Original Research
ISSN (Print): 0971-913X
ISSN (Online): 2455-1244
Journal of Endocrinology and Reproduction, *Vol 25(2), 133-144, December 2021, DOI: 10.18311/jer/2021/28983*

Regulation of H^+ and K^+ Gradients by *In Vitro* 3,5-Diiodothyronine in Hepatocyte Explants of Hypoxic Air-Breathing Fish *Anabas testudineus* Bloch

S. Aswathi, Valsa S. Peter and M. C. Subhash Peter*

Department of Zoology, Inter-University Centre for Evolutionary and Integrative Biology (iCEIB), School of Life Sciences, University of Kerala, Kariavattom, Thiruvananthapuram – 695581, Kerala, India; subashpeter@yahoo.com

Abstract

Thyroid hormone metabolite 3, 5-diiodothyronine (T_2) has been shown to possess physiological actions in vertebrates including fishes. It is, however, not certain if T_2 has a role in cation transport in fish hepatocytes, particularly in a stressed condition. We, therefore, tested the *in vitro* action of T_2 on the activities of ion transporters such as Na^+/K^+ ATPase, H^+/K^+ ATPase, Na^+/NH_4^+ ATPase, vacuolar H^+-ATPase, Plasma Membrane Ca^{2+} ATPase (PMCA), mitochondrial Ca^{2+} and mitochondrial H^+-ATPase as these ATPases are known for their roles in maintaining systemic and cellular cation gradients including proton and potassium gradients. Hepatocyte explants of air-breathing fish (*Anabas testudineus,* Bloch), either in non-stressed or hypoxic condition, were incubated with varied doses of T_2 (10^{-9}, 10^{-8} and 10^{-7} M) for 15 min and the specific activities of these cation-dependent ATPases were analyzed. We found that T_2 exposure evoked higher sensitivity to vacuolar and mitochondrial H^+-ATPases and H^+/K^+ ATPase and not to PMCA or mitochondrial Ca^{2+} ATPase. The data also indicated that T_2 has a similar sensitivity to vacuolar and mitochondrial H^+-ATPases and H^+/K^+ ATPase in the hepatocytes of both non-stressed and hypoxia-stressed fish. The data thus provide evidence for a direct action of T_2 on the regulation of proton and potassium gradients in the hepatocytes of both non-stressed and hypoxicair-breathing fish.

Keywords: Diiodothyronine, Fish, Hypoxia Stress, T_2, Na^+/K^+-ATPase, H^+-ATPase, Ca^{2+}-ATPase, Na^+/NH_4^+-ATPase

1. Introduction

Thyroid Hormones (THs) are critical in the regulation and coordination of many vital physiological processes in all vertebrates including fishes. They engage in genomic mechanisms that are mediated by the involvement of nuclear TH receptors[1,2] or non-genomic mechanisms that are initiated by the binding of hormone molecules to the receptors present in the plasma membrane, mitochondria or cytoplasm[3]. In addition, sharing of both non-genomic and genomic actions of THs have also been documented[4]. Thyroxine (T_4) and triiodothyronine (T_3) are the leading hormones of Hypothalamic-Pituitary-Thyroid (HPT) axis and are involved in many physiological actions in fishes including osmotic and ionic regulations[5-7]. THs perform both fundamental and modulator roles in the regulation of water and mineral balance in fishes particularly during stress conditions[8]. During stress response, many hormonal signals interact with an array of ion transporters that regulate ionic homeostasis in fishes[9]. As an important ion-transporter regulator and a stress modifier, THs have the ability to modify the stress-induced physiological responses in fishes. Furthermore, non-genomic actions of TH particularly on plasma membrane ion transporters and cytoskeleton have been found associated with homeostaticmechanisms[10]. However, a role for 3, 5– diiodothyronine (3, 5-T_2 or T_2) that mimics several biological effects of T_3[11,12] has not yet been delineated in fishes.

*Author for correspondence

Thyroid hormones are key regulators of cellular metabolism, and have influence over metabolic processes in almost all tissues. As the important metabolic center, hepatocytes are one of the major target cells of TH, a prime regulator of energy metabolism[13,14]. Moreover, hepatocytes play a crucial role in TH metabolism. THs are known for their metabolic and ion-regulatory actions where they utilize their differential and integrative mechanisms[5], involving regulation of ion transporter protein abundance and modulation of activity patterns[15-18]. Hepatic function has been found associated with TH function and, naturally, any alteration in TH status would affect hepatic activity[19]. Furthermore, THs would modulate ion cycling by altering membrane permeability, expression and characteristics of ion pumps[20-22]. Liver is an important site for the stress acclimation, demanding high energy requirement for homeostasis particularly during stress[23,24]. Induction of stress could influence the liver physiology of fishes and could alter the carbohydrate and lipid metabolism[25] and there exists an intricate relationship between THs and liver function[19,26,27].

Bioenergetic mechanisms appear to be a main machinery target of T_2[12,28,29] particularly in hepatocytes[30-33]. However, the action of T_2 on ion transporters that regulate cationic gradients necessary for normal hepatic functioning has not yet been studied especially in fishes. In the present study we, thus, examined the *in vitro* action of T_2 on ion-dependent ATPases such as Na^+, K^+-ATPase (NKA), H^+, K^+-ATPase (HKA), Na^+, NH^{4+} ATPase (NNA), vacuolar H^+-ATPase (vHA), plasma membrane Ca^{2+} ATPase (PMCA), mitochondrial Ca^{2+} ATPase and mitochondrial H^+-ATPase in the hepatic explants of the air-breathing fish *Anabas testudineus* which were kept either in non-stressed or hypoxia-stressed state to address how T_2 modulates cationic gradients in hepatocytes during hypoxia-stress condition.

2. Materials and Methods

2.1 Fish Holding Conditions

Climbing perch (*Anabas testudineus* Bloch), an obligate air-breathing tropical freshwater fish belonging to order Perciformes and family Anabantidae, was selected as the test species. This fish that inhabit the backwaters of Kerala in Southern India, have well defined physiological and biochemical mechanisms to live in demanding environmental conditions[34,35]. As an excellent model to study the advanced physiological mechanisms[6,34,36,37], wild *Anabas* (approx. 40 ± 5 g body weight) were collected locally and held for three weeks in the laboratory conditions. They were kept under natural photoperiod (12 L/D cycle) at water temperature ranging from 28°C to 29°C, with a mean water pH of 6.2. Before the start of the experiments, fish were transferred to 50 L glass tanks and kept for another two weeks of acclimation. They were fed with dry commercial fish feed at 1.5% of body mass, and feeding was discontinued for 24 h prior to experiment, and care was taken to minimize the effect of stress such as handling to ensure standardized experimental conditions. The regulations of Institutional Animal Ethics Committee of the University were followed and we found no mortality during the course of the experiments.

2.2 Animal Care and Experiments

Two independent experiments were carried out. The first experiment tested the dose-responsive *in vitro* action of T_2 on the specific activity of ion-dependent ATPases such as Na^+, K^+-ATPase (NKA), H^+, K^+-ATPase (HKA), Na^+, NH^{4+} ATPase (NNA), vacuolar H^+-ATPase (vHA), plasma membrane Ca^{2+} ATPase (PMCA), mitochondrial Ca^{2+} ATPase (mit.CA) and mitochondrial H^+-ATPase (mit.HA) in the hepatocyte explants of fish. Hepatocyte explants were prepared from laboratory-acclimated fish (n=24). A set of fish hepatocyte explants (n=6) were kept as control and the remaining three sets of explants were exposed to various doses of T_2 such as 10^{-9}, 10^{-8} and 10^{-7} M, respectively, for 15 min under *in vitro* conditions. T_2 (Sigma-Aldrich, USA) was dissolved in 0.04 M NaOH and subsequently diluted with 0.65% saline solution. This stock solution was kept at 4°C[38].

In the second experiment, the *in vitro* action of T_2 on the activity of hepatocyte ion-dependent ATPases was analyzed either in non-stressed or immersion-stressed fish. Twenty four laboratory-acclimated fish were held as four groups (4×6). Fish in the first and second groups were kept as non-stressed fish. Fish in the third and fourth groups were subjected to immersion-stress for 30 min before *in vitro* treatment. Induction of stress was practiced in these fish by placing them in water under an iron-mesh that prevented them from gulping air as reported earlier[9,39]. To ensure standardized experimental conditions, feeding was discontinued for 24 hours prior to the experiment, and care was taken to minimize the effect of handling stress on experimental fish.

2.3 Preparation of Hepatocyte Explants and in Vitro Conditions

After the experimental treatment, fish were anesthetized using 0.2% 2-phenoxyethanol (SRL, Mumbai, India) solution for a brief period of two minutes and blood was immediately drawn from the caudal vein. The fish were sacrificed immediately by spinal trans-section and the lower lobes of liver tissue were extracted and placed in Cortland saline (CS) containing 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 of pH 7.4[40,41]. The hepatic explants were cut into small pieces (5 mm^2) that provided enough surface area for the absorption. The explants were washed thrice in Cortland saline to remove the tissue debris before the treatment. Then, these explants were held for 10 min in Cortland saline for equilibration on an orbital shaker platform at room temperature (28ºC) as described earlier[42]. In the first dose-responsive experiment, graded concentrations of T_2 (10^{-9}, 10^{-8}, or 10^{-7} M) prepared in Cortland saline were added to the medium *in vitro* that contained the hepatic tissue explants and incubated for 15 min. In the second stress experiment, a selected dose of T_2 (10^{-7} M) was added *in vitro* to the explants obtained from both non-stressed and hypoxia-stressed fish. Similarly, control hepatocyte explants were maintained concurrently in incubation medium. After *in vitro* exposure of T_2 or vehicle, incubation was terminated by placing the hepatic explants at 4^0C and the explants were washed with ice-cold Cortland saline three times. These explants were then stored in 0.25M Sucrose-EDTA-Imidazole (SEI) buffer (pH 7.1) and kept at −80°C for further analysis.

2.4 Sample Preparation and Quantification of Ion-specific ATPases

Frozen hepatocyte explants were thawed on ice and a homogenate was prepared in SEI buffer (0.05 M; pH 7.1) using a glass homogenizer (Remi, Mumbai) with Teflon pestle giving three strokes, as described previously[7]. A portion of the homogenate was centrifuged at 700×g for 10 minutes at 4°C (Eppendorf 5430R, Germany) to obtain membrane fraction (H_0) for quantifying the activity of NKA, HKA and NNA. The remaining portion of the supernatant was centrifuged at 10,000 x g for 10 min at 4°C to separate mitochondrial fraction and post-mitochondrial (PMS) fraction. Vanadate-sensitive PMCA and baffilomycin-sensitivev HA-specific activities were quantified in PMS as described earlier[7,43]. The mitochondrial pellets thus obtained were centrifuged and repeatedly washed and suspended in fresh ice-cold 0.25 M SEI buffer (pH 7.1)[43]. Vanadate-sensitive mitochondrial Ca^{2+} ATPase- and baffilomycin-sensitive mitochondrial H^+ATPase-specific activities in the mitochondrial suspension were quantified. Modified Biuret assay was used to determine protein concentration in sample preparations utilizing bovine serum albumin as the standard[44].

2.4.1 NKA-Specific Activity

The ouabain-sensitive NKA-specific activity was quantified adopting the method of Peter *et al.* (2000) modified for microplateassay[34]. To optimize substrate accessibility, saponin (0.2 mg $protein^{-1}$) was added and samples in duplicates containing 1.0 µg protein were added to a 96-well microplate containing 100 mM NaCl, 30 mM imidazole (pH 7.4), 0.1 mM EDTA, and 5 mM $MgCl_2$. KCl 0.13 mM and ouabain 0.14 mM were used as the promoter and inhibitor, respectively, for NKA. The assay mixture was vortexed and then incubated at 37°C for 15 min. The reaction was initiated by the addition of 0.3 mM ATP and terminated with the addition of 8.6% TCA. The liberated inorganic phosphate (Pi) was measured against phosphate standard at 700 nm in Synergy HT Biotek Microplate Reader, USA. The change in absorbance was calculated and regression analysis was used to derive the rate of activity of NKA and expressed in micromoles of Pi liberated per hour per milligram of protein.

2.4.2 HKA-Specific Activity

SCH28080-sensitive HKA-specific activity was quantified based on the NKA microplateassay[34]. Saponin-treated samples in duplicates (1.0 µg protein) were added to a 96-well microplate containing the above reaction mixture, and the reaction was initiated by the addition of 0.3 mM ATP and incubated at 37°C for 15 minutes. About 0.1 mM SCH28080 (2-methyl-8-(phenylmethoxy)-imidazo [1,2-a] pyridine-3-acetonitrile) was used as inhibitor and 0.13 mM KCl was used as promoter. The inorganic phosphate content was measured in Synergy HT Biotek Microplate Reader after termination of reaction by adding 8.6% TCA and the change in absorbance at 700 nm between promoter and inhibitor assays was calculated using regression analysis. The rate of HKA activity was

expressed as micromoles of Pi liberated per hour per milligram of protein.

2.4.3 NNA-Specific Activity

An ouabain-sensitive NNA-specific activity was quantified based on the NKA microplate assay[34] but here 0.02 mM NH_4Cl, instead of 0.13 mM KCl, was used as promoter and 0.14 mM ouabain was used as inhibitor. Saponin (0.2 mg protein−1) treated samples in duplicates, containing 1.0 µg protein, were added to a 96-well microplate containing 100 mM, NaCl, 30 mM imidazole, 0.1 mM EDTA, and 5 mM $MgCl_2$. After vortexing, the assay mixture was incubated for 15 minutes at 37°C and the reaction was initiated by the addition of 0.3 mM ATP and terminated with addition of 8.6% TCA. The inorganic phosphate liberated was measured against phosphate standard at 700 nm in Synergy HT Biotek Microplate Reader. The change in absorbance between promoter and inhibitor assays was calculated. The rate of activity was derived by regression analysis and expressed in micromoles of Pi liberated per hour per milligram of protein.

2.4.4 PMCA and Mitochondrial Ca^{2+}ATPase-Specific Activity

The specific activity of PMCA and mitochondrial Ca^{2+}ATPase was measured in post-mitochondrial and mitochondrial fractions, respectively, as described for NKA-specific activity. Here, 10 mM $CaCl_2$ was used as the promoter. Sodium deoxycholate (1% DOC; Sigma-Aldrich)-treated samples in duplicates were added to a 96-well microplate containing 60 mM imidazole, 0.2 mM EGTA and 75 mMKCl. The reaction was initiated by the addition of 0.3 mM ATP and the inorganic phosphate liberated was measured against phosphate standard at 700 nm in Synergy HT Biotek Microplate Reader. The rate of activity was expressed in micromoles of Pi liberated per hour per milligram of protein[43].

2.4.5 H^+-Dependent vsHA- and Mitochondrial HA-Specific Activities

The baffilomycin-sensitive H^+-ATPase activity was measured as described for NKA microplate assay using an inhibitor 32nM or 64 nM baffilomycin A[7]. Samples in duplicates containing 1.0 µg protein were added to a 96-well microplate containing 100 mM NaCl, 30 mM imidazole (pH 7.4), 0.1 mM EDTA, 5 mM $MgCl_2$ and 0.14 mM ouabain. The assay mixture was incubated with 0.3 mM ATP for 15 min at 37^{0}C. The inorganic phosphate content released was determined as above and expressed in micromoles of Pi liberated per hour per milligram of protein.

2.5 Statistical Analysis

Data, after checking for normal distribution and variance homogeneity, were subjected to one-way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls test (SNK comparison test). Significance between the groups was analyzed with the help of GraphPad InStat-3; GraphPad Software, Inc., San Diego, CA, USA, and statistical differences between the means were accepted as significant if $p<0.05$.

3. Results

3.1 Dose-Responsive *In Vitro* Action of T_2 on Hepatocyte Explants of Non-Stressed Fish

In vitro exposure of varied doses (10^{-9},10^{-8}, 10^{-7}M) of T_2 for 15 min showed no response to the activities of NKA, HKA and NNA in hepatocyte explants collected from non-stressed fish (Figure 1A). Likewise, PMCA activity in hepatocyte explants remained unaffected after varied doses of T_2 exposure (Figure 2A). In contrast, vacuolar H^+-ATPase activity in hepatocyte explants showed substantial increase after T_2 treatment (Figure 2A). Likewise, mitochondrial Ca^{2+}-ATPase activity showed significant ($p<0.001$) decline after the treatment of low and medium doses of T_2, whereas the high dose did not affect its activity (Figure 2B). Similarly, high dose of T_2 treatment produced a significant increase ($p<0.05$) in mitochondrial H^+-ATPase activity in hepatocyte explants of non-stressed fish (Figure 2B).

3.2 *In Vitro* Action of T_2 in Hepatocyte Explants of Immersion-Stressed Fish

The activity of NKA increased significantly ($p<0.05$) in hepatocyte explants of hypoxia-stressed fish and its activity significantly increased ($p<0.05$) further by T_2 addition (Figure 1B). In contrast, the NNA activity in hepatocytes remained unaffected after T_2 addition in both non-stressed and hypoxia-stressed fish (Figure 1C).

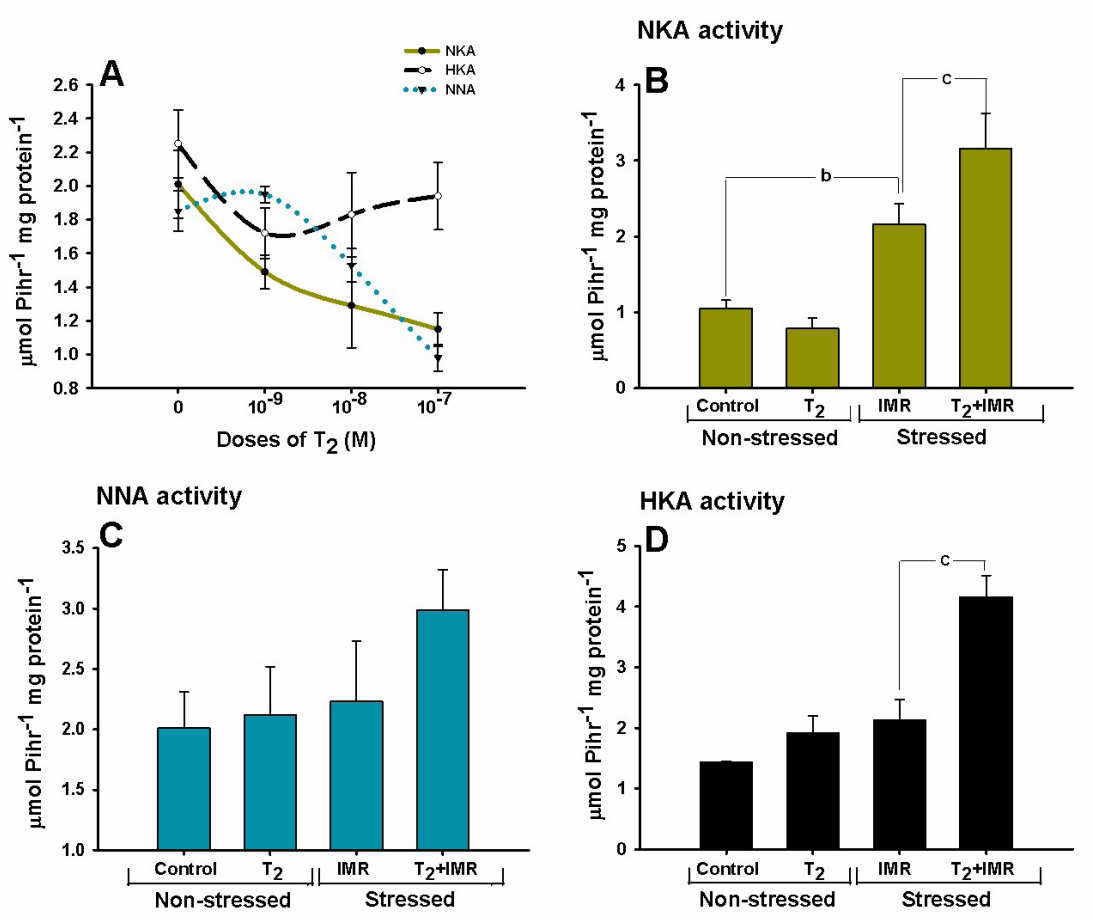

Figure 1. Dose-responsive *in vitro* action of T_2 (10^{-9}, 10^{-8} and 10^{-7} M) treatment for 15 min on the activities of Na^+/K^+-ATPase (NKA), H^+/K^+-ATPase (HKA) and Na^+/NH_4^+-ATPase(NNA) in the hepatocyte explants of *Anabas testudineus*. **(A)**. The activity pattern of Na^+/K^+-ATPase, H^+/K^+-ATPase and Na^+/NH_4^+-ATPase are presented in **B, C & D,** respectively. These activities were obtained from non-stressed and immersion-stressed fish after T_2 treatment (10^{-7} M). Each bar is mean ± SE for 6 fish. The significance levels of figures 1B-D are represented as "**b**" when compared between non-stressed control and immersion-stressed fish (IMR) and "**c**" represents significance between immersed control fish (IMR) and T_2-treated stressed (T_2+IMR) fish.

The activity of HKA activity that remained unresponsive to hypoxia but increased significantly (p<0.01) in hepatocytes of stressed fish following T_2 challenge (Figure 1D). In hepatocytes obtained from both non-stressed and stressed fish treatment of 10^{-7}M T_2 did not produce any alteration in PMCA activity (Figure 2C). Mitochondrial Ca^{2+}-ATPase activity showed a significant increase (p<0.01) in hypoxia-stressed hepatocytes, whereas T_2 treatment failed to produce any modification in its activity in both non-stressed and stressed fish (Figure 2D). A significant rise in (p<0.01) in vH^+-ATPase activity occurred after T_2 exposure in hepatocytes of both non-stressed and hypoxia-stressed fish (Figure 2E). Similarly, the activity of mitochondrial H^+-ATPase increased significantly (p<0.01) after T_2 treatment in the hepatocytes of non-stressed and hypoxia-stressed fish (Figure 2F).

4. Discussion

Thyroid Hormones (THs) play vital role in vertebrate physiology as key regulators of growth, development, metamorphosis, metabolism, osmoregulation and reproduction[34,45-47]. It is known that the principal THs, T_4 and T_3, as the active hormones are responsible for these activities. However, evidences have showed that T_2 also has important biological actions[12,32,48] either through

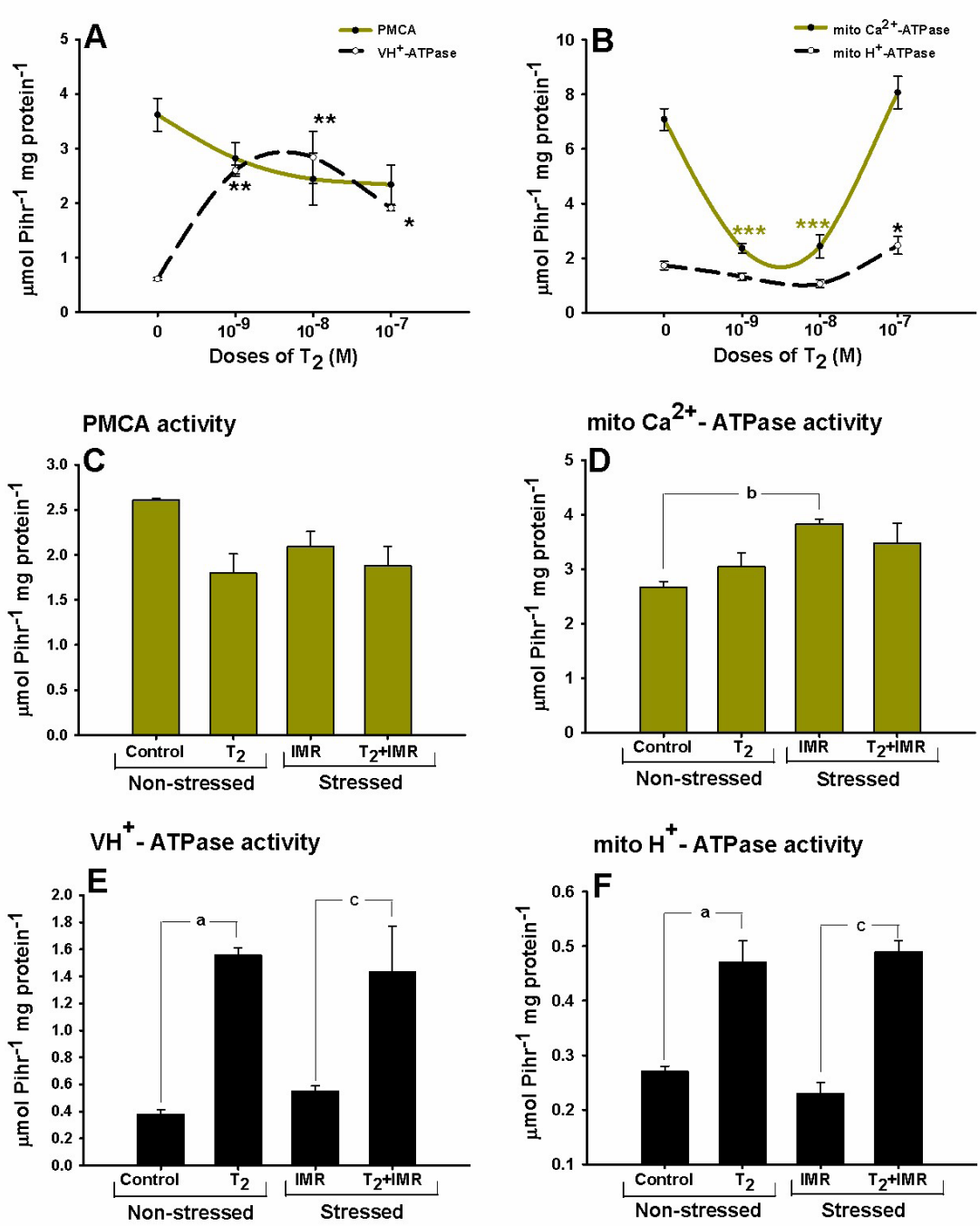

Figure 2. Dose-responsive *invitro* action of T_2 (10^{-9}, 10^{-8} and 10^{-7} M) treatment for 15 min on the activities of PMCA, VH^+-ATPase, mitochondrial Ca^{2+}-ATPase and mitochondrial H^+-ATPase in the hepatocyte explants of *Anabas testudineus.* **(A)**. The activity patterns of PMCA, VH^+-ATPase, mitochondrial Ca^{2+}-ATPase and H^+-ATPase are presented in **B, C, D, E & F,** respectively. These activities were obtained from non-stressed and immersion-stressed fish after T_2 treatment (10^{-7} M). Each bar is mean ± SE for 6 fish. In figure 2A, the significance levels are represented as "*" (p<0.05), "**" (p<0.01) and "***" (p<0.001) when compared with control fish. significance levels in figures 2B-F are represented as "**a**" when compared between control and T_2-treated fish, "**b**" when compared between non-stressed control and immersion-stressed fish (IMR) and "**c**" represents significance between immersed control fish (IMR) and T_2-treated stressed (T_2+IMR) fish.

non-genomic[3,12] or genomic actions[49,50]. These effects of T_2 are not limited to mammals but reported in fishes as well[51]. For example, mitochondria and bioenergetics mechanisms have been shown to be the main targets of the TH metabolite T_2[52,53].

Physiological response to a stressor involves the induction of neuro-endocrine cascade[54]. Fishes have evolved mechanisms to respond to various stress factors and rely on complex network of neuro-endocrine and physiologic responses to maintain their homeostasis[8]. The role of cortisol and adrenaline as major stress hormones during stress response have been well documented in fishes[55]. Similarly, THs have been shown to act as a stress modifiers due to their unique modulatory role in fish physiological processes and their ability to modify stress-induced response[5,6,8].

Fishes have evolved transepithelial transport mechanisms to achieve ionic and osmotic homeostasis[56]. Various transporter proteins that are located in the trans-epithelium move specific solutes across the membrane with the aid of transmembrane ATPases which utilize energy from the hydrolysis of ATP[57]. NKA, a key transporter that acts as the driving force for Na^+-coupled ion transport is abundant in active ion-transporting epithelia of fishes[58-60]. Similarly, Ca^{2+}, that controls many critical cellular responses, is also essential for the cell functioning. Plasma membrane Ca^{2+} ATPase (PMCA) has a high Ca^{2+} affinity and acts as the fine-tuner of cytosolic Ca^{2+}[61]. Likewise, PMCA maintains a gradient across the plasma membrane by regulating cellular Ca^{2+} extrusion[62].

NKA and PMCA are the membrane ATPases critical for the normal physiology and functioning of hepatocytes[63,64]. A unique Na^+-dependent NH_4^+-ATPase, contributes to basolateral ammonia transport[65] and it takes place via NKA[66]. Since NH_4^+ and K^+ have similar hydrated radii, NH_4^+ binds to K^+ binding sites on the NKA[9,67-69]. HKA, another P-type transporter, is responsible for ATP-dependent translocation of H^+ for K^+ across plasma membranes[70]. Gastric type HKA in the stomach and kidneys of mammals mediates acid secretion[71] and the presence of nongastric type HKA has been reported in the gills of Atlantic stingray, an elasmbranch[72] and in the osmoregulatory epithelia of our test fish[9].

Vacuolar H^+-ATPase or proton pump, an oligomeric protein responsible for electrogenic H^+ secretion, is another important ATPase involved in fish ion regulation[73-75]. Primarily, this transporter is responsible for acidification of intracellular compartments and proton transport across the plasma membrane[76]. Likewise, proton- or H^+-ATPases in mitochondria are responsible for the transport of protons by utilizing the energy through the hydrolysis of ATP[77]. Mitochondria, as an important Ca^{2+} reservoir, rely on Ca^{2+} ATPase of the mitochondrial membrane to transport Ca^{2+} from cytosol into mitochondrial matrix[77]. Further, mitochondrial Ca^{2+} regulation has a direct link with bioenergetics and cellular signalling[78].

As a multifunctional organ, liver plays an important role in fish physiology[79]. Liver appears to one of the most important target organs of THs due to its role in energy metabolism[80]. Further, hepatic tissue is also a major target for T_2 action like active THs, particularly due to its action on energy metabolism[52,53]. The roles played by T_4 and T_3 in physiological processes are well documented. In contrast, the role of T_2 in ion-dependent ATPases that drives cation gradients across membrane remains unknown. We, thus, analyzed the *in vitro* action of T_2 on the ion-transport dynamics that would affect the hepatocyte function. We found that T_2 exposure could affect the activity pattern of various ATPases in this important metabolically active organ. The pattern of dose-dependent *in vitro* action of T_2 on ATPase activity in non-stressed and immersion-stressed hepatocyte explants revealed a direct action of T_2 on hepatocyte cation transport.

It is known that THs affect several fundamental ion-transporter systems in many osmoregulatory tissues as evident in *in vitro* and *in vivo* studies[7,81,82]. *In vitro* studies on primary culture of rat hepatocytes and rat liver cell lines proved that NKA is a major target of THs and are able to produce both short-term and long-term modulation on this ion-transporter[83-85]. 3,5-T_2 exposure increased the hypothyroid liver mitochondrial F_0F_1-ATP synthase activity and expression, and this activation could be observed in both directions i.e., towards synthesis as well as hydrolysis of ATP[86]. In teleost fish liver, T_2 regulates the transcription of classical TH-regulated genes[49,51,87]. All these studies indicate that liver is one of the main targets of T_2 and this TH metabolite influences physiology of the liver.

In chick embryo hepatocytes, 3,5-T_2 decreased the activity of NKA through the activation of PKA, PKC and PI3K, which was comparable to that exerted by T_3[84]. NKA activity has been shown to be elevated by T_3; however, 3,5-T_2 decreased its activity in fish liver[88]. In liver, NKA plays a key role in the physiology and structure of hepatocytes, and Na^+ and K^+ gradients produced by NKA was essential

to regulate the transport of bile acid and water[89]. We found increased NKA and HKA activities after T_2 treatment in hepatocytes of immersion-stressed fish which indicated its role in the regulation of H^+, Na^+ and K^+ gradients for the normal functioning of liver. Furthermore, we also found that T_2 facilitates these gradients especially in stress-induced hepatocytes.

Vacuolar H^+-ATPase activity showed significant increase after T_2 exposure which was also evident in stressed hepatocytes. T_2 has an influence on cellular acidification and proton gradient *via* modulating the activity of H^+-ATPase was evident from the increased activity of this ATPases in both non-stressed and stressed fish livers followed by T_2 treatment. Mitochondrial Ca^{2+} concentration regulation is more critical in cellular function that ranges from ATP production to cell death[90]. In the present study, T_2 exposure did not influence Ca^{2+}-ATPase activity in either cytoplasmic or mitochondrial origin, which point to insensitivity of this divalent cation to T_2 in hepatocytes of both non-stressed and stressed fish. An increase in intracellular calcium level in mitochondria that activates several dehydrogenase enzymes, increase in the respiratory rate, H^+ extrusion, and ATP production; however, prolonged increase leads to cell death by apoptosis[90]. T_2 has been shown to increase mitochondrial activity and respiration through an increase in mitochondrial Ca^{2+} uptake[91]. In pituitary GH3 cells, T_2 rapidly affects intracellular Ca^{2+} via plasma membrane and mitochondrial pathways[92].

A role for proton-dependent ATPase, particularly in maintaining mitochondrial H^+ concentration, which could affect the mitochondrial respiratory function and ATP production, could be seen in hepatocytes of both non-stressed and stressed conditions. Increased activity of this transporter in hypoxia stress may indicate the increased need of energy metabolism particularly during stress. Mitochondrial proton gradient was rapidly affected by T_2 through modulating the activity of H+ ATPase. We found a rise in this ATPase activity in non-stressed and stressed fish. Previous studies showed that mitochondria and bioenergetics mechanisms become the central target of T_2. This was supported by our *in vitro* study, and further points to a rapid action of T_2 on both cellular and mitochondrial proton gradients they would directly affect mitochondrial respiratory function.

Overall, the present data indicate that T_2 can directly induce high proton and potassium gradients in the hepatocytes of both non-stressed and immersion-stressed fish. Furthermore, this regulatory action of T_2 suggests a role for T_2 in mitochondrial ion transport activity particularly during immersion-stress, probably for meeting out the energetics associated with a physiological response of this metabolite of TH in a way similar to the active THs.

5. Acknowledgments

We gratefully acknowledge the financial support received from the Higher Education Department, Government of Kerala, for establishing the iCEIB at the University of Kerala. Thanks are due to UGC-SAP-DRS II facility in the Department of Zoology of the University of Kerala. SA acknowledges the Research Fellowship of Backward Commission of Government of Kerala. VSP acknowledges the Emeritus Scientistship of Kerala State Council of Science, Technology and Environment (KSCSTE) and Visiting Scientistship of iCEIB project. MCSP wishes to acknowledge the support of UGC-BSR Faculty Fellowship.

6. References

1. Lazar MA. Thyroid hormone action: a binding contract. J Clin Invest. 2003; 112:497-499. https://doi: 10.1172/JCI19479. PMid:12925689 PMCid: PMC171396.
2. Brent GA. Mechanisms of thyroid hormone action. J Clin Invest. 2012; 122:3035-3043. https://doi.org/10.1172/JCI60047. PMid:22945636 PMCid: PMC3433956.
3. Davis PJ, Goglia F, Leonard JL. Nongenomic actions of thyroid hormone. Nat Rev Endocrinol. 2016; 12:111-121. https://doi.org/10.1038/nrendo.2015.205. PMid:26668118.
4. Hammes SR, Davis PJ. Overlapping nongenomic and genomic actions of thyroid hormone and steroids. Best Pract Res Clin Endocrinol Metab. 2015; 29(4):581-593. https://doi.org/10.1016/j.beem.2015.04.001. PMid:26303085 PMCid:PMC4976293.
5. Peter VS, Peter MCS. The interruption of thyroid and interrenal and the inter-hormonal interference in fish: does it promote physiologic adaptation or maladaptation? Gen Comp Endocrinol. 2011; 174(3):249-258. https://doi.org/10.1016/j.ygcen.2011.09.018. PMid:22001502.
6. Peter MCS. Understanding the adaptive response in vertebrates: The phenomenon of ease and ease response during post-stress acclimation. Gen Comp Endocrinol. 2013; 181:59-64. https://doi.org/10.1016/j.ygcen.2012.09.016. PMid: 23063668.

7. Peter MCS, Lock RAC, Wendelaar Bonga SE. Evidence for an osmoregulatory role of thyroid hormones in the freshwater *Mozambique tilapia*, *Oreochromis mossambicus*. Gen Comp Endocrinol. 2000; 120:157-167. https://doi.org/10.1006/gcen.2000.7542. PMid:11078627.
8. Peter MCS. The role of thyroid hormones in stress response of fish. Gen Comp Endocrinol. 2011; 172(2):198-210. https://doi.org/10.1016/j.ygcen.2011.02.023. PMid:21362420.
9. Simi S, Peter VS, Peter MCS. Zymosan-induced immune challenge modifies the stress response of hypoxic air-breathing fish (*Anabas testudineus* Bloch): Evidence for reversed patterns of cortisol and thyroid hormone interaction, differential ion transporter functions and non-specific immune response. Gen Comp Endocrinol. 2017; 251:94-108. https://doi.org/10.1016/j.ygcen.2016.11.009. PMid:27871800.
10. Incerpi S, Davis PJ, Pedersen JZ, Lanni A. Nongenomicactions of Thyroid Hormones. In: Belfiore A., LeRoith D. (eds) Principles of Endocrinology and Hormone Action. Endocrinology. Springer, Cham; 2016. https://doi.org/10.1007/978-3-319-27318-1_32-1.
11. Orozco A, Navarrete-Ramírez P, Olvera A, García-G C. 3,5-Diiodothyronine (T2) is on a role. A new hormone in search of recognition. Gen Comp Endocrinol. 2014; 203:174-180. https://doi.org/10.1016/j.ygcen.2014.02.014. PMid: 24602963.
12. Goglia F. Biological effects of 3, 5-diiodothyronine (T2). Biochem (Mosc). 2005; 70(2):164-172. https://doi.org/10.1007/s10541-005-0097-0. PMid:15807655.
13. Sinha RA, Singh BK, Yen PM. Direct effects of thyroid hormones on hepatic lipid metabolism. Nat Rev Endocrinol. 2018; 14(5):259-269. https://doi.org/10.1038/nrendo.2018.10. PMid:29472712 PMCid:PMC6013028.
14. Mullur R, Liu YY, Brent GA. Thyroid hormone regulation of metabolism. Physiol Rev. 2014; 94:355-382. https://doi.org/10.1152/physrev.00030.2013. PMid:24692351 PMCid:PMC4044302.
15. Awais D, Shao Y, Ismail-Beigi F. Thyroid hormone regulation of myocardial Na/K-ATPase gene expression. J Mol Cell Cardiol. 2000; 32:1969-1980. https://doi.org/10.1006/jmcc.2000.1229. PMid:11040102.
16. Lei J, Wendt CH, Fan D, *et al*. Developmental acquisition of T3-sensitive Na-K-ATPase stimulation by rat alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2007; 292:L6-L14. https://doi.org/10.1152/ajplung.00078.2006. PMid:16951134.
17. Lei J, Mariash CN, Bhargava M, *et al*. T3 increases Na-K-ATPase activity via a MAPK/ERK1/2-dependent pathway in rat adult alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2008; 294:L749-L754. https://doi.org/10.1152/ajplung.00335.2007. PMid:18223161.
18. Lei J, Ingbar DH. Src kinase integrates PI3K/Akt and MAPK/ERK1/2 pathways in T3-induced Na-K-ATPase activity in adult rat alveolar cells. Am J Physiol Lung Cell Mol Physiol. 2011; 301:L765-L771. https://doi.org/10.1152/ajplung.00151.2011. PMid:21840963.
19. Khanam S. Impact of thyroid on liver metabolism. Endocrinol Res Metab. 2017; 1(2):6.
20. Cicatiello AG, Di Girolamo D, Dentice M. Metabolic effects of the intracellular regulation of thyroid hormone: Old players, new concepts. Front Endocrinol. 2018; 9:474. https://doi.org/10.3389/fendo.2018.00474. PMid: 30254607. PMCid: PMC6141630.
21. Silva JE. Thermogenic mechanisms and their hormonal regulation. Physiol Rev. 2006; 86:435-464. https://doi.org/10.1152/physrev.00009.2005. PMid:16601266.
22. Haber RS, Loeb JN. Stimulation of potassium efflux in rat liver by a low dose of thyroid hormone: evidence for enhanced cation permeability in the absence of Na,K-ATPase induction. Endocrinology. 1986; 118:207-211. https://doi.org/10.1210/endo-118-1-207. PMid:2416552.
23. Dhanasiri AKS, Fernandes JMO, Kiron V. Liver Transcriptome changes in zebrafish during acclimation to transport-associated stress. PLoS One. 2013; 8(6):e65028. https://doi.org/10.1371/journal.pone.0065028. PMid:23762281 PMCid:PMC3677916.
24. Mommsen TP, Vijayan MM, Moon TW. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish. 1999; 9:211-268. https://doi.org/10.1023/A:1008924418720.
25. Hernández-Pérez J, Naderi F, Chivite M, *et al*. Influence of stress on liver circadian physiology. A study in rainbow trout, Oncorhynchus mykiss, as fish model. Front Physiol. 2019; 10. https://doi.org/10.3389/fphys.2019.00611. PMid:31164837 PMCid:PMC6536609.
26. Chi HC, Tsai CY, Tsai MM, *et al*. Molecular functions and clinical impact of thyroid hormone-triggered autophagy in liver-related diseases. J Biomed Sci. 2019; 26:24. https://doi.org/10.1186/s12929-019-0517-x. PMid:30849993 PMCid: PMC6407245.
27. Malik R, Hodgson H. The relationship between the thyroid gland and the liver. QJM. 2002; 95(9):559-569. https://doi.org/10.1093/qjmed/95.9.559. PMid:12205333.
28. Moreno M, de Lange P, Lombardi A, *et al*. Metabolic effects of thyroid hormone derivatives. Thyroid. 2008; 18(2):239-253. https://doi.org/10.1089/thy.2007.0248. PMid:18279024.
29. Lanni A, Moreno M, Lombardi A, *et al*. Control of energy metabolism by iodothyronines. J Endocrinol Invest. 2001; 24:897-913. https://doi.org/10.1007/BF03343949. PMid:11817716.

30. Silvestri E, Lombardi A, Coppola M, *et al*. Differential effects of 3,5-diiodo-L-thyronine and 3,5,3'-triiodo-L-thyronine on mitochondrial respiratory pathways in liver from hypothyroid rats. Cell Physiol Biochem. 2018; 47:2471-2483. https://doi.org/10.1159/000491620. PMid:29990992.
31. Coppola M, Glinni D, Moreno M *et al*. Thyroid hormone analogues and derivatives: Actions in fatty liver. World J Hepatol. 2014; 6(3):114-129. https://doi.org/10.4254/wjh.v6.i3.114. PMid:24672641 PMCid:PMC3959112.
32. Mangiullo R, Gnoni A, Damiano F, *et al*. 3,5-diiodo-L-thyronine upregulates rat-liver mitochondrial FoF1-ATP synthase by GA-binding protein/nuclear respiratory factor-2. Biochim Biophys Acta (BBA) - Bioenergetics. 2010; 1797(2):233-240. https://doi.org/10.1016/j.bbabio.2009.10.009. PMid:19878644.
33. Grasselli E, Canesi L, Voci A, *et al*. Effects of 3,5-diiodo-L-thyronine administration on the liver of high fat diet-fed rats. Exp Biol Med. (Maywood). 2008; 233(5):549-557. https://doi.org/10.3181/0710-RM-266. PMid:18375830.
34. Peter MCS, Leji J, Peter VS. 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. 2011; 171:225-231. https://doi.org/10.1016/j.ygcen.2011.01.013. PMid: 21295572.
35. Peter MCS, Leji J, Rejitha V, *et al*. Physiological responses of African catfish (*Clarias gariepinus*) to water-borne ferric iron: Effects on thyroidal, metabolic and hydromineral regulations. J Endocrinol Reprod. 2008; 12:24-30.
36. Peter MCS, Peter VS. Action of thyroid inhibitor propyl thiouracil on thyroid and interrenal axes in freshwater tilapia, *Oreochromis mossambicus* Peters. J Endocrinol Reprod. 2009; 13:37-44. https://doi.org/10.18519/JER/2009/V13/77712.
37. 37. Peter VS, Joshua EK, Bonga WSE, Peter MCS. Metabolic and thyroidal response in air-breathing perch (*Anabas testudineus*) to water-borne kerosene. Gen Comp Endocrinol. 2007; 152:198-205. https://doi.org/10.1016/j.ygcen.2007.05.015. PMid:17574248.
38. Padron AS, Neto RAL, Pantaleão TU, *et al*. Administration of 3,5-diiodothyronine (3,5-T2) causes central hypothyroidism and stimulates thyroid-sensitive tissues. J Endocrinol. 2014; 221(3):415-427. https://doi.org/10.1530/JOE-13-0502 PMid:24692290 PMCid:PMC4045230.
39. Gayathry R, Peter VS, Peter MCS. Nitric oxide drives mitochondrial energetics in heart and liver mitochondria of hypoxia-stressed climbing perch (*Anabas testudineus* Bloch). J Endocrinol Reprod. 2018; 22(1):1-14.
40. Babitha GS, Peter MCS. 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. 2010; 168(1):14-21. https://doi.org/10.1016/j.ygcen.2010.03.021. PMid: 20347823.
41. Rejitha V, Peter MCS. Adrenaline and triiodothyronine modify the iron handling in the freshwater air-breathing fish *Anabas testudineus* Bloch: Role of ferric reductase in iron acquisition. Gen Comp Endocrinol. 2013; 181:130-138. https://doi.org/10.1016/j.ygcen.2012.11.008. PMid: 23168087.
42. Sheetal G, Valsa SP, Peter MCS. In vitro action of matrix metalloproteinases 2 and 9 Inhibitors on Na^+ /K^+ -ATPase, H^+ /K^+ -ATPase and PMCA activities in the osmoregulatory epithelia of climbing perch (*Anabas testudineus* Bloch). 2020; 22. https://doi.org/10.18311/jer/2018/24711.
43. Peter MCS, Mini VS, Bindulekha DS, Peter VS. Short-term in situ effects of prolactin and insulin on ion transport in liver and intestine of freshwater climbing perch (*Anabas testudineus* Bloch). J Endocrinol Reprod. 2014; 18(1):47-58. https://doi.org/10.18519/JER/2014/V18/71719.
44. Alexander JB, Ingram GA. A comparison of five of the methods commonly used to measure protein concentration in fish sera. J Fish Biol. 1980; 16:I5-122. https://doi.org/10.1111/j.1095-8649.1980.tb03691.x.
45. Janz DM. Endocrine System. In: The Laboratory Fish. GK Ostrander (ed.), Chap. 25. Academic Press, San Diego; 2000. https://doi.org/10.1016/B978-012529650-2/50016-0.
46. Power DM, Llewellyn L, Faustino M, *et al*. Thyroid hormones in growth and development of fish. Comp Biochem Physiol. C. 2001; 130:447-459. https://doi.org/10.1016/S1532-0456(01)00271-X.
47. Blanton ML, Specker JL. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. Crit Rev Toxicol. 2007; 37:97-115. https://doi.org/10.1080/10408440601123529. PMid:17364706.
48. Giudetti AM, Leo M, Geelen MJH, Gnoni GV. Short-term stimulation of lipogenesis by 3,5-L-diiodothyronine in cultured rat hepatocytes. Endocrinology. 2005; 146(9):3959-3966. https://doi.org/10.1210/en.2005-0345. PMid:15932927.
49. García-G C, López-Bojorquez L, Nuñez J, *et al*. 3,5-diiodothyronine in vivo maintains euthyroidal expression of type 2 iodothyroninedeiodinase, growth hormone, and thyroid hormone receptor beta1 in the killifish. Am J Physiol Regul Integr Comp Physiol. 2007; 293(2):R877-883. https://doi.org/10.1152/ajpregu.00101.2007. PMid:17522123.
50. Mendoza A, Navarrete-Ramírez P, Hernández-Puga G, *et al*. 3,5-T2 is an alternative ligand for the thyroid hormone receptor β1. Endocrinology. 2013; 154:2948-2958. https://doi.org/10.1210/en.2013-1030. PMid:23736295.

51. Navarrete-Ramírez P, Luna M, Valverde-R C, Orozco A. 3,5-di-iodothyronine stimulates tilapia growth through an alternate isoform of thyroid hormone receptor β1. J Mol Endocrinol. 2014; 52(1):1-9. https://doi.org/10.1530/JME-13-0145. PMid:24031088.
52. Senese R, de Lange P, Petito G, *et al*. 3,5-diiodothyronine: A novel thyroid hormone metabolite and potent modulator of energy metabolism. Front Endocrinol. 2018; 9:427. https://doi.org/10.3389/fendo.2018.00427. PMid:30090086 PMCid:PMC6068267.
53. Goglia F. The effects of 3,5-diiodothyronine on energy balance. Front Physiol. 2015; 5:528. https://doi:10.3389/fphys.2014.00528. PMid:25628573 PMCid:PMC4292545.
54. Schreck CB, Tort L. The concept of stress in fish. Fish Physiol. 2016; 35:1-34. https://doi.org/10.1016/B978-0-12-802728-8.00001-1.
55. Wendelaar Bonga SE. The stress response in fish. Physiol Rev. 1997; 77:591-625. https://doi.org/10.1152/physrev.1997.77.3.591. PMid: 9234959.
56. Guh YJ, Lin CH, Hwang PP. Osmoregulation in zebrafish: ion transport mechanisms and functional regulation. EXCLI Journal. 2015; 14:627-659. https://doi.org/10.17179/excli2015-246.
57. Griffith MB. Toxicological perspective on the osmoregulation and ionoregulation physiology of major ions by freshwater animals: Teleost fish, crustacea, aquatic insects, and Mollusca. Environ Toxicol Chem. 2017; 36(3):576-600. https://doi.org/10.1002/etc.3676. PMid:27808448 PMCid:PMC6114146.
58. Wong MK, Pipil S, Ozaki H, *et al*. Flexible selection of diversified Na(+)/K(+)-ATPase α-subunit isoforms for osmoregulation in teleosts. Zool Lett. 2016; 2(1):15. https://doi.org/10.1186/s40851-016-0050-7. PMid: 27489726 PMCid:PMC4971688.
59. Hiroi J, McCormick SD. New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. Respir Physiol Neurobiol. 2012; 184(3):257-68. https://doi.org/10.1016/j.resp.2012.07.019. PMid:22850177.
60. Peter MCS, Gayathry R. Nitric oxide synthase (NOS) inhibitor L-NAME activates inducible NOS/NO system and drives multidimensional regulation of Na^+ /K^+ -ATPase in ionocyte epithelia of immersion-stressed air-breathing fish (*Anabas testudineus* Bloch). J Exp Zool A: Ecol Integr Physiol. 2021; 335:396-416. https://doi.org/10.1002/jez.2454. PMid:33734617.
61. Brini M, Carafoli E. The plasma membrane Ca^{2+} ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. Cold Spring Harb Perspect Biol. 2011; 3(2):a004168. https://doi.org/10.1101/cshperspect.a004168. PMid:21421919 PMCid:PMC3039526.
62. Shull GE. Gene knockout studies of Ca2+-transporting ATPases. Eur J Biochem. 2000; 267:5284-5290. https://doi.org/10.1046/j.1432-1327.2000.01568.x. PMid:10951186.
63. Delgado-Coello B, Mas-Oliva J. Relevance of the plasma membrane calcium-ATPase in the homeostasis of calcium in the fetal liver. Organogenesis. 2014; 10(4):333-339. https://doi.org/10.1080/15476278.2015.1011918. PMid:25836032 PMCid:PMC4594366.
64. Benkoel L, Dodero F, Hardwigsen J, *et al*. Effect of ischemia-reperfusion on Na^+, K^+-ATPase expression in human liver tissue allograft: image analysis by confocal laser scanning microscopy. Dig Dis Sci. 2004; 49(9):1387-1393. https://doi.org/10.1023/B:DDAS.0000042235.72622.16. PMid:15481308.
65. Salama A, Morgan IJ, Wood CM. The linkage between Na^+ uptake and ammonia excretion in rainbow trout: Kinetic analysis, the effects of $(NH_4)_2SO_4$ and NH_4HCO_3 infusion and the influence of gill boundary layer pH. J Exp Biol. 1999; 202:697-709. PMID: 10021323. https://doi.org/10.1242/jeb.202.6.697.
66. Randall DJ, Wilson JM, Peng KW, Kok TWK, Kuah SSL, Chew SF, Lam TJ, Ip YK. The mudskipper, *Periophthalmodon schlosseri*, actively transports NH^{4+} against a concentration gradient. Am J Physiol. 1999; 277(6):R1562-R1567. https://doi.org/10.1152/ajpregu.1999.277.6.R1562. PMid:10600900.
67. Knepper MA, Packer R, Good DW. Ammonium transport in the kidney. Physiol Rev. 1989; 69:179 -249. https://doi.org/10.1152/physrev.1989.69.1.179. PMid:2643123.
68. Randall DJ, Ip YK, Chew SF, Wilson JM. Air breathing and ammonia excretion in the giant mudskipper *Periophthalmodon schlosseri*. Physiol Biochem Zool. 2004; 77:783-788. https://doi.org/10.1086/423745. PMid:15547796.
69. Weihrauch D, Wilkie MP, Walsh PJ. Ammonia and urea transporters in gills of fish and aquatic crustaceans. J Exp Biol. 2009; 212(11):1716-1730. https://doi:10.1242/jeb.024851. PMid:19448081.
70. Song I, Yamada T, Trent JM. Mapping of the gene encoding the α-subunit of the human H^+, K^+-ATPase to chromosome 19q13.1 by fluorescent in situ hybridization. Genomics. 1992; 14:547-548. https://doi.org/10.1016/S0888-7543(05)80266-6.
71. Faller L, Jackson R, Malinowska D, *et al*. Mechanistic aspects of gastric (H^{++} K^+)-ATPase. Ann N Y Acad Sci. 1982; 402:146-63. https://doi.org/10.1111/j.1749-6632.1982.tb25738.x. PMid:6301328.
72. Choe KP, Verlander JW, Wingo CS, Evans DH. A putative H^+-K^+-ATPase in the Atlantic stingray, *Dasyatis sabina*: primary sequence and expression in gills. Am J Physiol

Regul Integr Comp Physiol. 2004; 287:R981-R991. https://doi.org/10.1152/ajpregu.00513.2003. PMid:15217793.

73. Guffey S, Esbaugh A, Grosell M. Regulation of apical H^+-ATPase activity and intestinal HCO3− secretion in marine fish osmoregulation. Am J Physiol Regul Integr Comp Physiol. 2011; 301(6):R1682-R1691. https://doi.org/10.1152/ajpregu.00059.2011. PMid:21865541.
74. Piermarini PM, Evans DH. Immunochemical analysis of the vacuolar proton-ATPase B-subunit in the gills of a euryhaline stingray (*Dasyatis sabina*): Effects of salinity and relation to $Na(^+)/K(^+)$-ATPase. J Exp Biol. 2001; 204(Pt 19):3251-3259. PMID: 11606599. https://doi.org/10.1242/jeb.204.19.3251.
75. Perry S, Fryer J. Proton pumps in the fish gill and kidney. Fish Physiol Biochem. 1997; 17:363-369. https://doi.org/10.1023/A:1007746217349.
76. Cipriano DJ, Wang Y, Bond S, *et al*. Structure and regulation of the vacuolar ATPases. Biochim Biophys Acta. 2008; 1777(7-8):599-604. https://doi.org/10.1016/j.bbabio.2008.03.013. PMid: 18423392 PMCid:PMC2467516.
77. Baoping W. Analysis of ATPase activity of mitochondria intima in exercise-induced fatigue. Chem Eng Trans. 2018; 64:199-204. https://doi.org/10.3303/CET1864034.
78. Pathak T, Trebak M. Mitochondrial Ca^{2+} signaling. Pharmacol Ther. 2018; 192:112-123. https://doi.org/10.1016/j.pharmthera.2018.07.001. PMid:30036491 PMCid:PMC6263837.
79. Bruslé J, Anadon GG. The Structure and Function of Fish Liver. In: Munshi J S D. & Dutta HM. (eds.). Fish Morphology. North-Holland, Science Publishers; 1996. p. 77-93. https://doi.org/10.1201/9780203755990-6.
80. Chi H-C, Tsai C-Y, Tsai M-M, *et al*. Molecular functions and clinical impact of thyroid hormone-triggered autophagy in liver-related diseases. J Biomed Sci, 2019; 26(1). https://doi:10.1186/s12929-019-0517-x. PMid:30849993 PMCid:PMC6407245.
81. Klaren PHM, Geven EJW, Flik G. The Involvement of the Thyroid Gland in Teleost Osmoregulation. In: Baldisserotto B, Mancera JM, Kapoor BG. (Eds.) Fish Osmoregulation, Enfield, NH: Science Publishers; 2007. Chapter 2, p. 35-65. https://doi.org/10.1201/b10994-3.
82. McCormick SD. Endocrine control of osmoregulation in teleost fish. Am Zool. 2001; 41(4):781-794. https://doi.org/10.1093/icb/41.4.781.
83. Cheng S-Y, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocr Rev. 2010; 31(2):139-170. https://doi:10.1210/er.2009-0007. PMid:20051527 PMCid: PMC2852208.
84. Scapin S, Leoni S, Spagnuolo S, *et al*. Short-term effects of thyroid hormones on Na^+-K^+-ATPase activity of chick embryo hepatocytes during development: focus on signal transduction. Am J Physiol Cell Physiol. 2009; 296(1):C4-C12. https://doi.org/10.1152/ajpcell.90604.2007. PMid: 18829895.
85. Incerpi S. Actions of thyroid hormone on ion transport. Curr Opin Endocrinol Diabetes. 2002; 9(5):381-386. https://doi.org/10.1097/00060793-200210000-00004.
86. Cavallo A, Gnoni A, Conte E, *et al*. 3,5-diiodo-L-thyronine increases FoF1-ATP synthase activity and cardiolipin level in liver mitochondria of hypothyroid rats. J Bioenerg Biomembr. 2011; 43(4):349-57. https://doi.org/10.1007/s10863-011-9366-3. PMid:21739248.
87. Olvera A, Martyniuk CJ, Buisine N, *et al*. Differential transcriptome regulation by 3,5-T2 and 3′,3,5-T3 in brain and liver uncovers novel roles for thyroid hormones in tilapia. Sci Rep. 2017; 7:15043. https://doi.org/10.1038/s41598-017-14913-9. PMid:29118400 PMCid:PMC5678081.
88. Shameena B, Sheelu V, Leena S, Oommen OV. 3,5,3'-Triiodothyronine (T3) and 3',5'-diiodothyrone (T2) have short-term effects on lipid metabolism in a teleost *Anabas testudineus* (Bloch): Evidence from enzyme activities. Endocr Res. 2000; 26:431-44. https://doi.org/10.3109/07435800009066178. PMid: 11019906.
89. Benkoel L, Dodero F, Hardwigsen J, *et al*. Effect of ischemia-reperfusion on Na+,K+-ATPase expression in human liver tissue allograft: Image analysis by confocal laser scanning microscopy. Dig Dis Sci. 2004; 49(9):1387-1393. https://doi.org/10.1023/B:DDAS.0000042235.72622.16. PMid:15481308.
90. Giorgi C, Romagnoli A, Pinton P, Rizzuto R. Ca2+ signaling, mitochondria and cell death. Curr Mol Med. 2008; 8(2):119-130. https://doi.org/10.2174/156652408783769571. PMid:18336292.
91. Hummerich H, Soboll S. Rapid stimulation of calcium uptake into rat liver by L-tri-iodothyronine. Biochem J. 1989; 258(2):363-367. https://doi.org/10.1042/bj2580363. PMid:2705987 PMCid:PMC1138370.
92. Viscovo AD, Secondo A, Esposito A, *et al*. Intracellular and plasma membrane-initiated pathways involved in the $[Ca^{2+}]i$ elevations induced by iodothyronines (T3 and T2) in pituitary GH3 cells. Am J Physiol Endocrinol Metab. 2012; 302(11):E1419-4130. https://doi.org/10.1152/ajpendo.00389.2011. PMid:22414808.