish were all in the same order of magnitude, but were generally highest in liver preparations, followed by kidney and gills. A notable exception was the branchial specific UGT activity, which was 50 to 100% higher than in kidney and liver (Fig. 2).

The most pronounced effects were measured in hepatic sulfotransferase and branchial UGT activities, and these were further investigated using T_4 and T_3 as endogenous acceptor substrates. Fig. 3 shows that, upon the addition of the sulfate group donor PAPS to hepatic homogenates, a small but consistent amount of iodothyronine sulfates was produced. The amounts of T_4 sulfate produced in hepatic homogenates from control seabream and those acclimated to low salinity water were 0.7 ± 0.3 and 0.9 ± 0.4% ($P = 0.40$, $n = 4$). Similarly, the amounts of T_3 sulfate produced were 0.3 ± 0.2 and 0.4 ± 0.2% ($P = 0.45$).

No UDPGA-dependent glucuronidation of T_4 and T_3 by gill homogenates could be demonstrated as no water-soluble ^{125}I-radioactivity was detected (Fig. 4). Instead, outer-ring deiodination (ORD) activities were detected. The amount of T_4-derived free ^{125}I-radioiodide was 71 ± 3 and 48 ± 9% ($P < 0.001$, $n = 5$) for control fish and low salinity water-acclimated fish, respectively. Similarly, upon incubation with T_3 the amount of free ^{125}I-radioiodide was 65 ± 4 and 44 ± 3% ($P < 0.0001$). In-vitro assays with rT_3, the preferred substrate for many vertebrate D1 enzymes, showed rT_3 ORD activities in liver, kidney and, also, in gills (Table 2). Highest activities were measured in the gills, and were approximately 5 times higher than those in liver and kidney homogenates. ORD activities in liver and gills were decreased by 20 and 30%,

respectively, upon acclimation to low salinity water. Although consistent with the 32% reduction in branchial T_4 and T_3 ORD observed upon acclimation to low salinity, these results did not reach statistical significance. Renal rT_3 ORD activity did not change upon acclimation to low salinity water. In a seawater-adapted seabream , saD1 expression was detected in kidney and liver, not in gills, pituitary gland, brain, skin, intestine, heart, and skeletal muscle (results not shown).

4. Discussion

We show here, in *Sparus auratus*, that the plasma free T_4 concentration and branchial ORD activity, two key role players of the thyroid hormone status of fish, respond to the acclimation to low salinity conditions. This indicates the involvement of the thyroidal system in seabream osmoregulation. The in-vitro ORD capacity in the gills in particular is substantial, and adjusted to ambient salinity. This indicates an important role of the gills in the metabolism of thyroid hormones and, hence, the modulation of plasma thyroid hormone levels in the acclimation of seabream to lowered salinity conditions.

Despite the high rT_3 ORD activities measured in branchial preparations, which were approximately 5 times higher than in liver and kidney preparations, no saD1 mRNA expression could be detected in gills of seawater-adapted seabream. By deduction, it must be concluded that a deiodinase type 2 (D2) is involved in branchial ORD in seabream. D2 mRNA transcripts have been detected in Australian lungfish (*Neoceratodus forsteri*) gills (Sutija et al., 2003), but not in gills of rainbow trout (Sambroni et al., 2001). Due to the scarcity of data, it is difficult to construct a generalised tissue distribution of deiodinases in fishes. Indeed, the tissue distribution of seabream D1 mRNA we show here, with positive signals only for liver and kidney preparations, differs distinctly from that in Nile tilapia (*Oreochromis niloticus*) where abundant expression and activity were measured in the kidney, but intermediate levels in the gills and none in the liver (Sanders et al., 1997). The high ORD activities towards T_4, T_3 and rT_3 measured in seabream gills, that were responsive to a hypoosmotic challenge, are indicative for an important role of branchial tissue in

thyroid hormone metabolism in seabream. A similar role, but then in response to a mild hyperosmotic challenge, has been suggested for rainbow trout gills (Orozco et al., 2002).

The substantial branchial sulfotransferase and UGT activities, of which only sulfotransferase accepted thyroid hormones as acceptor substrates, confer a conjugating capacity to the gills of seabream. Conjugating enzyme activities were not responsive to low salinity water acclimation of seabream, however. The sulfation of *p*NP in liver homogenates had significantly decreased upon acclimation of seabream to low salinity water, whereas the hepatic sulfation of T_4 and T_3 remained unchanged. This indicates that different sulfotransferase isozymes are involved in the conjugation of *p*NP and iodothyronines. Indeed, sulfotransferases from zebrafish (*Danio rerio*) display markedly different affinities for phenolic compounds, thyroid hormones, and steroids alike (Ohkimoto et al., 2003; Sugahara et al., 2003). The increased arylsulfatase activity in livers and kidneys, and the decreased sulfotransferase activity in livers of low salinity water-acclimated seabream, point to a shift in the equilibrium between conjugation and deconjugation reactions in favour of non-sulfated, native compounds. However, the 96%-decrease in hepatic *p*NP-sulfotransferase activity observed upon acclimation to low salinity water was not reflected in a reduced hepatic sulfation of T_4 or T_3, and it appears therefore that thyroid hormone levels are not regulated via sulfating and desulfating reactions.

Sulfotransferase and UGT isozymes show overlapping but distinct acceptor substrate specificities, accepting thyroid hormones, steroids, phenols, and monoamines as conjugate group acceptors (Burchell and Coughtrie, 1989; Falany, 1997). The physiological importance of conjugated hormones, although their precise role is still poorly understood, is reflected in a number of different observations. Estrogen sulfotransferase deficient mice exhibited spontaneous fetal loss, which pathology was associated with elevated systemic and amniotic free estrogen levels (Tong et al., 2005). When the testis from the elasmobranch *Squalus acanthias* was perfused through the genital artery, androgens and estrogen were extensively metabolized and appeared as sulfates and glucuronides in the perfusate (Cuevas et al., 1992). Rat cardiac

fibroblasts, but not myocytes, synthesize and secrete glucuronidated iodothyronine conjugates *in vitro*, and it was subsequently shown that cells from the embryonic rat heart ventricle cell line H9c2(2-1) preferentially take up glucuronidated T_4 and T_3 over the native, unconjugated hormones (van der Heide et al., 2007; van der Heide et al., 2002). These findings support the role of conjugating and deconjugating enzymes in the modulation of hormone bioactivities in an endocrine or paracrine fashion. It remains to be investigated whether the activities of sulfotransferases and UGTs, and their deconjugating counter-enzymes, similarly affect hormone bioactivity in seabream.

Olson (1998) described a number of enzymes that are involved in the branchial metabolism of regulatory molecules such as angiotensin I, catecholamines, and arachidonic acid metabolites. In seabream, specifically, several peptidases have been detected in gill tissue (Agirregoitia et al., 2005), and this confers a capacity to metabolize bioactive peptides to the gills. The demonstration of branchial ORD *in vitro* by our work on seabream, and that of colleagues on other teleost species (Adams et al., 2000; Eales et al., 1993; Mol et al., 1998; Moore VanPutte et al., 2001; Orozco et al., 2000; Orozco et al., 2002), demonstrate that thyroid hormones, too, are subject to activating and deactivating pathways in the gills, and emphasises that the gills play an important role in modulating plasma hormone titers.

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References

Adams, B.A., Cyr, D.G., Eales, J.G., 2000. Thyroid hormone deiodination in tissues of American plaice, *Hippoglossoides platessoides*: characterization and short-term responses to polychlorinated biphenyls (PCBs) 77 and 126. Comp. Biochem. Physiol. C 127, 367-378.

Agirregoitia, N., Laiz-Carrión, R., Varona, A., Martín del Río, M.P., Mancera, J.M., Irazusta, J., 2005. Distribution of peptidase activity in teleost and rat tissues. J. Comp. Physiol. B 175, 433-444.

Burchell, B., Coughtrie, M.W., 1989. UDP-glucuronosyltransferases. Pharmacol. Ther. 43, 261-289.

Cuevas, M.E., Miller, W., Callard, G., 1992. Sulfoconjugation of steroids and the vascular pathway of communication in dogfish testis. J. Exp. Zool. 264, 119-129.

Daniel, W.L., Leonard, L., Carmody, T., 1987. Comparative biochemistry of fish and amphibian arylsulfatases. Comp. Biochem. Physiol. B 87, 385-390.

De, S., Ray, A.K., Medda, A.K., 1987. Nuclear activation by thyroid hormone in liver of Singi fish: changes in different ion-specific adenosine triphosphatases activities. Horm. Metab. Res. 19, 367-370.

DiStefano, J.J., III, Ron, B., Nguyen, T.T., Weber, G.M., Grau, E.G., 1998. 3,5,3'-Triiodothyronine (T_3) clearance and T_3-glucuronide (T_3G) appearance kinetics in plasma of freshwater-reared male tilapia, *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 111, 123-140.

Eales, J.G., Morin, P.P., Tsang, P., Hara, T.J., 1993. Thyroid hormone deiodination in brain, liver, gill, heart and muscle of Atlantic salmon (*Salmo salar*) during photoperiodically-induced parr-smolt transformation. II. Outer- and inner-ring 3,5,3'-triiodo-L-thyronine and 3,3',5'-triiodo-L-thyronine (reverse T3) deiodination. Gen. Comp. Endocrinol. 90, 157-167.

Esfandiari, A., Gavaret, J.-M., Lennon, A.M., Pierre, M., Courtin, F., 1994. Sulfation after deiodination of 3,5,3'-triiodothyronine in rat cultured astrocytes. Endocrinology 135, 2086-2092.

Falany, C.N., 1997. Enzymology of human cytosolic sulfotransferases. FASEB J. 11, 206-216.

Falany, J.L., Azziz, R., Falany, C.N., 1998. Identification and characterization of cytosolic sulfotransferases in normal human endometrium. Chem.-Biol. Interact. 109, 329-339.

Finnson, K.W., Eales, J.G., 1996. Identification of thyroid hormone conjugates produced by isolated hepatocytes and excreted in bile of rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 101, 145-154.

Finnson, K.W., Eales, J.G., 1998. Sulfation of thyroid hormones by liver of rainbow trout, *Oncorhynchus mykiss*. Comp. Biochem. Physiol. C 120, 415-420.

Fishman, W.H., 1974. β-Glucuronidase. In: Bergmeyer, H. U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 929-943.

Flik, G., Wendelaar Bonga, S.E., Fenwick, J.C., 1983. Ca^{2+}-dependent phosphatase and ATPase activities in eel gill plasma membranes. I. Identification of Ca^{2+}-activated ATPase activities with non-specific phosphatase activities. Comp. Biochem. Physiol. B 76, 745-754.

Hays, M.T., Hsu, L., 1987. Preparation and separation of the glucuronide and sulfate conjugates of thyroxine and triiodothyronine. Endocr. Res. 13, 215-228.

Horowitz, B., Hensley, C.B., Quintero, M., Azuma, K.K., Putnam, D., McDonough, A.A., 1990. Differential regulation of Na,K-ATPase α1, α2, and β subunit mRNA and protein levels by thyroid hormone. J. Biol. Chem. 265, 14308-14314.

Klaren, P.H.M., Haasdijk, R., Metz, J.R., Nitsch, L.M.C., Darras, V.M., Van der Geyten, S., Flik, G., 2005. Characterization of an iodothyronine 5'-deiodinase in gilthead seabream (*Sparus auratus*) that is inhibited by dithiothreitol. Endocrinology 146, 5621-5630.

Mol, K.A., Van der Geyten, S., Burel, C., Kühn, E.R., Boujard, T., Darras, V.M., 1998. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean fishes. Fish Physiol. Biochem. 18, 253-266.

Moore VanPutte, C.L., MacKenzie, D.S., Eales, J.G., 2001. Characterization of hepatic low-K_m outer-ring deiodination in red drum (*Sciaenops ocellatus*). Comp. Biochem. Physiol. B 128, 413-423.

Ohkimoto, K., Sugahara, T., Sakakibara, Y., Suiko, M., Liu, M.-Y., Carter, G., Liu, M.-C., 2003. Sulfonation of environmental estrogens by zebrafish cytosolic sulfotransferases. Biochem. Biophys. Res. Commun. 309, 7-11.

Olson, K.R., 1998. Hormone metabolism by the fish gill. Comp. Biochem. Physiol. A 119, 55-65.

Orozco, A., Linser, P., Valverde-R, C., 2000. Kinetic characterization of outer-ring deiodinase activity (ORD) in the liver, gill and retina of the killifish *Fundulus heteroclitus*. Comp. Biochem. Physiol. B 126, 283-290.

Orozco, A., Villalobos, P., Valverde-R, C., 2002. Environmental salinity selectively modifies the outer-ring deiodinating activity of liver, kidney and gill in the rainbow trout. Comp. Biochem. Physiol. A 131, 387-395.

Peter, M.C.S., Lock, R.A.C., Wendelaar Bonga, S.E., 2000. Evidence for an osmoregulatory role of thyroid hormones in the freshwater Mozambique tilapia *Oreochromis mossambicus*. Gen. Comp. Endocrinol. 120, 157-167.

Pinto, J.P., Conceição, N., Gavaia, P.J., Cancela, M.L., 2003. Matrix Gla protein gene expression and protein accumulation colocalize with cartilage distribution during development of the teleost fish *Sparus aurata*. Bone 32, 201-210.

Sambroni, E., Gutieres, S., Cauty, C., Guiguen, Y., Breton, B., Lareyre, J.-J., 2001. Type II iodothyronine deiodinase is preferentially expressed in rainbow trout (*Oncorhynchus mykiss*) liver and gonads. Mol. Reprod. Dev. 60, 338-350.

Sanders, J.P., Van der Geyten, S., Kaptein, E., Darras, V.M., Kühn, E.R., Leonard, J.L., Visser, T.J., 1997. Characterization of a propylthiouracil-insensitive type I iodothyronine deiodinase. Endocrinology 138, 5153-5160.

Sangiao-Alvarellos, S., Arjona, F.J., Martín del Río, M.P., Miguez, J.M., Mancera, J.M., Soengas, J.L., 2005. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. J. Exp.Biol. 208, 4291-4304.

Sinclair, D.A., Eales, J.G., 1972. Iodothyronine-glucuronide conjugates in the bile of brook trout, *Salvelinus fontinalis* (Mitchill) and other freshwater teleosts. Gen. Comp. Endocrinol. 19, 552-598.

Sugahara, T., Liu, C.-C., Pai, T.G., Liu, M.-C., 2003. Molecular cloning, expression, and functional characterization of a novel zebrafish cytosolic sulfotransferase. Biochem. Biophys. Res. Commun. 300, 725-730.

Sutija, M., Longhurst, T.J., Joss, J.M., 2003. Deiodinase type II and tissue specific mRNA alternative splicing in the Australian lungfish, *Neoceratodus forsteri*. Gen. Comp. Endocrinol. 132, 409-417.

Tong, M.H., Jiang, H., Liu, P., Lawson, J.A., Brass, L.F., Song, W.C., 2005. Spontaneous fetal loss caused by placental thrombosis in estrogen sulfotransferase-deficient mice. Nature Med. 11, 153-159.

van der Heide, S.M., Joosten, B.J.L.J., Dragt, B.S., Everts, M.E., Klaren, P.H.M., 2007. A physiological role for glucuronidated thyroid hormones: preferential uptake by H9c2(2-1) myotubes. Mol. Cell. Endocrinol. 264, 109-117.

van der Heide, S.M., Joosten, B.J.L.J., Everts, M.E., Klaren, P.H.M., 2004. Activities of UDP-glucuronyltransferase, β-glucuronidase and deiodinase types I and II in hyper- and hypothyroid rats. J. Endocrinol. 181, 393-400.

van der Heide, S.M., Visser, T.J., Everts, M.E., Klaren, P.H.M., 2002. Metabolism of thyroid hormones in cultured cardiac fibroblasts of neonatal rats. J. Endocrinol. 174, 111-119.

Visser, T.J., 1994. Role of sulfation in thyroid hormone metabolism. Chem.-Biol. Interact. 92, 293-303.

Visser, T.J., 1996. Pathways of thyroid hormone metabolism. Acta Med. Austr. 23, 10-16.

Wu, S.-y., Green, W.L., Huang, W.-s., Hays, M.T., Chopra, I.J., 2005. Alternate pathways of thyroid hormone metabolism. Thyroid 15, 943-958.

Legends to Figures

Figure 1. Specific Na^+/K^+-ATPase activities in hepatic, renal, and branchial homogenates from control (closed bars) and low salinity water-acclimated (open bars) seabream. Mean values ± SD ($n = 5$) are shown.

Figure 2. Specific enzyme activities in hepatic, renal, and branchial homogenates from control seabream (closed bars) and low salinity water-acclimated seabream (open bars). Mean values ± SEM ($n = 5$) are shown. Specific activities of UGT, arylsulfatase, and sulfotransferase are expressed in nmol *p*NP/h per mg protein, the specific ß-glucuronidase activity is expressed in nmol phenolphthalein/h per mg protein. Asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$) compared to the SW control group.

Figure 3. Sephadex LH-20 chromatograms of incubates obtained from hepatic homogenates incubated with the sulfate group donor PAPS and T_4 (left hand panels) or T_3 (right hand panels). The horizontal bar indicates the fractions collected with 0.1 M HCl, distilled water (dH_2O), and 1 M NH_3 in ethanol (NH_3/EtOH) that contain liberated iodide, water-soluble conjugates (here: sulfates) and native iodothyronines, respectively. Closed (●, ▲) and open symbols (○, △) indicate liver homogenates from control animals and animals acclimated to low salinity water, respectively. Triangles (▲, △) and dotted lines indicate control incubations in the absence of PAPS; circles (●, ○) and solid lines indicate incubations in the presence of PAPS ($n = 4$, mean ± SD).

Figure 4. Sephadex LH-20 chromatograms of incubates obtained from branchial homogenates incubated with the glucuronyl group donor UDPGA and T_4 (top panel) or T_3 (bottom panel). The horizontal bar indicates the fractions collected with 0.1 M HCl, distilled water (dH_2O), and 1 M NH_3 in ethanol (NH_3/EtOH) that contain liberated iodide, water-soluble conjugates (here: glucuronides) and native iodothyronines, respectively (●, control (seawater); ○, low salinity water) ($n = 4$, mean ± SD).

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Table 1. Primer sequences with corresponding DDBJ/EMBL/GenBank database accession numbers and *Sparus auratus* genes.

Gene	Accession number	Primer	Sequence 5' → 3'
Deiodinase type 1	AJ619717	saD1.fw	CGCTAGCAGGCATATTTTGC
		saD1.rv	GGTCCAACATTTGGCTCGAA
β-actin	X89920	ACT.fw	TTCCTCGGTATGGAGTCC
		ACT.rv	GGACAGGGAGGCCAGGA

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Table 2. Outer ring deiodination (ORD) activities towards rT_3 in hepatic, renal, and branchial homogenates from control and low salinity water-acclimated seabream. Activities are expressed as fmol/min per mg homogenate protein ($n = 5$, mean ± SD).

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Preparation	Seawater (control)	Low salinity water	*P*
Liver	91 ± 33	64 ± 22	0.17
Kidney	83 ± 25	80 ± 20	0.84
Gills	447 ± 111	356 ± 63	0.15

Fig.1

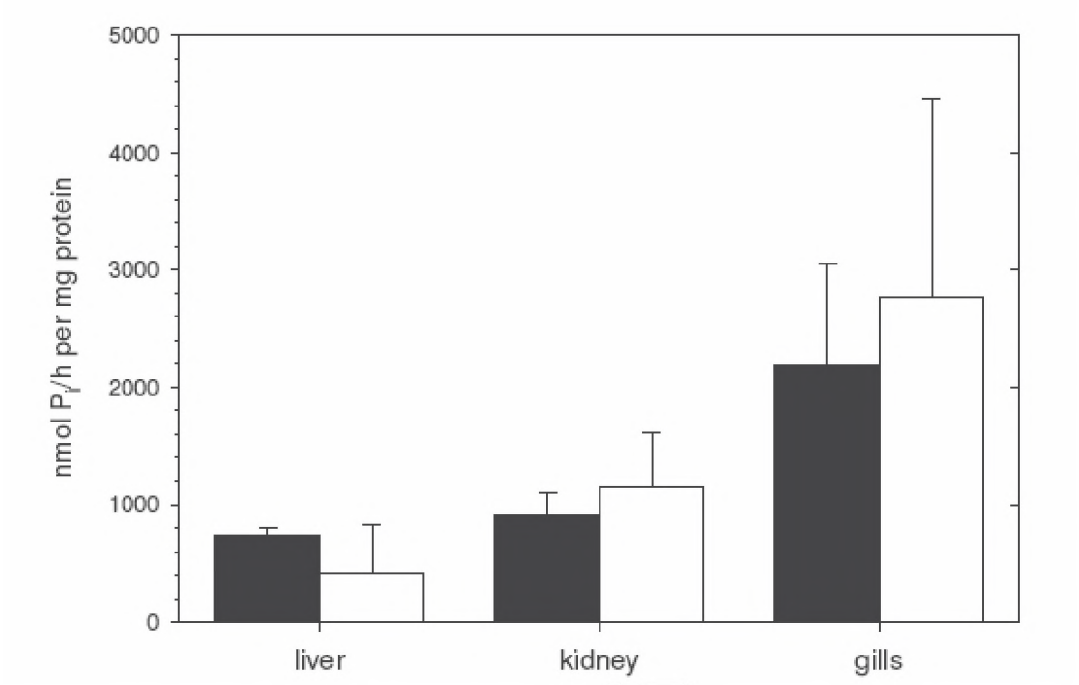
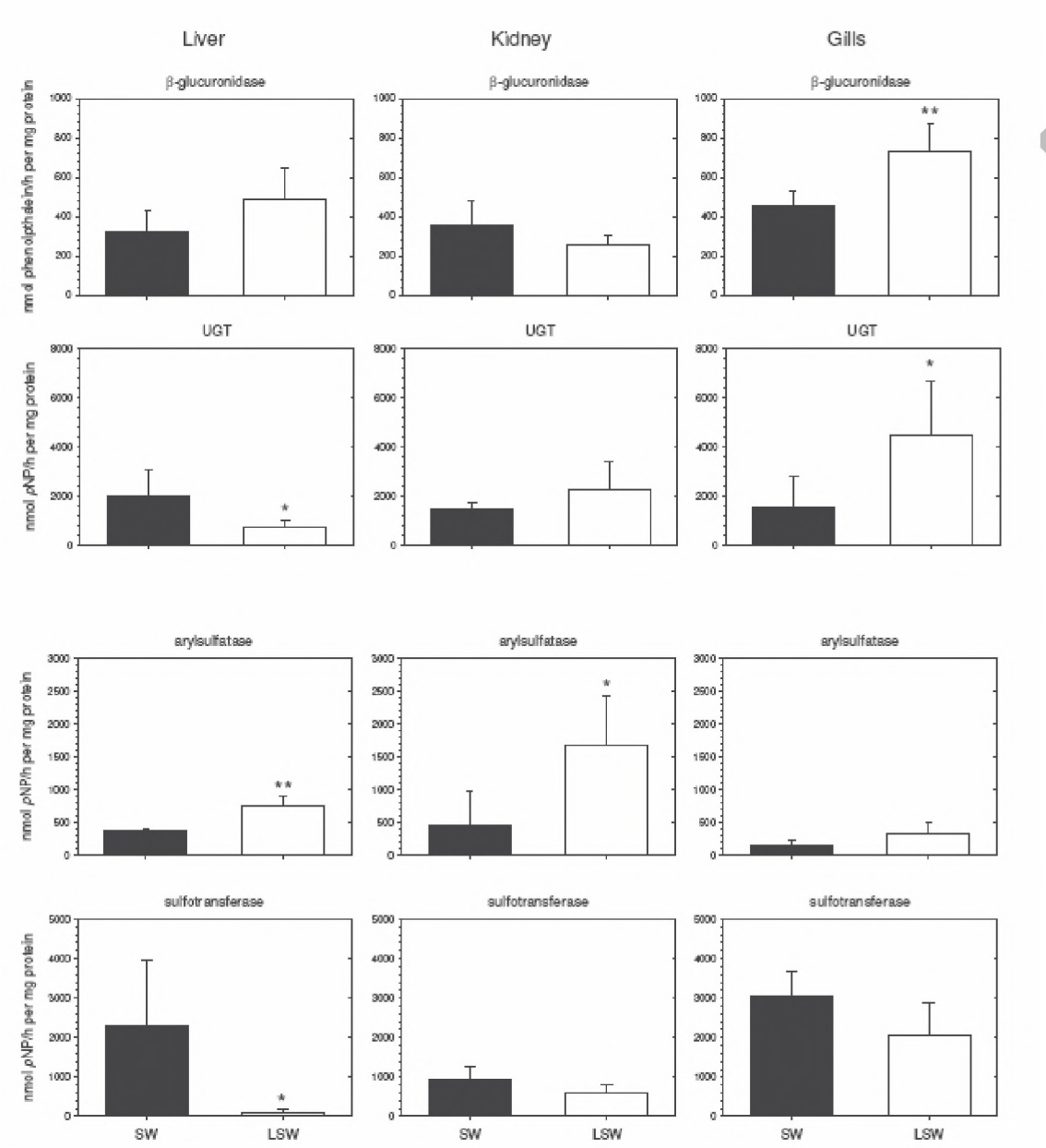


Fig.2



490

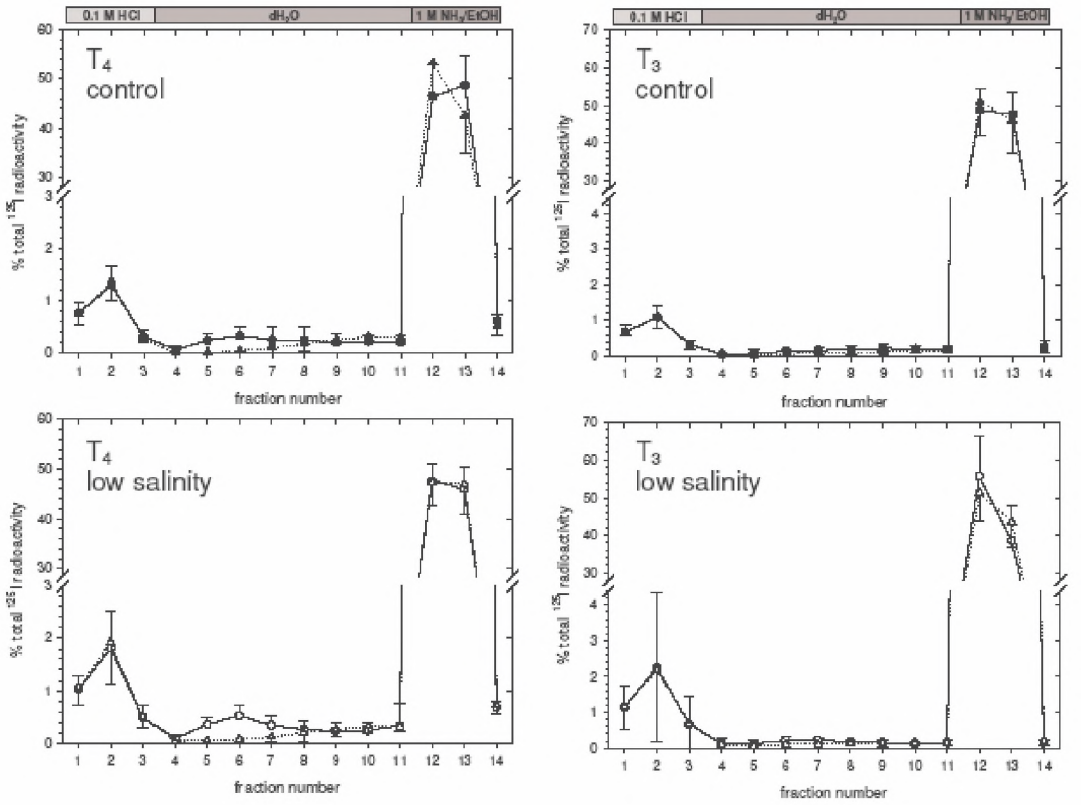


Figure 3

Fig.4