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# Short-term *In situ* effects of prolactin and insulin on ion transport in liver and intestine of freshwater climbing perch (*Anabas testudineus* Bloch)

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## Summary

The short-term *in situ* effects of prolactin and insulin on the transport of monovalent and divalent cations were examined in climbing perch *Anabas testudineus* to infer upon how these hormones regulate ion transport in the metabolic and osmoregualtory tissues of freshwater fish. Varied doses ($10^{-9}$, $10^{-8}$ and $10^{-7}$ M) of ovine prolactin (oPRL) and insulin were infused in these fish for 20 min and the specific activities of ion-specific ATPases were analyzed. The $Na^+$, $K^+$-ATPase activity in the intestine and liver showed significant increase after oPRL infusion. Infusion of oPRL significantly decreased the cytosolic $H^+$-ATPase activity in the intestine and liver, but increased the cytosolic $Ca^{2+}$-ATPase activity in these tissues. Infusion of oPRL decreased the mitochondrial $Mg^{2+}$-ATPase and $H^+$-ATPase activities in the liver but the activities of these transporters increased in the intestine. Similar to oPRL, insulin infusion produced dose-dependent effects on the transporter activities in the liver and intestinal tissues. The mitochondrial $Mg^{2+}$-ATPase activity in the liver significantly decreased at $10^{-9}$ and $10^{-7}$ M insulin infusion whereas its activity increased significantly in intestine at $10^{-8}$ M insulin. Likewise, insulin infusion produced significant increases in the activities of all tested transporters except the cytosolic $H^+$-ATPase activity in the intestine. A direct action of insulin on ion transport was found in both liver and intestinal tissues, as evident in the activity patterns of ion-specific ATPases. Taken together the results point to vital roles of PRL and insulin in ion transport in both liver and intestinal tissues of climbing perch.

**Key words**: Fish, prolactin, insulin, ATPase, osmoregulation.

## Introduction

In teleosts, ionic and osmotic homeostasis is achieved by the integrated functions of osmoregulatory organs (Evans, 1993). Several hypophyseal and extrahypophyseal hormones control the activities of the osmoregulatory organs and maintain hydromineral balance in fish under varying environmental conditions. Prolactin (PRL) is considered as one of the major osmoregulatory hormones in freshwater teleost species (McCormick, 2001; McCormick and Bradshaw, 2006; Seale et al., 2014). Unlike this osmoregulatory hormone, insulin as a widely recognized metabolic hormone is known for its metabolic action in fish (Polakof et al., 2010). It is of interest to study the role of these hormones in the ion transport in fish tissues as every hormone acts basically by modifying some aspects of cellular metabolism (Frye, 1967).

Fish that reside in a freshwater environment face two primary challenges *viz.,* loss of ions to the external hypoosmotic environment and the influx of water into the body (Manzon, 2002). PRL plays a central role in the control of these activities during the adaptation of fish to freshwater as evidenced by its ability to increase plasma $Na^+$ and $Cl^-$ concentrations and decrease the permeability to water (Seale et al., 2014). Prolactin receptor belongs to the super-family of cytokines and produces its biological effects by interacting and dimerizing with single transmembrane-domain receptors (Forsyth and Wallis, 2002). PRL as a pituitary polypeptide hormone exerts a wide spectrum of functions in vertebrates (Bole-Feysot et al., 1998) and has been shown to favor Na- and Cl-retaining activity in a variety of freshwater and euryhaline teleosts where it promotes the morphology of ion uptake cells (Herndon et al., 1991; Pisam et al., 1993).

On the other hand insulin, a polypeptide hormone, has a multitude of actions on a wide range of cellular processes in vertebrates including fishes. Insulin acts on a variety of tissues *via* insulin receptors which are ubiquitously distributed in the plasma membrane of cells (Borge and Wolf, 2003**)**. In vertebrates, this polypeptide belongs to a super-family of structurally related proteins

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(Schulingkamp et al., 2000) and is involved in the uptake of glucose, stimulation of $Na^+$, $K^+$-ATPase and regulation of cellular metabolism at the level of mitochondria (Standaert and Pollet, 1988). Insulin stimulates the resting membrane potential of insulin-sensitive cells, while increasing the cells' permeability to glucose (Stark et al., 1980). Likewise, the insulin-induced hyperpolarization of the membrane potential in both muscle and adipose tissues may account for a fall in intracellular $Na^+$ and subsequent increase in intracellular $K^+$ (Lantz et al., 1998). It is now known that these electrochemical changes associated with the decline in $Na^+$ may further account for the initiation of changes in insulin-regulated metabolic pathways. However, the interactive role of insulin in metabolism and osmoregulation has been poorly studied in teleosts (Morgan et al., 1997; Nakano et al., 1998).

Teleost fishes possess a wide variety of ion transport proteins that mediate the active and passive movement of ions (Evans et al., 2005; Lorin-Nebel et al., 2006). Adenosine triphosphatases (ATPases), a complex set of enzyme systems found in invertebrates and vertebrates (Carfagna et al., 1996), play a central role in the physiological functions of a cell as main energy transducers (Kodama, 1985). The activity response of ATPases, which are coupled with pumping of cations ($Na^+$, $K^+$, $Ca^{2+}$ and $Mg^{2+}$) across the membranes, reflects the rate of transport of these cations and contributes to the understanding of the functional status of cell (Peter, 2007). Despite the osmoregulatory actions of PRL and the metabolic actions of insulin on their respective osmoregulatory and metabolic organs, the actions of these hormones on ion transport in liver and intestine are not yet well understood in fish. The *in situ* effects of PRL or insulin on membrane-bound and mitochondrial ion transporters were therefore examined in the freshwater climbing perch, *Anabas testudineus* Bloch.

## Materials and methods

### Fish holding conditions

Tropical freshwater (FW) air-breathing fish, commonly known as climbing perch (*A. testudineus* Bloch) belonging to order Perciformes and family Anabantidae, was used as the test species. This native teleost fish inhabiting the backwaters of Kerala in Southern India is an obligate air-breathing fish equipped to live in demanding environmental conditions with their well-defined physiological and biochemical mechanisms (Peter et al., 2007, 2011). These fish in their post-spawning phase were collected from the wild and maintained under laboratory conditions for three weeks under natural photoperiod (12 h L:12 h D) and at freshwater temperature ranging from 28 to $29^0$C with a mean water pH of 6.2. Fish fed with dry commercial fish feed at 1.5% of body mass were used in the experiment. The regulations of Institutional Animal Ethics Committee of the University were followed.

### Experimental protocol

The dose-responsive *in situ* effects of oPRL and insulin on ion transporter activities were studied in the intestine and liver of freshwater climbing perch to understand the short-term actions of these hormones. The *in situ* effects of varied concentrations of oPRL (Sigma, USA) and insulin (Novo Nordisk) infusions were investigated in the test species. Two independent experiments were conducted, and the laboratory-acclimated FW climbing perch were held as two batches containing four groups each. In first set, the effects of varied concentrations ($10^{-9}$, $10^{-8}$ and $10^{-7}$ M) of oPRL and, in second set, the effects of varied concentrations of insulin ($10^{-9}$, $10^{-8}$ and $10^{-7}$ M), respectively, were tested in the $2^{nd}$, $3^{rd}$ and $4^{th}$ group fish in each set. First group of fish in each batch (n=6) were infused with infusion medium alone and they served as the control. oPRL or insulin was dissolved in infusion medium and all fish were infused for 20 minutes.

Feeding was restricted for 24 h prior to infusion procedure to ensure optimum experimental conditions. *In situ* infusion was done as described earlier (Babitha and Peter, 2010). Briefly, fish were first anesthetized in 2-phenoxyethanol solution and blood was drawn from the caudal artery with the help of a heparinized syringe. A ventral incision was made to each fish from the anus to the pectoral girdle and a cannula (PE-50 tubing) was inserted into the ventricle through the bulbus arteriosus. Infusion was done with the help of a peristaltic pump (ENPD-100 EnterTech, Mumbai) using an infusion medium (Cortland saline; 119 mM NaCl, 5 mM $NaHCO_3$, 5.4 mM KCl, 0.35 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 0.81 mM $MgSO_4$, 1.25 mM $CaCl_2$ and 5 mM D-glucose; pH 7.4) at a rate of 0.3 ml $min^{-1}$ for 20 min as demonstrated earlier in *Clarias gariepinus* (Babitha and Peter, 2010). After 20 min of vehicle, oPRL or insulin infusion, anterior portion of intestine (approximately 1cm)

and a part of lower lobe of liver were excised and stored at 80°C for further analysis.

### Isolation of mitochondria

Mitochondria were isolated from intestine and liver tissues. Briefly, the tissues were kept in ice-cold 0.25M sucrose. A 10% tissue homogenate was prepared and subjected to differential centrifugation at $4^0$C (Eppendorf R3435). First, it was centrifuged at 700 xg for 10 minutes to separate the cell debris and nuclei. The supernatant obtained was spun at 10,000 xg for 10 minutes and the pellets recovered were washed twice by repeating the centrifugation. This mitochondrial pellet was later suspended in fresh ice-cold 0.25 M SEI buffer containing 0.25 M sucrose, 10 mM $Na_2EDTA$, 0.1 M imidazole. The supernatant collected first was taken as post-mitochondrial supernatant (PMS) and it served as the material for cytosolic transporter assays. The protein concentrations in mitochondria as well as in cytosol were determined using modified Biuret Assay (Alexander and Ingram, 1980) with bovine serum albumin as standard.

### Quantification of ion-specific ATPases

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

A portion of the tissue homogenized in SEI buffer (pH 7.1) and centrifuged at 700 xg for 10 minutes was used for analyzing the $Na^+$, $K^+$-ATPase activity. The ouabain-sensitive $Na^+$, $K^+$-ATPase-specific activity in the intestinal and liver tissue homogenates was quantified adopting the method of Peter et al. (2000). Saponin (0.2 mg $protein^{-1}$) was routinely added to optimize substrate accessibility. The samples in duplicate were added to a 96-well microplate containing 100 mM $L^{-1}$ NaCl, 30 mM $L^{-1}$ immidazole (pH 7.4), 0.1 mM $L^{-1}$ EDTA and 5 mM $L^{-1}$ $MgCl_2$ with or without ouabain and incubated at $37^0$C. The reaction was initiated by the addition of ATP and was terminated with addition of 8.6% TCA. The liberated inorganic phosphate was determined in Autoreader 4011 (Spam Diagnostics Ltd., Surat, India) at 700 nm and expressed in µM Pi $h^{-1}$ mg $protein^{-1}$.

#### *$H^+$-ATPase specific activity*

The baffilomycin-sensitive $H^+$-ATPase activity in the intestine and liver cytosolic and mitochondrial fractions was measured as described for $Na^+$, $K^+$-ATPase but baffilomycin A was used as inhibitor. The samples in duplicate were added to a 96-well microplate containing baffilomycin A. The reaction was initiated by the addition of ATP and terminated by adding 8.6% TCA and the inorganic phosphate content was determined as above and expressed in µM Pi $h^{-1}$ mg $protein^{-1}$.

#### *$Ca^{2+}$-dependent ATPase specific activity*

The vanadate-dependent $Ca^{2+}$-ATPase activity in the intestine and liver cytosolic and mitochondrial fraction was determined as described for $Na^+$, $K^+$-ATPase but vanadate was used as inhibitor. Samples in duplicate were added to a 96-well microplate containing either $CaCl_2$ or vanadate. The inorganic phosphate content released was determined as above and expressed in µM Pi $h^{-1}$ mg $protein^{-1}$.

#### *$Mg^{2+}$-dependent ATPase specific activity*

The specific activity of oligomycin-sensitive $Mg^{2+}$-ATPase in the intestine and liver mitochondrial fraction was determined as described for $Na^+$, $K^+$ATPase but oligomycin was used as inhibitor. Mitochondrial samples in duplicate were added to a 96-well microplate with or without oligomycin. The inorganic phosphate content released was measured and expressed in µM Pi $h^{-1}$ mg $protein^{-1}$.

### Statistical analysis

Data were collected from six animals in each group. Statistical difference among groups was tested by means of one-way analysis of variance (ANOVA) followed by SNK comparison test. Significance between the groups was analyzed with the help of Graphpad Software (Graphpad Instat-3, San Diego) and the level of significance was accepted if $P < 0.05$.

## Results

### *In situ* effects of PRL

Infusion of $10^{-7}$ M oPRL for 20 min significantly increased ($P<0.001$) the $Na^+$, $K^+$-ATPase activity in the intestine, though $10^{-9}$ and $10^{-8}$ M oPRL decreased its activity (Fig. 1). The $Na^+$, $K^+$-ATPase activity showed an increase after $10^{-9}$ and $10^{-8}$ M oPRL but not with $10^{-7}$ M of oPRL in the liver (Fig. 2). The cytosolic $H^+$-ATPase activity in the intestine and liver decreased significantly ($P<0.001$) after oPRL infusion (Fig. 1, 2). The liver mitochondrial $H^+$-ATPase activity showed a significant decrease ($P<0.001$), whereas its activity in the intestine showed an increase ($P<0.01$) after $10^{-7}$ M of oPRL infusion (Fig. 1, 2).

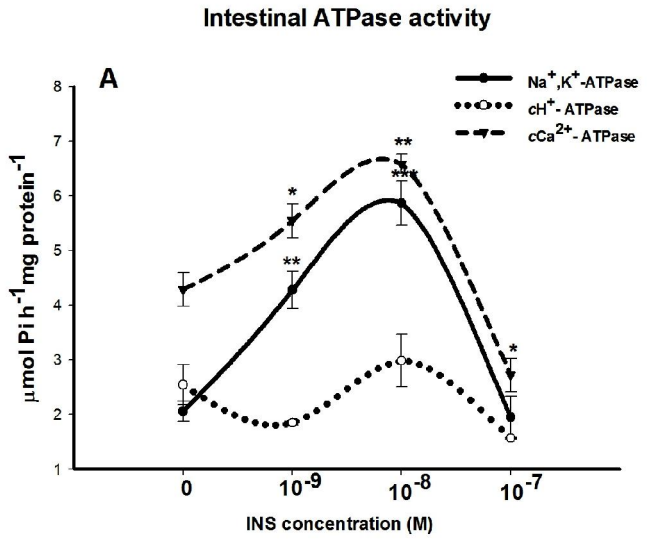


Fig. 1. Responses of ion-specific ATPase activities in the intestine of climbing perch to infusion of varied doses of prolactin (oPRL) for 20 min. Each point represents mean ± SEM for six tests. Statistical differences between fish groups were quoted after SNK test. *(P<0.05), **(P<0.01) and ***(P<0.001) denote significant difference from control.

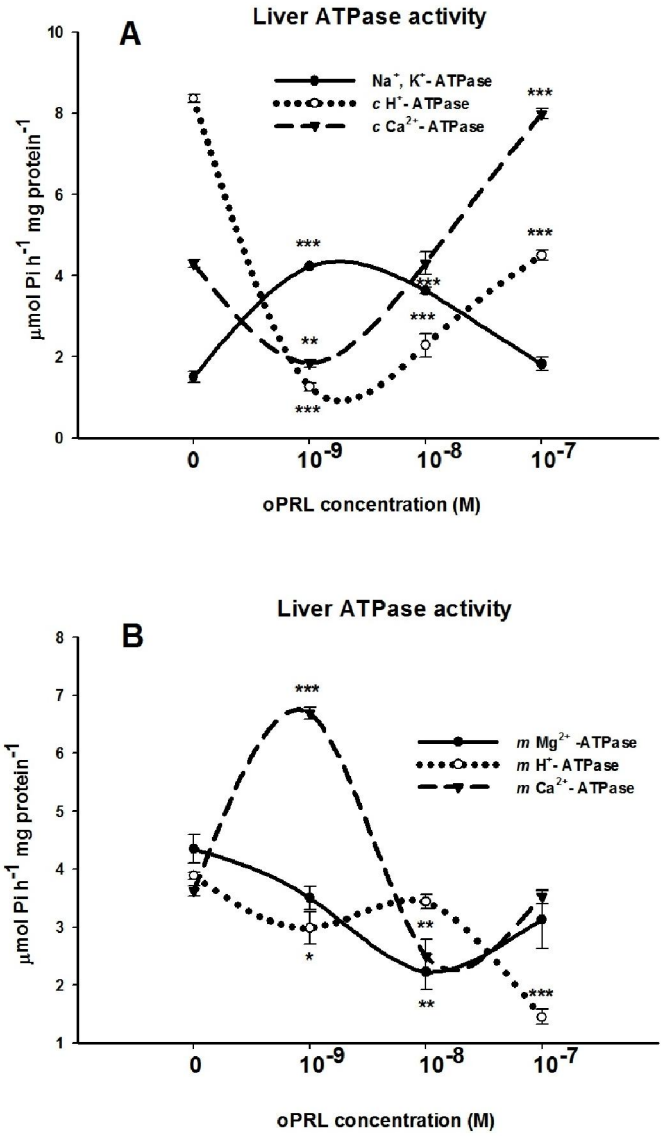


Fig. 2. Responses of ion-specific ATPase activities in the liver of climbing perch to infusion of varied doses of prolactin (oPRL) for 20 min. Each point represents mean ± SEM for six tests. Statistical differences between fish groups were quoted after SNK test. *(P<0.05), **(P<0.01) and ***(P<0.001) denote significant difference from control.

The cytosolic $Ca^{2+}$-ATPase activity showed substantial increase ($P$<0.001) in the intestine after oPRL infusion (Fig. 1). But in the liver, its activity decreased after $10^{-9}$ M oPRL infusion though the activity increased after $10^{-7}$ M oPRL infusion (Fig. 2). The mitochondrial $Ca^{2+}$-ATPase activity showed a decrease in the intestine ($P$<0.01) after a high dose of oPRL infusion, though its activity in the liver showed no response (Fig. 1, 2). The mitochondrial $Mg^{2+}$-ATPase activity significantly decreased ($P$<0.01) in the liver but its activity in the intestine showed significant increases ($P$<0.001) after $10^{-8}$ and $10^{-7}$ M oPRL infusion (Fig. 1, 2).

## *In situ* effects of insulin

Infusion of $10^{-8}$ M insulin for 20 min increased the $Na^+$, $K^+$-ATPase activity in the liver ($P$<0.05), though $10^{-9}$ M and $10^{-7}$ M insulin had no effect on its activity (Fig. 3). Low doses ($10^{-9}$ M and $10^{-8}$ M) of insulin infusion

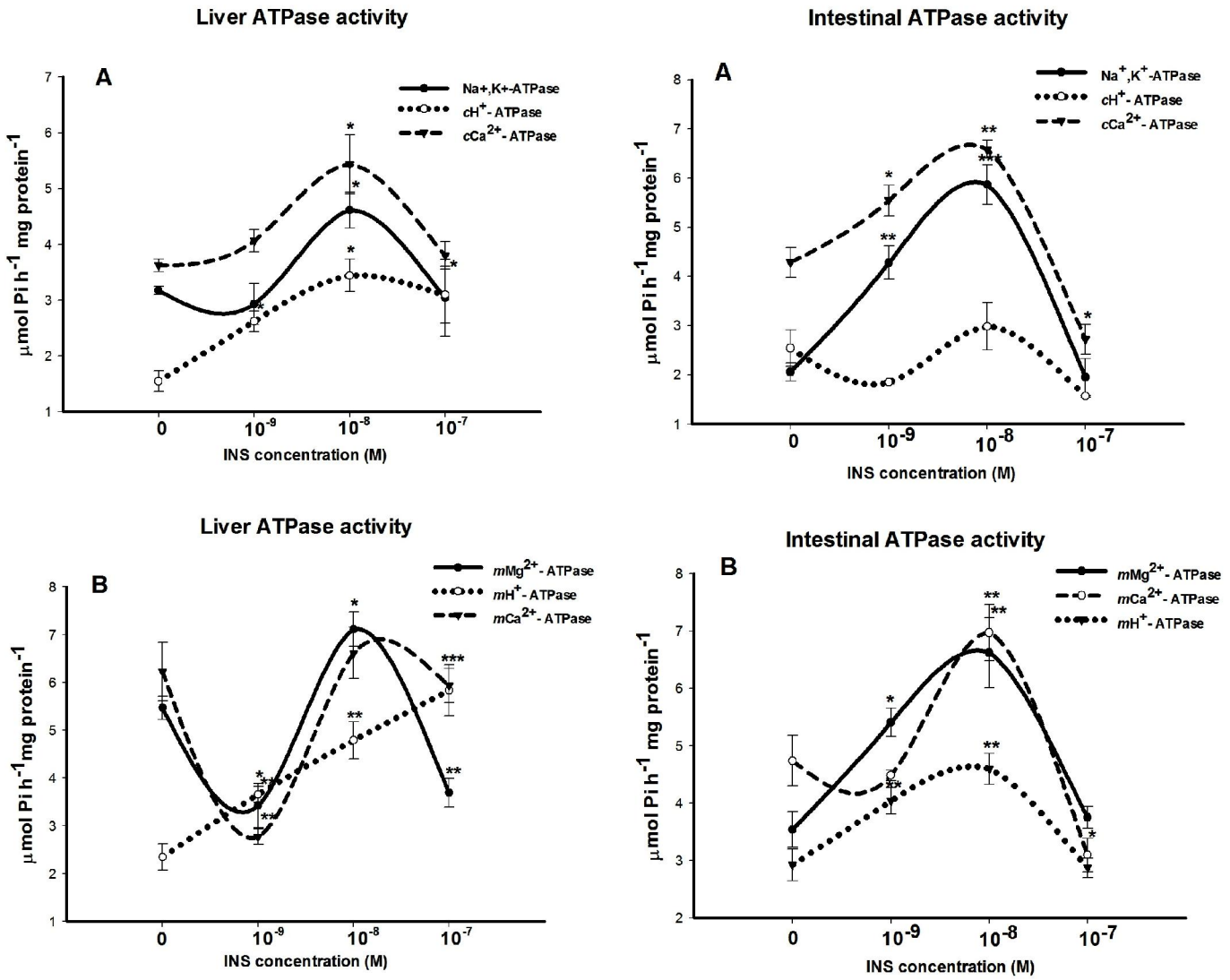


Fig. 3. Responses of ion-specific ATPase activities in the intestine of climbing perch to infusion of varied doses of insulin (INS) for 20 min. Each point represents mean ± SEM for six tests. Statistical differences between fish groups were quoted after SNK test. *(P<0.05), **(P<0.01) and ***(P<0.001) denote significant difference from control.

Fig. 4. Responses of ion-specific ATPase activities in the liver of climbing perch to infusion of varied doses of insulin (INS) for 20 min. Each point represents mean ± SEM for six tests. Statistical differences between fish groups were quoted after SNK test. *(P<0.05), **(P<0.01) and ***(P<0.001) denote significant difference from control.

significantly increased ($P< 0.01$) the intestinal $Na^+$, $K^+$-ATPase activity (Fig. 4). Cytosolic $H^+$-ATPase showed a significant increase ($P<0.05$) in the liver after all doses of insulin infusion (Fig. 3), whereas its activity remained unaffected in the intestine (Fig. 4). Mitochondrial $H^+$-ATPase activity in the liver increased ($P<0.01$) after all doses of insulin infusion (Fig. 3). Infusion of $10^{-9}$ M and $10^{-8}$ M insulin increased the mitochondrial $H^+$-ATPase activity in the intestine, whereas $10^{-7}$ M insulin had no effect on this pump activity (Fig. 4).

The cytosolic $Ca^{2+}$-ATPase activity in the liver increased ($P<0.05$) after $10^{-8}$ M insulin infusion (Fig. 3). However, significant decrease ($P<0.01$) in the cytosolic $Ca^{2+}$- ATPase activity was found in the intestine after $10^{-7}$ M insulin infusion (Fig. 4). The mitochondrial $Ca^{2+}$-ATPase activity in the liver showed a significant decrease ($P<0.05$) after $10^{-9}$ M of insulin infusion, whereas both $10^{-8}$ and $10^{-7}$ M insulin had little effect on its activity (Fig. 3). $10^{-9}$ and $10^{-8}$ M insulin infusion increased the $Ca^{2+}$ ATPase activity in intestine, but $10^{-7}$ M insulin decreased

its activity (Fig. 4). Insulin infusion at $10^{-9}$ and $10^{-7}$ M concentration showed significant decrease ($P<0.01$) in the liver mitochondrial $Mg^{2+}$- ATPase activity, whereas its activity significantly increased ($P<0.05$) after $10^{-8}$ M insulin infusion (Fig. 3). The mitochondrial $Mg^{2+}$-ATPase activity in the intestine increased ($P<0.05$) after $10^{-9}$ and $10^{-8}$ M insulin infusion (Fig. 4).

## Discussion

Intestine as a major osmoregulatory organ of fish coordinates the hydromineral homeostasis (McCormick, 2001; Marshall et al., 2002; Evans, 2002, 2008), whereas liver as the major non-osmoregulatory organ directs metabolite homeostasis under varied physiologic conditions (Mommsen et al., 1999; Iwama et al., 2006; Soengas et al., 2007; Peter and Peter, 2007; Babitha and Peter, 2010). In fish, intestine is involved in seawater adaptation as it can absorb water to compensate for dehydration (Marshall and Grosell 2005). The rich $Na^+$, $K^+$-ATPase in the intestinal epithelia absorbs $Na^+$ from the lumen and exogenous PRL decreases intestinal permeability to water and ions (Collie et al., 1987) and $Na^+$, $K^+$-ATPase activity (Manzon, 2002) and can absorb $Na^+$, $Cl^-$, $Ca^{2+}$, and $Mg^{2+}$ present in food (Baldisserotto and Mimura, 1995; Bijvelds et al., 1998).

Cellular $Na^+$ and $K^+$ gradients are essential for the ion transport across the plasma membrane and $Na^+$, $K^+$-ATPase is delicately involved in this transport (Evans, 2002, 2008). $Na^+$, $K^+$-ATPase that represents the sodium pump, is an index of osmoregulation in fish (Evans et al., 2005). $Na^+$, $K^+$-ATPase contributes to basic metabolic reactions in mammals and is also responsible for regulation of membrane polarization and thermogenesis (Ismail-Beigi, 1988). On the contrary, the $Na^+$, $K^+$-ATPase activity in fishes mainly accounts for the maintenance of ionic gradients across epithelial membranes. The elevated intestinal $Na^+$, $K^+$-ATPase activity observed after $10^{-7}$ M oPRL treatment indicates a role of PRL in promoting luminal $Na^+$ absorption, though low dose of oPRL inhibited sodium pump activity. These dose-responsive actions of PRL on intestinal ion-selective ATPase activities point to a direct action of PRL on intestinal mitochondrial and cytosolic ion transporters in this fish. It has been shown that $Na^+$ uptake across the gill epithelium of freshwater fishes relies on the apical vacuolar $H^+$-ATPase that generates a negative membrane potential to drive $Na^+$ entry into the epithelium *via* an apical $Na^+$ channel (Guffey et al., 2011). It is likely that oPRL exerts an inhibition of vacuolar pump transport activity in the intestine and liver of fish as evident in its decreased $H^+$-ATPase activity in these tissues.

As systemic organ, liver harbors major metabolic machineries and plays a critical role in energy homeostasis (Babitha and Peter, 2010). Despite the limited contribution of liver to whole body osmoregulation, it is found that oPRL infusion significantly alters the ion-specific ATPase activities ($Na^+$, $K^+$-, $H^+$, $Ca^{2+}$ and $Mg^{2+}$) emphasizing a key role of oPRL in ion transporters in liver. The decreased liver $Na^+$, $K^+$-ATPase activity that reflects a lowered Na pump activity thus appears to be an osmotic response to PRL that may ensure a PRL-mediated energy metabolism in this organ. This osmotic response of liver as evident in $Na^+$, $K^+$-ATPase activity to PRL underscores the vital role of this organ to drive the osmotic function and to exert a substantial role in metabolic processes.

Fish absorb calcium directly from their aquatic environment (Payan et al., 1981) and regulate its levels very tightly (Bailey and Fenwick, 1975). Transport of calcium from the exterior of the cell into the cytosol is passive and is regulated through channel- or carrier-proteins. A hormone-regulated carrier mechanism for $Ca^{2+}$ entry could also be found in the apical membrane (Flik and Verbost, 1993). On the other hand, transport of calcium from the cytosol to the exterior requires energy-consuming extrusion mechanisms, involving $Ca^{2+}$-ATPase and $Na^+$/$Ca^{2+}$ exchange (Payan et al., 1981). Dose-responsive action of cytosolic and mitochondrial $Ca^{2+}$-ATPase activity in the intestine and liver clearly signifies a hypercalcemic action of PRL in intestine and its regulatory role in liver calcium homeostasis. It appears that PRL exerts a dose-responsive calcium handling in the intestine where it favors cytosolic $Ca^{2+}$ transport while inhibiting $Ca^{2+}$ entry in to the mitochondria. This further points to the important role of PRL in calcium mobilization across the intestinal epithelia, as similar observations have been found in other fishes (Flik and Verbost, 1993; Charoenphandhu et al., 2010).

$Mg^{2+}$-ATPase plays an essential role in energy synthesis and in oxidative phosphorylation in mitochondria (Mohiyuddin et al., 2010). Transepithelial regulation of $Mg^{2+}$ ions is essential for the integrity of the cellular membrane, intracellular signaling and the stabilization of branchial permeability (Parvez et al., 2006). As an index

of general ATPase activity, $Mg^{2+}$-ATPase makes abundant distribution and dual localization in mitochondria and cytosol (Lehninger, 1988). The elevated $Mg^{2+}$-ATPase activity in the intestine after oPRL infusion emphasizes a direct action of PRL in Mg transport. The decreased liver $Mg^{2+}$-ATPase activity reflects a decreased Mg transport in liver and this may be linked with altered cellular energy metabolism.

The osmotic response of liver to insulin has not been addressed adequately, though liver plays critical roles in intermediary metabolism (Mommsen, et al., 1999; Peter, et al., 2007). It is likely that, with the help of $Na^+$,$K^+$-ATPase activity, liver cells can maintain cellular Na/K gradient which directs cellular biochemical functions (Babitha and Peter, 2010). Liver being a metabolic organ possesses its own osmotic competence and that makes them metabolically active (Lang et al., 1998), since the regulation of $Na^+$ and $K^+$ gradients and its integrated control through hormones are fundamental to organ function (Babitha and Peter, 2010). Our results show that insulin has a role in the regulation of $Na^+$,$K^+$-ATPase activity in both liver and intestine. Similar dose-dependent changes in $Na^+$,$K^+$-ATPase activity has been reported in rat liver cells (Arora and Kaur, 1993). Activation of hepatocyte fuction by insulin could be ascribed as these cells show upregulated $Na^+$,$K^+$-ATPase activity following insulin infusion. The ionic response of liver mitochondria to insulin further points to a direct action of insulin on the ion transporters since these transporters compete with cellular energy metabolism in the liver of climbing perch.

A direct action of insulin on intestine can be seen as insulin increased $Na^+$,$K^+$-ATPase activity. Furthermore, this organ is involved in the whole body osmoregulation where it favors the absorption of $Na^+$ from the intestinal lumen. This stimulatory role of insulin in $Na^+$,$K^+$-ATPase activity in both liver and intestine of the test fish thus clearly suggests a role for insulin in Na homeostasis. Fehlmann and Freychet (1981) reviewed that insulin can increase the entry of $Na^+$ into hepatocytes, where it can enhance the pump activity by increasing the availability of $Na^+$ ions to internal $Na^+$ transport sites of the $Na^+$,$K^+$-ATPase. These authors also reported that the activation of the $Na^+$,$K^+$-ATPase transport activity by insulin in isolated hepatocytes is secondary to a rapid and transient increase in $Na^+$ influx. Insulin has been shown to stimulate the electrogenic Na transport in a variety of cells including isolated rat proximal convoluted tubule (Feraille et al., 1994). It is well documented that the mechanism of insulin stimulation of $Na^+$,$K^+$-ATPase activity varies among different cells and tissues (Longo et al., 2001). In primary adipocytes insulin does not increase $Na^+$ entry (McGill and Guidotti, 1991) but directly stimulates the $\alpha_2$ isoform of the pump by increasing its affinity toward intracellular $Na^+$ (Marette et al., 1993). Longo et al. (2001) suggested that stimulation of $Na^+$,$K^+$-ATPase activity in human fibroblasts occurs without an increase in the number of membrane-associated ouabain binding sites, indicating that insulin can increase the turnover rate of existing transporters.

Magnesium plays an important role in the stability of nucleic acids and is involved in the release of energy from ATP (Rude, 1989). A tight regulation of Mg transport, therefore, exists across the membranes (Gardner, 2003; Warren et al., 2004). $Mg^{2+}$- ATPase as a transporter of Mg is localized in the mitochondria with a unique role in energy synthesis. The increased $Mg^{2+}$ATPase activity after insulin infusion suggests a role of insulin in supplying energy for iono-osmoregulation. Similar increase in the transport of $Mg^{2+}$ by insulin has been reported earlier (Jarret and Smith, 1974).

Calcium acts as a mediator for insulin action (Clausen and Everts, 1989). Carbohydrate metabolites appear to play a major role in the energy supply for iono- and osmoregulation, and the liver is the major source supplying carbohydrate metabolites to osmoregulatory organs (Tseng and Hwang, 2008). The increased cytosolic and the decrease in the mitochondrial $Ca^{2+}$ATPase activities in the liver after insulin clearly signify a role for insulin in liver calcium homeostasis. Mitochondrial calcium is also having an impact on the synthesis of factors other than ATP that couple mitochondrial metabolism to insulin secretion (Maechler et al., 1998). The regulation of cellular $Ca^{2+}$ signals by mitochondria has been found to depend on the mitochondrial energetic status (Goldnathal and Marin-Garcia, 2004). Further, insulin has been shown to reduce the influx of $Ca^{2+}$ through receptor-operated channels and to decrease the voltage-mediated $Ca^{2+}$ influx (Standley et al., 1991). It is known that insulin can raise cytosolic calcium in liver as a possible mechanism of the insulin-mediated stimulation of glucose uptake in tissues (Draznin et al., 1988).

The rise in increased intestinal $Ca^{2+}$ATPase activity after insulin challenge clearly provides evidence

for an integrative role of insulin in exerting both metabolic and osmotic actions in this fish. It appears that this rise could be either due to the increase in calcium permeability of the plasma membrane and /or by releasing intracellular calcium from its binding sites. Furthermore, the increased $Ca^{2+}$-ATPase activity in the metabolic and osmoregulatory organs also indicates increased calcium signaling in these tissues.

Relatively little is known about the hormonal regulation of the proton pump in fish tissues. A role of insulin in regulation of the electrochemical proton gradient for generating an appropriate membrane potential could be seen in our study. Insulin as an important regulator of energy metabolism regulates mitochondrial ATP synthase gene expression and enzymatic activity (Mei et al., 2007). There are reports on the feasibility of different mechanisms of insulin action on ATPase system (Serhan and Kreydiyyeh, 2010). However, the physiological importance of rapid alteration of ion distribution by insulin in mediating its action on metabolic processes is still poorly understood (Fehlmann and Freychet, 1981).

Overall, the data indicate that both PRL and insulin have direct actions on ion transport in liver and intestine of fish which further point to the integrative actions of these osmoregulatory and metabolic hormones in fish tissues. The results further indicate that PRL has both dose-responsive and differential actions on monovalent and divalent cation pump activities in liver and intestine tissues. Insulin, on the other hand, is equally effective in promoting cation transport in both intestine and liver tissues. It is concluded that both PRL and insulin can rapidly regulate cellular ion transport and thus maintain ion homeostasis in this fish.

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## References

Alexander JB, Ingram GA. (1980) A comparison of five methods commonly used to measure protein concentration of fish sera. *J Fish Biol.* **16**: 115-122.

Arora SK, Kaur G. (1993) Effect of insulin induced hypoglycemia on acetylcholinesterase and $Na^+$,$K^+$-ATPase activity of rat heart, liver and kidney. *Biochem Mol Biol Int.* **31**: 413-420.

Babitha GS, Peter MCS. (2010) Cortisol promotes and integrates the osmotic competence of the organs in North African catfish (*Clarias gariepinus* Burchell): Evidence from *in vivo* and *in situ* approaches. *Gen Comp Endocrinol.* **168**: 14-21.

Bailey JR, Fenwick JÑ. (1975) Effect of angiotensin II and the corpuscles of Stannius extract on total and ionic plasma calcium levels and blood pressure in intact eels (*Anguilla rostrata* LeSueur). *Gen J Zool.* **53**: 630-633.

Baldisserotto B, Mimura OM. (1995) Ion and water transport in the gut of the freshwater teleost *Prochilodus scrofa*. *J Braz Ass Adv Sci.* **47**: 83-85.

Bijvelds MJC, Van der Velden JA, Kolar, ZI, Flik G. (1998) Magnesium transport in freshwater teleosts. *J Exp Biol.* **201**: 1981-1990.

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. (1998) Prolactin (PRL) and its receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev.* **19**: 225–268.

Borge PD Jr, Wolf BA. (2003) Insulin receptor substrate 1 regulation of sarco-endoplasmic reticulum calcium ATPase 3 in insulin-secreting beta-cells. *J Biol Chem.* **278**: 11359-11368.

Carfagna MA, Ponsler GD, Muhoberac BB. (1996) Inhibition of ATPase activity in rat synaptic plasma membranes by simultaneous exposure to metals. *Chem Biol Interact.* **100**: 53-65.

Charoenphandhu N, Wongdee K, Krishnamra N. (2010) Is prolactin the cardinal calciotropic maternal hormone? *Trends Endocrinol Metab.* **21**: 395–401.

Clausen T, Everts ME. (1989) Regulation of the $Na^+$, $K^+$-pump in skeletal muscle. *Kidney Int.* **35**: 1–13.

Collie NL, Hirano T. (1987) Mechanisms of hormone actions on intestinal transport. *In*: Pang PKT, Schreibman MP (Eds.) *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*. **2**: 239–270. San Diego: Academic Press.

Draznin B, Sussman KE, Eckel RH, et al. (1988) Possible role of cytosolic free calcium concentrations in mediating insulin resistance of obesity and hyperinsulinemia. *J Clin Invest*. **82:** 1848-1852.

Evans DH. (1993) Osmotic and ionic regulation. *In*: Evans DH (Ed) *The Physiology of Fishes*. pp. 315-341. Boca Raton, Florida: CRC Press.

Evans DH. (2002) Cell signalling and ion transport across the fish gill epithelium. *J Exp Zool*. **293**: 336-347.

Evans DH. (2008) Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. *Am J Physiol Regul Integr Comp Physiol.* **295**: R704-R713.

Evans DH, Piermarini PM, Choe KP. (2005) The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. *Physiol Rev.* **85**: 97-177.

Fehlmann M, Freychet P. (1981) Insulin and glucagon stimulation of $Na^+$,$K^+$-ATPase transport activity in isolated rat hepatocytes. *J Biol Chem*. **256**: 7449-7453.

Feraille E, Carranza ML, Rousselot M, Favre H. (1994) Insulin enhances sodium sensitivity of Na,K-ATPase in isolated rat proximal convoluted tubule. *Am J Physiol.* **267**: F55–F62.

Flik G, Verbost PM. (1993) Calcium transport in fish gills and intestine. *J Exp Biol.* **184**: 17–29.

Forsyth IA, Wallis M. (2002) Growth hormone and prolactin-molecular and functional evolution. *J Mammary Gland Biol Neoplasia.* **7**: 291-312.

Frye BE (1967) *Hormonal Control of Vertebrates.* Current Concepts in Biology Series. New York: Macmillian Co.

Gardner RC. (2003) Genes for Magnesium Transport. *Curr Opin Plant Biol*. **6**: 263-267.

Goldenthal MJ, Marin-Garcia J. (2004) Mitochondrial signaling pathways: A receiver/ integrator organelle. *Mol Cell Biochem*. **262**: 1-16.

Guffey S, Esbaugh A, Grosell M. (2011) Regulation of apical $H^+$-ATPase activity and intestinal $HCO_3^-$ secretion in marine fish osmoregulation. *Am J Physiol Regul Integr Comp Physiol*. **301**: R1682–R1691.

Herndon TM, McCormick SD, Bern HA. (1991) Effects of prolactin on chloride cells in opercular membrane of seawater adapted tilapia. *Gen Comp Endocrinol.* **83**: 283–289.

Ismail-Beigi F. (1988) Thyroid thermogenesis: Regulation of ($Na^+$,$K^+$)- adenosine triphosphatase and active Na, K transport. *Amer Zool.* **28:** 363-371.

Iwama GK, Alfonso LOB, Vijayan MM. (2006) Stress in fish. *In*: Evans DH, Claiborne JB (Eds) *The Physiology of Fishes*, 3rd ed. pp. 319-342. Boca Raton, Florida: CRC Press.

Jarett L, Smith RM (1974) The stimulation of adipocyte plasma membrane magnesium ion-stimulated adenosine triphosphatase by insulin and concanavalin A. *J Biol Chem.* **249**: 5195-5199.

Kodama T. (1985) Thermodynamic analysis of muscle ATPase mechanisms. *Physiol Rev.* **65**: 467-551.

Lang F, Busch GL, Ritter M, et al. (1998) Functional significance of cell volume regulatory mechanisms. *Physiol Rev.* **78**: 247–306.

Lantz RC, Elsas LJ, DeHaan RL. (1980) Ouabain-resistant hyperpolarization induced by insulin in aggregates of embryonic heart cells. *Proc Natl. Acad Sci. USA* **77**: 3062-3066.

Lehninger AL. (1988) *Biochemistry: The Molecular Basis of Cell Structure and Function*. 2nd Edn. Ludhiana, New Delhi: Kalyani Publishers.

Longo N, Scaglia F, Wang Y. (2001) Insulin increases the turnover rate of $Na^+$-$K^+$ATPase in human fibroblasts. *Am J Physiol Cell Physiol.* **280**: 912-919.

Lorin-Nebel C, Boulo V, Bodinier C, Charmantier G. (2006) The $Na^+/K^+/2Cl^-$ cotransporter in the sea bass *Dicentrarchus labrax* during ontogeny: involvement in osmoregulation. *J Exp Biol*. **209**: 4908-4922.

Maechler P, Kennedy ED, Wang H, Wollheim CB. (1998) Desensitization of mitochondrial $Ca^{2+}$ and insulin secretion responses in the beta cell. *J Biol Chem*. **273**: 20770-20778.

Manzon LA. (2002) The role of prolactin in fish osmoregulation: a review. *Gen Comp Endocrinol.* **125**: 291-300.

Marette A, Krischer J, Lavoie L, et al. (1993) Insulin increases the $Na^+$-$K^+$-ATPase alpha-2 subunit in the surface of rat skeletal muscle: morphological evidence. *Am J Physiol Cell Physiol.* **265**: C1716- C1722.

Marshall WS, Grosell M (2005). Ion transport, osmoregulation, and acid-base balance. *In*: Evans D, Claiborne JB. (Eds) *The Physiology of Fishes*. 3rd Edition. Pp. 177-230. Bosta racon, Florida: CRC Press.

Marshall WS, Howard JA, Cozzi RRF, Lynch EM. (2002) NaCl and fluid secretion by the intestine of the teleost *Fundulus heteroclitus*: involvement of CFTR. *J Exp Biol.* **205**: 745-758.

McCormick SD. (2001) Endocrine control of osmoregulation in teleost fish. *Amer Zool.* **41**: 781-794.

McCormick SD, Bradshaw D. (2006) Hormonal control of salt and water balance in vertebrates. *Gen Comp Endocrinol.* **147**: 3-8.

McGill DL, Guidotti G. (1991) Insulin stimulates both the á -1 and the á-2 isoforms of the rat adipocyte Na+-$K^+$ ATPase. *J Biol Chem.* **266**: 15824–15831.

Mei J, Wood C, L'Abbe MR, et al. (2007) Consumption of soy protein isolate modulates the phosphorylation status of hepatic ATPase/ATP synthase b protein and increases ATPase activity in rats. *J Nutr*. **137**: 2029–2035.

Mohiyuddin SS, Reddy RS, Kumar AL, Doss JP. (2010) Acephate induced alterations in $Mg^{2+}$-ATPase and $Na^+$-$K^+$-ATPases of different brain regions of albino rats. *The Bioscan.* **5**: 153–156.

Mommsen TP, Vijayan MM, Moon TW. (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fish.* **9**: 211-268.

Morgan JD, Sakamoto T, Grau EG, Iwama GK. (1997) Physiological and respiratory responses of the Mozambique tilapia (*Oreochromis mossambicus*) to salinity acclimation. *Comp Biochem Physiol.* **117**: 391-398.

Nakano K, Tagawa M, Takemura A, Hirano T. (1998) Temporal changes in liver carbohydrate metabolism associated with seawater transfer in *Oreochromis mossambicus*. *Comp Biochem Physiol.* **119**: 721-728.

Parvez S, Sayeed I, Raisuddin S. (2006) Decreased gill ATPase activities in the freshwater fish *Channa punctata* exposed to a diluted paper mill effluent. *Ecotoxicol Enviorn Saf.* **65**: 62–66.

Payan P, Mayer-Gostan N, Pang PKT. (1981) Site of calcium uptake in the freshwater trout gill. *J Exp Zool*. **216**: 345-347.

Peter MCS. (2007) *Thyroid hormones and hydromineral regulation during stress in fish*, D.Sc. Thesis, Radboud University, Nijmegen, The Netherlands.

Peter MCS, Lock RAC, Wendelaar Bonga SE. (2000) Evidence for an osmoregulatory role of thyroid hormones in the freshwater Mozambique tilapia, *Oreochromis mossambicus*. *Gen Comp Endocrinol*. **120**: 157–167.

Peter MCS, Leji J, Peter VS. (2011) Ambient salinity modifies the action of triiodothyronine in the air-breathing fish *Anabas testudineus* Bloch: effects on mitochondria-rich cell distribution, osmotic and metabolic regulations. *Gen Comp Endocrinol*. **171**: 225–231.

Peter VS, Peter MCS. (2007) Influence of coconut husk retting effluent on metabolic, interrenal and thyroid functions in the air-breathing perch, *Anabas testudineus* Bloch. *J Endocrinol Reprod.* **11**: 8-14.

Peter VS, Joshua EK, Wendelaar Bonga SE, Peter MCS. (2007) Metabolic and thyroidal response in air-breathing perch (*Anabas testudineus*) to water-borne kerosene. *Gen Comp Endocrinol.* **152**: 198-205.

Pisam M, Auperin B, Prunet P, et al. (1993) Effects of prolactin on a and b chloride cells in the gill epithelium of the saltwater adapted tilapia *Oreochromis niloticus*. *Anat Rec.* **235**: 275–284.

Polakof S, Moon TW, Aguirrel P, et al. (2010) Effects of insulin infusion on glucose homeostasis and glucose metabolism in rainbow trout fed a high-carbohydrate diet. *J Exp Biol*. **213**: 4151-4157.

Rude RK. (1989) Physiology of magnesium metabolism and the important role of magnesium in potassium deficiency. *Am J Cardiol.* **63**: 31G-34G.

Schulingkamp RJ, Pagano TC, Hung D, Raffa RB. (2000) Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci Biobehav Rev.* **24:** 855–872.

Seale AP, Moorman BP, Inokuchi M, et al. (2014) The osmoregulatory effects of rearing Mozambique tilapia in a tidally changing salinity. *Gen Comp Endocrinol*. **207**: 94–102.

Serhan MF, Kreydiyyeh SI (2010) Insulin down-regulates the Na+/K+ ATPase in enterocytes but increases intestinal glucose absorption. *Gen Comp Endocrinol*. **167**: 228–233.

Soengas JL, Sangiao-Alvarellos S, Laiz-Carrion R, Mancera JM (2007) Energy metabolism and osmotic acclimation in teleost fish. *In*: Baldisserotto B, Mancera JM, Kapoor BG. (Eds) *Fish Osmoregulation*. pp. 277-307. Enfield NH: Science Publishers.

Standaert ML, Polet RJ. (1988) Insulin-glycerolipid mediators and gene expression *FASEB J.* **2**: 2453-2461.

Standley PR, Zhang F, Ram JL, et al. (1991) Insulin attenuates vasopressin-induced calcium transients and voltage-dependent calcium current in rat vascular smooth muscle cells. *J Clin Invest*. **88**: 1230-1236.

Stark RJ, Read PD, O'doherty J. (1980) Insulin does not act by causing a change in membrane potential or intracellular free sodium and potassium concentration of adipocytes. *Diabetes* **29**: 1040-1043.

Tseng YC, Hwang PP. (2008) Some insights into energy metabolism for osmoregulation in fish. *Comp Biochem Physiol C Toxicol Pharmacol*. **148**: 419-429.

Warren MA, Kucharski LM, Veenstra A, et al. (2004) The CorA $Mg^{2+}$ transporter is a homotetramer. *J Bacteriol*. **186**: 4605-4612.