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# Stress response in Mozambique tilapia (*Oreochromis mossambicus*): Temporal and inverse interaction of cortisol and thyroid hormone when confined to net

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## Summary

Thyroid hormones and cortisol are vital for the regulation of metabolic and hydromineral homeostasis in fish. The levels of triiodothyronine ($T_3$), thyroxine ($T_4$) and cortisol in the plasma and the indices of metabolic and hydromineral regulations were quantified in fresh water tilapia after confining them to net for varied time intervals (2, 6, 12, 24 h) to examine whether thyroid and interrenal interact during net-confinement. A time-dependent increase ($P< 0.001$) in plasma cortisol occurred after net-confinement with a maximum increase at 12 h, indicating an induction of stress response in this fish. Confinement of tilapia to net for 6 and 12 h did not alter plasma $T_3$ but significantly decreased ($P< 0.05$) its level at 24 h. Plasma $T_4$ remained unaffected at all intervals tested. Net-confinement produced a substantial increase in the plasma glucose ($P< 0.01$) at all intervals tested and a maximum rise was found at 6 h. Branchial $Na^+$, $K^+$-ATPase activity increased ($P< 0.01$) and renal $Na^+$, $K^+$-ATPase activity decreased ($P< 0.01$) after 12 and 24 h net-confinement, with the maximum rise at 12 h. Plasma $Na^+$ and plasma osmolality declined significantly ($P< 0.05$) at 24 h net-confinement. Overall, the results indicate that net-confinement evokes stress response in tilapia, which includes a temporal and inverse interaction between $T_3$ and cortisol. The data thus support the hypothesis of a lead role of cortisol in stress response of tilapia.

***Key words***: Fish, tilapia, net-confinement, stress, cortisol, thyroid hormones, metabolism, osmoregulation

## Introduction

Fishes are equipped to perceive stressors and able to respond to the stressors with a complex network of neuroendocrine and physiological responses. Cortisol, a stress hormone released from the interrenal gland, in the hypothalamo-pituitary-interrenal axis (HPI-axis), is capable of directing many physiological processes (Wendelaar Bonga, 1997; Bowers et al., 2000; Dang et al., 2001; Lock and Wendelaar Bonga, 2008). This corticosteroid regulates hydromineral and metabolic processes including proliferation of mitochondria-rich cells of gill epithelia in many fish species (Specker et al., 1994; Wendelaar Bonga, 1997; Perry, 1997; Mancera and McCormick, 1999; Mommsen et al., 1999). Moreover, in tilapia cortisol has been shown to regulate the $Na^+$, $K^+$-ATPase activity in the osmoregulatory epithelia of tilapia in both seawater and freshwater (Nolan et al., 1999; Dang et al., 2000). Thyroid hormones on the other hand, are known for their control on many physiological processes related to energy metabolism and growth (Leatherland, 1994; Oommen and Matty, 1997; Power et al., 2001; Peter, 2007). Like cortisol, triiodothyronine ($T_3$) and thyroxine ($T_4$), the primary thyroid hormones, released from hypothalamo-pituitary-thyroid (HPT) axis, regulate water and mineral balance, although some uncertainty exists for their role in hydromineral regulation (Leatherland, 1994; Mancera and McCormick, 1999; Schreiber and Specker, 2000; Peter, 2007).

In fishes, gills, kidneys and intestine, the major osmoregulatory organs, integrate the osmotic functions and maintain an optimal hydromineral balance. Thyroid hormones and cortisol, as the main signaling molecules, promote the osmotic capacity of these osmoregulatory tissues (Peter, 2007; Babitha and Peter, 2010). In freshwater teleosts, active ion uptake is essential to compensate for the constant losses by diffusion of ions through the gill epithelia (McCormick, 1995; Perry, 1997; Marshall and Bryson, 1998; Marshall and Grosell, 2006) and this leakage of ions through gill epithelium is more severe during stressor exposure (Wendelaar Bonga, 1997; Lock and Wendelaar Bonga, 2008). The activity pattern of $Na^+$, $K^+$-ATPase, the driving force for transepithelial $Na^+$ transport, has been used extensively as a measure of hydromineral capacity (Dang et al., 2000; McCormick, 2001). Many stressors are vulnerable to alter this Na transporter mainly because of its sensitivity to the regulation of cellular Na and K gradients.

Fishes experience stressors such as handling and confinement during aquaculture practices that disturb their

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physiologic homeostasis. As fishes are constantly exposed to stressful conditions in the aquatic medium they have evolved different stress response systems which have been expressed at all levels of biological organization (Dini et al., 2006; Iwama et al., 2006). This response of fishes to stressors evokes a multitude of integrated physiological responses which include primary, secondary and tertiary responses (Barton and Iwama, 1991; Iwama et al., 2006). The activation of the neuroendocrine system that bring about biochemical and physiological adaptations ultimately favour the animal to acclimate to the hostile environment (Wendelaar Bonga, 1997; Peter, 2007; Lock and Wendelaar Bonga, 2008).

This study was undertaken to examine whether thyroid gland and interrenal gland interact during stress response in fresh water tilapia. Net-confinement was practiced in this fish since this handling stressor is known for its effect to induce stress without any toxic manipulation (Nolan et al., 1999). The effects of varied intervals of net-confinement on thyroid hormone and cortisol production and their interaction on metabolic and hydromineral regulations were examined in this fish.

## Materials and Methods

### Animals

Adult Mozambique tilapia, in their pre-spawning phase and approximately 45 g body mass, were collected and acclimated in tap water at 28 ± 2°C under natural photoperiod (12L/12D) for three weeks prior to experiment. They were fed with commercial fish feed at a ration of 1.5% of body mass per day. The animal care and the experimentation were strictly according to the regulation of Animal Ethical Committee of the University and there was no mortality during the experiments.

### Experimental protocol

Laboratory-acclimated fish were divided into five groups of six each. Fish in the untreated group 1 were taken as control. The remaining fish in groups 2 to 5 were held in a dip-net and made confined for varied intervals of 2, 6, 12 and 24 h, respectively. Food was withdrawn 24 h prior to killing to ensure optimum experimental conditions.

### Sampling and analyses

Fish in all the groups were sampled on the same day at the specific time interval after net- confinement. Fish were collected from the net and anesthetized in a 2-phenoxyethanol solution (1: 2,000; Sigma Aldrich, St Louis, MO), and blood samples were collected by caudal puncture with heparinized syringes fitted with 23 gauge needle. Plasma was separated by centrifugation (3 min, 5,000 x g) and stored at $^{-}20^{o}$C. Fish were then killed by spinal transection 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 7.1) and stored at -20° C.

### Plasma cortisol, $T_3$ and $T_4$

Cortisol concentrations in plasma samples were measured by competitive immunoenzymatic assay (DiaMetra, Foligno, Italy) and the values were expressed as ng $ml^{-1}$. The sensitivity and reliability of this method was examined and the values were comparable to RIA method reported earlier (Peter, 2007; Peter and Peter, 2007). Plasma $T_3$ (Catalog # 3810-96) and $T_4$ (Catalog # 2210-96) concentrations were measured by microwell enzyme immunoassay (EIA: magnetic solid phase) with kits (Syntron Bioresearch Inc, Carlsbad, California) and the values were expressed in nmoles $L^{-1}$. The sensitivity of this method was checked by comparison of results from RIA based on competitive binding of $^{125}$I-labelled $T_3$ or $T_4$ (Peter et al., 2000) with the EIA results (Peter et al., 2007).

### Plasma glucose and minerals

Plasma glucose levels were measured using a glucose assay kit (Sigma, St Louis, Missouri, USA). The plasma [Na] and [K] concentrations were measured with a flame-photometric auto- analyzer (Systronics, New Delhi). Plasma osmolality (mOsm.$kg^{-1}$) was measured using a micro-osmometer (Gonotec, Germany).

### $Na^+$, $K^+$-ATPase activity

The specific activity of ouabain-sensitive $Na^+$, $K^+$-ATPase was measured in homogenates (Ho) prepared from branchial and renal tissues as described earlier (Peter et al., 2000). The protein concentration in homogenates was measured using a commercial Biuret protein-assay kit (Bio-Rad, Hercules, USA) with bovine serum albumin as the standard. Phosphate release was quantified spectrophotometrically and the specific activity expressed as μmol $P_i$. $h^{-1}$. mg $protein^{-1}$.

### Statistical analysis

Before statistical analyses, the data were checked for normal distribution and variance homogeneity. Two-way analysis of variance (ANOVA) and Student-Newman-Keul's test were employed to test the significance of the difference between the treatment groups using Instat-3 Software (GraphPad Software Inc.,

San Digeo, California). Significant difference between groups was accepted if $P<0.05$ and the values are in mean ± SEM (n = 6).

## Results

### Plasma cortisol, $T_3$ and $T_4$

The plasma cortisol increased to significant ($P<0.05$) levels in the fish with increasing duration of net-confinement (Fig 1B). Net-confinement, on the other hand, produced a significant ($P<0.01$) reduction in the level of plasma $T_3$ at 24 h confinement though its level remained unaffected at 2, 6 and 12 h net-confinement and it showed a tendency to rise at 6 h (Fig. 2). The plasma $T_4$, however, remained unaffected after net-confinement for varied intervals (Fig. 2).

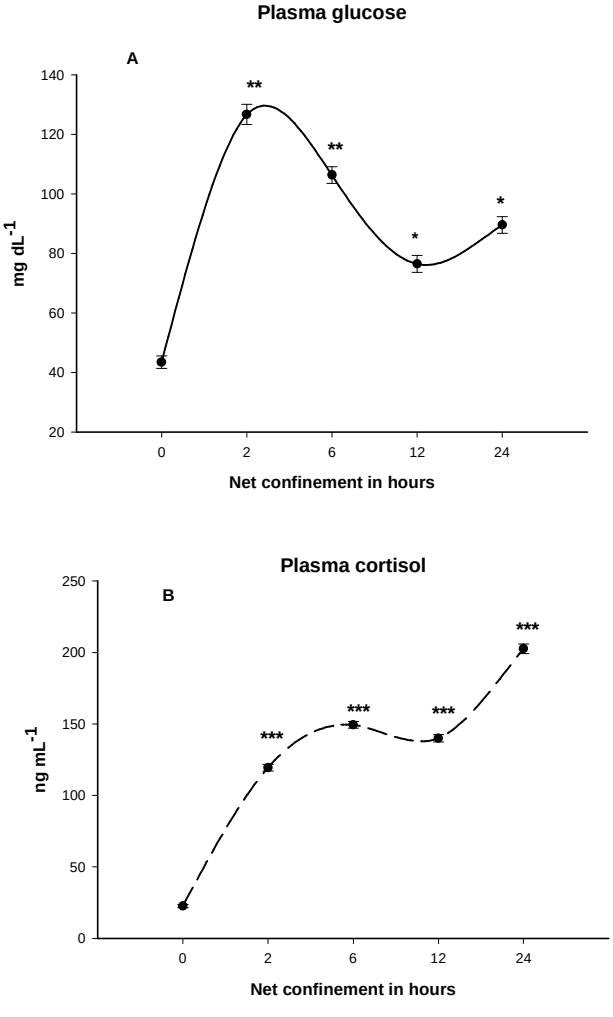


**Fig. 1.** Plasma glucose (mg $dL^{-1}$) and cortisol (ng $mL^{-1}$) levels in tilapia exposed to net-confinement for varied time slots. Each point is mean ± SEM for six fish.

* P<0.05; ** P<0.01; *** P<0.001

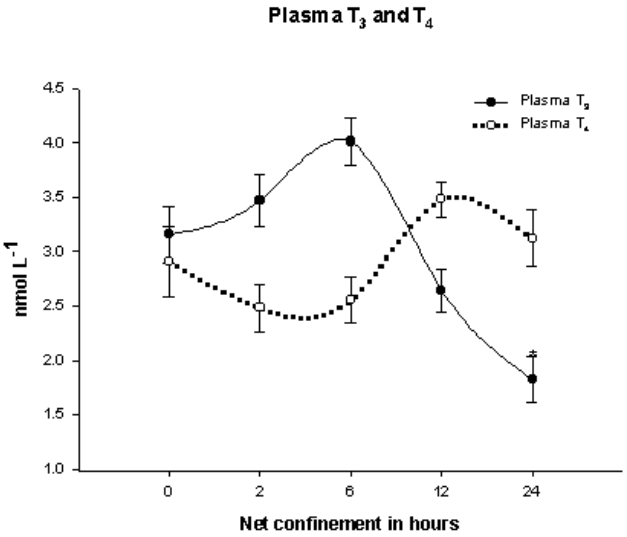


**Fig. 2.** Plasma $T_3$ and $T_4$ (nmol $L^{-1}$) in tilapia exposed to net confinement for varied time slots. Each point is mean ± SEM for six fish.

* Denotes (P<0.05) significant when compared with control (0).

### Plasma glucose and minerals

Significant hyperglycaemia ($P< 0.001$) occurred in fish at varied tested intervals of net-confinement (Fig. 1A). The plasma Na showed a reduction ($P<0.05$) at 24 h net-confinement (Table 1). The plasma osmolality showed a reduction at this time of confinement whereas plasma K remained unaffected (Table 1).

### $Na^+$, $K^+$-ATPase activity

Branchial $Na^+$, $K^+$-ATPase activity showed significant ($P<0.05$) increase at 12 and 24 h net-confinement, whereas the renal $Na^+$, $K^+$-ATPase activity decreased ($P<0.05$ and $P<0.0$) at these intervals (Fig.3).

## Discussion

Evidences are presented to the effect that confining tilapia to net induces stress response and that involves a temporal and inverse interaction between cortisol and $T_3$, the active thyroid hormone. The elevated cortisol and glucose in the plasma of tilapia clearly indicate a classic stress response due to stress induction in this fish. As a common response to acute stress, hyperglycaemia occurs due to the rapid effects of catecholamines on glycogenolysis and the long-term effects of cortisol on gluconeogenesis (Van der Boon et al., 1991; Wendelaar

Bonga, 1997). Mobilization of energy substrates including glucose in response to the stressor thus becomes an

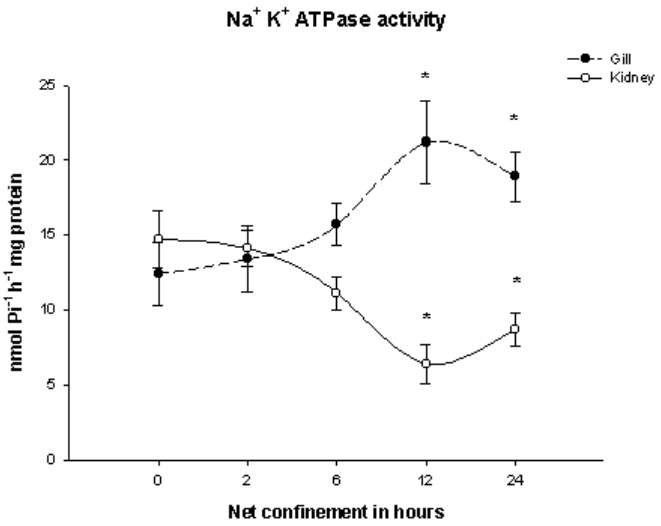


**Fig. 3.** Gill and kidney $Na^+$, $K^+$ ATPase activities in tilapia exposed to net confinement for varied time slots. Each point is mean ± SEM for six fish.
* Denotes (P<0.05) and ** denotes (P<0.01) significant when compared with control (0).

essential metabolic strategy during stress adaptation. Cortisol is known for its stimulatory action on gluconeogenesis in common carp (Janssens and Waterman, 1988) probably because of the activation of gluconeogenic enzyme like glucose-6-phosphatase (Mommsen et al., 1999). Glucose appears to be the main energy source the fish always relies on (Ruane et al., 2001) and the elevated plasma glucose has been reported in carp *Cyprinus carpio* during transfer, though these responses were not consistent (Pottinger, 1998). Hyperglycaemia during confinement has been linked to gluconeogenesis or enhanced glycogenolysis or a decreased clearance of glucose from the blood as reported earlier in tilapia (Vijayan et al., 1997), sea raven (Vijayan and Moon, 1994) and sea bream (Arends et al., 1999). The significant metabolic role of cortisol in fish includes the stimulation of pathways that increase blood glucose levels (Leach and Taylor, 1980; Vijayan et al., 1997; Diouf et al., 2000). Therefore, glycogenolysis and gluconeogenesis, in which metabolites such as amino acids (Milligan, 1997) and lactate (Young and Cech, 1993, 1994) are used as substrates, are activated during stress. Alternately, the rapid rise in plasma glucose in response to stressors may also be attributed to activation of the brain-sympathetic-chromaffin (BSC) axis and the release of catecholamines by the chromaffin cells (Barton and Iwama, 1991; Arends et al., 1999; Ruane et al., 2001). Therefore, the rise in glucose and cortisol are indirectly considered as the indicators of sympathetic activation and the activation of HPI axis during stress (Rotlland et al., 2000; Wendelaar Bonga, 1997; Peter and Peter, 2009).

Fishes respond to stressors by eliciting physiological responses that include elevation of cortisol and adrenaline

**Table. 1:** Levels of plasma minerals (mmol $L^{-1}$) and osmolality (mOsmol $kg^{-1}$) in tilapia exposed to net-confinement for varied time slots. Values are mean ± SEM for six fish.

| | Na | K | Osmolality |
|---|---|---|---|
| 0 | 142.8 ± 1.6 | 4.61 ± 0.2 | 315 ±0.2 |
| 2 | 141.3 ± 1.9 | 4.62 ± 0.3 | 311 ± 0.2 |
| 6 | 134.7 ± 1.7 | 4.55 ± 0.2 | 308 ± 0.2 |
| 12 | 132.4 ± 1.5 | 4.56 ± 0.3 | 306 ± 0.3 |
| 24 | 128.9 ± 1.4* | 4.12 ± 0.2 | 303 ± 0.2* |

* Denotes (P<0.05) significant when compared with control (0).

(Wendelaar Bonga 1997). The time-dependent rise in plasma cortisol in tilapia establishes that these fish are stressed as reported earlier (Vijayan et al., 1997; Dini et al., 2006). The rise in plasma cortisol after net-confinement has also been reported in many fishes including striped bass (Noga et al., 1994), paddlefish (Barton et al., 1998), gilthead sea bream (Arends et al., 1999), juvenile pallid and sturgeons (Barton et al., 2000), rainbow trout (Trenzado et al., 2003; Pankhurst et al., 2008) and the olive flounder (Hur et al., 2007). Many extrinsic and intrinsic factors have been known to induce the cortisol release in fishes which include age, sex and maturity of the fish (Sumpter, 1997), the environmental temperature (Sumpter et al., 1986), the species and strain of fish (Pickering and Pottinger, 1989) and the chemical composition of the water (Pickering and Pottinger, 1987).

Cortisol exerts multiple physiologic actions in fish that include hydromineral and metabolic regulations (Mommsen et al., 1999; Laiz-Carrion et al., 2002, 2003; Gallo and Civinini, 2003; Sangiao-Alvarellos et al., 2005; McCormick et al., 2008). The plasma level of cortisol is often considered as a measure of the magnitude of stress response (Wendelaar Bonga, 1997) and under acute stress it can easily shoot up many-folds to enhance the mobilization of energy reserves and metabolic rate (Wendelaar Bonga, 1997; Flik et al., 2006). Likewise, cortisol contributes to hydromineral regulation in freshwater fish, though it is often referred to as a seawater hormone (McCormick, 2001). Branchial $Na^+$, $K^+$-ATPase activity, a measure of hydromineral capacity, increases in freshwater tilapia after cortisol treatment (Dang et al., 2000). Similar to cortisol, TH's also direct metabolic and osmoregulatory function in fish (Peter et al., 2000, 2007; Peter and Peter, 2007). Studies on freshwater tilapia have provided evidence that physiological concentrations of both $T_3$ and $T_4$ enhance branchial $Na^+$, $K^+$-ATPase activity and chloride cell (CC) dynamics (Peter et al., 2000). The importance of TH's to maintain Na and water balance during an osmotic challenge in the mummichog has been reported by Knoeppel et al. (1982) and Grau (1987). However, in teleosts such as *Salmo salar* (Saunders et al., 1985; Shrimpton and McCormick, 1998) and *Salmo gairdneri* (Madsen, 1990), no effect of TH was found on $Na^+$, $K^+$-ATPase activity. In the present study, on the contrary, stress induction appears to reduce the plasma $T_3$ substantially, suggesting a lack of $T_3$ action during net-confinement.

The present data thus point to specific metabolic and hydromineral actions of cortisol during stress in freshwater tilapia. As we observed decrease of $T_3$ action in tilapia after prolonged net- confinement, the possibility of TH involvement in the metabolic and hydromineral regulation is doubtful. In this context, it is reasonable to presume that cortisol may take up the lead of regulating the metabolic and hydromineral actions in the absence of $T_3$ action. Changes in the deiodination activity in the peripheral tissues are important mechanisms to modulate TH activity in mammals (Kuiper et al., 2005) and fish (Eales, 1985; Eales et al., 1990; Van der Geyten et al., 2005; Walpita et al., 2007).

There are indications that thyroid activity and the cortisol release are interrelated in fishes (Walpita et al., 2007; Peter, 2007; Peter and Peter, 2009). In this study, confinement stress brought about decreased plasma $T_3$ and not plasma $T_4$, supporting the view that alterations of thyroid function occur particularly on the actions of $T_3$ during stress in fish. A modification of $T_3$ metabolism and its availability in our net-confined tilapia could thus be ascribed to an adaptive strategy of fish to combat stress. Similar decline of plasma $T_3$ has also been reported in rainbow trout (Himick and Eales, 1990) and in perch (*Anabas testudineus*) exposed to kerosene (Peter et al., 2007). Modification of peripheral deiodination and thyroid axis during stress were also demonstrated in Nile tilapia (Walpita et al., 2007).

In tilapia, thus, a temporal and inverse yet functional relationship exists between the interrenal and thyroidal axis in the face of a stressor challenge, suggesting a lead role of cortisol in stress response. This idea is consistent with a negative interaction of the thyroid and interrenal axes reported earlier for salmonids (Young et al., 1989; Vijayan et al., 1997) though no correlation between thyroid activity and cortisol has been observed in the rainbow trout (Madsen, 1990; Gomez et al., 1997) and in mummichog (Mancera and McCormick, 1999). In some studies the possibility of a rapid clearance of TH's after cortisol treatment has been proposed (Vijayan et al., 1988;

Brown et al., 1991). For example, in brook trout, cortisol increased the hepatic conversion of $T_4$ to $T_3$ (Vijayan et al., 1988). It seems that in tilapia cortisol is contributing to the metabolic and hydromineral regulations during net confinement. The hyperglycemic effects observed in the net-confined tilapia may thus support the metabolic role of cortisol, though TH's also direct metabolic processes in fish tissues (Leatherland, 1994; Power et al., 2001; Peter et al., 2007; Peter and Peter, 2009).

$Na^+$, $K^+$-ATPase activity is under multiple hormonal control with cortisol in a dominant role (Young et al., 1995; McCormick, 1995, 2001; Dang et al., 2000; Evans, 2002). The concomitant rise in cortisol and the upregulation of branchial $Na^+$, $K^+$-ATPase activity in tilapia indicate a cortisol-directed Na pump activity in this stressed fish (Nolan et al., 1999). On the contrary, the decline of renal $Na^+$, $K^+$-ATPase in tilapia after 24 h net confinement indicates a disturbed osmotic function of kidney tubules to retain $Na^+$ as this Na transporter energizes $Na^+$ reabsorption and also the transport of other ions or uncharged solutes in the kidney tubules. Similar inhibition of renal $Na^+$, $K^+$-ATPase activity has also been observed in tilapia after seawater acclimation (Nolan et al., 1999) and in mummichog (Epstein et al., 1967). Interestingly, these differential actions of $Na^+$, $K^+$-ATPase activity on gills and kidney further point to the ability of cortisol to integrate the osmotic functions of these organs as evident in catfish organs (Babitha and Peter, 2010).

Overall, the present results indicate that tilapia shows a pattern of stress response to net- confinement with characteristic rises in plasma glucose and cortisol associated with compensatory metabolic and hydromineral modifications. Evidences are also presented to the effect that net- confinement produces temporal and inverse interaction of cortisol and $T_3$ which ultimately permits cortisol to direct the compensatory stress response in this fish.

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