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Action of thyroid inhibitor propyl thiouracil on thyroid and interrenal axes in the freshwater tilapia *Oreochromis mossambicus* Peters

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SUMMARY

The actions of propyl thiouracil (PTU), a thyrostatic drug, on thyroid hormones (THs) and cortisol production were examined in freshwater tilapia (*Oreochromis mossambicus*) to examine whether the hypothyroid state affects the function of interrenal axes in this fish. The plasma levels of triiodothyronine (T_3), thyroxine (T_4), and cortisol along with branchial and renal Na^+, K^+-ATPase activities were measured after feeding varied doses (5-20 µg g^{-1}) of PTU for over 10 or 15 days. Feeding low dose (5 µg g^{-1}) of PTU produced significant rise in plasma T_4 level but both T_3 and T_4 levels were decreased significantly after feeding a high dose (20 µg g^{-1}) of PTU for 15 days. All doses of PTU failed to alter the plasma cortisol. No significant correlation was found between the plasma levels of T_3, T_4 and cortisol after varied doses of PTU. Treatment of T_3 or T_4 (40 ng g^{-1}) to the high dose PTU-fed tilapia produced a significant rise in plasma cortisol, suggesting a link to the thyroid and interrenal axes in this fish. The rise in T_3 or T_4 level in the PTU-treated tilapia after TH injections correlated with the branchial and renal Na^+, K^+ -ATPase activities, which imply an effective Na handling, though a tight regulation of Na^+ and K^+ transport was maintained in the plasma of these fish. The data show that high dose of PTU inactivates the thyroid axis resulting in the decreased production of T_3 and T_4, though a low dose of PTU activates the thyroid axis. Our results provide evidence that exogenous T_3 activates interrenal axis to produce cortisol and both exogenous T_3 and T_4 promote branchial and renal Na handling in PTU-induced hypothyroid tilapia.

Key words: Cortisol, fish, propyl thiouracil, *Oreochromis mossambicus*, osmoregulation, thyroid hormones, tilapia, stress.

Introduction

Triiodothyronine (T_3) and thyroxine (T_4), the principal thyroid hormones (THs) of the hypothalamo-pituitary-thyroid (HPT) axis, are involved in development and growth of fishes (Leatherland, 1994; Power et al., 2001, Peter, 2007; Geven et al., 2009). Evidences for an involvement of THs in hydromineral regulation are available, though there is some ambiguity on its role in hydromineral balance (Leatherland, 1994; Shrimpton and McCormick, 1998; Schreiber and Specker 1999, 2000; Mancera and McCormick, 1999). Our earlier studies on freshwater tilapia provided evidence that physiological concentrations of both T_3 and T_4 enhance branchial sodium pump activity and chloride cell functions (Peter et al., 2000). On the contrary, there are studies that report a lack of THs effect on branchial Na^+, K^+-ATPase activity in fishes including tilapia (Dange, 1986).

Cortisol, the product of hypothalamo-pituitary-interenal (HPI) axis, controls many physiological functions in teleosts including hydromineral regulation (Wendelaar Bonga, 1997; Bowers et al., 2000; Dang et al., 2001). This major corticosteroid regulates chloride cell function (Li et al., 1997; Wendelaar Bonga, 1997; Perry, 1997) and stimulates branchial Na^+, K^+-ATPase activity in many species of fishes (Mommsen et al., 1999; Mancera and McCormick, 1999). However, cortisol replacement in hypophysectomized coho salmon failed to restore completely Na^+, K^+-ATPase activity, highlighting the involvement of other hormones in hydromineral regulation (Bjornsson et al., 1987, Richman et al., 1987). Cortisol regulates plasma Na^+ levels in both seawater and freshwater fish and suppresses the rise of plasma Na^+ during seawater acclimation of Coho salmon (Redding et al., 1991).

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Na^+, K^+-ATPase activity provides the driving force for Na^+ transport in fishes. It is essential for reducing the urinary loss of Na^+ in the kidney tubules of freshwater fish (Madsen et al., 1995) and enhances the loss of Na^+ through the urine in seawater-adapted fish (Nolan et al., 1999). This enzyme system in the branchial chloride cells is involved in the absorption of Na^+ and Cl^- in freshwater fish (Perry, 1997; Fenwick et al., 1999). In tilapia THs maintain hydromineral balance by regulating branchial and renal tissue Na^+, K^+-ATPase activities, which increased and decreased respectively after TH treatment (Peter et al., 2000; Peter, 2007).

The interrelationship between the thyroid and the interrenals in teleosts has been the subject of many studies (Leatherland, 1987; Redding et al., 1986; Brown et al., 1991; Vijayan et al., 1997; Larsen et al., 1998; Geven et al., 2006; De Groef et al., 2006). For example, an activation of T_4 release from the carp renal tissue has been reported recently (Geven et al., 2009). It is believed that some of the actions of THs on hydromineral regulation may be mediated through cortisol (Leatherland, 1994; De Groef et al., 2006), though concrete evidences for such an interaction is not available (Peter, 2007). For example, T_4 has been shown to potentiate the stimulatory action of cortisol on Na^+, K^+-ATPase activity (Dange, 1986). Shrimpton and McCormick (1998) demonstrated a positive interaction of T_3 with corticosteroid receptors in juvenile Atlantic salmon. Redding et al. (1986) provided evidence of a cortisol-stimulated increase in T_3 clearance and not T_4 clearance in eels. Conversely, no correlation between thyroid and cortisol has been observed in salmon (Madsen, 1990), rainbow trout (Gomez et al., 1997) and mummichog (Mancera and McCormick, 1999). The physiological interaction of THs and cortisol is still unclear in spite of certain studies that have shown an association between the HPT and HPI axes in fishes (*e.g.*, Young et al., 1989; De Groef et al., 2006).

Lack of conclusive evidence for an interaction between thyroid and interrenal axes in fish (McCormick, 2001; Evans et al., 2005; Peter, 2007) prompted us to undertake this study to gather more information on the function of interrenal axis in a hypothyroid fish. Propylthiouracil (PTU), a thyroid inhibitor, was used in this study to induce hypothyroidism in the tilapia. PTU inhibits intrathyroidal synthesis of TH by interfering with thyroid peroxidase-mediated iodine utilization and the coupling of mono- and diiodotyrosines (MIT and DIT) which are required for the synthesis of T_3 and T_4 (Cooper, 2005; van der Ven et al., 2006). Furthermore, it blocks the peripheral conversion of T_4 to T_3 (Cooper, 2005). PTU administration has been shown to reduce THs levels in peripheral tissues and feedback stimulation of the thyroid in zebrafish (van der Ven et al., 2006). Similar hypothyroidic function has been reported in climbing perch after thiouracil treatment (Peter and Oommen, 1989; Nair and Oommen, 1997).

Materials and Methods

Fish and maintenance

Adult mozambique tilapia *Oreochromis mossambicus* (body mass 50 ± 5 g) of both sexes in pre spawning phase were kept in separate 100 L glass aquaria (density 3 gm L^{-1}) containing adequately aerated well water of 28 °C at 12h:12 h L:D cycle. Fish were fed daily with commercial fish feed at 1.5 % total body weight.

Experimental protocol

Experimental hypothyroidism was tested by feeding varied doses (5, 10 and 20 μg g^{-1}) of PTU (Sigma, St Louis, Missouri, USA) for 10 or 15 days. Fish were divided into groups of six each and kept in separate tanks. Two sets of experiment were conducted. Control feed at a ration of 1.5% body weight was fed to control fish daily for 10 or 15 days. Test groups of fish were fed with PTU meal (5 and 10 μg g^{-1}) over 10 or 15 days. On day 11 fish groups containing both control-fed and PTU-fed were sampled. Similarly, the other fish groups including control fish were sampled on day 16. In the second set of experiment, fish groups were given high dose (20 μg g^{-1}) of PTU for 15 days. On day 16 fish in these three groups were injected with saline, T_3 or T_4 (40 ng g^{-1}) for 24 h. All fish were sampled at 24 h after feeding or injection. No mortality was observed in any fish groups during the experiments.

Sampling procedure

Fish in each set of experiment were netted and anaesthetized in a 2-phenoxyethanol solution (0.1% SRL, Mumbai) and blood samples were collected by caudal puncture using heparinised syringe fitted with 23 gauge needle. Plasma was separated by centrifugation (3 min, 5,000 xg) and stored at –20 °C. Fish were then killed by spinal transsection and the gill arches and the kidney were excised and placed in 2 ml of ice cold SEI buffer (0.3 M sucrose, 20 mM Na_2EDTA, 0.1 M imidazole, pH 7.4) and stored at -20 °C.

Analyses

Plasma cortisol, and plasma total T_4 and T_3 were quantified by RIA (Peter, 2007). The specific activity of ouabain-sensitive Na^+, K^+-ATPase was measured in homogenates prepared from branchial and renal tissues as described earlier (Peter et al., 2000). The protein concentration in homogenates was measured with a commercial Biuret protein-assay kit (Bio-Rad, Hercules, USA) using bovine serum albumin as standard. Phosphate release was quantified spectrophotometrically and the specific activity expressed as mmol *Pi* h^{-1} mg $protein^{-1}$. Plasma Na^+ and K^+ concentrations were measured with a flame-photometer (Systronics 129, New Delhi, India). The Cl^- concentration was determined spectrophotometrically in terms of formation of ferrothiocyanate complex. Plasma osmolality (mOsm Kg^{-1}) was measured with a micro-osmometer (Gonotech 030, GmBH, Germany). Heparinized capillaries were filled with blood, centrifuged for 3 min at 5000 xg and the haematocrit value was determined.

Statistical analyses

Data were presented as means ± S.E.M. for six fish and checked for normal distribution and variance homogeneity. One-way analysis of variance followed by Student-Newman-Keuls (SNK) multiple range tests (Instat-3, Graphpad Software Inc., San Digeo, USA) were used to assess the significance between treatments and significance was accepted if $P < 0.05$.

Results

Plasma T_3, T_4 and cortisol

Feeding tilapia with low doses of PTU (5 µg g^{-1}) for 10 and 15 days resulted in significant increase ($P< 0.01$) in plasma T_4 without affecting plasma T_3, compared with the control fish (Fig. 1A and B). However, feeding fish with 20 µg g^{-1} of PTU resulted in significant decrease ($P< 0.01$) of plasma T_3 and T_4 levels (Fig. 1A). Replacement of T_3 (40 ng g^{-1}) brought about significant increase of plasma T_3 ($P < 0.01$) level after 24 h without any change in T_4 level (Fig. 2 A). Treatment of T_4 brought about significant increase in the plasma T_4 ($P < 0.01$) level in the PTU-fed tilapia (Fig. 2A).

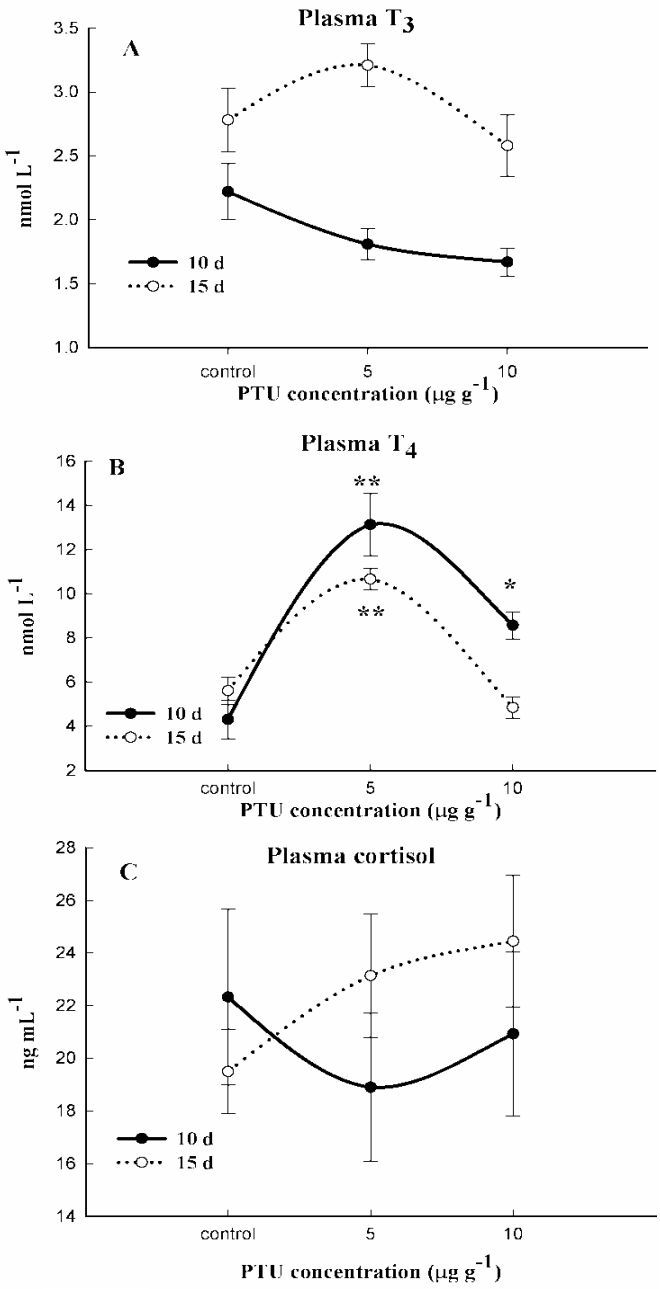

Fig. 1. Plasma T_3 (A), T_4 (B) and cortisol (C) levels in freshwater tilapia after low doses (5 and 10 µg g^{-1}) of PTU feeding for 10 and fifteen days. Each point is mean ± SEM. The data were tested for statistical significance between treatments with the SNK test. * ($P<0.05$) shows significance between control and PTU fed tilapia .

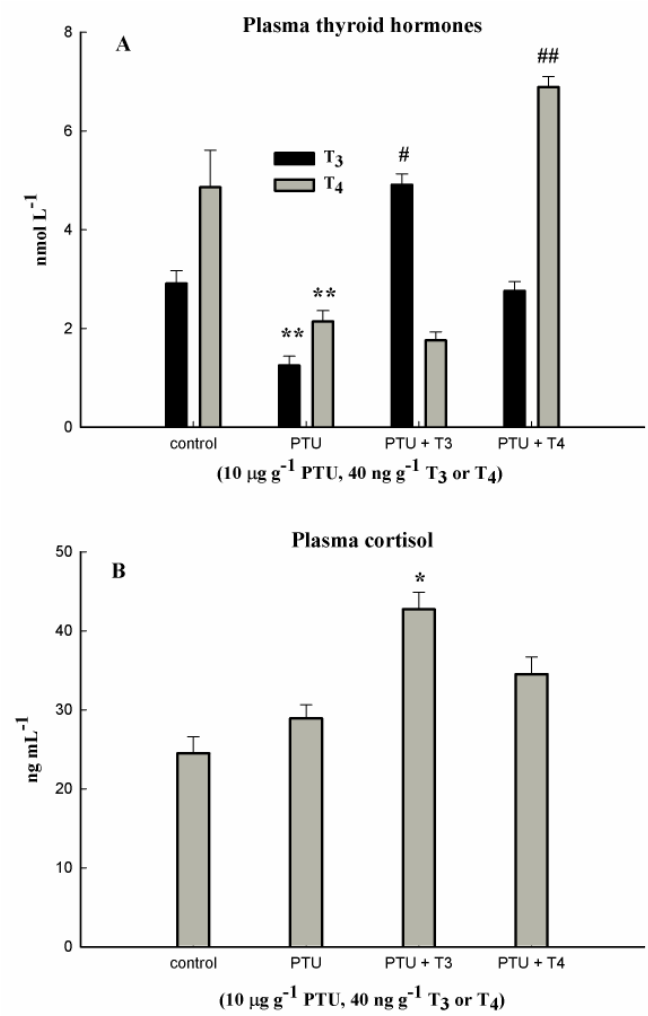

Fig. 2. Plasma T_3, T_4 and cortisol levels in freshwater tilapia after PTU feeding (20 µg g^{-1}) for 15 days with or without a single injection of T_3 (40 ng g^{-1}) or T_4 (40 ng g^{-1}) for 24 h. Each column, mean ± SEM. The data were tested for statistical significance between treatments with the SNK test. * ($P<0.05$) shows significance between control and PTU fed tilapia # ($P<0.05$) and ## ($P<0.01$) show significance between PTU fed tilapia and PTU fed + TH treated fish.

The plasma cortisol level in the fish fed 5 and 10 µg g^{-1} of PTU failed to show any change (Fig. 1C). However, a significant increase in plasma cortisol ($P < 0.01$) occurred in the 20 µg g^{-1} PTU-treated tilapia after a single T_3 injection (Fig. 2B).

Na^+, K^+-ATPase activity

The branchial and renal Na^+, K^+-ATPase activities remained unchanged following feeding of low doses of PTU for either 10 or 15 days (data not shown). Feeding PTU (20 µg g^{-1}) for 15 days altered neither branchial nor renal Na^+, K^+-ATPase activity (Fig. 3). However, treatment of T_3 or T_4 to PTU-treated fish brought about significant ($P<0.01$) increase in the branchial Na^+, K^+-ATPase activity ($P < 0.05$) and decrease in the renal Na^+, K^+-ATPase activity (Fig. 3).

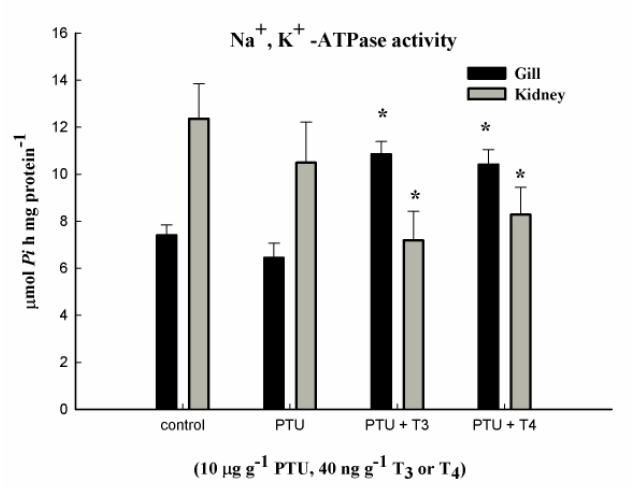

Fig. 3. Branchial and renal Na^+, K^+-ATPase activities in freshwater tilapia after PTU feeding (20 µg g^{-1}) for 15 days with or without a single injection of T_3 (40 ng g^{-1}) or T_4 (40 ng g^{-1}) for 24 h. Each column, mean ± SEM. The data were tested for statistical significance between treatments with the SNK test. * ($P<0.05$) shows significance between PTU fed fish and PTU fed and TH treated fish.

Plasma parameters

Neither PTU-treatment nor treatment of T_3 or T_4 in PTU-fed tilapia altered the plasma Na^+ and K^+ contents (Table 1). Similarly, plasma osmolality and haematocrit were not different from the control groups and were considered normal (Table 2).

Discussion

Data to the effect that PTU-feeding brings about hypothyroidism in tilapia but produces no effect on interrenal activity, are presented. An activation of interrenal axis occurred in the hypothyroid tilapia supplemented with T_3. We also demonstrate that replacement of THs stimulate hydromineral capacity of the PTU-induced hypothyroid tilapia.

This study indicates a direct and specific effect of PTU on thyroid activity in tilapia as this goitrogen in its low dose activates thyroid axis by releasing T_4, whereas in its high dose, it inactivates thyroid by inhibiting T_3 and T_4 production. It is likely that the low doses of PTU might have induced hyperplasia of thyroid follicles and releasing its stored T_4 into the blood. This view is consistent with

Table 1 Plasma Na^+, K^+ and Cl^- levels in freshwater tilapia after 20 μg g^{-1} of PTU feeding for fifteen days, and 40 ng g^{-1} TH for 24 h. Each value is mean ± SEM. The data were tested for statistical significance between treatments with the SNK test.

	Na^+ (mmol L^{-1})	K^+ (mmol L^{-1})	Cl^- (mmol L^{-1})
Control + saline	155 ± 2.15	4.91 ± 0.36	133.4 ± 1.18
PTU 20 μg g^{-1} for15 d + saline	151 ± 1.84	5.14 ± 0.41	132.6 ± 1.71
PTU 20 μg g^{-1} for15 d + 40 ng T_3	153 ± 1.69	5.29 ± 0.51	134.8 ± 1.69
PTU 20 μg g^{-1} for15 d + 40 ng T_4	152 ± 1.79	5.89 ± 0.34	131.4 ± 1.72

Table 2 Plasma haematocrit and osmolality levels in freshwater tilapia after 20 μg g^{-1} of PTU feeding for fifteen days, and 40 ng g^{-1} TH for 24 h. Each value is mean ± SEM. The data were tested for statistical significance between treatments with the SNK test.

	Haematocrit value (%)	Osmolality (mOsmol Kg^{-1})
Control + saline	30.4 ± 1.21	312 ± 1.42
PTU 20 μg g^{-1} for15 d + saline	32.8 ± 1.32	318 ± 1.23
PTU 20 μg g^{-1} for15 d + 40 ng T_3	29.6 ± 1.19	314 ± 1.51
PTU 20 μg g^{-1} for15 d + 40 ng T_4	30.4 ± 1.32	315 ± 1.65

the report on the thyroid hypertrophy observed in zebrafish after PTU feeding (van der Ven et al., 2006). The unaffected plasma T_3 in the low dose PTU-treated fish, on the contrary, supports the notion that PTU would impair the T_4 conversion to T_3. Such impaired TH synthesis was also found in zebrafish (van der Ven et al., 2006). High doses of PTU feeding in tilapia, on the contrary, substantially inhibited TH production and made these tilapia experimentally hyperthyroidic as demonstrated in zebrafish (van der Ven et al., 2006). This hyperthyroid tilapia, however, failed to show any impairment in their hydromineral effects, probably because of the involvement of other hormones in the absence of THs.

A close functional relationship exists between TH and corticosteroids in mammals, though little evidence for such an interaction is available in fish (Leatherland, 1994; Peter, 2007). There is no activation of interrenal axis in PTU-fed fish and it is likely that some other hormone(s) are involved in the maintenance of the hydromineral regulation in the PTU-induced hypothyroid tilapia. Surprisingly, an up-regulated interrenal axis, as indicated by high levels of plasma cortisol, occurred in hypothyroid tilapia following T_3 replacement. This positive correlation of exogenous T_3 and cortisol levels in hypothyroid tilapia is probably due to a direct action of T_3 on the interrenal axis which becomes apparent only in the PTU-induced hypothyroid fish. This interpretation is in agreement with a report on the stimulatory effects of THs on the interrenal axis of coho salmon (Young, 1988). It appears that the exogenous T_3 that stimulates the interrenal tissue to produce more cortisol might lead to an increased clearance of T_3, as cortisol-stimulated increase in T_3 clearance has been reported in European eels (Redding et al., 1986) and coho salmon (Redding et al., 1984). The results of other studies also indicate a rapid clearance of T_3 after cortisol treatment. For instance, an increased hepatic conversion of T_4 to T_3 by cortisol has been found in brook trout (Vijayan et al., 1988). However, reduction in plasma T_3 level by enhancing its clearance, without changing the hepatic conversion of T_4 to T_3, occurs in rainbow trout (Brown et al., 1991). It appears that a decreased TSH production might occur in the PTU fed tilapia due to the increase in plasma T_3 availability through a negative feedback. This might activate corticotrophin-releasing hormone (CRH), which is responsible for cortisol release

in these hypothyroid fish. It is likely that a sort of central interaction between thyroid and interrenal axis might occur in the tilapia. Besides these central regulations of interrenal and thyroid axes, peripheral interaction between TH and cortisol has been reported in common carp (Geven et al., 2009). The release of cortisol from head kidney fragments does not happen in carp tissues after T_4 treatment (Geven et al., 2006). On the contrary, exposure of head kidney and kidney fragments to cortisol and ACTH stimulates the release of T_4 from these carp tissues (Geven et al., 2009). Similar interaction of thyroid and interrenal axis at the central level has been found in the carp after T_4 injection that decreased the plasma cortisol (Geven et al., 2006).

Our data clearly indicate that replacement of T_3 in PTU-fed hypothyroid tilapia stimulates Na handling as it stimulated branchial Na^+, K^+-ATPase activity and decreased renal Na^+, K^+-ATPase activity. This is in line with our earlier report on the hydromineral regulatory action of THs in freshwater tilapia (Peter et al., 2000) and, thus, confirms the role of THs in sodium handling in tilapia. Madsen and Korsgaard (1989) have shown that long-term treatment of T_4 increased branchial Na^+, K^+-ATPase activity in juvenile Atlantic salmon. Injection of T_3 or T_4 for five days, on the contrary, failed to produce a stimulatory action on branchial Na^+, K^+-ATPase activity (Peter, 2007). Similarly, branchial enzyme activity remained unresponsive to T_4 treatment in freshwater acclimated rainbow trout (Madsen, 1990). Since Na^+, K^+-ATPase activity is often considered as the biomarker of chloride cell function (Dang et al., 2000), our data further point to the effect of TH on chloride cell activity in tilapia. Thus, it may be argued that THs are involved in the uptake of ions in freshwater tilapia as the chloride cells in the branchial epithelium of freshwater fish have been implicated in the uptake of ions (Na^+, Cl^-, Ca^{2+}) from the surrounding water (Flik et al., 1994; Perry, 1997). Induction of a hypothyroid state by PTU treatment in freshwater tilapia did not affect the hydromineral parameters measured. This observation stands against the prominent effect of THs reported earlier on hydromineral regulation of tilapia (Peter et al., 2000). It appears that compensatory adaptations mediated by other osmoregulatory hormones such as prolactin, growth hormone or cortisol might occur in these hypothyroid tilapia as evidences have suggested a synergistic action of THs with other hormones (Leatherland, 1994; Peter and Oommen, 1993). For example, growth hormone and cortisol, along with T_3, have been reported to promote the hypo-osmoregulatory ability of coho salmon during smoltification and seawater adaptation (Young et al., 1989). Similarly, T_4 treatment in tilapia had been shown to exert an additive effect on the stimulatory action of cortisol on Na^+, K^+-ATPase activity, though T_4 alone failed to elicit a response (Dange, 1986).

Overall, our results demonstrate the sensitivity of interrenal axis to TH availability, an indication of a positive correlation between the exogenous TH and interrenal activity in the PTU-fed fish. It was found that high dose of PTU inactivates the thyroid axis of tilapia though a low dose activates the thyroid axis. Further, stimulatory action of THs on Na^+ handling is clearly evident in the PTU-induced hypothyroid tilapia.

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