Iodothyronine deiodinases and the control of plasma and tissue thyroid hormone levels in hyperthyroid tilapia (*Oreochromis niloticus*)

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† We dedicate this paper to the memory of Prof. N Byamungu

Abstract

Thyroid status is one of the most potent regulators of peripheral thyroid hormone metabolism in vertebrates. Despite this, the few papers that have been published concerning the role of thyroid hormones in the regulation of thyroid function in fish often offer conflicting data. We therefore set out to investigate the effects of tetraiodothyronine (thyroxine) (T_4) or tri-iodothyronine (T_3) supplementation (48 p.p.m.) via the food on plasma and tissue thyroid hormone levels as well as iodothyronine deiodinase (D) activities in the Nile tilapia (*Oreochromis niloticus*). T_4 supplementation did not induce a hyperthyroid state and subsequently had no effects on the thyroid hormone parameters measured, with the liver as the sole notable exception. In T_4 -fed tilapias, the hepatic T_4 levels increased substantially, and this was accompanied by an increase in *in vitro* type I deiodinase (D1) activity. Although the lack of effect of T_4 supplementation could be partially explained by an inefficient uptake of T_4 from the

gut, our current data suggest that also the increased conversion of T_4 into reverse (r) T_3 by the D1 present in the liver plays an important role in this respect. In addition, T_3 supplementation increased plasma T_3 and decreased plasma T_4 concentrations. T_3 levels were also increased in the liver, brain, kidney, gill and white muscle, but without affecting local T_4 concentrations. However, this increase in T_3 availability remained without effect on D1 activity in liver and kidney. This observation, together with the 6-*n*-propylthiouracyl (PTU) insensitivity of the D1 enzyme in fish, sets the D1 in teleost fish clearly apart from its mammalian and avian counterparts. The changes in hepatic deiodinases confirm the role of the liver as an important T_3 -regulating tissue. However, the very short plasma half-life of exogenously administered T_3 implies the existence of an efficient T_3 clearing/degradation mechanism other than deiodination.

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Introduction

In fish, as in other vertebrates, the thyroid secretes predominantly 3,5,3',5'-tetraiodothyronine (thyroxine) (T_4), which has to be converted into 3,5,3'-tri-iodothyronine (T_3) in order to bind to a nuclear receptor and exert its full biologic activity. This activation, as well as the inactivation of thyroid hormones, occurs not in the thyroid, but in the periphery through the action of iodothyronine deiodinases (Eales & Brown 1993, Mol *et al.* 1997, 1998). Although it has long been an issue of debate, recent molecular studies have indicated that in fish, as in mammals, at least three different selenocysteine-containing deiodinases exist, with similar properties to their mammalian counterparts. The type I deiodinase (D1), which is capable of both activation (outer-ring

deiodination (ORD)) and inactivation (inner-ring deiodination (IRD)), is a high K_m (μ M range for reverse (r) T_3), high V_{max} enzyme which is, in contrast to D1 in mammals and birds, insensitive to inhibition by 6-*n*-propylthiouracyl (PTU) (Sanders *et al.* 1997, Orozco *et al.* 2003). However, the type II deiodinase (D2) is a low K_m (nM range for T_4), low V_{max} enzyme that only has ORD activity and is also not inhibited by PTU (Valverde-R *et al.* 1997, Orozco *et al.* 2002). The type III deiodinase (D3), like D2, is also a selective, low K_m (nM range for T_3), low V_{max} enzyme that is not inhibited by PTU, but, unlike D2, it is capable only of IRD (Sanders *et al.* 1999).

Although a large number of factors (endocrine and others) are known to affect peripheral thyroid hormone metabolism, thyroid status itself is one of the most potent regulators of iodothyronine deiodinase expression and

activity (Bianco *et al.* 2002). Despite this, the literature records surprisingly few data on the role of thyroid hormones in the regulation of fish deiodination. Mol *et al.* (1999) reported that in the Nile tilapia, induction of hyperthyroidism by feeding the animals with T_3 -supplemented food (12 p.p.m.) for 11 days resulted in a prominent decrease in hepatic D2 activity, whereas hepatic D3 activity increased. However, neither brain nor gill D3 activity was affected, nor was kidney D1 activity. Similarly, in rainbow trout (Finsson & Eales 1999) and sturgeon (Plohman *et al.* 2002), administration of T_3 -supplemented (12 p.p.m.) food also resulted in a decrease in *in vitro* hepatic D2 activity. However, only in rainbow trout was this also accompanied by a decrease in hepatic D3 activity, whereas liver D1 remained unaffected (Finsson & Eales 1999). In addition, hyperthyroidism had no effect on D3 levels in the brain of sturgeon (Plohman *et al.* 2002) and Atlantic salmon (Morin *et al.* 1995), whereas in the gills of rainbow trout (MacLachy & Eales 1993) hyperthyroidism had no effect on D3 activity levels. The only case so far in which hyperthyroidism induced an increase in *in vitro* D3 activity, other than in the liver, was noted by Plate *et al.* (2002), who observed that in 1-year-old rainbow trout that had been immersed for 6 weeks in 100 p.p.m. T_4 , liver and brain D3 activities were increased. However, so far, no data are available on the regulation of iodothyronine deiodinases in T_4 -fed hyperthyroid fish, making it difficult to come to any clear-cut conclusions. In addition, in most of these T_3 supplementation studies, plasma T_3 concentrations were only slightly elevated, so that it is possible that intracellular T_3 levels in the brain and the gills were not sufficiently high to affect D3 in these tissues. This would probably not be the case in the liver, since it directly receives the T_3 absorbed by the intestine, thereby making it more prone to react to T_3 supplementation. On the other hand, the different effects of T_3 -induced hyperthyroidism on liver D3 versus brain and gill D3 might point to differences in D3 regulation between these tissues. In order to resolve this controversy, it is necessary to determine the intracellular thyroid hormone concentrations in these tissues during hyperthyroidism, and this, to our knowledge has never been done.

We therefore decided to study the effects of experimentally induced hyperthyroidism on plasma T_3 and T_4 levels, as well as T_3 and T_4 concentrations, in liver, kidney, brain, gill and white muscle of tilapia (*Oreochromis niloticus*). Also the *in vitro* activity of the different deiodinases expressed in liver, kidney, brain and gill was measured. Since previous studies in tilapia had demonstrated that the use of 12 p.p.m. T_3 -supplemented food resulted in only a modest increase in plasma T_3 levels (Mol *et al.* 1999), we decided to induce hyperthyroidism by feeding the fish food supplemented with 48 p.p.m. T_3 or 48 p.p.m. T_4 for 2 weeks. This was done to ensure that, in addition to the plasma thyroid hormone levels, the intracellular T_3 and T_4 levels

would also increase significantly, thereby expanding the hyperthyroid state to the cellular level. For a better insight into the dynamics of thyroid hormone metabolism in fish, we also measured the same thyroid-related parameters during the 2 weeks following the treatment period, when thyroid-supplemented food was replaced with a control diet. In this respect, also the half-life of exogenously administered T_3 was measured.

Materials and Methods

Experimental design

A total of 360 adult tilapia (*O. niloticus*) of both sexes were procured from a commercial fish farm (Maryn Donkers, Boerdonk, The Netherlands). Three weeks before the start of the experiment, the fish were divided into nine groups of 40 fish and were acclimatized in 60 l tanks supplied with aerated tap water under a 12 L:12D photoperiod and an average temperature of 27 °C. The average weight of the fish at the start of the experiment was 190.4 ± 2.9 g. Fish were fed four times a day (0800, 1200, 1600 and 2000 h) to satiety with commercial tilapia pellets containing 49% crude protein, 11% crude fat and 1.5% cellulose, enriched with vitamin A, vitamin D₃ and vitamin E (TI-3 tilapia start feed, Trouw Nutrition Belgium, Ghent, Belgium). Three groups of fish ($n=120$) were made hyperthyroid by treatment with 48 p.p.m. T_3 in the food during 14 days, while three other groups ($n=120$) were given food containing 48 p.p.m. T_4 . For supplementation of the food, T_3 and T_4 were dissolved in ethanol and sprayed over the food with a Wurster-coater (Glatt, Binzen, Germany). Therefore, the remaining three groups, which served as controls, received food treated with an identical amount of ethanol. Treatment with the hormone-supplemented food was applied during the first 2 weeks of the experiment (days 1–15). Immediately after sampling at day 14, hormone-supplemented food was withdrawn, and all groups were fed the control diet for an additional 14 days (days 15–28). Just before the start of the experiment (day 0), blood and tissue samples were taken from 10 animals, while an additional 10 animals per treatment group were sampled on days 1–3, 7, 14–17, 21 and 28. To exclude variation due to circadian changes in hormone levels or deiodinase activities, all samples were taken between 1400 and 1600 h. Blood was collected in heparinized tubes by puncture of the caudal vein within 1 min after netting. After centrifugation, plasma was collected and stored at –20 °C until assayed for hormone levels. Fish were subsequently measured, weighed and killed by decapitation. Liver, brain, gill, kidney and white muscle were isolated, frozen in liquid nitrogen and stored at –80 °C when used to determine deiodinase activity or –20 °C when used to measure thyroid hormone levels.

For determination of the plasma T_3 half-life, eight fish from the batch used in the first experiment were housed in

individual 60 l glass tanks supplied with aerated tap water under a 12 L:12D photoperiod and an average temperature of 27 °C. Before the start of the experiment (0 min), a 200 µl blood sample was taken, as described before. Subsequently, the fish were injected intravenously with 200 µl T_3 solution (1 ng/µl). Additional 200 µl blood samples were collected 2, 5, 10, 20, 40, 60, 90, 120, 180 and 300 min after the injection. Plasma was stored at -20 °C until assayed for T_3 .

The University Ethical Committee for Animal Experiments approved all experimental animal manipulations.

Determination of T_4 and T_3 levels in plasma and tissues

T_3 and T_4 levels in plasma and in liver, kidney, brain, gill and muscle extracts were measured by radioimmunoassay (RIA) as described previously (Van der Geyten *et al.* 2001). Tissues were extracted as described in detail elsewhere (Reyns *et al.* 2002). In short, 200 mg tissue were homogenized in methanol, and 1500–2000 c.p.m. of outer-ring $^{131}\text{I}-T_3$ and $^{125}\text{I}-T_4$ were added as internal recovery tracers. Thereafter, chloroform was added to form a solvent mixture of chloroform:methanol (v/v=2:1). After centrifugation (15 min, 1900 g, 4 °C), the pellet was re-extracted with the same solvent. Both supernatants were combined and further extracted with chloroform:methanol:water (8:4:3) and 0.05% CaCl_2 . The mixed solution was centrifuged (10 min, 800 g, 4 °C) to stimulate the separation of the apolar and the polar phases. The lower or apolar phase was re-extracted with pure upper layer, chloroform:methanol:water (3:49:48), in an amount equal to that removed. The obtained upper or polar layers were pooled and further purified by anion-exchange chromatography on AG 1-X2 resin columns (Bio-Rad). The columns were washed with solutions of decreasing pH. Finally, thyroid hormones were eluted with 70% acetic acid, evaporated to dryness and resuspended in RIA buffer (0.05 M sodium diethylbarbiturate, 0.05 M sodium azide, 0.05 M EDTA disodium salt dihydrate and 1% bovine serum albumin). Recoveries of extracted thyroid hormones were usually 50–75% for $^{131}\text{I}-T_3$ and 40–60% for $^{125}\text{I}-T_4$. Plasma T_3 and T_4 levels were expressed as pmol/ml, whereas tissue thyroid hormone levels were expressed as pmol/g wet tissue.

Deiodinase assays

Preparation of homogenates or microsomal fractions and measurement of D1, D2 and D3 activities were performed as described by Van der Geyten *et al.* (1998, 2001). For D1 activity, incubation mixtures contained 50 000 c.p.m. $3',5'-^{125}\text{I}-\text{r}T_3$, 1 µM $\text{r}T_3$, 0.5 mg/ml homogenate or microsomal tissue protein, and 15 mM dithiothreitol (DTT). For D2 activity, incubation mixtures contained 50 000 c.p.m. $3',5'-^{125}\text{I}-T_4$, 1 nM T_4 , 0.25–1 mg/ml homogenate or microsomal protein, and 30 mM DTT. To detect

possible interference from residual D1 activity, the ORD of T_4 was also measured in the presence of 100 nM T_4 . At this substrate concentration, the D2 enzyme is saturated, so that all Γ measured in the assay is derived from the ORD of $3',5'-^{125}\text{I}-T_4$ by D1. Only when Γ production was minimal in the presence of 100 nM T_4 was enzyme activity in the assay using 1 nM T_4 considered to be true D2 activity. For D3 activity, incubation mixtures contained 150 000 c.p.m. $3'-^{125}\text{I}-T_3$, 1 nM T_3 , 0.5–1 mg/ml homogenate or microsomal tissue protein, and 30 mM DTT. All deiodinase activities were calculated as the amount of substrate deiodinated per milligram of protein per minute.

Statistical analysis

Data were processed and analyzed by PRISM 4.02 software (Graphpad Software, San Diego, CA, USA), using two-way ANOVA followed by Bonferroni post-tests, in which data from the T_3 -fed and T_4 -fed groups were compared with the control group. Statistically significant differences are indicated as follows: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$. For determination of the plasma T_3 half-life, a one-phase exponential decay curve ($Y=a \times \exp(-K \times X)+b$) was fitted through the data, and the half-life was calculated as $t_{1/2}=0.69/K$.

Results

Analysis of the data revealed what appeared to be sex-related differences in the parameters studied, in the control group as well as in the thyroid hormone-treated groups. However, since only 15% of all animals in the studied cohort were female, these differences could not be verified statistically. Therefore, only the data from the male population (85% of the samples) were taken into account and presented in the results.

T_3 and T_4 levels in plasma and tissues

For plasma thyroid hormone levels, as well as brain, muscle and gill T_3 concentrations, significant changes were observed for treatment ($P<0.01$), time ($P<0.05$) and time $\times$ treatment interaction ($P<0.05$). For liver and kidney T_3 levels, only treatment-related changes were observed ($P<0.001$ and $P<0.01$ respectively). Except for liver T_4 ($P<0.001$), no treatment-related changes were observed in tissue T_4 levels. In contrast, significant changes in tissue T_4 content were observed for time ($P<0.05$) in all tissues examined, but not for the time $\times$ treatment interaction.

Plasma T_3 levels were elevated in the T_3 -fed fish during week 1 of treatment, although this increase became significant only at day 3 (Fig. 1A). By day 14, circulating T_3 concentrations had returned to control values. However, after withdrawal of the T_3 -supplemented food,

Figure 1 Plasma T₃ levels (pmol/ml) in control, T₃-fed and T₄-fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. ($n=10$). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ (black stars refer to the T₃-fed group).

plasma T₃ levels rapidly fell, reaching a minimum 3 days later (day 17), after which they gradually returned to control values (Fig. 1B). T₄ supplementation, in contrast,

had no effect on plasma T₃ concentrations (Fig. 1). Plasma T₄ levels were also not affected by feeding the T₄ supplemented food, while T₃ supplementation resulted in decreased plasma T₄ concentrations during the entire treatment period, although this was only statistically significant at day 14 (Fig. 2A). After we changed to the control diet, plasma T₄ in the T₃-fed fish returned to control levels within 1 day, whereas it tended to increase in the T₄-fed groups, although this was only significant at day 21 (Fig. 2B).

Like plasma T₃, T₃ levels in the liver, kidney and brain were increased in the T₃-fed groups. While in the liver (Fig. 3A) and brain (Fig. 5A) these levels remained above control levels during the entire first week of T₃ supplementation, this increase was short-lived in the kidney (Fig. 4A). After withdrawal of the T₃-supplemented food, T₃ levels in liver (Fig. 3B) and brain (Fig. 5B) fell below control levels during the first 3 days (until day 17), although this decrease was only significant in the brain. One week after the switch to the control diet (day 21), liver and brain T₃ concentrations had reached control values again. Neither liver, kidney, nor brain T₃ concentrations were affected in the T₄-fed groups (Figs 3–5). T₃ concentrations in muscle were, on average, higher in the T₃-fed group than in the control group, although this was only significant at days 2 and 15. As in liver and brain, withdrawal of the T₃-supplemented food resulted in a transient decrease of muscle T₃ levels, although this decrease never became statistically significant. T₄ supplementation had no effect on thyroid hormone levels in muscle (Table 1). In the gills, T₃ supplementation induced higher T₃ levels only at the end of the treatment period (day 14), while T₄ treatment remained without effect (Table 1). Tissue T₄ concentrations in kidney, brain, muscle and gills remained largely unaffected by T₃ and T₄ supplementation, although they tended to be somewhat lower in the kidney of T₃-fed fish (Table 1). Hepatic T₄ levels also tended to be lower than control values in the T₃-supplemented fish, but they were higher in the T₄-fed fish (significant at day 7) (Table 1).

Deiodinases in liver, kidney, brain and gill

For hepatic D1, D2 and D3, as well as gill D3, significant changes were observed for treatment ($P<0.01$), time ($P<0.001$) and time $\times$ treatment interaction ($P<0.001$). Gill D2 showed significant changes only for time ($P<0.01$) and time $\times$ treatment interaction ($P<0.05$). Kidney D1 demonstrated only time-related changes ($P<0.05$), while kidney D3 and brain D3 were not affected by the experimental procedure.

T₃ supplementation had no effect on *in vitro* D1 activity in the liver (Table 2). T₄ supplementation, however, significantly increased hepatic D1 activity during week 1 of treatment (starting at day 3), whereas withdrawal of the T₄-supplemented food resulted in a decrease of hepatic D1

Figure 2 Plasma T_4 levels (pmol/ml) in control, T_3 -fed and T_4 -fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. ($n=10$). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ (black stars refer to the T_3 -fed group; white stars to the T_4 -fed group).

activity that proved to be significant after 2 and 7 days (days 16 and 21) (Table 2). In contrast, *in vitro* hepatic D2 activity was significantly decreased in the T_3 -fed fish after 1 and 2 weeks of treatment (Fig. 6A), and withdrawal of

Figure 3 Liver T_3 levels (pmol/g) in control, T_3 -fed and T_4 -fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. ($n=5$). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ (black stars refer to the T_3 -fed group).

the T_3 -supplemented food resulted in a gradual return to control values that was complete after about 1 week (day 21) (Fig. 6B). T_4 supplementation, in turn, did not affect *in vitro* hepatic D2 activity (Fig. 6). Neither T_3 nor T_4

Figure 4 Kidney T_3 levels (pmol/g) in control, T_3 -fed and T_4 -fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. (n=5). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * P <0.05, ** P <0.01 and *** P <0.001 (black stars refer to the T_3 -fed group).

Figure 5 Brain T_3 levels (pmol/g) in control, T_3 -fed and T_4 -fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. (n=5). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * P <0.05, ** P <0.01 and *** P <0.001 (black stars refer to the T_3 -fed group).

supplementation had any effect on *in vitro* renal D1 activity or on *in vitro* gill D2 activity (Table 2). Although D2 activity was also measured in kidney and brain, under the

experimental conditions described in this paper and with the kinetic assays used, no D2 activity could be detected in these tissues.

Table 1 Effects of thyroid hormone supplementation via the food on T₃ and T₄ concentrations (pmol/g) in liver, kidney, brain, gill and muscle. Data presented are the mean $\pm$ s.e.m. (*n* = 5)

		Time (days)										
		0	1	2	3	7	14	15	16	17	21	28
Liver T4	control	8.4 ± 2.0	7.7 ± 1.5	6.1 ± 1.6	5.7 ± 0.6	5.9 ± 0.6	5.0 ± 1.3	7.9 ± 1.8	3.13 ± 0.3	7.6 ± 2.8	4.9 ± 0.8	7.3 ± 1.6
	T ₃ -fed		3.6 ± 0.6	3.7 ± 0.5	6.2 ± 1.0	6.0 ± 1.4	3.3 ± 0.6	5.4 ± 0.4	6.0 ± 1.0	3.3 ± 0.8	7.1 ± 1.4	3.7 ± 0.5
	T ₄ -fed		4.2 ± 0.9	7.7 ± 2.1	10.8 ± 2.5	14.8 ± 3.7**	10.1 ± 0.9	4.5 ± 0.8	5.7 ± 0.7	8.1 ± 1.9	9.6 ± 1.8	6.3 ± 2.0
Kidney T4	control	11.1 ± 3.5	12.2 ± 5.4	9.5 ± 0.3	12.9 ± 5.1	10.1 ± 3.2	10.3 ± 5.1	6.3 ± 1.7	4.3 ± 1.6	5.4 ± 4.2	5.0 ± 3.4	6.9 ± 3.4
	T ₃ -fed		4.8 ± 1.6	5.4 ± 0.7	6.4 ± 0.9	4.3 ± 1.4	4.1 ± 0.8	6.3 ± 3.2	11.2 ± 3.8	1.2 ± 1.1	6.4 ± 3.1	6.4 ± 5.8
	T ₄ -fed		7.5 ± 2.0	9.5 ± 2.9	8.4 ± 2.6	7.5 ± 0.6	3.5 ± 2.9	6.4 ± 0.8	10.0 ± 5.0	11.2 ± 4.5	11.6 ± 2.6	5.6 ± 3.6
Brain T4	control	5.4 ± 0.8	4.6 ± 0.3	4.3 ± 0.8	5.3 ± 0.9	4.0 ± 0.6	3.6 ± 1.1	6.0 ± 1.0	4.1 ± 0.2	3.4 ± 1.4	4.5 ± 0.5	2.8 ± 1.3
	T ₃ -fed		4.0 ± 0.4	3.0 ± 0.4	4.5 ± 0.6	3.9 ± 0.6	1.9 ± 0.3	2.9 ± 0.54	4.7 ± 0.9	1.9 ± 0.4	3.8 ± 0.7	4.2 ± 0.6
	T ₄ -fed		4.2 ± 0.7	4.5 ± 1.2	3.9 ± 0.5	3.1 ± 0.3	3.1 ± 0.6	2.9 ± 0.4	5.8 ± 1.0	4.3 ± 1.0	6.4 ± 0.8	4.6 ± 1.5
Gill T4	control	0.49 ± 0.10	0.39 ± 0.03	0.34 ± 0.04	0.36 ± 0.02	0.58 ± 0.06	0.62 ± 0.10	0.45 ± 0.04	0.47 ± 0.10	0.46 ± 0.02	0.56 ± 0.07	0.54 ± 0.08
	T ₃ -fed		0.40 ± 0.03	0.30 ± 0.02	0.43 ± 0.02	0.42 ± 0.09	0.76 ± 0.15	0.40 ± 0.02	0.52 ± 0.06	0.40 ± 0.03	0.49 ± 0.03	0.60 ± 0.11
	T ₄ -fed		0.36 ± 0.06	0.42 ± 0.05	0.48 ± 0.09	0.69 ± 0.17	0.51 ± 0.05	0.80 ± 0.23	0.51 ± 0.10	0.51 ± 0.03	0.42 ± 0.02	0.52 ± 0.05
Muscle T4	control	2.2 ± 0.5	1.1 ± 0.1	1.7 ± 1.2	1.6 ± 0.3	1.2 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	0.9 ± 0.3	0.9 ± 0.1	1.0 ± 0.1
	T ₃ -fed		1.2 ± 0.5	2.7 ± 0.6	0.7 ± 0.2	1.3 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
	T ₄ -fed		1.8 ± 0.2	1.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
Gill T3	control	0.89 ± 0.15	0.86 ± 0.21	0.41 ± 0.05	0.34 ± 0.02	1.03 ± 0.59	0.43 ± 0.16	0.52 ± 0.11	0.68 ± 0.08	0.39 ± 0.05	0.43 ± 0.19	0.41 ± 0.02
	T ₃ -fed		1.41 ± 0.05	0.39 ± 0.02	0.48 ± 0.03	0.93 ± 0.47	2.41 ± 0.87**	0.46 ± 0.06	0.54 ± 0.05	0.40 ± 0.06	0.60 ± 0.10	1.31 ± 0.74
	T ₄ -fed		0.63 ± 0.03	0.49 ± 0.03	0.32 ± 0.02	1.08 ± 0.52	0.45 ± 0.02	1.18 ± 0.80	0.56 ± 0.04	0.59 ± 0.14	0.53 ± 0.05	0.50 ± 0.08
Muscle T3	control	5.5 ± 0.6	4.9 ± 0.4	7.2 ± 1.4	5.4 ± 0.5	5.9 ± 0.6	5.6 ± 0.4	3.7 ± 1.3	5.3 ± 1.0	5.9 ± 0.9	5.5 ± 0.2	4.7 ± 0.5
	T ₃ -fed		7.6 ± 1.4	16.8 ± 2.6***	6.6 ± 1.1	9.8 ± 0.5	8.1 ± 1.5	6.8 ± 1.9*	3.9 ± 0.3	2.9 ± 0.3	4.8 ± 0.2	4.6 ± 0.9
	T ₄ -fed		6.3 ± 0.2	8.5 ± 3.6	9.9 ± 5.7	4.1 ± 0.7	4.8 ± 0.5	4.4 ± 0.5	4.5 ± 0.5	4.3 ± 0.3	5.1 ± 0.8	4.9 ± 0.5

Statistically significant differences from the control group are indicated as follows: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. || indicates the moment thyroid hormone-supplemented food was withdrawn and replaced with the control diet.

Table 2 Effects of thyroid hormone supplementation via the food on *in vitro* D1 activity (pmol T₃/mg × min) in liver and kidney, *in vitro* D2 activity (fmol T₄/mg × min) in gill, and *in vitro* D3 activity (fmol T₃/mg × min) in kidney and brain. Data presented are the mean ± s.e.m. (n=5).

		Time (days)											
		0	1	2	3	7	14	15	16	17	21	28	
Liver D1	control	2.06 ± 0.44	2.46 ± 0.34	1.84 ± 0.54	1.46 ± 0.35	0.88 ± 0.09	0.95 ± 0.15	1.14 ± 0.25	2.22 ± 0.18	1.90 ± 0.17	1.92 ± 0.46	1.46 ± 0.34	
	T ₃ -fed		0.95 ± 0.28*	1.10 ± 0.24	2.02 ± 0.24	1.26 ± 0.25	1.06 ± 0.13	0.96 ± 0.12	1.20 ± 0.25*	1.00 ± 0.38	1.18 ± 0.28	1.96 ± 0.47	
	T ₄ -fed		2.12 ± 0.14	1.38 ± 0.35	3.09 ± 0.35*	2.04 ± 0.43*	0.94 ± 0.17	1.38 ± 0.17	1.67 ± 0.17*	1.48 ± 0.21	0.85 ± 0.22*	1.19 ± 0.26	
Kidney D1	control	1.40 ± 0.45	1.94 ± 0.53	0.90 ± 0.17	1.21 ± 0.25	0.74 ± 0.19	0.93 ± 0.12	0.55 ± 0.07	0.75 ± 0.14	0.78 ± 0.08	0.95 ± 0.28	0.96 ± 0.04	
	T ₃ -fed		1.41 ± 0.40	1.62 ± 0.34	1.85 ± 0.68	1.22 ± 0.29	1.25 ± 0.23	0.70 ± 0.17	1.02 ± 0.23	0.49 ± 0.10	1.03 ± 0.12	1.40 ± 0.46	
	T ₄ -fed		2.05 ± 0.08	1.65 ± 0.41	1.67 ± 0.39	1.30 ± 0.24	0.70 ± 0.06	0.70 ± 0.08	0.74 ± 0.10	0.55 ± 0.10	0.60 ± 0.17	0.92 ± 0.08	
Gill D2	control	0.84 ± 0.13	0.80 ± 0.03	0.80 ± 0.04	0.70 ± 0.04	0.74 ± 0.02	0.70 ± 0.02	0.76 ± 0.04	0.88 ± 0.05	0.75 ± 0.06	0.73 ± 0.08	0.71 ± 0.04	
	T ₃ -fed		0.84 ± 0.03	0.65 ± 0.08	0.91 ± 0.07	0.82 ± 0.02	0.79 ± 0.03	0.90 ± 0.01	0.79 ± 0.03	0.76 ± 0.03	0.74 ± 0.03	0.89 ± 0.04	
	T ₄ -fed		0.74 ± 0.11	0.85 ± 0.01	0.60 ± 0.07	0.76 ± 0.05	0.70 ± 0.01	0.90 ± 0.04	0.81 ± 0.04	0.68 ± 0.05	0.74 ± 0.04	0.76 ± 0.01	
Kidney D3	control	0.07 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.04 ± 0.01	0.07 ± 0.02	0.04 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.08 ± 0.03	0.07 ± 0.04	0.04 ± 0.03	
	T ₃ -fed		0.06 ± 0.01	0.11 ± 0.01	0.07 ± 0.01	0.02 ± 0.01	0.08 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.03 ± 0.02	
	T ₄ -fed		0.08 ± 0.02	0.07 ± 0.02	0.08 ± 0.01	0.09 ± 0.03	0.07 ± 0.01	0.03 ± 0.01	0.06 ± 0.02	0.02 ± 0.02	0.01 ± 0.01	0.06 ± 0.02	
Brain D3	control	8.0 ± 0.7	5.9 ± 1.6	7.9 ± 0.4	7.2 ± 0.5	7.9 ± 0.6	7.6 ± 1.4	5.7 ± 0.5	6.3 ± 1.1	6.6 ± 0.3	7.8 ± 0.9	8.8 ± 0.4	
	T ₃ -fed		7.3 ± 2.2	5.8 ± 1.1	6.3 ± 1.9	9.3 ± 0.8	8.7 ± 0.8	9.6 ± 0.9	7.1 ± 0.6	6.6 ± 1.3	8.7 ± 1.1	8.8 ± 1.1	
	T ₄ -fed		9.4 ± 1.3	7.6 ± 0.4	5.3 ± 1.6	9.1 ± 0.6	9.4 ± 1.0	5.1 ± 0.3	6.2 ± 1.6	8.6 ± 0.9	9.5 ± 1.1	7.8 ± 0.7	

Statistically significant differences from the control group are indicated as follows: *P<0.05. || indicates the moment thyroid hormone supplemented food was withdrawn and replaced with the control diet.

Figure 6 Liver D2 activities (fmol T₄/mg per min) in control, T₃-fed and T₄-fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. ($n=5$). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ (black stars refer to the T₃-fed group; white stars to the T₄-fed group).

In contrast to hepatic D2, *in vitro* hepatic D3 activity increased significantly in the T₃-fed fish by the end of the treatment period (Fig. 7A), and decreased rapidly the moment the T₃-supplemented food was replaced with the

Figure 7 Liver D3 activities (fmol T₃/mg per min) in control, T₃-fed and T₄-fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. ($n=5$). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ (black stars refer to the T₃-fed group; white stars to the T₄-fed group).

control diet (Fig. 7B). These low levels were maintained until the end of the experiment. Overall, T₄ supplementation had no effect on hepatic D3 activity, although activity levels were highly variable, especially after

Figure 8 Gill D3 activities (fmol T₃/mg per min) in control, T₃-fed and T₄-fed fish (A) during the treatment period (days 1–14) and (B) after changing to the control menu (days 15–28). Data are presented as the mean $\pm$ S.E.M. ($n=5$). The arrow ($\uparrow$) indicates the first sampling after all groups had switched to the control diet. Statistically significant differences from the control group are indicated as follows: * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ (black stars refer to the T₃-fed group; white stars to the T₄-fed group).

withdrawal of the T₄-supplemented food (Fig. 7). As in the liver, gill *in vitro* D3 activity was higher after 2 weeks of treatment in the T₃-fed fish (Fig. 8A). After the change to the control diet, gill D3 activity remained elevated for

Figure 9 T₃ clearance in plasma of fish after acute T₃ challenge. Data are presented as mean $\pm$ S.E.M. ($n=8$).

1 additional day (day 15), before falling below control levels until day 21 (Fig. 8B). T₄ supplementation had no effects on gill D3 activity (Fig. 8). Thyroid hormone supplementation also had no effect on *in vitro* D3 activity in kidney and brain (Table 2).

Plasma T₃ half-life

The plasma half-life of exogenously administered T₃ was determined to be 74.37 ± 4.37 min (Fig. 9).

Discussion

Two weeks of T₄ supplementation (48 p.p.m.) had no effect on the plasma T₃ and T₄ concentrations in tilapia. These observations agree with data obtained in rainbow trout, where 3 days of T₄ supplementation (24 and 48 p.p.m.) also did not affect circulating thyroid hormone levels (Sweeting & Eales 1992). In contrast, however, long-term immersion in T₄-supplemented water (6 weeks, 100 p.p.m. T₄) has been shown to increase plasma T₃ and T₄ levels significantly in rainbow trout (Plate *et al.* 2002). In addition to plasma T₃ and T₄, the tissue T₃ and T₄ concentrations, as well as the different deiodinases expressed in these tissues, also remained unaffected by feeding the T₄-supplemented food, with the liver as the sole notable exception. In T₄-fed tilapia, the hepatic T₄ levels increased substantially, and this was accompanied by an increase in *in vitro* D1 activity, while hepatic T₃ concentrations, as well as D2 and D3 activity, were not affected. This increase in intrahepatic T₄ levels was also observed in killifish immersed for 12–24 h in 100 nM T₄,

although in this experiment hepatic T_3 levels were also significantly increased, while D1 activity remained unaffected (García-G *et al.* 2004). The reason that T_4 supplementation via the food does not result in elevated plasma hormone levels is not readily apparent. Despite the difference in the amount of T_4 used between the experimental approaches, the observation that immersion in T_4 -supplemented water increases both plasma and hepatic T_3 and T_4 levels, while feeding of T_4 -supplemented food does not, suggests that the T_4 ingested with the food is not taken up efficiently from the gut, as has been proposed by several authors (Collicutt & Eales 1974, Higgs *et al.* 1979, Sweeting & Eales 1992). Although this mechanism is probably involved, it does not explain why in the current experiment the increase in hepatic T_4 is not reflected at the plasma level. However, Byamungu *et al.* (1990) demonstrated that in tilapia stimulation of endogenous T_4 levels, as well as intravenous injection of T_4 , was each time accompanied by a 20-fold increase in plasma rT_3 concentrations. These results therefore suggest that the lack of effect of T_4 supplementation via the food on circulating T_3 and T_4 might also be due to a rapid conversion of T_4 into rT_3 . This hypothesis is supported by our finding that the increase in hepatic T_4 is accompanied by an increase in hepatic D1 activity, which in turn is able to increase the conversion of T_4 into rT_3 via IRD.

However, in accordance with previous data, administration of T_3 -supplemented food resulted in a significant increase in plasma T_3 levels (MacLachy & Eales 1993, Finnson & Eales 1999, Mol *et al.* 1999), which in the present study was accompanied by a decrease in plasma T_4 . Although this T_4 decrease was not always observed earlier (Eales *et al.* 1990, Sweeting & Eales 1992), more recent studies indicate that it is, as in other vertebrates, a logical consequence of the T_3 -induced negative feedback at the level of pituitary thyroid-stimulating hormone (TSH) production and release (Larsen *et al.* 1997, Moriyama *et al.* 1997, Mol *et al.* 1999). Our present study supports this conclusion, since, after withdrawal of the T_3 -supplemented food, when plasma T_3 levels decreased and the negative feedback was abolished, plasma T_4 levels also returned to control values. In contrast, intracellular T_4 concentrations in the tissues examined do not seem to be affected by the drop in circulating T_4 levels, thereby providing further support for the negative feedback hypothesis. Besides plasma T_3 , T_3 in the different tissues also increased substantially, thereby demonstrating that the ingested T_3 reached all tissue compartments. In the gills, however, T_3 concentrations are, on average, 80% lower than plasma T_3 levels, while in the other tissues these levels are equal to or higher than the levels found in circulation. This could be explained by the relatively high D3 levels in the gills, were it not that in the brain, where D3 activity is at least twofold higher, T_3 concentrations are similar to what is present in the plasma. These observations therefore suggest that the gills, in contrast to the other

tissues, do not accumulate T_3 ; rather, they function as a thyroid hormone metabolizing relay station that is probably involved in regulating plasma T_3 availability. This hypothesis is supported by the observation that in the T_3 -supplemented fish, gill D3 activity reaches maximum levels at the same time that intracellular gill T_3 levels do.

The T_3 -induced hyperthyroidism affected neither hepatic nor renal D1 activity, confirming previous data in fish (Mol *et al.* 1999, Plohman *et al.* 2002). In addition, García-G *et al.* (2004) demonstrated in killifish that, although acute hyperthyroidism did not affect hepatic D1 activity, it readily decreased D1 mRNA expression levels in this tissue. This is in contrast to mammals, where hyperthyroidism stimulates both D1 activity and mRNA expression (Berry *et al.* 1990). These data indicate that in the substrate-induced regulation of D1 in the liver and kidney, the mechanisms at work in mammals do not seem to apply to fish. This hypothesis is further supported by the observation that in hypothyroid tilapia, hepatic D1 activity, as well as D1 mRNA expression, is upregulated (Van der Geyten *et al.* 2001), again in contrast to what is observed in mammals (Berry *et al.* 1990). In contrast, the decrease in D2 activity, as well as the increase in D3 activity, in the liver of T_3 -supplemented fish conforms more to what is observed in respectively the mammalian brain and liver (Kaplan & Yaskoski 1980, St Germain 1988, Tu *et al.* 1999). After withdrawal of the T_3 -supplemented food, hepatic D2 and D3 levels gradually return to control values, in concordance with the normalizing plasma T_3 levels. Although the liver is considered to be the main source of circulating T_3 , these changes in hepatic deiodination do not explain how plasma and tissue T_3 levels can drop so low so quickly, once the exogenous source of T_3 has been removed. In order for this to happen, a fast and efficient mechanism for clearing the excess T_3 would be required. This could be achieved by stimulating thyroid hormone sulfation and/or glucuronidation, thereby increasing thyroid hormone metabolic clearance rates and hence reducing circulating and tissue T_3 levels (Finnson & Eales 1996, Finnson *et al.* 1999). Although we did not measure thyroid hormone conjugation in the current study, our finding that exogenously administered T_3 resides in the plasma, with a half-life of only 74.37 ± 4.37 min (Fig. 9), suggests that tilapia possess a very efficient means of clearing the excess T_3 . This, together with the slowly restoring hepatic T_3 production capacity (via D2), may explain why plasma T_3 levels drop to only 31% of control values after removal of dietary T_3 supplementation.

Previous studies have demonstrated that hyperthyroidism has no effect on D3 activity in the brain and the gills (MacLachy & Eales 1993, Morin *et al.* 1995, Mol *et al.* 1999, Plohman *et al.* 2002). In accordance with these data, we also did not observe any effect of T_3 supplementation on *in vitro* brain D3 activity, while gill D3 activity increased together with gill T_3 levels. Nor did kidney D3

react to T_3 supplementation. Taken together, these data suggest that in the previously published studies T_3 levels in the gills probably never reached levels high enough to elicit an effect on gill D3 activity. However, this cannot be the case for lack of effect on brain and kidney D3, since the current data demonstrate that, in both these tissues, T_3 supplementation boosts intracellular T_3 concentrations to levels that are equivalent to the levels present in the plasma or the liver. Therefore, we must conclude that T_3 regulates D3 activity in brain and kidney differently from D3 activity in liver and gill.

In conclusion, the presented results demonstrate that in hyperthyroid tilapia the increase in plasma T_3 levels is also reflected in the liver, brain, kidney, gill and white muscle without affecting local T_4 concentrations. However, this increase in T_3 availability remains without effect on D1 activity in liver and kidney. This observation, together with the PTU insensitivity of the D1 enzyme in fish, sets the D1 in teleost fish clearly apart from its mammalian and avian counterparts. The changes in hepatic deiodinases confirm the role of the liver as an important T_3 -regulating organ. However, the very short plasma half-life of exogenously administered T_3 implies the existence of an efficient T_3 clearing/degradation mechanism other than deiodination. In addition, fish are remarkably insensitive to exogenous T_4 administration via the food. Although this could be partially explained by an inefficient uptake of T_4 from the gut, our current data suggest that the increased conversion of T_4 into rT_3 by the D1 present in the liver also plays an important role in this respect.

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