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RESEARCH ARTICLE

Osmoregulatory Genes Expression Analysis and Chloride Cells Activity in Common Carp: Insight from Fish Exposed to Salinity

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ABSTRACT

The common carp, *Cyprinus carpio*, is a stenohaline species but can tolerate some changes in environmental salinity. This study aimed to better understand their osmoregulatory response to salinity increases. Messenger RNA expression of the Na^+K^+ ATPase α3 (*NKAα3*) and Aquaporin 3α (*AQP3α*) genes and histomorphological changes of the chloride cells were investigated on common carp gills, as an osmoregulatory organ in experimental and control groups. The salinity of the experimental group gradually increased to 10 ppt by 3 ppt NaCl per day. The experiment continued for 14 days. Gill samples were preserved in RNA later for RT-qPCR analysis. For histomorphological changes, gill tissue was fixed in glutaraldehyde and processed for electron microscopic examination. RT-qPCR analysis further confirmed that mRNA expression levels of *NKAα3* and *AQP3α* were significantly up-regulated with increasing concentration of NaCl. The tissue-specific *NKAα3* and *AQP3α* transcript response in the gill suggests a critical role of *NKAα3* and *AQP3α* expression in fish for successful acclimation to increased salinity. Also, chloride cells demonstrated hypertrophy with an increased number and size of mitochondria. The current findings clarified that increasing salinity modulates *NKAα3* and *AQP3α* genes expression in common carp, and the gill chloride cells manifest profound histomorphological alteration. These findings are critical for the future application to cultivate the common carp in water with high salinity, especially in countries suffering from scarce freshwater.

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INTRODUCTION

Water homeostasis and osmotic adjustment are critical for maintaining physiological homeostasis in aquatic life. A fish's internal water balance and ion concentration must be adjusted for its survival in a hypotonic or hypertonic environment (Sheida *et al.*, 2010). Hence, salinity is a significant environmental factor directly influencing fish's physiological state and functions. Appropriate salinity is crucial to the survival and development of fish. Complex regional differences make it hard to predict salinity levels resulting in complex salinity variation for fish (Brocker *et al.*, 2012; Xu *et al.*, 2018). For this reason, marine inhabitants face significant challenges in establishing appropriate ion balance. In marine fish ionocytes or chloride cells participate in removing excess ions, and in freshwater fish, chloride cells help in ion absorption (Florkin, 2014; Kiran *et al.*, 2022). Generally, fish exposed to a chronic increase in environmental salinity requires the differentiation of epithelial transporter cells and an increase in the number of chloride cells rich in mitochondria (Fielder *et al.*, 2007), as well as changes in transporter gene expression (McCormick, 2001).

Common carp, *Cyprinus carpio* (*C. carpio*) is a stenohaline freshwater fish that belongs to the order Cypriniformes and the family Cyprinidae. It is native to

Asia and Eastern Europe, but it has spread globally and today lives in North America, Africa, and Europe (Nelson, 2006). Because it can grow quickly in eutrophic environments and survive severe environmental conditions, common carp is good for brackish water aquaculture (Váradi, 2014) Additionally, it is one of economically essential fish species that s play a crucial role in the human diet as a source of protein and omega-3 polyunsaturated fatty acids (Al-Saeed *et al.*, 2023).

The primary function of *NKA* is ion pump by active transport; it exports three Na^+ ions extracellular and imports two K^+ ions intracellular against a concentration gradient using energy through ATP hydrolysis (Apell, 2019). Many previous studies have manipulated the correlation between salinity environment modification and *NKA* in fish (Pan *et al.*, 2014; Pham *et al.*, 2016). They recorded increased activity and mRNA expression of gills *NKA3* (Mancera and McCormick, 2000; Richards *et al.*, 2003) as a response to the high salinity.

Aquaporins (AQPs) are transmembrane proteins that are essentially considered primarily as water-selective channels, simplifying the transmembrane movement of water across the cell membrane and, subsequently, osmoregulation in mammals and aquaculture. According to their preferential permeability to water and solutes, the 13 discovered mammalian AQPs have been divided into two groups (Hara-Chikuma and Verkman, 2008). Last decades, many studies shed light on the role of AQPs in aquaculture with the change of salinity; they found gills *AQP3* downregulated after exposure to the high salinity of Japanese Medaka, (Ellis *et al.*, 2019).

There is scarce data about the molecular mechanism of salinity stress tolerance in common carp. Therefore, identifying and characterizing the genes and regulatory factors involved in this process is essential. In this study, *NKAα3* and *AQP3α* mRNA expression and changes in the chloride cells through histomorphological methods were evaluated on the gill tissues of common carp after 14 days of exposure to 10 ppt salinity.

MATERIALS AND METHODS

Experimental design and sample collection: Healthy Common carp, *C. carpio* fingerlings (average weight 10 ± 2 g) were obtained from a private fish farm and delivered to the Aquatic Laboratory of Veterinary Medicine, South Valley University, Qena, Egypt. The fish were reared under laboratory conditions (salinity = 0.2 ppt) in a porcelain tank recirculation system for three weeks. Then, the fish were divided into two experimental groups, with 30 fish/each in three replicates, and stocked in six fiberglass aquaria containing 120 L of dechlorinated tap water. The first group served as a control (salinity = 0.2 ppt); however, the salinity-exposed group was reared at 10 ppt. Salinity was obtained by dissolving commercial-grade NaCl into dechlorinated tap water. Salinity in the tanks increased gradually by 3 g/L per day until the final concentration was reached. An electrical conductivity meter was used to monitor a constant salinity concentration throughout the experiment. Fish were maintained in their final salinity for 14 days. The fish were fed twice daily with commercial floating pellets (Grand Aqua, Egypt) containing 45% protein at a feeding rate of 3% of their body weight.

After 14 days, the fish were euthanized using eugenol, and the gills of both groups were carefully excised (n = 9 fish/each group). For qPCR gene expression analysis, 30 mg of gill tissue was used with three biological repeats. The samples were stored in RNAlater (Qiagen) and then transferred to a -80°C freezer. For histological examination, gill samples were preserved in glutaraldehyde (90 mL, 0.1 M Na-phosphate buffered formalin and 10 mL, 2.5% glutaraldehyde).

RNA extraction, cDNA Synthesis, and Real-Time qPCR: Following the manufacturer's recommendations, RNeasy® Mini kit (QIAGEN, Germany, Cat. No. 74104) was used to extract total RNA from gill tissue. The quantity of RNA obtained was evaluated by using Nanophotometer (NanoDrop Technologies, Wilmington, DE) (Implen GmbH, Germany), and purity was assessed by an OD260/OD280 nm absorption ratio >1.95. Messenger RNA expression of *C. carpio NKAα3* and *AQP3α* genes was measured using real-time RT-PCR. For this purpose, 1 μg of each RNA sample was reverse transcribed to complementary DNA (cDNA) in a Vetiti™ 96 well thermal cycler, using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNA was kept at -80°C until further use. For real-time PCR amplification, each reaction included 1.5 μl of cDNA. SYBR Green from HERA PLUS SYBR® Green qPCR Kit (Willowfort, England) was used to detect specific PCR products. Real-time RT-PCRs were performed using Stratagene Mx3005p® real-time qPCR detection system (Agilent Technologies, USA). The reaction contained a total of 20 μl consisting of 10μl of 2× SYBR® Green, a final volume of 1 μl of each primer (10 μM of forward and reverse) of the gene of interest (Table 1), 1.5 μl of cDNA template, and the balance of sterile nuclease-free PCR grade water.

C. carpio beta-actin and elongation factor 1-α were used as housekeeping references to normalize expression levels between samples. All reactions were run in duplicate (3 samples per tank). The fold change was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Preparation of resin embedding specimens for semithin and ultrathin sectioning: This was performed according to Soliman (2021) and Abd-Elhafeez *et al.* (2021). Ultrathin sections were stained after dissolving the resin with a saturated alcoholic solution of sodium hydroxide for 10 minutes at room temperature. H&E staining was performed according to the method of using 1% glacial acetic acid for 2 minutes after hematoxylin staining. Other semithin sections were stained with toluidine blue, Heidenhain's iron hematoxylin, and methylene blue (Suvarna *et al.*, 2013). H&E and toluidine blue were used as general stains. Methylene blue (Lee *et al.*, 2000) and Heidenhain's iron hematoxylin were used for the identification of mitochondria in the osmoregulating cells (chloride cells).

Chloride cell counting: Chloride cell count was performed using Image J software (Version 1.53i). Cell count was estimated/at 6000 μm^2 according to the following procedure:

Table 1: Primers used in the analysis of qPCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature	References
AQP3α	TCCCTGTGTACTTTCTGTTCCAAAC	CAGCATATTCATACATTGCATCATGGT	60	(Monsang *et al.*, 2019)
NKAα3	GGCAAGAGATGGGCCAA	GCTGGCTCATCTTCGGT	58	(Sinha *et al.*, 2012)
β-actin	GATTCGCTGGAGATGATGCT	GATGGGGTACTTCAGGGTCA	60	(Schyth *et al.*, 2006)
EF1α	GGAGCCCAGCACAAACATG	TTACCCTCCTTGCGCTCAAT	60	(Mráz, 2012)

1- In the open window of Image J software; select" analyze menu" then" Set scale" to detect known distance.
2- The selection tools were used to mark the used area.
3- From Analyze menu," choose "measure" to estimate the surface area.
4- From the "Plugin menu," choose "Analyze: and "Grid" was used to divide the section for counting.
5- From the "Plugin menu," choose "Analyze: and "Cell count" to detect cells number.
6- The number of mitochondrial was estimated at 78.82 and 88.21 μm^2 for control and treated groups, respectively.

Coloring of transmission electron microscopy (TEM) images was performed according to previously described methods (Soliman, 2021).

Statistical analysis: Statistical analysis was conducted using Graph-Pad Prism (GraphPad 8.0.1 Software, San Diego, CA, USA). Significant differences between the control and saline group observations were analyzed using Student's *t-test*. Data were expressed as mean ± standard error of the mean, and statistical significance was P<0.05. The data were validated for normality and homogeneity of variance by the Shapiro-Wilk normality test and Kolmogorov-Smirnov normality test.

RESULTS

Na^+K^+ ATPase and Aquaporin 3 genes mRNA expression: Exposure to elevated saline level, 10 ppt for 14 days, markedly affected the osmoregulation of common carp. Homeostasis and osmoregulation are functions of the gills, the main osmoregulatory organ, and there was a significant (P<0.001) upregulation of *NKAα3* and *AQP3α* mRNA expression in gill tissue of saline-exposed fish compared to the control group (Fig. 1A and B, respectively).

Chloride cell counting: Fig. 2 illustrates the number of gill's chloride cells in control and treated groups after exposure to 0.2 and 10 ppt salinity, respectively. The number of chloride cells significantly increased in *C. carp* after high salinity exposure compared with a control group. Consequently, the increase of chloride cells is strong evidence of these cells' essentiality for osmoregulation and homeostasis.

Quantitative analysis of mitochondria per area (μm^2): Table 2 shows the number of mitochondria in chloride cells of the gill filament. Mitochondria showed a highly significant (P<0.05) increase in fish treated with 10 ppt compared with that treated with 0.2 ppt.

Table 2: Number of mitochondria in 0.02 and 10ppm salinity-treated fish

Group	Number	Area (μm^2)
0.2 ppt	18	78.82
10 ppt	97	88.21

Histomorphology of chloride cells: As shown in Fig. 3A and B, chloride cells increased visibly after 14 days of increased saline exposure in gill filaments stained by H&E compared with non-exposed fish. Furthermore, chloride cells in filaments of gills stained with toluidine blue are easily recognized by their large size and hypertrophic changes after saline exposure compared with control fish, as illustrated in Fig. 4A and B. Additionally, the bioactivity of chloride cells was evidenced by abundant mitochondria (granular cytoplasm), easily identified after staining with Heidenhain's iron hematoxylin (Fig. 5A and B) after raising the salinity to 10 ppt for 14 days.

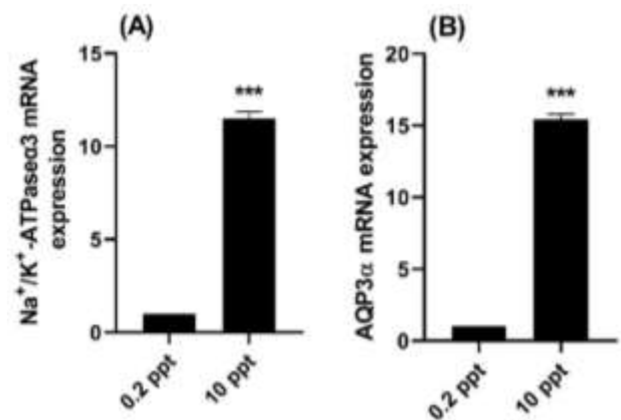

Fig. 1: Effect of salinity on gill Na+/K+-ATPase α3 (A) and *AQP3α* (B) expression in common carp after 14d of 10ppt saline exposure (0.2 ppt set for the control group). The level of each target gene was normalized to the geometric mean of the normalization genes *EF1α* and *β-actin*, and they are presented relative to the control group (value of 1.0). Data are presented as mean ± standard error of the mean (n = 3). Statistically significant factor effects (P<0.05) are noted by asterisks in the saline-exposed group (*** P<0.001).

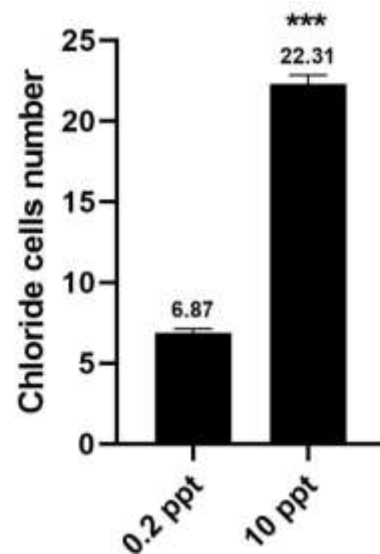

Fig. 2: Effect of salinity on chloride cell number in control (0.2 ppt) and treated (10 ppt) groups. Data are expressed as Mean ± SEM (n = 3 per treatment, independent *t-test*) *** P<0.01, vs. Control group).

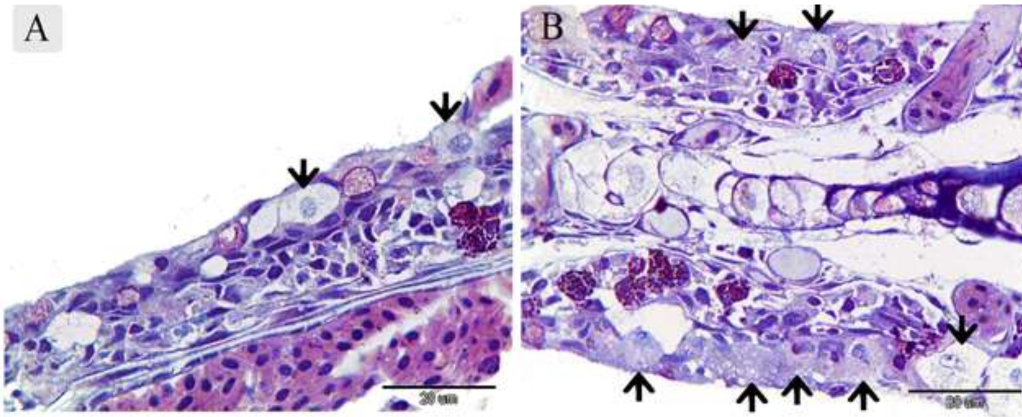

Fig. 3: Semithin sections of gill filaments stained by H&E. (A) A control sample of gill chloride cells (arrows). (B) Increased chloride cells (arrows) of gill filaments as a response to increased salinity.

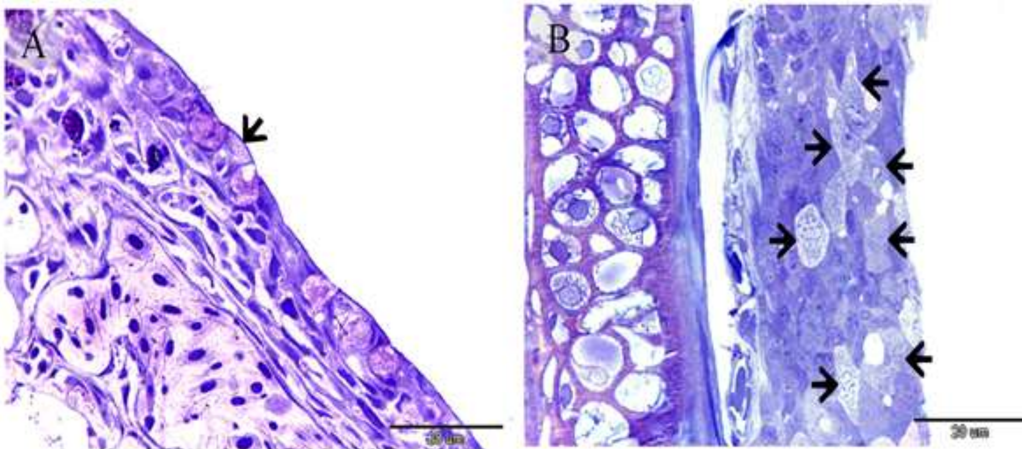

Fig. 4: Semithin sections of gill filaments stained by toluidine blue. (A) Control gill chloride cells (arrows). (B) Chloride cells (arrows) are hypertrophied and increase in number as a response to increased salinity.

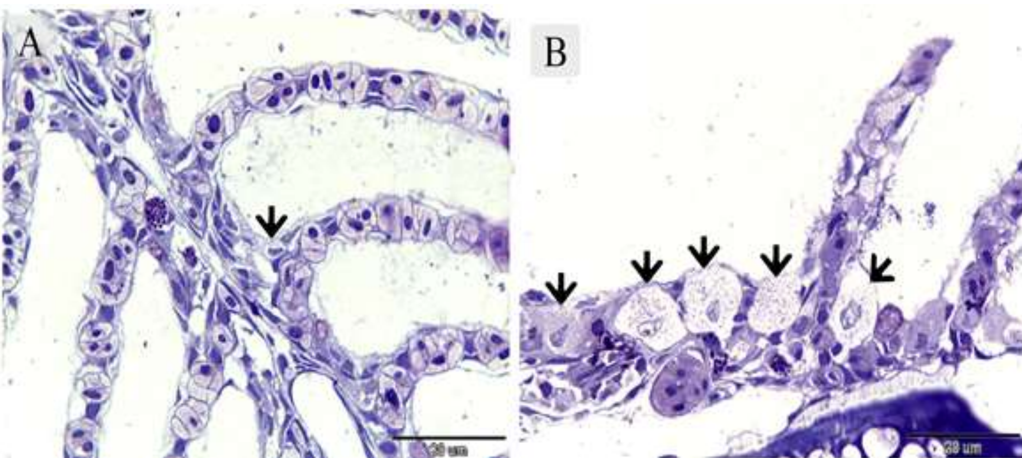

Fig. 5: Semithin sections of the gill filaments were stained by iron hematoxylin. (A) Control gill chloride cells (arrows). (B) Chloride cells (arrows) are hypertrophied and increase in number with increased mitochondria (granular cytoplasm) as a response to increased salinity.

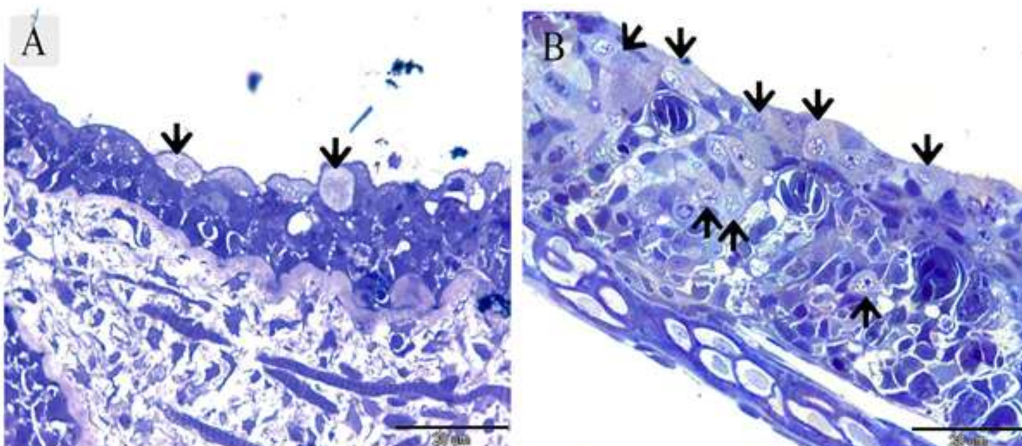

Fig. 6: Semithin sections of gill filaments stained by methylene blue. (A) Control gill chloride cells (arrows). (B) Chloride cells (arrows) undergo hypertrophy with an increase in number and an increase in mitochondria (dark granular cytoplasm) as a response to increased salinity.

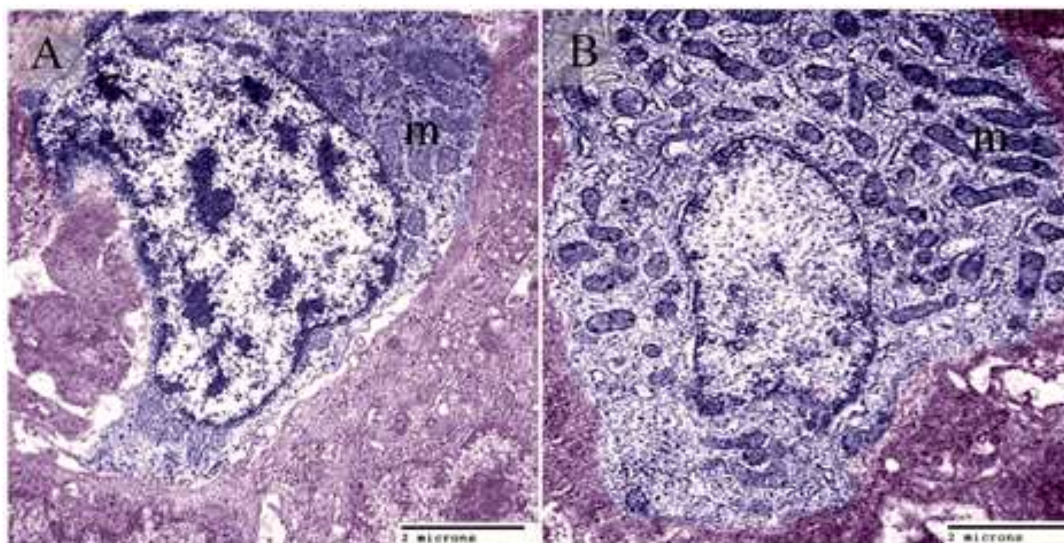

Fig. 7: Ultrathin sections of gill filaments. (A). Chloride cells (blue) are rich in mitochondria (m). (B) Chloride cells (blue) undergo hypertrophy with increased mitochondria (that acquired tubular cristae) in response to increased salinity.

Similarly, gill filaments stained by methylene blue show well-defined chloride cells with numerous mitochondria (dark granular cytoplasm) after salinity (0.2 and 10 ppt) exposure (Fig. 6A and B). For additional clarification, colored ultrastructural changes of the chloride cells were recorded by using TEM. Fig. 7A and B show numerous enlarged mitochondria with acquired tubular cristae in response to increased salinity tolerance.

DISCUSSION

Gills contain complicated channel epithelial functions such as regulating acid-base and nitrogenous waste excretion and water and gas exchange (Evans *et al*., 2005; Yasir *et al*., 2021; Aziz *et al*., 2022). Furthermore, gills are the most important osmoregulatory organs that essential for delivery of O2 to blood-forming organs (Kazmi *et al*., 2023). The number, size, and distribution of chloride cells in gill epithelium may be changed significantly as the need for ion transport and permeability change (Carmona *et al*., 2004). When fish are subjected to diverse abiotic stresses, such as a salinity challenge, gills are the primary site for sensing stress signals and initiating signaling cascades at the molecular level. Fish can regulate the gene expression of channel proteins and the morphology of gill epithelium to facilitate stress tolerance (Kültz *et al*., 1995). Salinity is an inherent physicochemical ecological factor of water and is extremely important for the survival, development, and growth of fish. According to previous studies, high salinity is the main contributor to the alteration of physiological function in aquamarine. For instant, exposure of Mrigal Carp (Cirrhinus mrigala) to high salinity for 90 days alters the antioxidants status by downregulation of glutathione (GSH), peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) along with total proteins were remarkably reduced in the kidneys, gills, and liver tissues (Kiran *et al*., 2022; Raza *et al*., 2022).

To better understand what limits the salinity tolerance of different fish species, it is essential to understand the regulation of gill NKA and AQP3, as both are known to be primary effectors of osmoregulation (Jeong *et al*., 2014). However, there is limited information on NKA and AQP3 in gills and their expression during salinity exposure in common carp. A study on the immune staining of NKA and AQP3 in the common carp kidney showed a direct relationship between environmental salinity and the intensity of the immune staining. Both are involved in the physiological response to environmental salinity (Salati *et al*., 2014). In the current study, gill tissues were examined by RT-qPCR analysis at two different degrees of salinity (0.2 ppt as a control group, and 10 ppt as a salinity-exposed group) in an importantly economical fish, the common carp.

Increased salinity exposure induces molecular and cellular changes in the gills of freshwater teleosts. During such a situation, the osmoregulatory action of NKA maintains homeostasis within the body by excreting Na+ from the cell and transferring K+ into the cell (Jeong *et al*., 2014). The function of NKA has been studied in the gill and kidney of multiple species of fish including tilapia (Hwang *et al*., 1998), spotted green pufferfish (Lin *et al*., 2004), eel (William *et al*., 2006), sea bass (Giffard-Mena *et al*., 2008), and medaka (Kang *et al*., 2008). Since NKA is in the gills, increased expression of NKA in gills after 14 days of exposure to 10 ppt saline is probably the result of the excretion of ions that flow into the body across the thin epithelia of the gills. The current findings revealed upregulation of NKA gene expression in the gills of fish exposed to hypersalinity compared with the corresponding control indicating the ability of common carp to acclimatize to hypersalinity. These results also signify a vital role in salinity acclimation (Singer *et al*., 2007). This upregulation of sodium pump transcripts in response to salinity exposure may be critical for maintaining elevated gill NKA activity over the 14 days of the salinity acclimation period (Shepherd *et al*., 2005). Salati *et al*. (2011) noted that the activity of NKA in the C. carpio gill was changed during exposure to different percentages of saline. Previous studies noted similar patterns of increase in NKA α-subunit mRNA expression in Nile Tilapia (Mohamed *et al*., 2021) at 10 ppt after 10 days and at 11 ppt salinity in rainbow trout (Singer *et al*., 2007). These findings are similar to the transient elevation seen in Atlantic salmon (D'Cotta *et al*., 2000), rainbow trout

(Richards *et al*., 2003), and killifish (Scott *et al*., 2004; Scott and Schulte, 2005). Furthermore, the osmoregulatory response varies in other carp species, for example Cyprinus carpio haematopterus (Amur carp) tolerates the change in salinity more than common carp as it showed better growth performance and can survive 100% up to salinity 5 ppm. In addition, they showed highest food conversion rate when reared in salinity 15 ppm for 90 days (Singh *et al*., 2020).

In contrast to the results of the current study, higher levels of NKA were noted in freshwater as opposed to brackish water (15 ppt) acclimated spotted green pufferfish (Lin *et al*., 2004). It should be noted that euryhaline fish, like the spotted green pufferfish, have physiological mechanisms to adapt themselves to different levels of salinity, but common carp, a stenohaline freshwater fish, can adjust to elevate levels of salinity. Therefore, increased NKA expression in gill tissue may be a compensatory response to the increased absorption of ions. While common carp can maintain ion balance upon gradual salinity exposure, the associated changes in gill function, including sodium pump activity, may be energetically expensive (Sangiao-Alvarellos *et al*., 2006); however, an increase in gill NKA activity is an integral part of successful acclimation to increased salinity.

AQP3 in the gills of teleosts provides water channels for the entrance and outflow of water. As a result, gills with a high concentration of AQP3 protein in the epidermal cells have a higher water flow rate than gills with a lower concentration of AQP3 protein. AQP3 may be involved in enhancing water absorption in stenohaline fish. It is also important in enabling water transfer between cells (Agre, 2006). The relocation of common carp to a greater saline environment is followed by an increase in AQP3 mRNA expression as a result of the gills sustaining effective osmotic homeostasis. However, Jeong *et al*. (2014) noted an elevated expression of AQP3 in gills 6 days after transfer from 5 to 30ppt saline in pufferfish. Similar results have been noted in studies investigating protein activation in teleost gills, such as saltwater tilapia (Watanabe *et al*., 2005) and European eel (Lignot *et al*., 2002), where AQP3 was expressed in the gills. At elevated levels of environmental salinity, fish drink large amounts of seawater to get water and monovalent ions across their intestinal epithelia; however, excretion of excess ions is primarily through the chloride cells of the gills (McCormick, 2001; Grosell, 2006).

In this study, salinity exposure stimulated marked changes in the number, morphology, and ultrastructure of chloride cells in gill tissue after 14 days of a saline challenge. This indicates the osmoregulatory capacity of these cells to maintain a hydromineral balance in the gills of common carp. Additionally, examination of the morphology and ultrastructure of gill tissue revealed increased mitochondria and elongation of the cristae and acquiring tubular profile. This indicates an increase in the cristae surface where ATPase synthase is necessary for the generation of ATP (Paumard *et al*., 2002). Generation of ATP is essential to maintain the function of energy-dependent channels, particularly AQP3, as well as the sodium pump which requires NKA activity. Reduction of glycolysis and ATP biosynthesis occur in AQP3-knockout mice (Hara-Chikuma and Verkman, 2008). Additionally, increased numbers of mitochondria in chloride cells are evidence for the availability of large amounts of energy for the Na+ and K+ pumps with the help of ATPase (Shikano and Fujio, 1998). Furthermore, an increased number and size of these cells confirm the ability of common carp to perform osmoregulation and homeostasis in a high-saline environment (Ghahremanzadeh *et al*., 2014).

Multiple studies support the results of the current study and note that a saline challenge is combined with an increase in the number and density of chloride cells in the gills of common carp (Al-Hilalli and Alkhshali, 2019), Oreochromis mossambicus (Lee *et al*., 2000), and juvenile Australian snapper (Fielder *et al*., 2007). However, another study demonstrated an increase in chloride cells at 15 –20% salinity, with cell numbers declining at a higher (36%) salinity level. The negative relationship with 36% salinity may result from acclimatization with the salinity change (Güner *et al*., 2005).

Conclusions: The current study is an attempt to estimate the response of common carp to hypersalinity (10 ppt) for 14 days. Common carp showed acclimatizing ability toward salinity challenge by upregulation of NKAα3 and AQP3α genes, which are responsible for ion and water maintenance and homeostasis, respectively. Furthermore, the chloride cells in gills tissue (The main osmoregulatory organ) increased in number in addition to the histomorphological changes of hypertrophy and an increase in the number and size of mitochondria. The present findings bridged the gap between hypersalinity and molecular, histomorphological mechanisms to hypersalinity exposure in common carp.

Ethics approval statement: The protocols of this study were approved by the Ethical Research Committee of the Faculty of Veterinary Medicine at South Valley University, Egypt with Approval number (NO. 8a/ 13.12.2020).

Authors contribution: WFAE Conceptualization, data curation, and methodology. SAS methodology of histochemistry and ultrastructure, AEA, AAA, and KMA Formal analysis, software, visualization, and writing – original draft. WFAE, HA Supervision, Validation. MEA performed statistical analyzes and Writing – reviews & editing. There is no conflict of interest among authors.

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REFERENCES

Abd-Elhafeez HH, Hassan AHS and Hussein MT, 2021. Melatonin administration provokes the activity of dendritic reticular cells in

the seminal vesicle of Soay ram during the non-breeding season. Sci Rep 11:872.

Agre P, 2006. The aquaporin water channels. Proc Am Thorac Soc 3:5-13.

Al-Hilalli H and Alkhshali M, 2019. Influence of transfer to high salinity on chloride cells, oxygen, and energy consumption in common carp Cyprinus carpio. J Animal Sci Prod 2:1-12.

Al-Saeed FA, Naz S, Saeed MH, *et al.*, 2023. Oxidative stress, antioxidant enzymes, genotoxicity and histopathological profile in Oreochromis niloticus exposed to lufenuron. 43:160-6. http://dx.doi.org/10.29261/pakvetj/2023.012

Apell HJ, 2019. Finding Na, K-ATPase II - From fluxes to ion movements. Substantia 3. https://doi.org/10.13128/ Substantia-207

Aziz S, Abdullah S, Anwar H *et al.*, 2022. DNA damage and oxidative stress in economically important fish, Bighead carp (Hypophthalmichthys nobilis) exposed to engineered copper oxide nanoparticles. Pak Vet J 42:1-8.

Brocker C, Thompson DC and Vasiliou V, 2012. The role of hyperosmotic stress in inflammation and disease. Biomolecular Concepts 3:345-64.

Carmona R, García Gallego M, Sanz A, *et al.*, 2004. Chloride cells and pavement cells in gill epithelia of Acipenser naccarii: ultrastructural modifications in seawater acclimated specimens. J Fish Biol 64:553-66.

D'Cotta H, Valotaire C, Le Gac F, *et al.*, 2000. Synthesis of gill Na+-K+-ATPase in Atlantic salmon smolts: differences in α-mRNA and α-protein levels. J Animal Physiol 278:101-10.

Ellis LV, Bollinger RJ, Weber HM, *et al.*, 2019. Differential Expression and Localization of Branchial AQP1 and AQP3 in Japanese Medaka (Oryzias latipes). Cells 8:422.

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

Fielder DS, Allan GL, Pepperall D, *et al.*, 2007. The effects of changes in salinity on osmoregulation and chloride cell morphology of juvenile Australian snapper Pagrus auratus. Aquaculture 272:656-66.

Florkin M, 2014. Deuterostomians, Cyclostomes and Fishes (Chemical Zoology), Elsevier 8:166.

Ghahremanzadeh Z, Namin JI, Bani A, *et al.*, 2014. Cytological comparison of gill chloride cells and blood serum ion concentrations in kutum (Rutilus frisii kutum) spawners from brackish (Caspian Sea) and freshwater (Khoshkrood River) environments. J Fisheries and Aquatic Life 22:189-96.

Giffard-Mena I, Lorin-Nebel C, Charmantier G, *et al.*, 2008. Adaptation of the sea-bass (Dicentrarchus labrax) to freshwater: role of aquaporins and Na+/K+-ATPases. Comparative Biochemistry and Physiology Part A. J Mol Integ Physiol 150:332-8.

Grosell M, 2006. Intestinal anion exchange in marine fish osmoregulation. J Exp Biol 209:2813-27.

Güner Y, Özden O, Çbç1rgan H, *et al.*, 2005. Effects of salinity on the osmoregulatory functions of the gills in Nile Tilapia (Oreochromis niloticus). Turk J Vet Animal Sci 29:1259-66.

Hara-Chikuma M and Verkman AS, 2008. Roles of Aquaporin-3 in the Epidermis. J Invest Dermatol 128:2145-51.

Hwang PP, Fang MJ, Tsai JC, *et al.*, 1998. Expression of mRNA and protein of Na+-K+-ATPase α subunit in gills of tilapia (Oreochromis mossambicus). Fish Physiol Biochem 18:363-73.

Jeong SY, Kim JH, Lee WO, *et al.*, 2014. Salinity changes in the anadromous river pufferfish, Takifugu obscurus, mediate gene regulation. Fish Physiol Bioch 40:205-19.

Kang CK, Tsai SC, Lee TH, *et al.*, 2008. Differential expression of branchial Na+/K+-ATPase of two medaka species, Oryzias latipes, and Oryzias dancena, with different salinity tolerances acclimated to fresh water, brackish water, and seawater. Comparative Biochemistry and Physiology Part A. J Mol Integ Physiol 151:566-75.

Kazmi SAH, Iqbal R, Al-Doaiss AA, *et al.*, 2023. Azoxystrobin-induced oxidative stress in gills, hematological biomarkers and histopathological ailments in freshwater fish. Pak Vet J 43:321-6. http://dx.doi.org/10.29261/pakvetj/2023.025

Kültz D, Jürss K and Jonas L, 1995. Cellular and epithelial adjustments to altered salinity in the gill and opercular epithelium of a cichlid fish (Oreochromis mossambicus). J Cell Tissue Res 279:65-73.

Kiran H, Kousar S, Ambreen F, *et al.*, 2022. Effect of plant-based feed on the antioxidant enzymes, biochemical and hematological parameters of Oreochromis niloticus. Cont Vet J 2:67-75.

Lee TH, Hwang PP, Shieh YE, *et al.*, 2000. The relationship between `deep-hole' mitochondria-rich cells and salinity adaptation in the euryhaline teleost, Oreochromis mossambicus. J Fish Physiol Biochem 23:133-40.

Lignot JH, Cutler CP, Hazon N, *et al.*, 2002. Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel *Anguilla anguilla* (L.). J Exp Biol 205:2653-63.

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. Comparative Biochemistry and Physiology Part A: J Mol Integ Physiol 138:287-95.

Livak KJ and Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. J Methods 25:402-8.

Mancera JM and McCormick SD, 2000. Rapid activation of gill Na+, K+-ATPase in the euryhaline teleost Fundulus heteroclitus. J Exp Zool 287:263-74.

McCormick SD, 2001. Endocrine control of osmoregulation in teleost fish. J Am Zoologist 41:781-94.

Mohamed NA, Saad MF, Shukry M, *et al.*, 2021. Physiological and ion changes of Nile tilapia (Oreochromis niloticus) under the effect of salinity stress. J Aquacul Reports 19:100567.

Monsang SJ, Parhi J, Choudhury J, *et al.*, 2019. Molecular characterization of carp aquaporin-3a: Expression profiling during hormone-induced spawning. J Aquacul 500:569-75.

Mráz J, 2012. Lipids in common carp (Cyprinus carpio) and effects on human health (Doctoral thesis, Faculty of Natural Resources and Agricultural Sciences Department of Food Science Uppsala).

Nelson JS, 2006. Fishes of the World. John Wiley and Sons, Inc 4th ed:140.

Pan L, Liu H and Zhao Q, 2014. Effect of salinity on the biosynthesis of amines in Litopenaeus vannamei and the expression of gill-related ion transporter genes. J Ocean Univ China 13:453-9.

Paumard P, Vaillier J, Coulary B, *et al.*, 2002. The ATP synthase is involved in generating mitochondrial cristae morphology. The EMBO J 21:221-30.

Pham D, Charmantier G, Boulo V, *et al.*, 2016. Ontogeny of osmoregulation in the Pacific blue shrimp, Litopenaeus stylirostris (Decapoda, Penaeidae): Deciphering the role of the Na+/K+-ATPase. Comparative Biochemistry and Physiology Part B. Biochem Mol Biol 196:27-37.

Raza GA, Ghaffar A, Hussain R, *et al.*, 2022. Nuclear and morphological alterations in erythrocytes, antioxidant enzymes, and genetic disparities induced by brackish water in mrigal carp (*Cirrhinus mrigala*). Oxid Med Cellul Long 2022:4972622.

Richards JG, Semple JW, Bystriansky JS, *et al.*, 2003. Na+/K+-ATPase α-isoform switching in gills of rainbow trout (Oncorhynchus mykiss) during salinity transfer. J Exp Biol 206:4475-86.

Salati AP, Baghbanzadeh A, Soltani M, *et al.*, 2011. Effect of different levels of salinity on gill and kidney function in common carp Cyprinus carpio (Pisces: Cyprinidae). Italian J Zool 78:298-303.

Salati AP, Ferrando S, Movahedinia A, *et al.*, 2014. Effect of different levels of salinity on immunolocalization of Na+/K+-ATPase and Aquaporin 3 in the kidney of common carp Cyprinus carpio. Iranian J Vet Res 15:45-9.

Sangiao-Alvarellos S, Arjona FJ, Míguez JM, *et al.*, 2006. Growth hormone and prolactin actions on osmoregulation and energy metabolism of gilthead sea bream (*Sparus auratus*). Comparative Biochemistry and Physiology Part A. J Mol Integ Physiol 144:491-500.

Schyth BD, Lorenzen N and Pedersen FS, 2006. Antiviral activity of small interfering RNAs: specificity testing using heterologous virus reveals interferon-related effects overlooked by conventional mismatch controls. Virology 349:134-41.

Scott GR, Richards JG, Forbush B, *et al.*, 2004. Changes in gene expression in gills of the euryhaline killifish Fundulus heteroclitus after abrupt salinity transfer. American J Physiol 287:C300-9.

Scott GR and Schulte PM, 2005. Intraspecific variation in gene expression after seawater transfer in gills of the euryhaline killifish Fundulus heteroclitus. Comparative Biochemistry and Physiology Part A. J Mol Integ Physiol 141:176-82.

Sheida A, Preeta K, Rahim P, *et al.*, 2010. Chloride cell morphometrics of Common carp, Cyprinus carpio, in response to different salinities. Comp Clin Pathol 20:363-7.

Shepherd BS, Drennon K, Johnson J, *et al.*, 2005. Salinity acclimation affects the somatotropic axis in rainbow trout. J Mol Integ Physiol 288:1385-95.

Shikano T and Fujio Y, 1998. Relationships of salinity tolerance to immunolocalization of Na+/K+-ATPase in the gill epithelium during seawater and freshwater adaptation of the Guppy Poecilia reticulata. Zool Sci 15:35-41.

Singer TD, Raptis S, Sathiyaa R, *et al.*, 2007. Tissue-specific modulation of glucocorticoid receptor expression in response to salinity

acclimation in rainbow trout. Comparative Biochemistry and Physiology Part B. Biochem Mol Biol 146:271-8.

Singh S, Krishna RA, Harikrishna V, *et al*., 2020. Growth and osmoregulatory response of Cyprinus carpio haematopterus (Amur carp) reared in inland saline water. Indian J Animal Sci 90:120–4.

Sinha AK, Diricx M, Chan LP, *et al*., 2012. Expression pattern of potential biomarker genes related to growth, ion regulation, and stress in response to ammonia exposure, food deprivation, and exercise in common carp (Cyprinus carpio). Aquatic Toxicol 122:93-105.

Soliman SA, 2021. Telocytes are major constituents of the angiogenic apparatus. Sci Reports 11:5775.

Suvarna K, Layton C and Bancroft J, 2013. Bancroft's Theory and Practice of Histological Techniques: Elsevier, Churchill Livingstone.

Váradi L, 2014. Cyprinus carpio (Common Carp). United Kingdom, CAB International, Wallingford.

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 208:2673-82.

William KF, Au DWT and Wong CKC, 2006. Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels. Biochem Biophy Res Commun 346:1181-90.

Xu C, Li E, Suo Y, *et al*., 2018. Histological and transcriptomic responses of two immune organs, the spleen and head kidney, in Nile tilapia (Oreochromis niloticus) to long-term hypersaline stress. Fish Shellfish Immunol 76:48-57.

Mahmood Y, Ghaffar A and Hussain R, 2021. New insights into Hemato-biochemical and Histopathological effects of Acetochlor in bighead carp (Aristichthys nobilis). Pak Vet J 41:538-44.