Effect of different levels of salinity on immunolocalization of Na^+-K^+ ATPase and Aquaporin 3 in kidney of common carp *Cyprinus carpio*

Salati, A. P.[1*]; Ferrando, S.[2]; Movahedinia, A.[3]; Gambardella, C.[2] and Gallus, L.[2]

[1]Department of Fisheries, Faculty of Marine Natural Resources, Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran; [2]Department of Earth, Environment and Life Sciences, School of Sciences, University of Genova, Genova, Italy; [3]Department of Marine Biology, Faculty of Marine Science and Oceanography, Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran

[*]***Correspondence:*** *A. P. Salati, Department of Fisheries, Faculty of Marine Natural Resources, Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran. E-mail: salatia@gmail.com*

(Received 5 Dec 2012; revised version 4 Sept 2013; accepted 7 Sept 2013)

Summary

Cyprinus carpio is a stenohaline species but can tolerate some ranges of changes in environmental salinities, so histomorphological methods and Na^+-K^+ ATPase and Aquaporin 3 immunohistochemistry were performed on common carp kidney as an osmoregulatory organ in experimental groups and control in order to investigate their possible roles during salinity challenge. Five groups of fish (n=25) with salinities ranging from 3, 6, 9 and 12 g/l marine salt and a control group (tap water) were used. The experiment was continued for two weeks. Kidney samples from control and experimental groups were fixed in 4% paraformaldehyde and were embedded in paraffin. The Na^+-K^+ ATPase and Aquaporin 3 intensity of the immunostaining and the renal tubules dilation had direct relation with environmental salinities, and showed the involvement of these proteins in physiological responses to environmental salinity. Furthermore, in the salinities 9 and 12 g/l epithelium of the renal tubules, profound histomorphological alteration was present.

Key words: Na^+-K^+ ATPase, Aquaporin 3, Salinity, Kidney, Common carp

Introduction

Common carp (*Cyprinus carpio*), a stenohaline freshwater fish (FW), is native to Asia and Eastern Europe but now has spread worldwide inhabiting various environments. It is one of the earlier species used in aquaculture and nowadays, one of the most important reared fish in the world. Common carp is a good candidate for culture in brackish water since it can tolerate a wider range of salinities in comparison to most of stenohaline FW fish, up to 15 g/l (Schwartz, 1964). For preservation of the homeostasis of its extracellular fluid (ECF), homeostasis might cause a further energy expenditure which reduces body growth, so a better knowledge on osmotic and ionic regulation in this new aquaculture medium could be useful to know whether the rearing in BW medium is economically favorable. Body fluid in the FW teleost is hyper-osmotic to the external environment, continuously gain water by osmosis and lose ions by diffusion across their permeable body surfaces, principally the gills. In such environments, fish produce large volumes of extremely dilute urine (Marshall and Grosell, 2005). A great deal of information has been gathered concerning the osmoregulatory physiology of the euryhaline fish (Karnaky, 1998; Evans, 2008) but there is not enough information about the stenohaline fish such as common carp. In fact, physiological mechanisms involved in response to changes in environmental salinity are less understood in stenohaline fish compared to euryhaline fish.

In general, adaptation to chronic increase of salinity requires differentiation of transport epithelia and synthesis of new transport proteins (McCormick, 2001). Numerous markers may be used for monitoring osmoregulatory functions in different fish organs. Na^+/K^+-ATPase (NKA), involved in ionic balance (Richards *et al*., 2003), and Aquaporin 3 (AQP3), implicated in the regulation of water fluxes (Verkman and Mitra, 2000) were examined. NKA plays a central role in ion transport in fish osmoregulatory organs (Marshall, 1995; McCormick, 1995). Immuno-cytochemical studies demonstrated that in euryhaline teleosts NKA is located in epithelia of renal tubules (Ura *et al*., 1996). Aquaporin 3 (AQP3) is a member of an extended family of water and small solute channels known as aquaporins or major intrinsic proteins. In the euryhaline fish tilapia, AQP3 mRNA was detected in major osmoregulatory organs including kidney using RT-PCR, suggesting a role for AQP3 in water/fluid re-absorption (Watanabe *et al*., 2005).

The aim of this work was to investigate the changes of kidney tissue in common carp experimentally exposed for two weeks to different salinities, in order to establish the renal mechanisms countering changes in environmental salinity.

Materials and Methods

Experimental design

Thirty healthy *C. carpio* (mean weight 68.43 ± 12.81 g and mean total length 14.16 ± 1.72 cm) were stocked in three tanks filled with 100 l of dechlorinated tap water. After one week acclimation, fish were randomly divided into six groups (N=5 fish at each experimental salinity: 3, 6, 9, 12 and 15 g/l of marine salt, and one control group reared in dechlorinated tap water). Salinities were made by adding the proper amount of marine salt (Seachem, USA) to dechlorinated tap water.

Salinity in the tanks was raised step by step, 3 g/l daily until the final concentration for each group. Fish were kept 14 days at the final salinity in each group. During the experiments, physicochemical parameters of water (temperature, pH, dissolved oxygen and nitrite) were monitored, and the aquaria were maintained at 20 ± 1°C under natural photoperiod conditions. Fish were fed by a commercial diet, once per day.

Histomorphology

After this period, fish were stunned by a sharp blow on the dorsocephalic region. Trunk part of kidney were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer saline pH = 7.4 (PBS). Subsequently samples were PBS rinsed, dehydrated in ascending series of alcohol, and embedded in paraplast (Bio-Optica, Italy). Transversal and longitudinal dewaxed serial sections (5 µm) were stained by haematoxylin and eosin (Bio-Optica, Italy) and by Alcian-PAS method (Bio-Optica, Italy).

Immunohistochemistry

For immunohistochemical investigation dewaxed serial sections were pre-incubated with normal goat serum (1:50), incubated overnight at room temperature with a rabbit polyclonal anti-Aquaporin 3 antiserum (1:100, Sigma, USA) as described previously by Lignot *et al*. (2002) or alternatively with a mouse monoclonal anti-NKA antibody (prediluted, α5, supernatant, 0.9 mg ml^{-1}, DSHB, Iowa city, IA, USA); the latter antibody is specific for the α-subunit of chicken Na^+-K^+ ATPase and has been used for a wide range of organisms, including elasmobranch fishes (Ferrando *et al*., 2006).

As secondary antiserum, a FITC conjugated anti-mouse antiserum (1:400 in PBS, DAKO, Glostrup, Denmark) was used. Negative controls were performed by omission of the primary antibody or using the NS1 hybridoma culture supernatant (DSHB) as primary antibody. The sections were examined with an Olympus BX60 microscope (light and epi-fluorescence microscope) and visualized through the Color-View Camera (Olympus, Tokyo, Japan). The images were acquired and analysed through the software analysis (Soft Imaging System, Lake Wood Co., USA).

Results

Surviving

In 15 g/l salinity, all fish died but in the other groups all animals survived during the experiment period and no mortality was recorded.

Histomorphology

The kidneys of common carp exhibit typical structure of the FW fish, nephrons consisting of glomerula, proximal convoluted tubules (PCTs) and distal convoluted tubules (DCTs), and collecting tubules. The epithelia of different tubules can be easily distinguished by morphology of their epithelia. PCTs are covered by columnar epithelial cells with basal nucleus, PAS positive granules in the supra-nuclear region, with high and dense brush border deeply protruding into the lumen (Fig. 1). The transition from the PCT to the DCT was marked by the abrupt disappearance of the brush border. Furthermore, the DCT displayed a narrow lumen and a small outer tubular diameter. The prismatic faintly stained epithelial cells were characterized by a round to ovoid nucleus, which was situated in the lower part of the cell body. The lumen of the collecting duct is larger in diameter than the preceding distal tubule. It is lined by columnar slightly eosinophilic cells, with basal nuclei and no brush border.

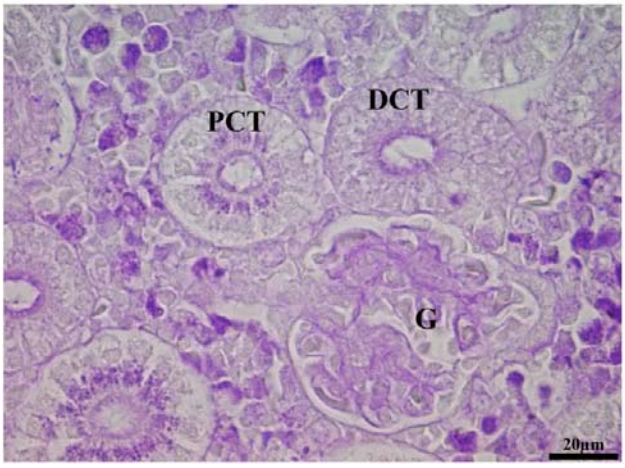


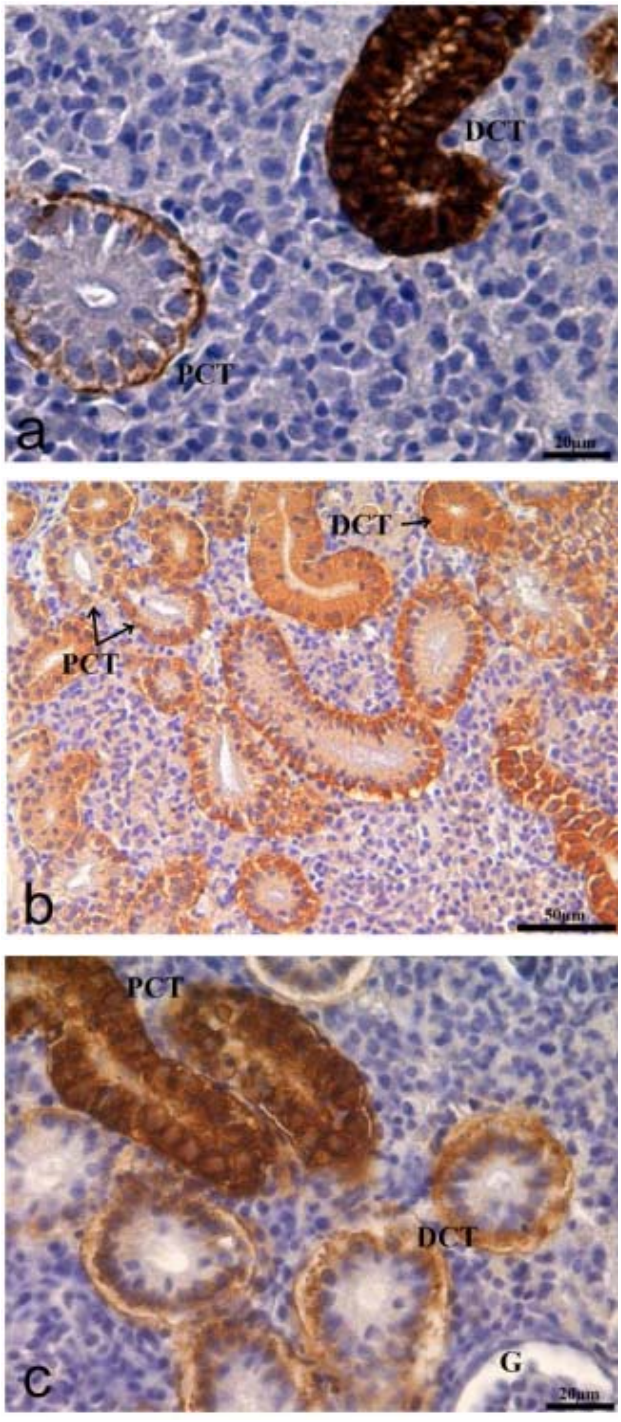
Fig. 1: Transverse sections of the kidney of common carp. In this PAS, stained sections PCT is easily distinguished by prominent brush border that is protruded into the lumen. In DCT, brush border is not as obvious as PCT that is covered by tall columnar epithelial cells with basal nuclei. DCT is lined by faintly stained epithelial cells characterized by a short brush border

Immunohistochemistry

In the control group, the immunoreactivities were distributed only in the nephron tubules, and glomeruli were never immunostained. Intensity of reaction was higher in DCT than in PCT. In particular, NKA immunoreactivity strongly stained the basolateral membrane and the cytoplasm of DCT cells, whereas in PCT cells only the basolateral membrane were stained (Figs. 2a-e). AQP3 immunolocalization was confined to apical membrane of both PCT and DCT cells (Figs. 3a-e).

The distribution and intensity of NKA immunoreactivity was different according to the utilized salinity. In particular, the NKA immunostaining of PCT

cells showed direct relation with environmental salinities and so, at the highest experimental salinity (12 ppt), the immunoreactions of the PCT cells were similar to the DCT cells in the control group (Figs. 2a-e). In fish acclimated to the lower salinities (salinities of 3 and 6 g/l) AQP3 immunostaining was more intense but showed similar localization like control group and was confined to apical membrane of tubule cells. At the higher salinities (9 and 12 ppt) both PCT and DCT cells appeared immunopositive with a more intense immunostaining in the PCT cells. Nevertheless, there were some series of nephrons completely AQP3 immunonegative.



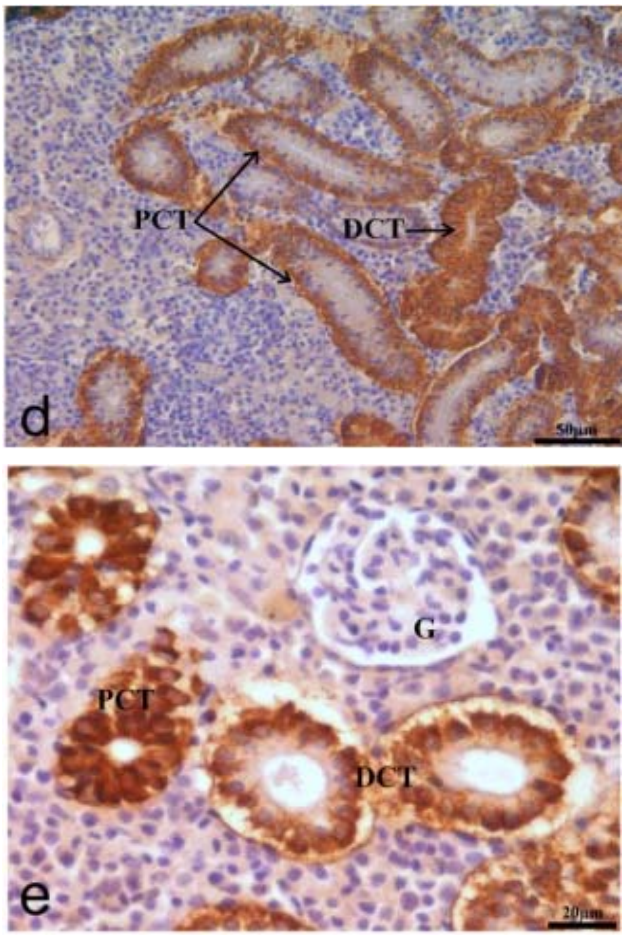


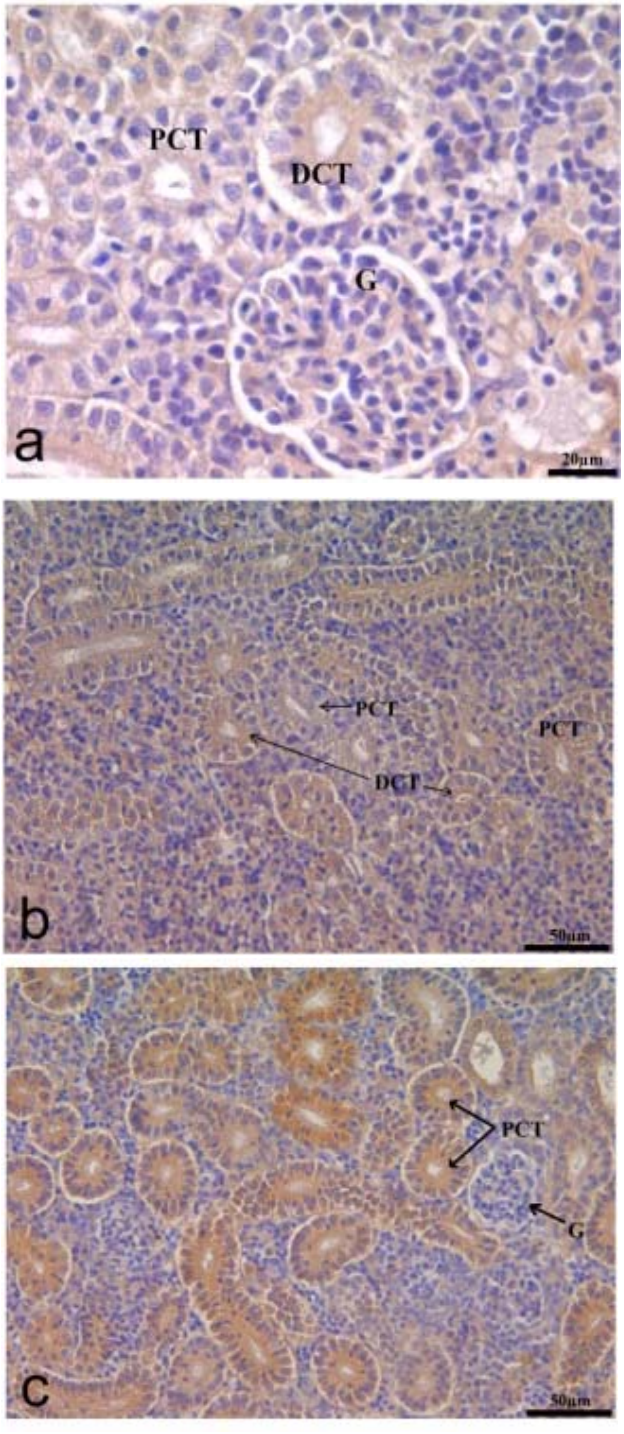
Fig. 2: (a) NKA immunoreactivity in control group is seen in both PCT and DCT basolateral membrane but in DCT NKA immunostaining is stronger. By increase in salinity, PCT NKA immunostaining showed an increase (b) 3 g/l salinity, (c) 6 g/l salinity, (d) 9 g/l salinity, and (e) 12 g/l salinity

Discussion

Results of the present study in the common carp showed a strong NKA immunoreactivity in the renal distal tubules in all control and experimental groups. This finding supports the importance of DCT in reabsorption of ions in distal tubules as Na^+ and Cl^- reabsorption in distal tubule is inhibited by serosal ouabaine, which indicates the involvement of NKA in this process (Marshall and Grosell, 2005). The different immunohistochemical distribution of NKA between proximal and distal tubules may be due to different extent of the tubular systems from basolateral membrane to the inside of cytoplasm, or to the highest concentration of the NKA in the same membrane extension (Lin *et al*., 2004). Lin *et al*. (2004) reported higher levels of NKA in FW than BW (15 ppt) acclimated spotted green pufferfish (*Tetraodon nigroviridis*). This differs from our results in which NKA immunoreactivity increased with the increase of environmental salinity; however, it must be noted that the spotted green pufferfish is a euryhaline fish with physiological mechanisms to adapt itself to different levels of salinity, but common carp is instead a stenohaline FW fish that is not able to adapt to higher

environmental salinities. Therefore, increase of NKA immunoreactivity in proximal tubules may be a compensatory response to increase reabsorption of ions. Increase in plasma ion levels, especially Na^+, is a common response of stenohaline fish to salinity challenges (De Boeck *et al*., 2000; Eckert *et al*., 2001; Yildiz and Uzbilek, 2001; Salati *et al*., 2011), so increase in NKA immunoreactivity in proximal tubule could be related to increase in plasma ion content.

AQP3 is a membrane water and small solute channel protein, expressed in various tissues in the body and, in particular, in the teleost kidney (Cutler *et al*., 2007). In control group only a faint AQP3 immunoreactivity in the



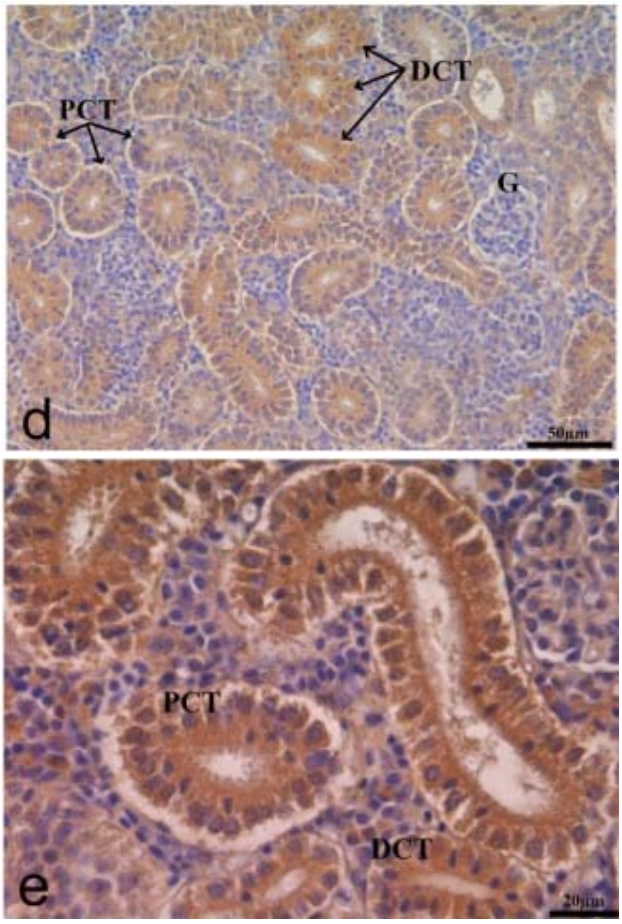


Fig. 3: AQP3 immunolocalization is confined to apical membrane of epithelial cells and is not similar in all nephrons of the same kidney. Some nephrons show more immunoreactivity in comparison to the others and, as seen in the figures, in some tubules there is no AQP3 immunoreactivity. With increase in environmental salinity AQP3 immunoreactivity increases (a) control group, (b) 3 g/l salinity, (c) 6 g/l salinity, (d) 9 g/l salinity, and (e) 12 g/l salinity

apical region of PCT cells was observed; this is in agreement with the low water permeability of PCT and the impermeability of DCT, which is characteristic of FW fish kidney (Marshall and Grosell, 2005). In low concentrations of salt (3 and 6 g/l) a more intense AQP3 immunostaining was observed but in the higher environmental salinities (9 and 12 g/l) a change in the situation can be observed, with more intense AQP3 immunostaining also in the DCT such as that found in SW fish. It has been found that in the eel kidney AQP3 immunostaining is distributed only in those renal tubules characterized by a well defined brush border (probably belonging to PCT), but only a slight difference has been observed in AQP3 immunostaining in FW-acclimated in comparison to SW-acclimated fish (Martinez *et al*., 2005). The distal parts of renal tubule are thought to have low permeability in FW fish, but higher permeability in SW fish where water needs to be conserved (Marshall and Grosell, 2005).

In stenohaline fish the most important adjustment in kidney function for tolerance of increased environmental

salinity is reduced urine flow rate (Hentschel *et al*., 1978), so in this species AQP3 may be involved in reduced urine flow by increasing water absorption.

The more intense immunoreactivity for both NKA and AQP3 in this study observed in response to increased environmental salinity suggests that these proteins play important roles in response to increased environmental salinity. At salinity of 9 g/l and higher, kidney morphology showed histomorphological changes (edema and detachment of basal lamina). Chemical assay of enzymes could be done to compare the amount of energy consumed in different levels of salinity in kidney, as changes in activity of NKA in *C. carpio* gill during exposure in different salinities has been shown (Salati *et al*., 2011). Numerous studies have shown that 20 to >50% of the total fish energy budget is dedicated to osmoregulation (Bœuf and Payan, 2001) so the effect of experimental salinities on fish energy budgets and therefore growth should be considered.

Acknowledgements

The hybridoma antibody developed by D. M. Fambrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242.

References

Bœuf, G and Payan, P (2001). How should salinity influence fish growth? Comp. Biochem. Physiol. C., 130: 411-423.

Cutler, C; Martinez, AS and Cramb, G (2007). The role of aquaporin 3 in teleost fish. Comp. Biochem. Physiol. A., 148: 82-91.

De Boeck, G; Vlaeminck, A; Van der Linden, A and Blust, R (2000). The energy metabolism of common carp (*Cyprinus carpio*) when exposed to salt stress: an increase in energy expenditure or effects of starvation? Physiol. Biochem. Zool., 73: 102-111.

Eckert, SM; Yada, T; Shepherd, BS; Stetson, MH; Hirano, T and Grau, EG (2001). Hormonal control of osmoregulation in the channel catfish *Ictalurus punctatus*. Gen. Comp. Endocrinol., 122: 270-286.

Evans, DH (2008). Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. Am. J. Physiol.: Comp. Evol. Physiol., 295: 704-713.

Ferrando, S; Bottaro, M; Gallus, L; Girosi, L; Vacchi, M and Tagliafierro, G (2006). Na^+-K^+ ATPase immuno-reactivity in olfactory epithelium of small-spotted catshark *Scyliorhinus canicula*, possible presence of ion exchanging cells? J. Fish Biol., 69: 278-282.

Hentschel, H; Jannke, C; Kaune, R and Elger, M (1978). Untersuchungen über Mucosubstanzen im Harnapparat des Giebels in Verbindung mit Experimenten zur renalen Osmo-und Ionenregulation. Verh. Dtsch. Zool. Ges., 71: 283-289.

Karnaky, KJ (1998). Osmotic and ionic regulation. In: Evans, DH (Ed.), *The physiology of fishes*. (2nd Edn.), Boca Raton, CRC Press. PP: 157-176.

Lignot, JH; Cutler, CP; Hazon, N and Cramb, G (2002). Immunolocalisation of Aquaporin 3 in the gill and the gastrointestinal tract of the European eel, *Anguilla anguilla* (L.). J. Exp. Biol., 205: 2653-2663.

Lin, CH; Tsai, RS and Lee, TH (2004). Expression and distribution of Na^+-K^+ ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comp. Biochem. Physiol. A., 138: 287-295.

Marshall, SM and Grosell, M (2005). Ion transport, osmoregulation and acid-base balance. In: Evans, DH and Claiborne, JB (Eds.), *The physiology of fishes*. (3rd Edn.), Boca Raton, CRC Press. PP: 177-230.

Marshall, WS (1995). Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. In: Wood, CM and Shuttleworth, TJ (Eds.), *Cellular and molecular approaches to fish ionic regulation*. (1st Edn.), New York, Academic Press Inc., PP: 1-23.

Martinez, AS; Cutler, CP; Wilson, G; Phillips, C; Hazon, N and Cramb, G (2005). Cloning and expression of three aquaporin homologues from the European eel (*Anguilla anguilla*): effects of seawater acclimation and cortisol treatment on renal expression. Biol. Cell. 9: 615-627.

McCormick, SD (1995). Hormonal control of gill Na^+-K^+-ATPase and chloride cell function. In: Wood, CM and Shuttleworth, TJ (Eds.), *Fish physiology*. (1st Edn.), Vol. 14, New York, Academic Press. PP: 285-315.

McCormick, SD (2001). Endocrine control of osmoregulation in Teleost fish. Am. Zool., 41: 781-794.

Richards, JG; Semple, JW; Bystriansky, JS and Schulte, PM (2003). Na^+-K^+-ATPase isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. J. Exp. Biol., 206: 4475-4486.

Salati, AP; Baghbanzadeh, A; Soltani, M; Peyghan, R and Riazi, G (2011). Effect of different level of salinity on gill and kidney function in common carp (*Cyprinus carpio*). Ital. J. Zool., 78: 298-303.

Schwartz, FJ (1964). Natural salinity tolerances of some freshwater fishes. Underwat. Nat., 2: 13-15.

Ura, K; Soyano, K; Omoto, N; Adachi, S and Yamauchi, K (1996). Localization of Na^+-K^+-ATPase in tissues of rabbit and teleosts using an antiserum directed against a partial sequence of the a-subunit. Zool. Sci., 13: 219-227.

Yildiz, HY and Uzbilek, MK (2001). The evaluation of secondary stress response of grass carp (*Ctnepharyngodon idella*; Val. 1844) after exposing to the saline water. Fish Physiol. Biochem., 25: 287-290.

Verkman, AS and Mitra, AK (2000). Structure and function of aquaporin water channels. Am. J. Physiol. Renal Physiol., 278: 13-28.

Watanabe, S; Kaneko, T and Aida, K (2005). Aquaporin-3 expressed in the basolateral membrane of gill chloride cells in Mozambique tilapia *Oreochromis mossambicus* adapted to freshwater and seawater. J. Exp. Biol., 208: 2673-2682.