Effects of cortisol and thyroid hormone on peripheral outer ring deiodination and osmoregulatory parameters in the Senegalese sole (*Solea senegalensis*)

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Abstract

The thyroid gland in fish mainly secretes the thyroid prohormone 3,5,3′,5′-tetraiodothyronine (T_4), and extra-thyroidal outer ring deiodination (ORD) of the prohormone to 3,5,3′-triiodothyronine (T_3) is pivotal in thyroid hormone economy. Despite its importance in thyroid hormone metabolism, factors that regulate ORD are still largely unresolved in fish. In addition, the osmoregulatory role of T_3 is still a controversial issue in teleosts. In this study, we investigated the regulation of the ORD pathway by cortisol and T_3 in different organs (liver, kidney, and gills) of *Solea senegalensis* and the involvement of T_3 in the control of branchial and renal Na^+, K^+-ATPase activity, a prime determinant of the hydromineral balance in teleosts. Animals were treated with i.p. slow-release coconut oil implants containing cortisol or T_3. Hepatic and renal ORD activities were up-regulated in cortisol-injected animals. T_3-treated fish showed a prominent decrease in plasma-free T_4 levels, whereas ORD activities did not change significantly. Branchial and renal Na^+, K^+-ATPase activities were virtually unaffected by T_3, but were transiently up-regulated by cortisol. We conclude that cortisol regulates local T_3 bioavailability in *S. senegalensis* via ORD in an organ-specific manner. Unlike T_3, cortisol appears to be directly implicated in the up-regulation of branchial and renal Na^+, K^+-ATPase activities.

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Introduction

Thyroid hormone regulates many important functions such as growth, salinity preference, oxygen consumption, nutrient metabolism, and metamorphosis in fish (reviewed by Eales (2006) and Blanton & Specker (2007)). Under normal conditions, the piscine thyroid gland mainly secretes the prohormone 3,5,3′,5′-tetraiodothyronine (T_4). T_4 must be converted, by removal of an iodine atom, to yield the biologically active hormone 3,5,3′-triiodothyronine (T_3; Eales & Brown 1993). This reaction is catalyzed by iodothyronine deiodinases, a family of selenoenzymes, of which three types exist. Deiodinases type 1 (D1) and type 2 (D2) catalyze the outer ring deiodination (ORD, or 5′-deiodination) pathway that converts the prohormone T_4 to T_3. The ORD pathway is thus of paramount importance for thyroid bioactivity in fish. Liver, gills, and kidney in particular have a high ORD capacity, and can function as an extrathyroidal source of bioactive T_3 (Arjona *et al*. 2010).

Thyroid hormones and the steroid hormone cortisol are thought to be implicated in osmoregulation in teleosts. Cortisol is a gluco- and mineralocorticoid in fish (Mommsen *et al*. 1999, McCormick 2001). In teleosts acclimating to hyperosmotic environments, cortisol regulates Na^+, K^+-ATPase activities which are prime determinants of osmoregulatory capacity (Mancera & McCormick 2007). On the other hand, the role of thyroid hormones in osmoregulation is generally much less well investigated (Klaren *et al*. 2007*a*).

Several studies have revealed effects of glucocorticoids on thyroid hormone metabolism. Cortisol stimulated the conversion, by ORD, of T_4 to T_3 in *Salvelinus fontinalis* liver *in vitro* (Vijayan *et al*. 1988), and enhanced liver D1 and D2 activities in *Fundulus heteroclitus* (Orozco *et al*. 1998). Negative results have also been reported, i.e. for the hepatic conversion of T_4 to T_3 in *Oncorhynchus mykiss* (Brown *et al*. 1991). I.v. administration of the synthetic glucocorticoid dexamethasone decreased liver D1 and D2 activities in *Oreochromis niloticus* but left ORD unaffected in gills, kidney,

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and brain (Walpita *et al.* 2007). It appears that the regulation of ORD by glucocorticoids in fish depends on the species and organ assessed, and a general picture cannot be constructed.

The Senegalese sole (*Solea senegalensis*) is a euryhaline species that is cultured at a commercial scale in Spain and Portugal (Dinis *et al.* 1999). Experimental results show that, following acclimation of *S. senegalensis* to different osmotic conditions, plasma cortisol and free T_4 (fT_4) concentrations change in a concerted manner, hinting at an interaction between the hypothalamus–pituitary–inter-renal (HPI) and the thyroidal axes (Arjona *et al.* 2008). Moreover, during the adjustment period that follows the transfer to a different ambient osmolarity, renal and hepatic ORD activities are elevated, and plasma cortisol concentrations are increased (Arjona *et al.* 2008).

Whether the bidirectional communication between HPI and thyroidal axes in *S. senegalensis* occurs at a central and/or peripheral level is as yet unknown. We here investigate the hypothesis that cortisol and T_3 affect the ORD pathway, a key metabolic route for thyroid hormones, in Senegalese sole. In particular, liver and kidney, two organs with considerable ORD capacity, and gills, the main osmoregulatory organ, were investigated. We have also analyzed Na^+, K^+-ATPase activities in gills and kidney, and plasma concentrations of glucose and lactate, which can fuel osmoregulatory processes.

Materials and Methods

Fish and animal care

Juvenile Senegalese sole (*S. senegalensis*) with a body weight of 38±9 g and a total length of 150±19 mm (mean±s.d., n=90) were provided by Planta de Cultivos Marinos (C.A.S.E.M., Universidad de Cádiz, Puerto Real, Cádiz, Spain). Fish were acclimated for 14 days to full strength seawater (SW, with a nominal salinity of 38‰ and a nominal osmolality of 1037 mOsm/kg) in three 400 l tanks in a flow-through system until the start of experimentation. Each tank, with a bottom surface of 0·81 m^2, contained 30 fish at an initial density of 1·4 kg/m^2. Fish were kept under a photoperiod of 10 h light:14 h darkness and at a constant water temperature of 18 °C. Fish were fed daily with commercial dry pellets (Dibaq-Diproteg SA, Segovia, Spain) at a ration of 1% of the total body weight, and were fasted 24 h before sampling. This ration did not lead to detectable nitrogenous waste build-up in the tanks. The experimental procedures comply with the Guidelines of the European Union Council (86/609/EU) and of the University of Cádiz (Spain) for the use of animals in research. No mortality was observed during the experiment.

Experimental design

SW-acclimated fish were randomly divided into three groups of 27 animals. Fish were caught by netting, lightly anesthetized with 0·05% (v/v) 2-phenoxyethanol (Sigma Chemical Co., St Louis, MO, USA.), weighed, and injected intra-peritoneally with slow-release coconut oil (Sigma Chemical Co.) implants. The injection volume was 5 μl/g body weight, and coconut oil was warmed to its melting point (24 °C) prior to injection. Experimental groups received cortisol (hydrocortisone 21-hemisuccinate) in a dose of 50 μg/g body weight, or T_3 (3,3′,5-triiodo-L-thyronine, ≥95% HPLC quality) in a dose of 2 μg/g body weight. The control group received only the coconut oil implant. Hormone preparations were obtained from Sigma Chemical Co. The use of coconut oil as a vehicle for i.p. hormonal implants has been shown to be an effective and practical method to raise plasma cortisol or thyroid hormone levels in different teleost species (Laiz-Carrión *et al.* 2003, Morgado *et al.* 2007). Nine fish did not receive any treatment and served as the pre-injection group. After injection, fish were returned to three designated 400 l tanks in a flow-through system with SW that was refreshed at a rate of 50 l/h. Per experimental group, nine animals were sampled on days 3, 7, and 14 post-implantation.

Sampling procedures

Fish were anesthetized in 0·1% (v/v) 2-phenoxyethanol and weighed. Mixed arterial and venous blood was collected from the caudal peduncle in 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 further analysis. After blood collection, fish were killed by spinal transection. From each fish, the first gill arch on the ocular side was excised as well as a small portion of the caudal zone of the kidney. Excess water was removed using absorbent paper, and tissues were frozen in liquid nitrogen and stored at −80 °C until analysis of Na^+, K^+-ATPase activities. Liver, the remaining kidney, and the rest of gill arches were removed, frozen in liquid nitrogen, and stored at −80 °C until analysis of ORD activities.

Plasma parameters

Six fish of each group were randomly selected for plasma analyses. Plasma osmolality was measured with a cryoscopic osmometer (Gonotec, Berlin, Germany). Plasma Na^+, Cl^-, K^+, glucose, and lactate concentrations were measured using a Stat Profile pHOx plus analyser (Nova Biomedical, Waltham, MA, USA). Plasma cortisol was measured by RIA as described by Metz *et al.* (2005). Plasma-free T_3 (fT_3) and fT_4 levels were determined by a commercially available solid-phase time-resolved fluoroimmunoassay (Wallac DELFIA from PerkinElmer Life and Analytical Sciences, Turku, Finland). Samples were diluted with charcoal-stripped plasma from SW-acclimated *S. senegalensis* prior to fT_3 determinations: plasma from T_3-injected fish was diluted 1:10 (v/v); plasma obtained from the other groups was diluted 1:3 (v/v). The DELFIA method has previously been validated for use with *S. senegalensis* plasma (Arjona *et al.* 2010).

Tissue preparations

To determine Na^+, K^+-ATPase activities, gills were thawed at room temperature. Branchial tissue was obtained by scraping the gill arch with a glass microscope slide and homogenized in 1 ml of ice-cold sucrose buffer (250 mM sucrose, 1 mM EDTA, and 100 mM trishydroxymethylaminomethane-HCl, pH 7·4) in a glass dounce homogenizer equipped with a tightly fitting Teflon pestle. Kidney fragments were homogenized in 0·5 ml sucrose buffer. To determine ORD activities, gills, liver, and the remaining kidney were homogenized in phosphate buffer (100 mM Na-phosphate, 2 mM EDTA, pH 7·0). Homogenates were stored at −80 °C until further analysis. Protein was measured with a commercial Coomassie Brilliant Blue reagent kit (Bio-Rad Laboratories) using BSA as a standard.

Gill and kidney Na^+, K^+-ATPase activities

The specific Na^+- and K^+-dependent, ouabain-sensitive ATPase activity was measured in triplicate in gill and kidney homogenates according to the method described by Flik *et al.* (1983). The method was adapted to 96-well microplate format by scaling down original volumes. Homogenates were diluted with ice-cold sucrose buffer in order to achieve maximally <15% ATP consumption during the incubation period. Triplicate 5 µl aliquots of diluted homogenates were incubated for 15 min at 37 °C. ATP consumption percentages were 11·8±0·4 for gills and 10·3±0·3 for kidney (n=60, mean±s.e.m.). The specific Na^+, K^+-ATPase activity is expressed as µmol inorganic phosphate per min per mg protein.

ORD activities

ORD activities were assayed following the method described by Klaren *et al.* (2005). We used reverse T_3 (rT_3, 3,3′,5′-triiodothyronine) and T_4 as the preferred substrates for 5′-deiodinases (Mol *et al.* 1998). The requirements of the rT_3-ORD and T_4-ORD reactions for dithiothreitol (DTT) in *S. senegalensis* have been determined previously (Arjona *et al.* 2008, 2010). As DTT inhibited ORD, it was excluded from the assay media. ORD activities were assayed in duplicate using 20–70 µg homogenate protein at 37 °C in 200 µl of 100 mM phosphate buffer (pH 7·0) to which were added: 5 µM of rT_3 or T_4 (Sigma Chemical Co.), 10^5 c.p.m. $[^{125}I]rT_3$ or $[^{125}I]$ T_4 (NEN Life Science Products, Inc., Boston, MA, USA), and 2 mM EDTA. Incubation period was set at 15 min for rT_3-ORD and 12 min for T_4-ORD. During this period, substrate consumption was <10%: rT_3 consumption percentages were 6·7±0·10 for gills, 5·8±0·19 for kidney, and 2·4±0·07 for liver; T_4 consumption percentages were 9·1±0·13 for gills, 6·4±0·18 for kidney, and 8·8±0·3 for liver (n=60, mean±s.e.m.). Measurements were corrected for non-enzymatic ORD activity that 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 & Visser (1985). The specific ORD rate was expressed as fmoles rT_3 or T_4 deiodinated per minute per mg protein. Our calculations

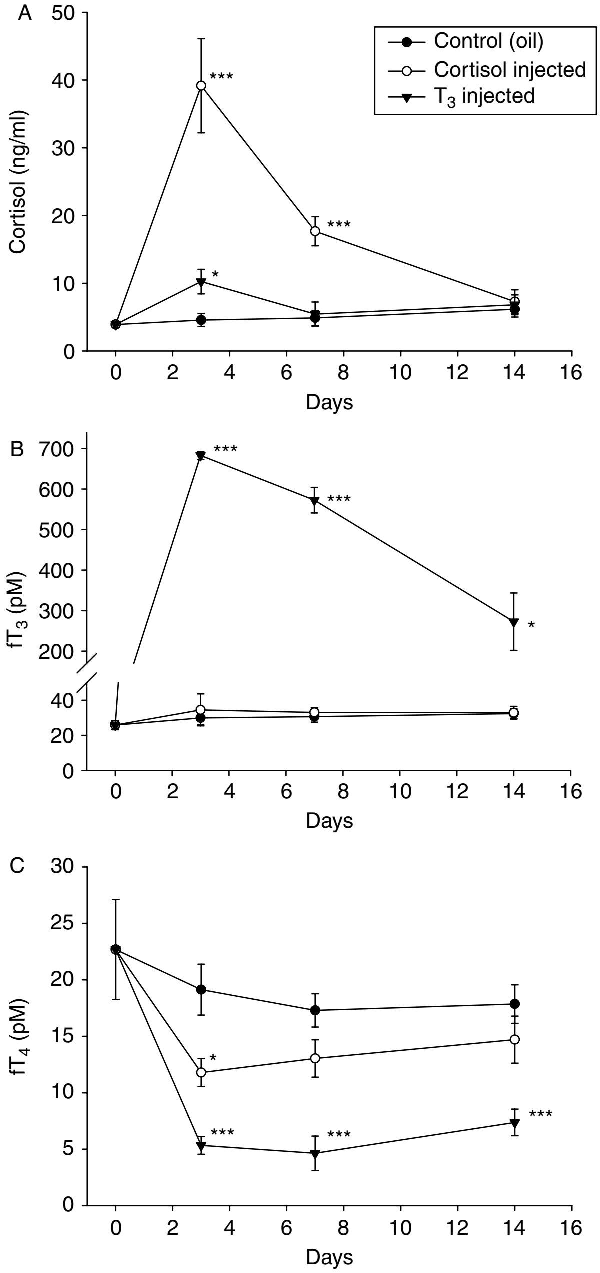

Figure 1 Time course of changes in plasma cortisol (A), fT_3 (B), and fT_4 (C) levels in juveniles of *S. senegalensis* after injections with coconut oil alone (control) or coconut oil containing cortisol (50 µg/g body weight) or T_3 (2 µg/g body weight). Values at day 0 refer to the pre-injection group. Asterisks indicate significant differences when comparing cortisol- or T_3-injected groups with control groups (injected with coconut oil alone) on days 3, 7, and 14 post-implantation (*$P<0·05$; ***$P<0·001$). Values are expressed as mean±s.e.m. (n=5–6).

included a correction factor of 2 to take into account the random radiolabeling of the 3′- and 5′-positions of [^{125}I]rT_3 and [^{125}I]T_4.

Statistical analysis

Experimental groups (cortisol- or T_3-injected animals) were compared with control groups (coconut oil-injected fish) on days 3, 7, and 14 post-implantation using Student's unpaired *t*-test or Mann–Whitney's non-parametric rank sum *U* test, where appropriate. Statistical significance was accepted at $P<0{\cdot}05$. The same statistical analyses were applied to test the effect of the coconut oil vehicle, but here groups injected with coconut oil alone were compared with the pre-injection group.

Results

No mortality or pathologies or differences in growth rates were observed in any group throughout the experimental period. No significant differences were observed between untreated fish (pre-injection group, 0 days) and those fish implanted with coconut oil alone (controls) for any parameter assessed.

Treatment with cortisol and T_3 implants effectively elevated plasma concentrations of cortisol and fT_3 respectively (Fig. 1A and B). Specifically, cortisol implants elicited a transient increase in plasma cortisol levels, while T_3 treatment resulted in sustained elevated fT_3 levels throughout. Treatment with T_3 also produced a ca. twofold increase in plasma cortisol levels on day 3 (Fig. 1A). Both cortisol and T_3 treatments decreased plasma fT_4 levels (Fig. 1C), where the effect of T_3 was more pronounced than that of cortisol.

Plasma Na^+, Cl^-, and K^+ concentrations in cortisol-treated animals decreased significantly on day 3 compared with the control group and returned to basal values from day 7 onwards (Table 1). Plasma osmolality did not vary significantly with the different experimental treatments. Cortisol treatment increased plasma glucose levels transiently on day 3 ($P<0{\cdot}001$) but did not change plasma lactate values. Treatment with T_3 did not produce statistically significant changes in plasma electrolyte, glucose, and lactate concentrations (Table 1).

Gill and kidney Na^+, K^+-ATPase activities were stable in the control fish, and reached a maximum on day 3 in the cortisol-treated fish (Fig. 2A and B), which coincided with

Table 1 Plasma osmoregulatory and metabolic parameters in *Solea senegalensis* specimens injected with coconut oil alone (control) or containing cortisol (50 µg/g body weight) or T_3 (2 µg/g body weight). Values are expressed as means±S.E.M. (n=5–6)

Parameters assessed	Days post-injection	Control (oil)	Cortisol	T_3
Osmolality (mOsm/kg)	3	346±4	335±3	340±2
	7	350±5	342±4	345±3
	14	351±5	341±1	342±4
Na^+ (mM)	3	154±4	137±2†	147±2
	7	158±4	154±2	153±1
	14	158±1	157±4	157±2
Cl^- (mM)	3	150±4	139±1*	141±4
	7	152±5	149±3	149±4
	14	152±4	150±7	150±4
K^+ (mM)	3	4·9±0·27	3·8±0·14†	4·3±0·18
	7	3·9±0·27	4·4±0·22	4·8±0·4
	14	4·2±0·16	4·4±0·4	4·5±0·28
Glucose (mM)	3	3·7±0·15	5·4±0·12‡	3·5±0·17
	7	3·9±0·3	4·4±0·14	3·5±0·18
	14	4·0±0·27	4·5±0·14	3·7±0·14
Lactate (mM)	3	3·1±0·08	3·3±0·4	3·3±0·20
	7	3·5±0·7	3·2±0·20	2·9±0·16
	14	3·2±0·20	3·0±0·6	3·2±0·27

Symbols indicate significant differences when comparing cortisol- or T_3-injected groups with control groups (injected with coconut oil alone) on days 3, 7, and 14 post-implantation (*$P<0{\cdot}05$; †$P<0{\cdot}01$; ‡$P<0{\cdot}001$).

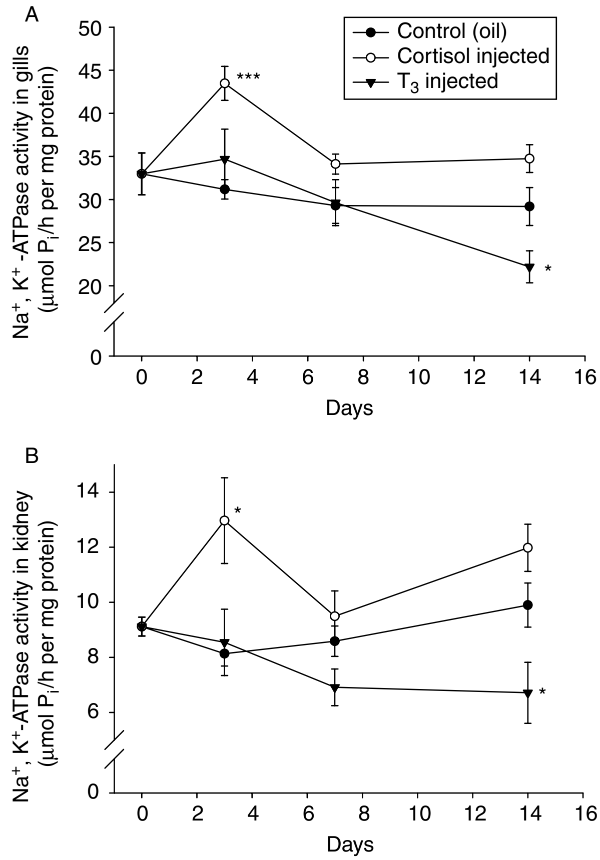

Figure 2 Time course of Na^+, K^+-ATPase activities in gills (A) and kidney (B) in juveniles of *S. senegalensis* after injections with coconut oil alone (control) or coconut oil containing cortisol (50 µg/g body weight) or T_3 (2 µg/g body weight). Values at day 0 refer to the pre-injection group. See the legend of Fig. 1 for an explanation of the symbols used. Values are expressed as mean±S.E.M. (n=5–6). *$P<0{\cdot}05$; ***$P<0{\cdot}001$.

the peak in plasma cortisol levels (Fig. 1A). Enzyme activities returned to control levels on day 7. Treatment with T_3 did not produce significant changes in Na^+, K^+-ATPase activities, except at the end of the experimental period (day 14) where lowered Na^+, K^+-ATPase activities were observed in gills and kidney ($P<0{\cdot}05$).

The effects of hormone treatments on kidney, liver, and gill rT_3-ORD activities were similar to the respective T_4-ORD activities in these organs (Figs 3 and 4). In fish treated with cortisol, renal and hepatic ORD activities were enhanced two- to three-fold at day 3 following injection, and decreased to control levels afterwards (Figs 3A and B, 4A and B). In T_3-treated fish, we only observed a significantly decreased renal T_4-ORD activity ($P<0{\cdot}01$) on day 7 (Fig. 4A). A transient and slight decrease in branchial T_4-ORD was observed in cortisol-injected fish (Fig. 4C).

Discussion

Our results demonstrate that cortisol stimulates the ORD pathway, an important peripheral source of T_3, in *S. senegalensis* liver and kidney. This observation supports our hypothesis, and indicates that the activity of thyroid hormones can be regulated not only at a central level in the brain, but also peripherally in extrathyroidal tissues. No effect of cortisol was seen on ORD activity in the gills. The constitutive branchial ORD capacity is comparable in magnitude to that in liver and kidney. It could well be that the control of intracellular thyroid hormone concentrations in gills occurs by means of up- or down-regulation of the activity of T_3 membrane transporters rather than *de novo* production of T_3. The increased ORD activities in kidney and liver following cortisol treatment in *S. senegalensis* (Figs 3 and 4) point to these organs as important determinants of thyroid bioactivity during a stress response when plasma cortisol concentrations increase (Wendelaar Bonga 1997, Barton 2002). It also suggests that at least some of cortisol's actions, i.e. increased renal Na^+, K^+-ATPase activity and liver gluconeogenesis (as deduced from the increased plasma glucose concentrations), are mediated via T_3. Treatment with T_3 failed to produce significant changes in renal Na^+, K^+-ATPase activity or plasma glucose levels, which is consistent with the suggestion that T_3 is a mediator of cortisol actions, without osmoregulatory or gluconeogenic activity *per se*.

A consequence of increased ORD activities is an increase in intracellular T_3 availability and, hence, an increased transcription of its genomic targets. The notion of intracellular actions of locally produced T_3 also helps explain why plasma fT_3 concentrations were unaltered by cortisol injection. Indeed, osmotic challenges changed peripheral ORD activities in *S. senegalensis* and *Sparus auratus* but did not result in altered plasma fT_3 levels (Klaren *et al.* 2007*b*, Arjona *et al.* 2008). In *S. fontinalis*, circulating T_4 and T_3 remained unaltered after a cortisol treatment that had increased hepatic T_4-ORD activities (Vijayan *et al.* 1988). A plausible explanation is that traffic of T_3 out of cells is regulated by the activity of membrane transporters, preventing T_3 from entering plasma.

The approximately eightfold increase in plasma cortisol levels following treatment with implants is within the physiological range, as it compares very well to the stressor-induced

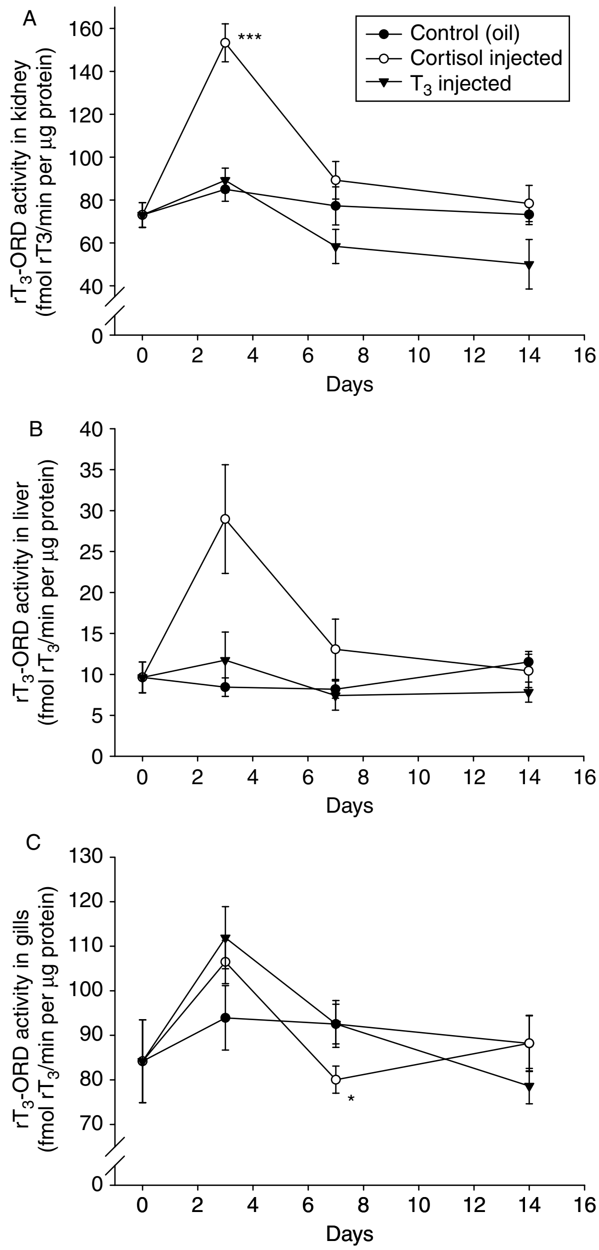

Figure 3 Time course of rT_3-ORD activities in kidney (A), liver (B), and gills (C) in juveniles of *S. senegalensis* after injections with coconut oil alone (control) or coconut oil containing cortisol (50 µg/g body weight) or T_3 (2 µg/g body weight). Values at day 0 refer to the pre-injection group. See the legend of Fig. 1 for an explanation of the symbols used. Values are expressed as mean ± S.E.M. (n=5–6). $^*P<0{\cdot}05$; $^{***}P<0{\cdot}001$.

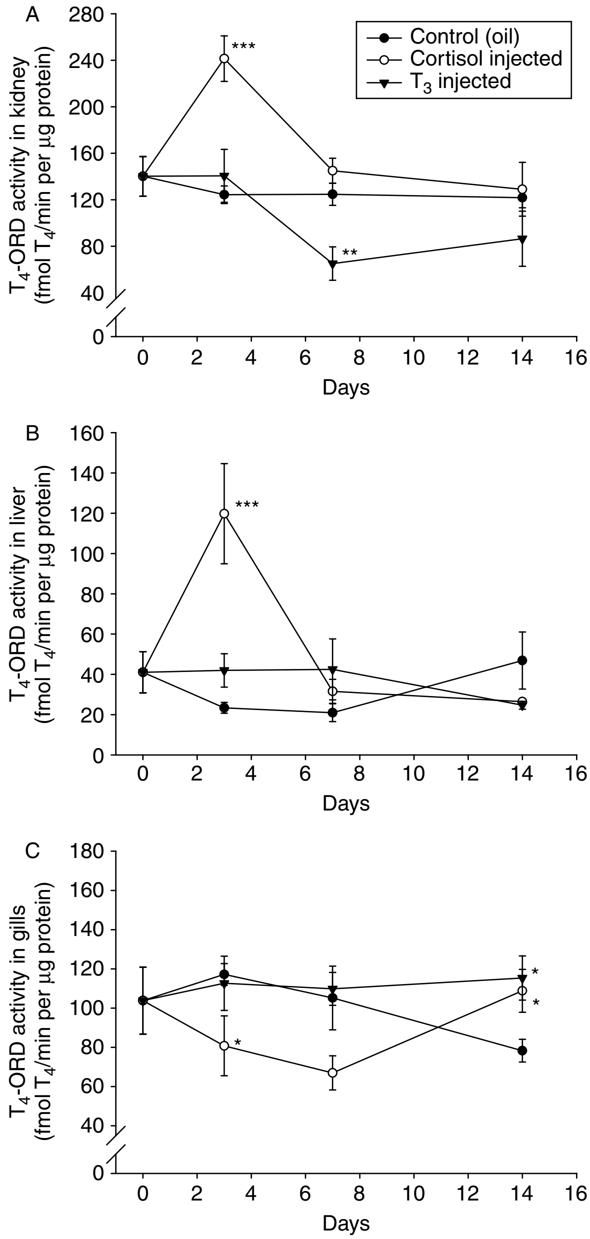

Figure 4 Time course of T_4-ORD activities in kidney (A), liver (B), and gills (C) in juveniles of *S. senegalensis* after injections with coconut oil alone (control) or coconut oil containing cortisol (50 µg/g body weight) or T_3 (2 µg/g body weight). Values at day 0 refer to the pre-injection group. See the legend of Fig. 1 for an explanation of the symbols used. Values are expressed as mean±s.e.m. (n=5–6). *P<0·05; **P<0·01; ***P<0·001.

4- to 40-fold increases observed in *S. senegalensis* and other teleost species (Woodward & Strange 1987, Goss & Wood 1988, van den Burg *et al.* 2005, Arjona *et al.* 2008). There are very few reports on the use of slow-release thyroid hormone implants in fishes. Morgado *et al.* (2007) used coconut oil to treat gilthead seabream (*S. auratus*) with a dose of 1 µg T_3/g body weight, resulting in a 25-fold increased T_3 plasma concentration. We found a similar 27-fold increase using a dose of 2 µg T_3/g. There are large differences between species in how plasma thyroid hormone levels respond to a stressor. Zebrafish (*Danio rerio*) can increase its plasma T_3 levels ca. 20-fold when challenged, whereas European flounder (*Platichthys flesus*) is refractory to a similar stimulus (Kuiper *et al.* 2008). We conclude that the experimentally increased plasma T_3 levels reflect an upper limit of a physiological range in *S. senegalensis*.

Cortisol affects the metabolism of carbohydrates, proteins, and lipids in fish (Mommsen *et al.* 1999). In our study, cortisol produced a glucocorticoid effect in *S. senegalensis*, evoking a mild hyperglycemia, probably by stimulation of gluconeogenic routes in the liver (Mommsen *et al.* 1999). In addition to its glucocorticoid actions, the role of cortisol as a mineralocorticoid is obvious in *S. senegalensis*, as cortisol-injected animals showed lowered plasma Na^+ and Cl^- levels and increased Na^+, K^+-ATPase activities in gills and kidney 3 days after injection. These results agree with the classical osmoregulatory role of cortisol in teleosts (Seidelin *et al.* 1999, Laiz-Carrión *et al.* 2003, Sherwani & Parwez 2008).

A drop in plasma fT_4 levels can be caused by a decrease in thyroidal T_4 production and secretion and/or changes in peripheral metabolism (Blanton & Specker 2007), a picture similar to that in mammals (Visser & Fliers 2007). The decrease in plasma fT_4 levels in cortisol-injected *S. senegalensis* (Fig. 1) coincided with increased ORD activities in liver and kidney and, hence, an increased T_4-to-T_3 conversion. However, we cannot exclude a feedback mechanism in which cortisol reduces the activity of hypothalamic corticotropin-releasing hormone (CRH), urotensin-I (UI), and thyrotropin-releasing hormone (TRH), factors with both corticotropic and thyrotropic activities in a number of fish species (reviewed by Bernier *et al.* (2009)). Indeed, in salmonids and eels, CRH has thyrotropic activity *in vitro* (Larsen *et al.* 1998, Rousseau *et al.* 1999), and, in carp, the interaction between thyroid hormones and the HPI axis is illustrated by the up-regulation of hypothalamic *crh-binding protein* gene expression after T_4 treatment (Geven *et al.* 2006). It could well be that hypothalamic factors of the HPI axis, namely CRH, UI, TRH, are altered after cortisol treatment in *S. senegalensis* and then, jointly with the hepatic and renal ORD pathway, have affected plasma fT_4 concentrations.

Treatment with T_3 reduced plasma fT_4 levels ca. fourfold. Besides a 47% reduction measured in the kidney 7 days after injection, no major changes in peripheral ORD activities were observed. It is very likely that the reduction in plasma fT_4 levels is the result of a reduced activity of the thyroid gland caused by a negative feedback of T_3 on pituitary thyrotropes. As in mammals, T_3 represses *tsh β-subunit* gene expression in fish pituitaries *in vitro* in a classical negative feedback system (Pradet-Balade *et al.* 1997, Schmitz *et al.* 1998, Sohn *et al.* 1999). Moreover, thyroid hormone economy is complicated by the presence of specific thyroid hormone-binding proteins that facilitate vectorial plasma transport (reviewed by Klaren *et al.* (2007*a*)). Morgado *et al.* (2007) have shown that treatment of *S. auratus* with T_3 increases the plasma concentration of the thyroid hormone-binding protein transthyretin ca. threefold. The concentration of free thyroid hormone levels in the plasma is a complex balance of hormonogenesis in the thyroid gland and the concentration of circulating binding proteins.

In *S. senegalensis*, T_4- and rT_3-ORD activities were mainly unaltered after T_3 treatment (Fig. 4). In some teleost species, deiodinase activity seems to be regulated by its iodothyronine substrate (Orozco *et al.* 1997, 2003, Sanders *et al.* 1997, Valverde-R *et al.* 1997). The ORD assays as performed in this study do not allow the discrimination between the 5′-deiodinases D1 and D2. Using rT_3, we have tested an iodothyronine that is the preferred substrate for many vertebrate D1 enzymes. T_4 is the preferred substrate for many deiodinases type 2, but, more importantly, is also the physiologically relevant iodothyronine since it is the endogenous prohormone that needs to be activated by ORD. Our results show that peripheral ORD is not responsive to *in vivo* T_3 treatment. Instead, cortisol appears to be a key regulator of peripheral ORD activity and, hence, extrathyroidal T_3 production in *S. senegalensis*.

This work confirms and extends previous results (Arjona *et al.* 2008), and provides evidence for the involvement of cortisol in the regulation of the ORD pathway in *S. senegalensis* in an organ-specific manner as well as in physiological processes related to the hydromineral balance.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the research reported.

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