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Short-term salinity acclimation demands thyroid hormone action in the climbing perch *Anabas testudineus* Bloch

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Summary

Fishes have developed many complex physiological mechanisms to combat osmotic challenges. In this study triiodothyronine (T_3), thyroxine (T_4) and cortisol in the plasma were quantified and the indices of metabolic and hydromineral regulations analyzed in the climbing perch *Anabas testudineus* after exposing the fish to a selected salinity (20 ppt) for varied intervals (1, 7, 14 and 21 days) to study the physiological basis of short- and long-term salinity acclimation. It was found that transfer of fish to 20 ppt salinity for a day after transient salinity changes, plasma T_4 was elevated, and plasma T_3 decreased whereas plasma cortisol remained unchanged. The levels of these hormones, however, returned to basal levels when these fish were kept for a prolonged acclimation of three weeks. Plasma glucose and lactate showed no change in response to salinity acclimation, whereas plasma urea showed an increase. Substantial increase in the gill Na^+, K^+-ATPase activity was found in these fish during salinity acclimation, which remained high even after three weeks. Salinity transfer for a day produced significant increase in the intestinal Na^+, K^+-ATPase activity, though it remained unaffected during the long-term acclimation. Kidney Na^+, K^+-ATPase activity decreased on day 1 salinity challenge, but remained unaltered after prolonged acclimation. Liver Na^+, K^+-ATPase activity increased upon transient salinity challenge but the levels were maintained during prolonged salinity challenge. Our results indicate that salinity acclimation in climbing perch demands thyroid hormone secretion and its action and not cortisol as part of co-ordinating the acclimation processes in the early phase of salinity acclimation. The results also point to the ability of climbing perch to tolerate osmotic challenge, and the fish becomes fully adaptive to brackish water salinity of 20 ppt after three weeks.

Key words: Climbing perch, cortisol, fish, Na^+, K^+-ATPase, salinity acclimation, thyroid hormones

Introduction

Teleost fishes possess multiple physiological mechanisms to combat osmotic challenges (Marshall and Grosell, 2006; Wang et al., 2009). Salinity adaptation in euryhaline teleosts is a complex process involving a set of physiological responses to varied environments with differing iono-regulatory requirements. Gill, as the most important osmoregulatory organ in fish (Hirose et al., 2003; Evans et al., 2005), absorbs Na and Cl in low salinity environments and rapidly secretes these ions in relatively high salinity environments (Karnaky 1998; Evans et al., 2005). The transport of Na and Cl across the teleost gill epithelium is facilitated by the chloride cells which absorb ion in freshwater while secrete ions in seawater (McCormick, 2001; Evans et al., 2005; Hwang and Lee, 2007). Na^+/K^+-ATPase (NKA), a universal membrane-bound enzyme, provides driving force for many transport systems and help the fish to maintain Na homeostasis with its presence in a variety of osmoregulatory epithelia including gills (Evans et al., 2005).

Fishes regulate metabolic and osmoregulatory processes with the support of many hormones (Leatherland, 1994; Evans et al., 2005; Babitha and Peter, 2010). Thyroxine (T_4) and triiodothyronine (T_3), the principal thyroid hormones (TH's), are known for their metabolic and osmoregulatory actions in fish tissues (Peter et al., 2000; Peter, 1996, 2007; Peter and Peter, 2009). Although, the involvement of thyroid hormones during salinity acclimation has been the subject of many studies, the exact role of these hormones has not yet been demonstrated (Geven et al., 2006; Peter, 2007; Soengas et al., 2007). A number of studies have, however, reported that TH's may not be involved in ion uptake or secretory mechanisms (Ayson et al., 1995; McCormick, 1995; Mancera and McCormick, 1999). Prolonged T_4 treatment has been shown to increase the number of chloride cells and gill Na^+, K^+-ATPase activity in Atlantic salmon smolts (Madsen and Korsgaard, 1989). Peter et al. (2000) reported that physiological levels of T_4 and T_3 increase chloride cell size, gill Na^+, K^+-ATPase activity and plasma $[Na^+]$ and $[Cl^-]$ in tilapia, suggesting a role of TH's in ion uptake in this fish.

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Inhibition of the thyroid axis with thiourea in mummichog caused increased plasma [Na^+] and osmolality in seawater but had no effect in freshwater (Knoeppel et al., 1982). A role of TH in seawater acclimation has been reported in rainbow trout (Lebel and Leloup, 1992; Leloup and Lebel, 1993) as it brought about decrease in the conversion of T_4 to T_3. Salinity acclimation demands increased Na^+, K^+-ATPase activity of the chloride cells (Dang et al., 2000; Hiroi and McCormick, 2007; Yada et al., 2008) and these changes have been correlated with increase in cortisol (Evans et al., 2005; McCormick et al., 2008) and TH's levels (Leatherland, 1994; Peter, 2007; Klaren et al., 2007).

Cortisol has a direct effect on the osmoregulatory functions of fish and this hormone is well known for its role in seawater acclimation (McCormick, 2001). Moreover, cortisol plays a dual role in ion secretion as well as ion uptake, indicating its plasticity in regulating osmoregulatory function in teleosts (McCormick, 2001; Evans et al., 2005; Flik et al., 2006; Babitha and Peter, 2010). The action of cortisol, however, in promoting ion uptake or secretion may depend, in part, on the relative activity of other hormones including growth hormone and prolactin (McCormick, 2001). Evidences are also presented on the role of GH/IGF-I axis in seawater acclimation, and cortisol interacts positively with these hormones to enhance salt secretion and promote the underlying physiological processes. Subsequently cortisol, along with a series of other hormones, was shown to regulate the development and differentiation of seawater-type and freshwater-type chloride cells in the branchial epithelia of euryhaline fish (Sakamoto and McCormick, 2006).

There are also evidences in fish that TH's exert some permissive action with cortisol. For example, an upregulation of the number of gill cortisol receptors by T_3 alone and its administration together with growth hormone in Atlantic salmon has been reported (Shrimpton and McCormick, 1998). T_3 has also been shown to increase the *in vitro* capacity of cortisol to increase gill Na^+, K^+-ATPase activity in rainbow trout, revealing the influence of TH's on ion secretion through their interaction with the GH/IGF-1 and cortisol axes (Shrimpton and McCormick, 1999). Notwithstanding these evidences that document the involvement of TH's and cortisol in osmoregulation in fishes, the endocrine physiology of salinity acclimation of air-breathing fish is less understood. We, therefore, examined the physiological mechanism of salinity acclimation in climbing perch and studied if TH's and cortisol interact in this process.

Materials and Methods

Animals

Adult air-breathing perch (*Anabas testudineus* Bloch; order Perciformes) of both sexes (45 ± 2 g body weight) in their pre-spawning phase were kept as groups and acclimated in 50 L glass tanks with tap water at 28 ± 1°C (pH 7.2) 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 done according to the regulation of Animal Ethical Committee of the University and there was no mortality during the experiments.

Experimental set-up

We examined the effects of salinity on thyroid hormone-cortisol interaction in our test species. We quantified the T_4 and T_3 and cortisol levels and also measured the indices of metabolic and hydromineral regulation and exposed the fish to a selected dilute seawater (20‰) at different intervals. Artificially-diluted seawater was prepared by dissolving natural sea salt (20 g L^{-1}) and aerated for 24 h. Fish were transferred to dilute seawater through transient transfer from 0, 5, 10, 15 and finally to 20‰ with a 12 h interval between two salinities. Fish groups exposed to 20‰ salinity were sampled after 1, 7, 14 and 21 days. A group of freshwater fish was held as control.

Sampling and Analysis

Food was withdrawn for 24 h prior to sampling to ensure optimum experimental conditions. After experimentation, the fish were anaesthetized in 0.1% 2-phenoxyethanol (SRL, Mumbai) and blood was obtained from caudal vessels using a heparinized syringe. Plasma was separated (5,000 xg for 5 min) immediately at 4°C and stored at -20°C until analysis. Fish were then sacrificed by spinal transsection and pieces of second gill arch, anterior intestine, lower lobe of liver and posterior kidney were excised, kept in ice-cold 0.25 *M* SEI buffer (pH 7.1) and stored at -80°C.

Plasma cortisol, T_3 and T_4

Cortisol concentrations in plasma samples were determined by competitive immunoenzymatic assay (DiaMetra, Foligno, Italy) and values were expressed as ng ml^{-1}. The sensitivity and reliability of this method were

examined and the values were comparable to RIA method reported earlier (Peter, 2007). In brief, plasma samples were treated with cortisol antibody (mouse anti-rabbit IgG) and incubated with cortisol-horseradish peroxidase (HRP) conjugate at 37°C for 1 h. Absorbance was recorded on a plate reader (Span Autoreader 4011, New Delhi) at 450 nm and the values are expressed in ng ml^{-1}

Plasma T_3 and T_4 concentrations were measured by microwell enzyme immunoassay (EIA: magnetic solid phase) with kits (Syntron Bioresearch Inc, California, Catalog # 3810-96 for T_3 and Catalog # 2210-96 for T_4). 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 with the EIA results (Peter et al., 2007). Briefly, the anti-T_4 or the anti-T_3 (goat anti-mouse IgG) coated wells were treated with standards, control and samples. T_4-HRP or T_3-HRP conjugate were incubated at 37°C for 1 h. Absorbance was read at 450 nm and the values are expressed in nmoles L^{-1}.

Plasma metabolites and minerals

Plasma glucose (GOD/POD test kit; Span Diagnostics Ltd., New Delhi), urea (DAM kit; Span Diagnostics, New Delhi) and lactate (PAP Fluid test; Radiant Diagnostics, New Delhi) concentrations were measured colorimetrically in a Systronics Spectrophotometer 2202 (Systronics, New Delhi) using commercial test kits. Plasma Na^+, K^+ and Ca^{2+} were measured in a flamephotometric analyser (Systronics 129, New Delhi) using standards of Na^+ and K^+ (Remedix diagnostics, Palakkad, India) and Ca^{2+} and values are expressed as mmol L^{-1}.

Na^+, K^+ ATPase specific activity

The ouabain-sensitive Na^+, K^+ ATPase specific activity was measured in tissue homogenates as described earlier (Peter et al., 2000). Saponin (0.2 mg mg $protein^{-1}$) was routinely added to optimize substrate accessibility. The liberated inorganic phosphate content was measured with a Systronics UV/Visible Spectrophotometer 2202 (New Delhi) at 700 nm and expressed in µmol P*i* h^{-1} mg $protein^{-1}$. The protein content in the tissues was measured using modified Biuret assay (Alexander and Ingram, 1980) with bovine serum albumin as standard.

Statistics

The data were collected from six fish from each group and presented as mean ± standard error. All data sets were analyzed using one-way analysis of variance (ANOVA) followed by a post-hoc multiple (all-pair-wise) SNK comparison test. Significance of differences between groups was accepted if $P<0.05$, and all the statistical tests were performed using a software package (Graphpad Instat-3, San Diego, USA).

Results

Plasma T_3, T_4 and cortisol

Plasma T_4 increased ($P<0.05$), but plasma T_3 decreased ($P<0.05$) on day 1 of salinity exposure but the hormones returned to the basal levels when these fish were kept for prolonged acclimation for 7 to 21 days (Fig. 1A). Plasma cortisol did not show any response upon salinity exposure except a tendency to increase on day 7 (Fig. 1B).

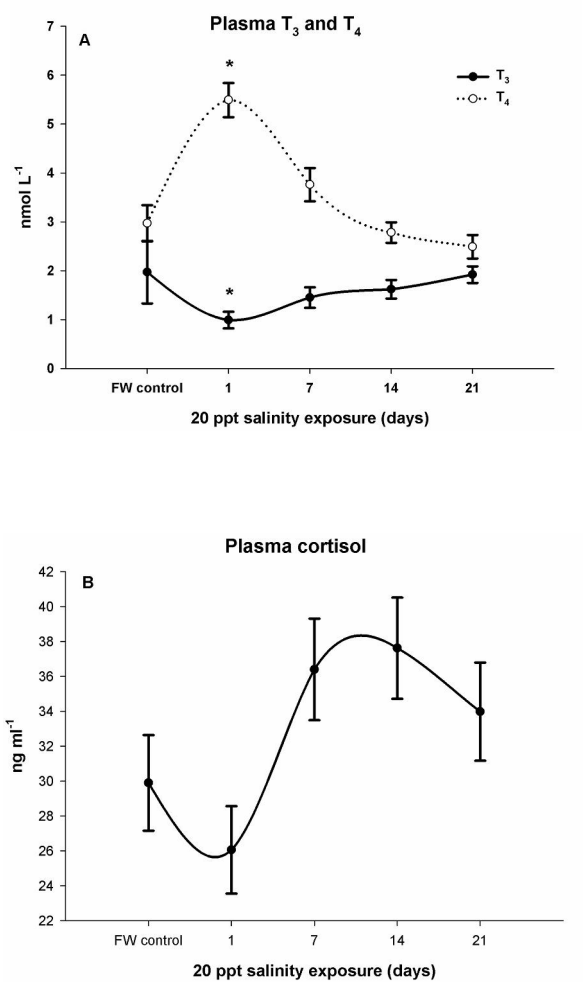

Fig. 1: Plasma T_3, T_4 (A) and cortisol (B) in the climbing perch after exposure to 20 ppt salinity for different time periods. Values are as mean ± SEM for 6 fish.
* ($P< 0.05$) denotes significant difference when compared with FW control

Plasma metabolites and minerals

Plasma glucose increased (P<0.01) on day 1 exposure but leveled off after prolonged exposure. Plasma urea increased (P<0.05) substantially in response to salinity acclimation at all the time points, whereas plasma lactate did change (Table 1). Plasma Na (P<0.05) increased with salinity acclimation but K decreased (P<0.05), while Ca remained unaffected (Table 2).

Na^+, K^+-ATPase activity

Gill Na^+, K^+-ATPase activity increased (P<0.05 and P<0.01) substantially after varied periods of salinity exposures and remained high after three weeks (Fig. 2A). Salinity acclimation produced significant increase (P<0.05) in the intestinal Na^+, K^+-ATPase activity (Fig. 2A), but remained unaffected with increasing time intervals. Kidney Na^+, K^+-ATPase activity decreased

Table 1: Plasma metabolites in the climbing perch after exposure to 20 ppt salinity for different time periods

Metabolite	Control	20 ‰ salinity exposure			
		1 day	7 days	14 days	21 days
Glucose (mg dL^{-1})	73.99 ± 4.69	149.50 ± 9.6**	97.40 ± 6.5	69.90 ± 3.8	58.41 ± 2.1
Lactate (mmol L^{-1})	1.53 ± 0.22	1.16 ± 0.30	2.15 ± 0.36	1.81 ± 0.31	1.99 ± 0.26
Urea (mg dL^{-1})	8.62 ± 1.22	16.15 ± 2.1*	17.54 ± 1.6*	16.42 ± 2.8*	18.5 ± 2.5*

Values are as mean ± SEM of 6 fish.
* (P< 0.05) and ** (P< 0.01) denote significant difference when compared with FW control

Table 2: Plasma minerals (mmol L^{-1}) in the climbing perch after exposure to 20 ppt salinity for different time periods

Parameter	Control	20 ‰ salinity exposure			
		1 day	7 days	14 days	21 days
$[Na^+]$	137.3 ± 2.39	143.3 ± 2.3	151.3 ± 2.8	152.6 ± 2.6	162.3 ± 1.8*
$[K^+]$	4.80 ± 0.44	3.40 ± 0.25*	3.91 ± 0.31	4.02 ± 0.19	4.12 ± 0.29
$[Ca^{2+}]$	2.03 ± 0.05	2.28 ± 0.15	2.21 ± 0.13	2.39 ± 0.21	2.98 ± 0.9

Values are as mean ± SEM of 6 fish.
* (P< 0.05) denotes significant difference when compared with FW control

(P<0.05 and P<0.01) in the early phase of salinity challenge, but remained unaltered after prolonged acclimation (Fig. 2A). Liver Na^+, K^+-ATPase activity increased (P<0.05) upon initial salinity challenge but these levels were maintained closer to normal during prolonged salinity challenges (Fig. 2B).

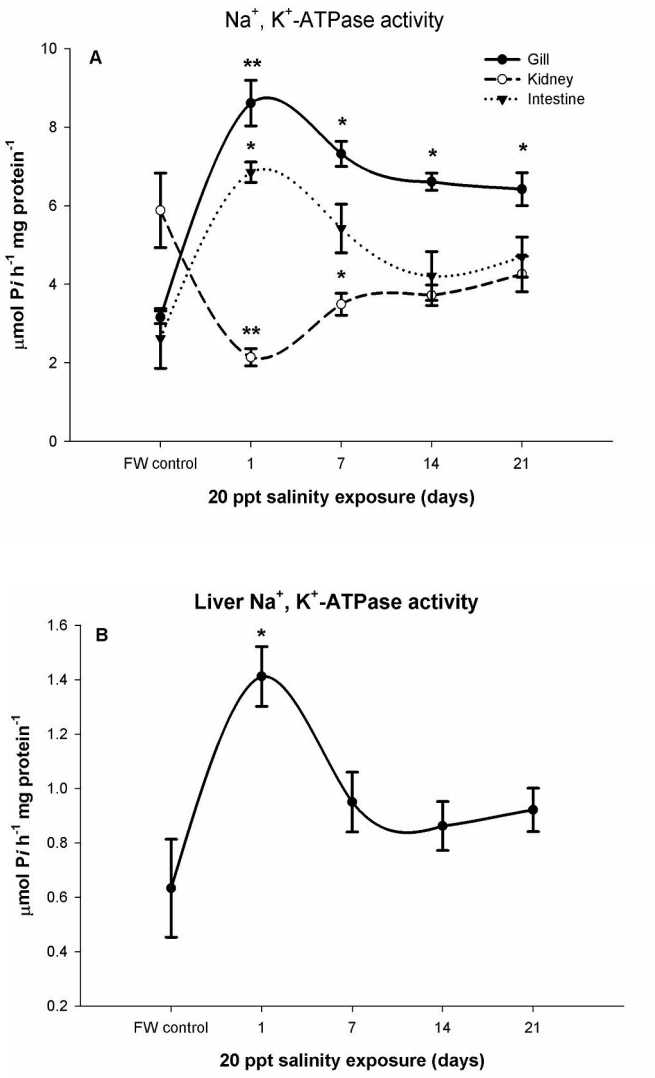

Fig. 2. Na^+, K^+-ATPase activity in the gill, intestine, kidney (A) and liver (B) of climbing perch after exposure to 20 ppt salinity for different time periods. Values are mean ± SEM of 6 fish.
* (P< 0.05) and ** (P< 0.01) denote significant difference when compared with FW control

Discussion

Our results indicate that thyroid hormones play pivotal roles in the initial days of salinity acclimation and contribute to the adaptability of the air-breathing fish to tolerate osmotic challenge as it becomes fully adaptive to the ambient salinity after three weeks of salinity exposure. The increased T_4 and the decreased T_3 in the fish on day 1 of salinity exposure, suggest the involvement of thyroid hormone in salinity acclimation probably due to the inhibition of outer ring deiodination of T_4, the key pathway in thyroid hormone metabolism. The elevated plasma T_4 also points to an increased T_4 synthesis which is required for the fish to cope with the salinity challenge. T_4, the end product of hypothalamo-pituitary-thyroid (HPT) axis, generally acts through the biologically active T_3 which is formed in the extrathyroidal tissues by the outer ring deiodination (ORD; Arjona et al., 2007). It is known that liver, kidney, and gills of fish possess high ORD activities (Mol et al., 1998; Klaren et al., 2007; Walpita et al., 2007). Similar increase in plasma T_4 and decrease in T_3 during salinity exposure has been reported in rainbow trout (Leloup and Lebel, 1993) and gilthead seabream (Klaren et al., 2007). It has been further demonstrated that thyroid hormones are involved in osmoregulation of this fish and deiodinases found in the gills, which would modulate the plasma TH levels (Klaren et al., 2007). Likewise, hypoosmoregulatory ability of T_4 in summer flounder larvae has also been reported (Schreiber and Specker, 1999). Elevated growth rates accompanied by higher plasma T_3 levels in females and lower plasma T_3 in males were observed in *Oreochromis niloticus* reared in 10‰ brackish water (Fontainhas-Fernandes et al., 2000). Thyroid hormones are important in salmonid parr-smolt transformation that allows acclimation to altered environmental salinities (Prunet et al., 1989; McCormick, 2001; McCormick et al., 2008).

Cortisol, the potent glucocorticoid in teleostean fish, plays a crucial role in the stress response and in osmoregulatory processes (Wendelaar Bonga, 1997; McCormick, 2001; Flik et al., 2006) and often this hormone is referred to as the seawater-adapting hormone (McCormick, 2001). Cortisol has been shown to interact with the thyroid axis in fishes as in other vertebrates (Kuhn et al., 1998; Peter, 2007; Klaren et al., 2007). In our study, plasma cortisol remained unaltered or tightly regulated during seawater acclimation of climbing perch, ruling out the possibility of a thyroid hormone-cortisol interaction during salinity acclimation in this fish. In contrast, an increased plasma cortisol and decreased plasma free T_4 were obtained in Senegalese sole during salinity exposure, suggesting an interaction between these hormones in this fish (Arjona et al., 2008).

Cortisol, a key multifunctional hormone in teleosts, acts to mobilize and synthesize metabolites and, thus, directly as well as indirectly influences many physiological processes during stress in fish (Vijayan et al., 1997; Wendelaar Bonga, 1997; Hontela, 2005; Peter, 2007; Babitha and Peter, 2010). Studies have also indicated the adaptive roles of cortisol in hypoosmotic and hyperosmotic acclimation (McCormick, 2001), immune function (Wendelaar Bonga, 1997; Flik et al., 2006) and intermediary metabolism (Vijayan et al., 1997). A dual role of cortisol in carbohydrate metabolism and hydromineral regulation has also been demonstrated in a number of fishes (Gallo and Civinini, 2003; Sangio-Alvarellos et al., 2005; Takahashi et al., 2006). Cortisol regulates glucose mobilization (Vijayan et al., 1997) by inducing liver glycogenolysis and gluconeogenesis to handle the high energy demand especially during stress conditions (Vijayan et al., 1997; Babitha and Peter, 2010). Hyperglycemia, the most common indicator of stress which is mainly due to the elevated cortisol and adrenaline levels (Wedemeyer, 1997; Barton et al., 2002) shows a positive correlation with T_4 in our fish, suggesting its direct effect on short-term salinity acclimation which is independent of plasma cortisol action. The return of glucose to the basal level in fish kept for a relatively longer period of salinity exposure indicates that this osmotic challenge does not pose any serious stress to this fish. The increase in glucose and T_4 levels in this fish is in accordance with our earlier observations on the ability of thyroid hormones to induce glucose production in freshwater (Peter, 1996, 2007) and seawater (Peter et al., unpublished) fish. The unaltered plasma lactate in the salinity-exposed fish also supports this view. In contrast, hyperglycaemia and a decreased liver lactate content have been reported in wedge sole (*Dicologoglossa cuneata*) kept at extreme salinities to 55‰ (Herrera et al., 2009) and in tilapia (*Oreochromis mossambicus*) exposed to double strength seawater (Fiess et al., 2009). Likewise, the rise in plasma urea in salinity-exposed fish provides evidence for the ability of air-breathing fish to switch over to ureogenesis as it is known that seawater challenge demands ureogenic potential (Anderson, 2001). Our results support the earlier understanding on the induction of ureogenic enzymes in this fish with increasing salinity exposure (Peter et al., unpublished). It is, thus, evident that the steady increase in plasma urea in this perch through out the period of salinity exposure implies that ambient salinity may not be in favour of ammonia excretion through the gills.

Gills, the main sites for exchange of gases, osmoregulation and excretion of nitrogenous waste products in fish, are vulnerable to even minor changes in the chemistry of surrounding water. In teleosts, gill Na^+, K^+-ATPase activity plays an important role in the acclimation to different environmental salinities (Marshall, 2002). Up-regulation of Na^+, K^+-ATPase has been used extensively as an index of transport capacity of fish during seawater acclimation (Mancera and McCormick, 2000; Dang et al., 2000). Most euryhaline teleosts exhibit modification of gill Na^+, K^+-ATPase activity during salinity changes (Marshall and Bryson, 1998; Peter, 2007). Gill Na^+, K^+-ATPase activity of many fishes show a linear relationship with increasing salinity (McCormick 1995; Sakamoto et al., 2001). In golden grey mullet (*Liza aurata*) fry, gill Na^+, K^+-ATPase activity was significantly higher when acclimated to 36‰ and 46‰ salinity compared to 12‰ (Kodabandeh et al., 2009). Increase in gill Na^+, K^+-ATPase activity produced due to salinity-challenge was observed in rainbow trout exposed to 66% seawater for 5 days (Shepherd et al., 2005). In the perch in this study, the gill Na^+, K^+-ATPase activity depicted a parabolic curve with a peak at seven days of salinity exposure but still has a steady increase even after three weeks of salinity acclimation. This clearly indicates the key role of gills to promote seawater tolerance in this fish and also demonstrates the ability of this freshwater air-breathing fish to tolerate ambient salinity by triggering adaptative responses to withstand in the hypersaline environment.

Similar modifying responses of kidney to ambient salinity have been observed in this fish where the enzyme activity decreased on the first day of exposure and gradually increased to become steady till three weeks. Environmental salinity has been shown to modify the morphology and physiology of kidney including the glomerular filtration rates, divalent ions' excretion capacity and Na^+, K^+-ATPase activity (Kelly and Woo, 1999; McDonald, 2007). Similar to gills, the intestinal and liver Na^+, K^+-ATPase activities of salinity-exposed fish increased on day 1. The increase in intestinal Na^+, K^+-ATPase activity of salinity-exposed tilapia has been reported earlier (Nolan et al., 1999). This up-regulated intestinal Na^+, K^+-ATPase activity indicates the contribution of intestine to maintain whole body osmoregulation by favoring the absorption of Na from the intestinal lumen.

The levels of plasma ions were generally not altered in the perch after salinity exposure except an increase in Na^+ after three weeks of exposure and a

decrease in K^+ after the first day of exposure. This indicates the capacity of fishes to regulate whole body mineral status during salinity exposure. The unaltered plasma Na^+ during the initial phases of salinity exposure and the downregulation of Na^+, K^+-ATPase activity in the kidney also give clues on the ability of kidney to handle Na. No change in plasma Na^+ level has been reported in rainbow trout upon exposure to salinity for five days (Shepherd et al., 2005).

Overall, our results provide evidence that during the initial days of salinity acclimation the climbing perch relies on thyroid hormones to coordinate the acclimation processes, and the fish becomes fully adaptive to brackish water salinity after three weeks. This successful salinity tolerance of the freshwater fish is due to its thyroid hormone-driven physiological mechanisms which they develop during salinity acclimation.

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