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ScienceDirect
General and Comparative Endocrinology 155 (2008) 796–803
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

The involvement of thyroid hormones and cortisol in the osmotic acclimation of *Solea senegalensis*

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Received 3 July 2007; revised 5 September 2007; accepted 6 September 2007
Available online 19 September 2007

Abstract

The peripheral conversion of the prohormone 3,5,3′,5′-tetraiodothyronine (T4) to the biologically active 3,5,3′-triiodothyronine (T3), via enzymatic deiodination by deiodinases, is an important pathway in thyroid hormone metabolism. The aim of this study was to test if thyroid hormones and cortisol, as well as the outer ring deiodination (ORD) metabolic pathway, are involved in the osmoregulatory response of Senegalese sole (*Solea senegalensis*, Kaup 1858). We measured osmoregulatory and endocrine parameters in immature juveniles *S. senegalensis* acclimated to seawater (SW, 38‰) and that were transferred and allowed to acclimate to different salinities (5‰, 15‰, 38‰ and 55‰) for 17 days. An adjustment and a chronic regulatory period were identified following acclimation. The adjustment period immediately follows the transfer, and is characterized by altered plasma osmolalities. During this period, plasma cortisol levels increased while plasma free T4 (fT4) levels decreased. Both hormones levels returned to normal values on day 3 post-transfer. In the adjustment period, renal and hepatic ORD activities had increased concomitantly with the decrease in plasma fT4 levels in fishes transferred to extreme salinities (5‰ and 55‰). In the chronic regulatory period, where plasma osmolality returned to normal values, plasma cortisol had increased, whereas plasma fT4 levels decreased in animals that were transferred to salinities other than SW. No major changes were observed in branchial ORD activity throughout the experiment. The inverse relationship between plasma cortisol and fT4 suggests an interaction between these hormones during both osmoregulatory periods while ORD pathway can be important in the short-term adjustment period.

Keywords: *Solea senegalensis*; Cortisol; Thyroid hormones; Outer ring deiodination; Osmoregulation

1. Introduction

The main end product from the hypothalamo-pituitary-thyroid (HPT) axis is the prohormone thyroxine (3,5,3′,5′-tetraiodothyronine, T4). In extrathyroidal tissues, enzymatic outer ring deiodination (ORD) removes an iodine atom from the phenolic ring of the T4 molecule to yield the potently biologically active hormone 3,5,3′-triiodothyronine (T3). Two iodothyronine deiodinases, type 1 (D1) and type 2 (D2), are involved in ORD. Fish liver, kidney, and gills express high ORD activities (Byamungu et al., 1992; Mol et al., 1997, 1998; Klaren et al., 2007; Walpita et al., 2007). Differential expression of deiodinases allows for the tissue-specific regulation of local, intracellular concentrations of T3 (Leonard and Köhrle, 2000; Orozco and Valverde-R, 2005). Deiodinases are thus important determinants of the thyroidal status of an animal, and are key parameters in assessing the activity of the thyroid system. Indeed, in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus auratus*), osmotic challenges alter ORD activities in gills, liver and kidney (Orozco et al., 2002; Klaren et al., 2007). Although scarce and not fully consistent, the available data on the response of the thyroid gland and associated peripheral metabolic pathways to alterations in environmental salinity suggest a close inter-

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doi:10.1016/j.ygcen.2007.09.007

action between the thyroid system and hydromineral regulation in teleosts, but a generalized picture cannot, as yet, be constructed.

Cortisol, secreted by the interrenal cells of the head kidney, is a potent gluco- and mineralocorticoid in teleostean fish. It plays a pivotal role in the stress response and in osmoregulatory processes (Wendelaar Bonga, 1997; McCormick, 2001; Flik et al., 2006). The hypothalamo-pituitary-interrenal axis also modulates the thyroid axis in fishes and other vertebrates (reviewed in Kühn et al., 1998). Indeed, heterologous CRH potently stimulated the release of TSH from cultured pituitary cells from coho salmon (*Oncorhynchus kisutch*) *in vitro* (Larsen et al., 1998). Also, thyrotropic actions of centrally administered CRH in goldfish (*Carassius auratus*) *in vivo* have been reported (de Pedro et al., 1995). Effects of peripherally administered hormones were observed in European eel (*Anguilla anguilla*) where cortisol treatment reduced plasma T4 levels; a similar same treatment had no effect in *O. mykiss*, however (Redding et al., 1986, 1991; Leatherland, 1987). Common carp (*Cyprinus carpio*) with experimentally induced hyperthyroidism developed a marked hypocortisolemia, and this correlated with increased hypothalamic mRNA levels of CRH-binding protein, an endogenous CRH antagonist (Geven et al., 2006). A synergistic effect of thyroid hormone and cortisol was observed on cultured pavement cell epithelia *in vitro* from *O. mykiss* where, compared with T3 alone, a co-incubation of T3 and cortisol decreased unidirectional Na^+- and Cl^--fluxes under symmetrical culture conditions (Kelly and Wood, 2001). However, in killifish (*Fundulus heteroclitus*) no evidence of cortisol-T3 synergism in osmoregulation could be established *in vivo* (Mancera and McCormick, 1999). The peripheral interactions of glucocorticoids with the thyroid axis in fishes, in particular in the context of osmoregulation, are far from completely understood.

The involvement of thyroid hormones proper in the regulation of hydromineral balance in non-mammalian vertebrates is not yet fully elucidated (Schreiber and Specker, 1999). Most information on the effects of osmoregulatory demands on thyroid function in fish stems from studies on smoltifying Atlantic salmon (*Salmo salar*). Thyroid hormones are pivotal in salmonid parr-smolt transformation (increasing branchial Na^+,K^+-ATPase activity and epithelial density of mitochondria-rich cells) to allow acclimation to altered environmental salinities (Dickhoff et al., 1978; Grau et al., 1981; Prunet et al., 1989; McCormick, 2001). These processes have been studied in feral, migrating populations where confounders other than environmental salinity (e.g. seasonality and photoperiod, nutritional state, sexual and somatic maturation) cannot be controlled, however.

The Senegalese sole (*Solea senegalensis*, Kaup 1858), is an euryhaline teleost from the coastal waters of the Eastern Atlantic. This species experiences considerable changes in water salinity and temperature in estuarine habitats (Imsland et al., 2003), and thus constitutes an appropriate model to study teleost osmoregulation (Arjona et al., 2007). *S. senegalensis* also becomes increasingly important in aquaculture which is centred in coastal ponds with a wide variation in salinities (Dinis et al., 1999). Therefore, an investigation into the involvement of thyroid hormones and cortisol in the osmoregulatory capacity of *S. senegalensis* is warranted. We here present results obtained in an experimental setup where salinity is the only independent variable, all other environmental factors are controlled.

2. Material and methods

2.1. Fish and experimental conditions

Sexually immature juvenile Senegalese sole ($n = 152$, 35 ± 9 g body weight) were provided by Planta de Cultivos Marinos (C.A.S.E.M., Universidad de Cádiz, Puerto Real, Cádiz, Spain). Fish were transferred to the wet facilities at the Faculty of Marine Sciences (Puerto Real, Cádiz), and acclimated for 14 days to seawater (SW, 38‰ salinity, 1090 mOsm/kg) in 400-l tanks in an open system until the onset of the experiment. Low salinity water (LSW, 5‰, 140 mOsm/kg H_2O) and brackish water (BW, 15‰, 364 mOsm/kg) were obtained by mixing SW with dechlorinated tap water (fresh water, FW). High salinity water (HSW, 55‰, 1546 mOsm/kg) was obtained by mixing full-strength SW with natural marine salt (Salina de La Tapa, Puerto de Santa María, Cádiz, Spain). During experiments the tank water in all systems was recirculated. Water quality criteria (hardness, and levels of O_2, CO_2, H_2S, NO_2^-, NO_3^-, NH_4^+, Ca^{2+}, Cl_2, suspended solids) were monitored; no major changes were observed during the experiments. Water salinity was checked daily and, when necessary, adjusted to the nominal salinity by addition of small volumes of FW or SW. The experiment was performed in December 2004. Fish were maintained under natural photoperiod and constant temperature (18 °C) and fed once daily with commercial dry pellets (Dibaq-Diproteg SA, Segovia, Spain) at a ration of 1% of the estimated body weight. Animals did not receive food during the 24 h before sampling. The experimental procedures described comply with the Guidelines of the European Union Council (86/609/EU) and of the University of Cádiz (Spain) for the use of laboratory animals. No mortality was observed during the experiment.

2.2. Experimental design

SW-acclimated animals were divided randomly into four groups ($n = 36$ animals per group) and transferred directly to 400-l tanks with the following environmental salinities: LSW, BW, SW (control group) and HSW. Fish ($n = 9$ per time point) were sampled on days 1, 3, 7 and 17 post-transfer. Before transfer, eight fish were sampled and constituted the day zero pre-transfer group and reference point (day 0) for the other groups.

2.3. Animal procedures

Fish were anaesthetized in 0.1% (v/v) 2-phenoxyethanol. Mixed arterial and venous blood was collected from the caudal peduncle into 1-ml heparinized syringes. Plasma was obtained by centrifugation (3 min at 10,000*g*), immediately frozen in liquid nitrogen and stored at −80 °C until analysis of osmolality and hormone levels. Fish were killed by spinal transection. From each fish the first caudal gill arch was excised, dried on absorbent paper and a biopsy was cut using fine-point scissors. The gill biopsies were placed in 100 µl of ice-cold sucrose–EDTA–imidazole (SEI) buffer: 150 mM sucrose, 10 mM ethylenediamine tetraacetic acid (EDTA), 50 mM imidazole, pH 7.3 (Zaugg, 1982); frozen in liquid nitrogen and stored at −80 °C. Liver, kidney and the remaining gill arches were removed, frozen in liquid nitrogen, and stored at −80 °C until further assay.

2.4. Analytical techniques

2.4.1. Water osmolality

Water samples were filtered through a 0.22-μm pore size filter prior to analysis. Water osmolality was measured using a freezing-point depression Osmomat-030 osmometer (Gonotec, Berlin, Germany) and expressed as mOsm/kg.

2.4.2. Plasma osmolality and cortisol

Plasma osmolality was measured as described above and expressed as mOsm/kg. Plasma cortisol was measured by radioimmunoassay (RIA) as described by Metz et al. (2005). Briefly, 10 μl 1:5 (v/v) diluted plasma was incubated overnight at 4 °C with 100 μl of primary antibody (IgG-F-1, Campro Scientific, Veenendaal, The Netherlands) in a 1:800 dilution, 2×10^3 cpm of ^{125}I-cortisol (Amersham, Buckinghamshire, United Kingdom) and 100 μl of secondary antibody (goat anti-rabbit gamma globulin, Campro Scientific, Veenendaal, The Netherlands) in a 1:320 dilution. All ingredients were dissolved in a buffer containing 63 mM Na_2HPO_4, 13 mM Na_2EDTA, 0.02% (w/v) NaN_3, 0.1% (w/v) 8-anilino-1-naphtalene sulfonic acid and 0.1% (w/v) bovine γ-globulin (Sigma Chemical Co., St. Louis, MO, USA). Immune complexes were precipitated by the addition of 1 ml ice-cold 5% (w/v) polyethylene glycol and 2% (w/v) bovine serum albumin (Fluka, St. Gallen, Switzerland) and subsequent centrifugation (20 min, 2000*g*, 4 °C). Pellets were counted in a 1272 Clinigamma gamma-counter (LKB Wallac, Turku, Finland). The cross-reactivity of the primary antibody, setting the reactivity to cortisol at 100%, is: 5.9% with 11-deoxycortisol, 2.6% with cortisone, 1.7% with corticosterone, 0.16% with cortisone acetate, 0.4% with 17α-OH-progesterone and 0.02% with progesterone.

2.4.3. Gill Na^+,K^+-ATPase activity

Gill Na^+,K^+-ATPase activity was determined following the method of McCormick (1993) that was adapted for microplates. Gill biopsies were homogenized in 125 μl of SEI buffer supplemented with 0.1% deoxycholic acid and centrifuged at 2000*g* for 30 s. Duplicate 10-μl homogenate samples were added to 200 μl assay mixture in the presence or absence of 0.5 mM ouabain, and were incubated in 96-well microplates at 25 °C on an EL340i microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Ouabain-sensitive ATPase activity was detected by the enzymatic coupling of ATP dephosphorylation to the oxidation of NADH to NAD^+ and expressed as μmol ADP per mg protein per hour. The absorbance of incubates was read at 340 nm for 10 min with intermittent stirring, and was analyzed using the Delta-Soft3 software for Macintosh (BioMetallics Inc., Princeton, NJ, USA). Protein was measured with the Pierce BCA Protein kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard.

2.4.4. Plasma fT3 and fT4

Plasma free T3 (fT3) and free T4 (fT4) levels were determined by enzyme linked immunosorbent assay (ELISA) using commercially available kits from Human Gesellschaft für Biochemica und Diagnostica mbH (Wiesbaden, Germany) according to the manufacturer's instructions. The reported cross reactivity of the monospecific ovine T4-antibody to D-T4 is 98% (the reactivity to L-T4 is set at 100% as a reference), 3% for L-T3 and 1.5% for D-T3. The reported cross reactivity of the ovine T3-antibody to L-T4 is <0.02% (the reactivity to L-T3 is set at 100% as a reference). Cross reactivities of both antibodies to diiodotyrosine and iodotyrosine are <0.01%. The fT4 ELISA test has a sensitivity of 0.05 ng/dl fT4 and for fT3 ELISA test this value is 0.5 pg/ml fT3. Absorbance was read at 450 nm in a Bio-Rad Model-680 microplate reader (Bio-Rad, Veenendaal, The Netherlands).

2.4.5. Tissue preparations

Branchial tissue was obtained by scraping gill arches with a glass microscope slide and homogenized in 1 ml of phosphate buffer (100 mM Na-phosphate, 2 mM EDTA, pH 7.0) in a glass dounce homogenizer equipped with a tightly fitting Teflon pestle. The same procedure was applied for the homogenization of liver and kidney but here 3 ml phosphate buffer was used. Homogenates were stored at −80 °C until further analysis. Protein was measured with a commercial Coomassie brilliant blue reagent kit (Bio-Rad Laboratories, München, Germany) using BSA as a reference.

2.4.6. Outer ring deiodination (ORD) assay

We used reverse T3 (rT3, 3,3′,5′-triiodothyronine) as the substrate for ORD activities. The requirements of the ORD reaction for dithiothreitol (DTT), and the optimum pH and temperature were determined first in kidney homogenates obtained from control (SW) animals (Fig. 4). DTT showed a clear inhibitory effect and was thus excluded from our assay media. We chose to perform the assay at pH 7.0 and 37 °C, at which values highest ORD activities were measured. Otherwise the assay was performed as described by Klaren et al. (2005). Outer ring deiodination activities were assayed on duplicate samples of 50 μg homogenate protein for 15 min at 37 °C in 200 μl of phosphate buffer (pH 7.0) to which were added: 5 μM of rT3 (Sigma Chemical Co., St Louis, LA, USA) and 10^5 cpm of [^{125}I]rT3 (NEN Life Science Products, Inc., Boston, MA, USA). Non-enzymatic ORD activity was determined in the absence of sample. Radiotracer was purified on a 10% (w/v) Sephadex LH-20 mini-column shortly before use, as described by Mol and Visser (1985). The incubation was quenched by adding 100 μl 5% (w/v) ice-cold BSA. Quenched incubates were deproteinized with 500 μl 10% (w/v) ice-cold trichloroacetic acid followed by precipitation of denatured proteins at 1400*g* (15 min, 4 °C). To 0.5 ml of the supernatant thus obtained, an equal volume of 1 M HCl was added, and liberated iodide was separated from the native iodothyronine with the use of Sephadex LH-20 mini-column chromatography, collecting $^{125}I^-$ in the first two 1-ml 0.1 M HCl eluates. ^{125}I radioactivity was measured in a LKB-1272 Clinigamma gamma-counter (Wallac, Turku, Finland). The specific ORD rate was expressed as fmoles rT3 deiodinated per minute per microgram protein. Our calculations included a correction factor of 2 to take into account the random labeling of the 3′- and 5′-positions of [^{125}I]rT3.

2.5. Statistics

Differences between experimental groups were tested by two-way analysis of variance (ANOVA) using environmental salinity (LSW, BW, SW and HSW) and days post-transfer (0, 1, 3, 7 and 17 days) as factors of variability. When appropriate, data were logarithmically transformed to fulfil the requirements for ANOVA but data are shown untransformed for clarity. When ANOVA yielded significant differences, the effect of each factor was analyzed and multiple comparisons were carried out with Tukey's test. Differences were considered to be statistically significant at $P < 0.05$. When samples were drawn from populations with non-equal variances or a non-normal distribution, a Kruskal–Wallis ANOVA on ranks was run. ELISA-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.

3. Results

Plasma osmolalities decreased in fish exposed to LSW (5‰) and BW (15‰), and increased in fish exposed to HSW (55‰), after one day post-transfer (Fig. 1). These changes were transient as they returned to control values from day 7 post-transfer onwards.

Branchial Na^+,K^+-ATPase activities in fish transferred to LSW and BW decreased on days 1 and 3, respectively, compared to the control group (i.e., fish transferred from SW to SW), and remained significantly lower throughout the experimental period ($P < 0.05$) (Fig. 2A). Na^+,K^+-

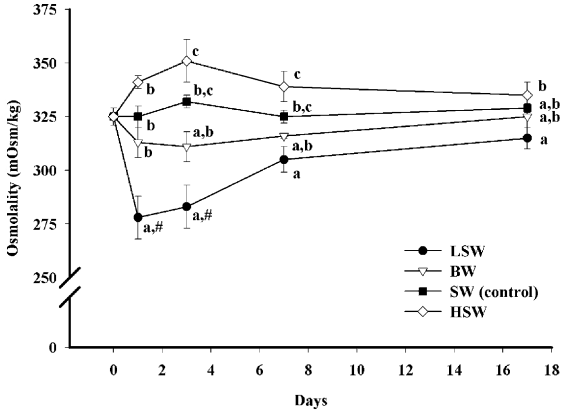

Fig. 1. Plasma osmolalities in juveniles of *S. senegalensis* after transfer to different environmental salinities. Values are mean ± S.E.M. ($n = 8$–9). Different letters indicate significant differences ($P < 0.05$) between groups within the same time point. # Indicates significant differences from undisturbed fish.

ATPase activities in fish transferred to HSW were similar to those in the control group until day 7 post-transfer. Only at day 17 post-transfer, branchial Na^+,K^+-ATPase activity in HSW had increased significantly by 30% compared to the control group. Significant differences ($P < 0.05$) between groups were observed at day 17 post-transfer, at which time-point branchial Na^+,K^+-ATPase activities correlated positively with salinity (Fig. 2B).

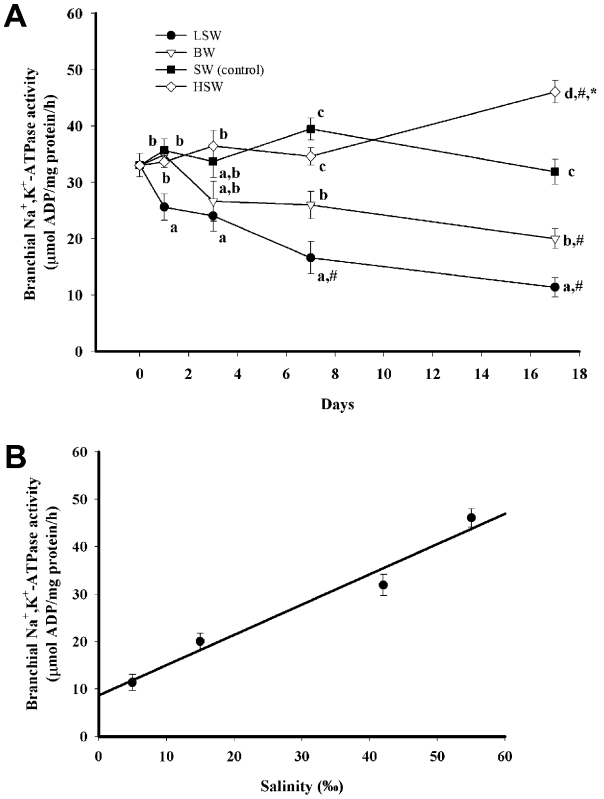

Fig. 2. (A) Time-course of branchial Na^+,K^+-ATPase activity in juveniles of *S. senegalensis* after transfer to different environmental salinities. (B) Correlation between branchial Na^+,K^+-ATPase activity, measured on day 17 post-transfer, and salinity. The straight line is calculated using the least squares criterion and is described by the regression equation $y = 0.64\,x + 8.7$ ($R^2 = 0.97$), the slope is significantly different from zero ($P = 0.016$). Data points represent mean ± S.E.M. ($n = 8$–9). * Indicates significant differences between the current and preceding time point. # Indicates significant differences from undisturbed fish.

Judged from the absence of a rise in plasma cortisol levels in the control fish, that were transferred from SW to SW, handling did not impose a stressor that confounded our data. Plasma cortisol levels changed transiently upon transfer to salinities other than SW: they had increased on day 1 post-transfer, and returned to normal values on day 3 (Fig. 3A). From day 3 post-transfer onwards, plasma cortisol levels in fish transferred to LSW, BW and HSW were elevated and remained so until the end of the experiment. Conversely, plasma fT4 levels had decreased on day 1 in fish transferred to LSW, BW and HSW, returned to normal values on day 3, and decreased again thereafter (Fig. 3B). Plasma fT3 levels clearly did not display the transient changes that were observed for cortisol and fT4 (Fig. 3C).

Hepatic ORD activities were generally one order of magnitude lower than ORD activities in gills and kidney. ORD activities in kidney and liver showed a peak at day 1 post-transfer in fish transferred to the extreme low and high salinities (LSW and HSW) and differed significantly ($P < 0.05$) compared to fish transferred to SW and BW (Fig. 5A and B). On day 3, renal and hepatic ORD activities had returned to control values. At the end of the experiment (day 17 post-transfer), significantly lowered ORD activities were observed in the liver of fish transferred to LSW and BW while significantly higher renal ORD activities were reached in fish transferred to HSW. No clear responsiveness was observed during the experiment in branchial ORD activity (Fig. 5C).

4. Discussion

The present findings demonstrate that the thyroid system, *viz*. the systemic fT4 concentrations and ORD activities in peripheral tissues, and the hypothalamo-pituitary-interrenal (HPI) axis in *S. senegalensis* are responsive to changes in environmental salinity. The negative correlation between plasma cortisol and plasma fT4 levels indicates an interaction between cortisol and the thyroid system. The increased hepatic and renal ORD activities observed on day 1 post-transfer (Fig. 5A and B) are consistent with the decreased plasma fT4 levels measured at that time point (Fig. 3B), and coincide with abrupt changes in plasma osmolality (Fig. 1). Our data suggest that the concerted actions of the thyroid system and cortisol are involved in the short-term salinity adaptation of *S. senegalensis*.

The time-course of acclimation in fish transferred to different environmental salinities is well-characterized in a number of teleostean species (Holmes and Donaldson, 1969; Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005) including *S. senegalensis* (Arjona et al., 2007). An initial adjustment period can be distinguished in which

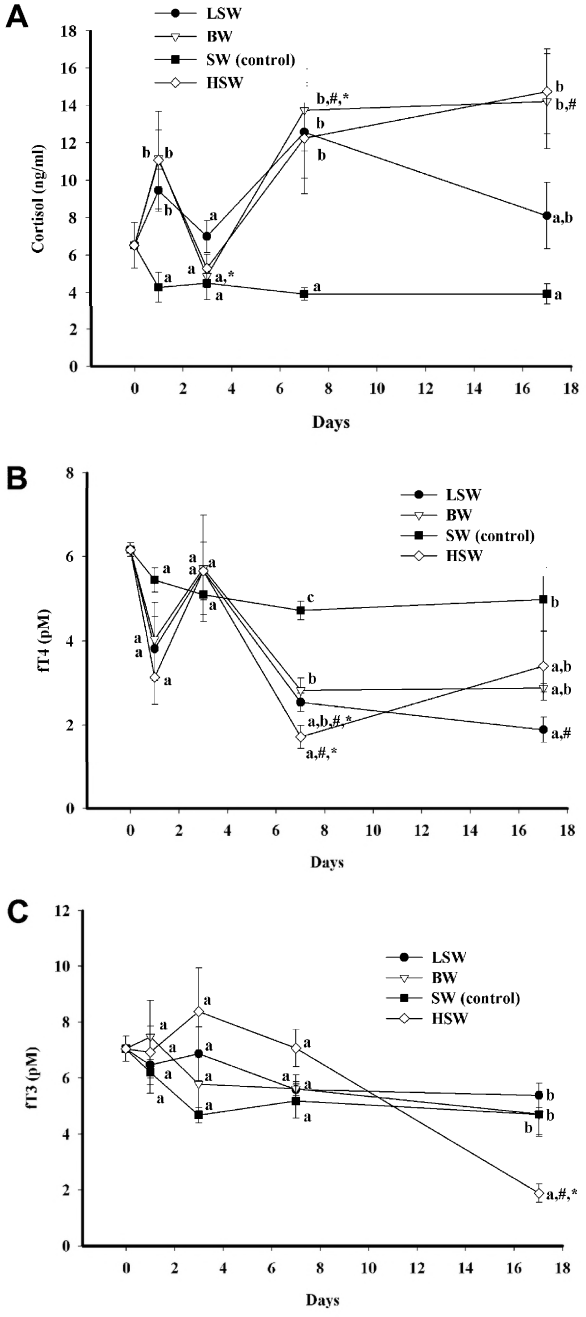

Fig. 3. Time-course of plasma cortisol (A), fT4 (B) and fT3 (C) levels in juveniles of *S. senegalensis* after transfer to different environmental salinities. Values are mean ± S.E.M. ($n = 8$-9 for cortisol, $n = 5$ for fT4 and fT3). See the legend to Fig. 2 for an explanation of the symbols.

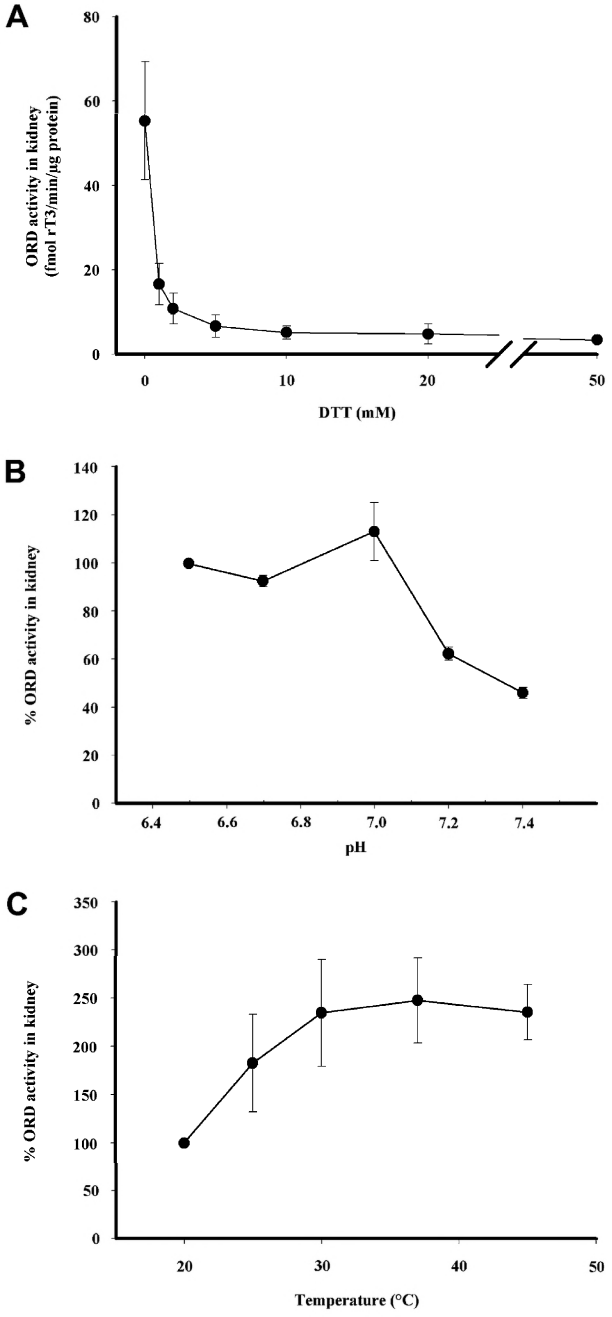

Fig. 4. ORD activities using rT3 as substrate in kidney homogenates of SW-acclimated juvenile *S. senegalensis*. (A) Effect of DTT. Mean values ± S.E.M. are shown ($n = 3$). (B) Effect of pH. ORD activities are here expressed relative to the activity measured at pH 6.5 (mean ± S.E.M., $n = 4$). (C) Effect of incubation temperature. ORD activities are expressed relative to the activity measured at 20 °C (mean ± S.E.M., $n = 4$).

transient changes in osmotic (plasma electrolyte levels and osmolality) and metabolic (plasma glucose and lactate levels) variables occur. This period is followed by a chronic regulatory period where these variables reach a new steady state and remain constant. In our time-course study, the chronology of changes in plasma parameters (osmolality, and fT4 and cortisol levels), together with the changes in hepatic and renal ORD activities indeed allows two phases to be distinguished in *S. senegalensis*: (i) an initial adjustment period, ending on day 3 post-transfer after which plasma osmolalities, fT4 and cortisol levels and peripheral ORD activities return to basal values; followed by (ii) a chronic regulatory period with sustained changes in branchial Na^+,K^+-ATPase activities.

As is cortisol, thyroid hormones are involved in the regulation of energy metabolism in vertebrates. Thyroid hormones stimulate basal metabolic rate and oxygen consumption in several teleostean tissues (Peter and Oom-

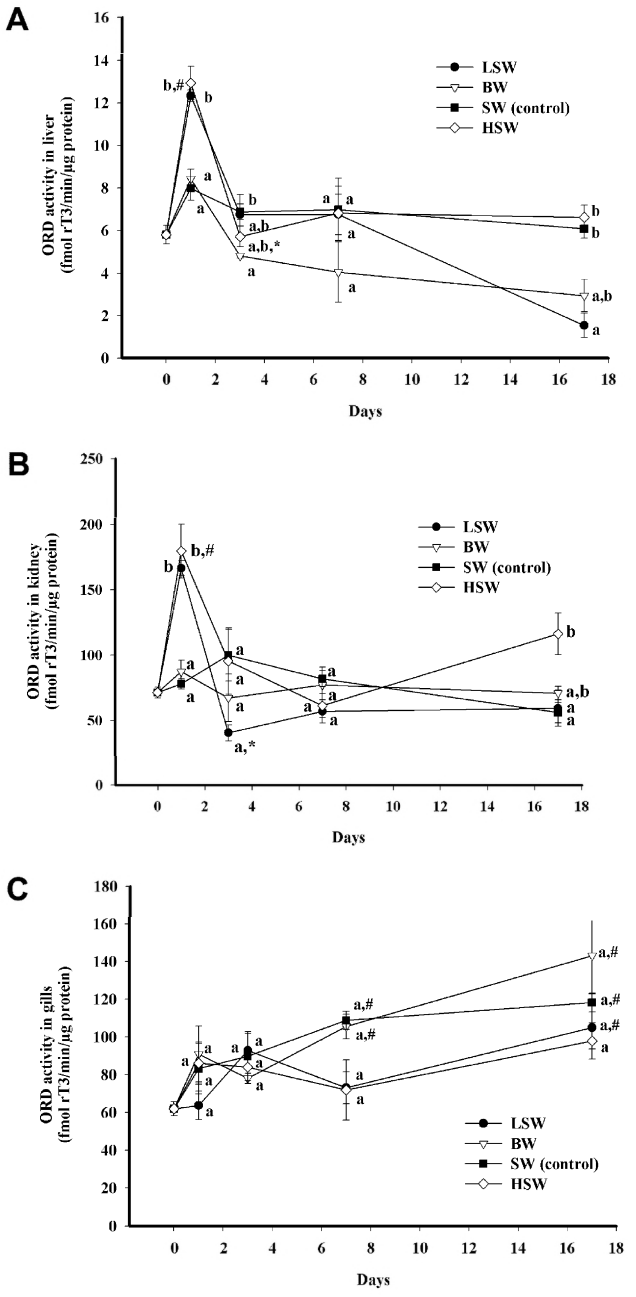

Fig. 5. Time-course of ORD activity in liver (A), kidney (B) and gills (C) in juveniles of *S. senegalensis* after transfer to different environmental salinities. Values are mean ± S.E.M. ($n = 5$). See the legend to Fig. 2 for an explanation of the symbols.

men, 1989; Lynshiang and Gupta, 2000), and have profound effects on lipid, carbohydrate and protein metabolism (Plisetskaya et al., 1983; Sheridan, 1986; Ballantyne et al., 1992; Scott-Thomas et al., 1992; Shameena et al., 2000). The involvement of catabolic pathways is exemplified by the increased plasma glucose, lactate and triglyceride levels that are observed in osmotically challenged fishes (Arjona et al., 2007; Laiz-Carrión et al., 2005; Sangiao-Alvarellos et al., 2005). The relevance, in this context, of the increased hepatic and renal ORD activities in the adjustment period following transfer (Fig. 5A and B) is to provide intracellular T3 by the conversion of T4. Bioactive T3 is then involved in the upregulation of intracellular enzymes that catalyze the metabolic pathways that are up-regulated following a transfer to LSW and HSW (Sangiao-Alvarellos et al., 2005). Cortisol, a gluco- and mineralocorticoid in fish (Wendelaar Bonga, 1997; Flik et al., 2006), and thyroid hormones can be envisaged to be jointly in control of the mobilization of energy to fuel osmoregulatory processes and in the up-regulated sodium-pump activity in the chronic regulatory period.

Whereas branchial Na^+,K^+-ATPase activity responded differentially following the transfer to different salinities, ORD activities in the gills did not, which would suggest that the local, branchial conversion of T4 to T3 is not involved in the regulation of sodium pump activity. However, although extrathyroidal conversion of T4 to T3 is of paramount importance, it is not the only determinant in the regulation of thyroid hormone activity. Indeed, the expression and activities of specific thyroid hormone transporters, conjugating enzymes, binding proteins and receptors are all critical to the biological activity of thyroid hormones. The differential regulation of peripheral components other than deiodinases of the thyroid system can well explain the increase in Na^+,K^+-ATPase activity in the absence of an increase of ORD in the gills.

In the chronic regulatory period, plasma fT4 and cortisol levels do not return to basal levels (Fig. 3A and B), again indicating the involvement of both hormones as primary mediators that sustain the allostatic state (McEwen and Wingfield, 2003) that allows *S. senegalensis* to cope with altered environmental salinities. Cortisol is classically viewed as a "seawater-adapting" hormone in fish (McCormick, 2001), but the elevated plasma cortisol levels observed in BW- and LSW-acclimated *S. senegalensis* here also indicate a role in the acclimation to iso- and hypoosmotic media (Fig. 3A). In *S. auratus*, the adjustment period that follows the transfer to a different salinity involves an increase in the plasma concentrations of metabolic fuels (glucose, lactate, triglycerides) and the use of these in organs that are directly or indirectly involved in osmoregulatory activities (Sangiao-Alvarellos et al., 2005). Similarly, we suggest that, in the adjustment period in *S. senegalensis*, cortisol mainly has a glucocorticoid action, which ultimately increases the availability of ATP for the Na^+,K^+-ATPase sodium pumps. In the chronic regulatory period cortisol mainly has a mineralocorticoid role to increase the activity and/or number of Na^+,K^+-ATPase pumps proper, as shown by the linear relationship between environmental salinity and branchial Na^+,K^+-ATPase activity at 17 days post-transfer (Fig. 2B). The differential expression of receptors with different affinities for glucocorticoids (Bury et al., 2003; Stolte et al., 2006) can confer the capacity to osmoregulatory tissues to discriminate between cortisol's gluco- and mineralocorticoid actions, and is worthy of further investigation.

This is, to our knowledge, the first study of the time-course of changes in plasma fT3 and fT4 levels during an

osmotic challenge in teleosts. The profiles of plasma fT4 and cortisol levels during osmotic acclimation point to an interaction between these hormones (Fig. 3A and B). This could indicate that, as in other teleosts, corticosteroids decrease plasma concentrations of thyroid hormones (Kühn et al., 1998). A drop in plasma fT4 levels can be caused by a decrease in thyroidal T4 production and secretion and/or changes in peripheral thyroid hormones metabolism (Van der Geyten et al., 2005). Changes in plasma fT4 levels during the adjustment period could be caused by an increase of the peripheral metabolism of thyroid hormones since hepatic and renal ORD activities were increased in fish transferred to LSW and HSW (Fig. 5A and B). Several activating effects of cortisol on thyroid function have been described: (i) in *Salvelinus fontinalis*, but not *O. mykiss*, cortisol stimulates the in-vitro hepatic ORD (Vijayan et al., 1988; Brown et al., 1991), and (ii) in *F. heteroclitus*, cortisol increases *in vitro* hepatic ORD activity (Orozco et al., 1998). In addition, other peripheral enzyme activities other than 5′-deiodinases that catalyze ORD are responsive to changes in environmental salinity and could cause or contribute to the changes in plasma fT4 levels observed in this experiment (Orozco et al., 2002; Klaren et al., 2007). On the other hand, experimental hyperthyroidism evoked low plasma cortisol levels in *C. carpio* (Geven et al., 2006). A negative correlation between plasma cortisol and T4 levels was also shown in *S. fontinalis* where increased plasma cortisol levels, obtained *via* injection or following a transfer from FW to 30% SW, resulted in lowered plasma T4 levels (Weisbart et al., 1987). Geven et al. (2006) also showed that hypocortisolemia following T4-treatment in carp correlated well with an enhanced expression of CRH-binding protein mRNA in the hypothalamus, resulting in a decreased activity of the HPI axis. These findings strongly indicate that the HPT and HPI axes interact, centrally as well as peripherally. More studies are warranted to unravel the interactions between both axes.

We found highest ORD activities in gills and kidney which were one order of magnitude higher than hepatic ORD (Fig. 5). In the gills, this activity was not responsive to changes in environmental salinity. Taken together, this points to the kidney as an important anatomical site for extrathyroidal thyroid hormone metabolism in *S. senegalensis*. In general, fish liver expresses relatively high D1 and D2 activities that confer a considerable ORD capacity to this organ (Mol et al., 1998; Orozco and Valverde-R, 2005). Specifically, D2 has been shown to determine hepatic ORD levels in some species (*F. heteroclitus*: Orozco et al., 1998; *O. mykiss*: Orozco et al., 2002; and *O. niloticus*: Walpita et al., 2007). In *in vitro* assays rT3 is commonly employed as the preferential substrate for ORD by mammalian D1, and is often used to discriminate between D1 and D2 activities. Our *in vitro* conditions would thus likely favor ORD by D1. However, teleostean deiodinases behave biochemically quite differently from those of mammals (Sanders et al., 1997; Klaren et al., 2005). A definitive identification of the deiodinase involved awaits an extensive biochemical characterization of D1 and D2 in *S. senegalensis*.

Acknowledgments

The authors are very grateful to Planta de Cultivos Marinos (C.A.S.E.M., Universidad de Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish. This study was partly supported by Grant BFU2004-04439-C02-01B (Ministerio de Educación y Ciencia and FEDER, Spain) to Juan Miguel Mancera, and by the pre-doctoral grant AP-2004-6829 (Ministerio de Educación y Ciencia, Spain) to Francisco Jesús Arjona. Selected results from this study were presented at the 23rd Conference of European Comparative Endocrinologists, August–September 2006, Manchester, United Kingdom.

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