Innovations

Effect of Freshwater-Seawater Transition on the Gill Atpase Activity of the Catadromous Euryhaline Mullet (Mugil Cephalus)

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Abstract: *Only a few studies have explored the expression of mullet gill ion transporters during the transition from freshwater to saltwater and back, a key stage in the mullet's life cycle.Low salinity (0, 5, 10, 15 ‰) acclimated grey mullets were sampled over 28days of acclimation for blood and gill tissue. Results revealed that mullets held in the controlexperiment with salinity maintained at 20‰ had constant sodium, chloride, and plasma osmolality across the experiment. Plasma sodium, chloride, and total osmolality levels improved markedly toward control values after seawater dilutions. Gill H+-ATPase activity increased by halfin 14days after exposure to low salt conditions, whereas H+-ATPase mRNA levels were not affected by the salinity change. Within 7 days of exposure to low salt conditions, gill Na+/K+-ATPase activity increasedto about 40% more than control levels, remaining significantly higher until the 28thday, when it reduced again to control levels. This increase in activity was accompanied by a more than 7-foldrisein the level of Na+/K+-ATPase isoform α1a mRNA level and a 6-foldfallin the amount of Na+/K+-ATPase isoform α1b mRNA. The mRNA levels of Na+/K+-ATPase isoforms α1c and α3 remained unchanged as a results of seawater dilutions. The time courses for mRNA expression of the small membrane protein FXYD 11 and the β1-subunit were very similar, with levels increasing significantly 14 days following exposure to low salt conditions before rising again to the control levels on the28thday. The findings of this study suggest an important role for Na+/K+-ATPase in seawater acclimation in grey mullet. The study recommends the α1a mRNA isoform of Na+/K+-ATPase as a suitable potential molecular biomarker for regulating and controlling genes in low-salinity aquatic environments.*

Keywords: *Grey mullet, Salinity acclimation, Na+/K+-ATPase, Gill physiology, Molecular biomarker*

Introduction

The grey mullet, scientifically referred to as Mugil cephalus, stands out as an extraordinary catadromous euryhaline fish, showcasing exceptional adaptability across a diverse range of salinities (Olukolajo, 2013). This adaptability empowers the grey mullet not only to traverse but also to thrive in environments spanning from freshwater to saltwater, a phenomenon extensively investigated by Kim et al. (2022). The catadromous nature of the grey mullet underscores its unique ability to migrate between distinct aquatic habitats, a phenomenon intricately linked with its physiological processes.Central to these physiological processes is osmoregulation, a complex biological mechanism vital for maintaining the delicate balance of fluids and salts within the fish's body (Herrera et al., 2022). This intricate dance of osmoregulation becomes particularly crucial during the mullet's migrations, demanding the orchestration of physiological mechanisms to ensure its well-being amidst changing salinity conditions.

At the heart of ion transport across gill membranes, a key facet of osmoregulation, lies the activity of ATPases—enzymes that harness the energy derived from ATP to facilitate the movement of ions across cell membranes (Hwang et al., 2011). Notably, Na+/K+-ATPase and H+-ATPase emerge as pivotal players in this dynamic process, with Na+/K+-ATPase primarily pumping sodium ions out of cells while importing potassium ions, creating a gradient crucial for ion movement across the gills (Evans, 2008; Bystrianskyet al., 2006). Simultaneously, H+-ATPase contributes to proton pumping, influencing the exchange of other ions across the gills and aiding in the overall regulation of osmolality (Evans et al., 2005; Young et al., 2022).

Existing research has shed light on the sensitivity of Na+/K+-ATPase and H+-ATPase activities in fish gills to variations in salinity levels, providing valuable insights into the osmoregulatory responses of various species (Gilmour & Perry, 2009; Lee et al., 2022). For instance, Liang et al. (2017) observed an increase in Na+/K+-ATPase activity in the gills of rainbow trout during the transition from freshwater to seawater, emphasizing the dynamic nature of ion transport mechanisms in response to environmental challenges. Similarly, investigations into H+-ATPase activity in fish gills, such as those conducted on tilapia by Garcia-Santos et al. (2015), revealed a decrease in activity when the fish moved from seawater to freshwater.

Despite this wealth of knowledge, the specific mechanisms governing Na+/K+-ATPase and H+-ATPase activities during the transition phases of grey mullet—from freshwater to seawater and vice versa—remain inadequately explored (Takvamet al., 2021; Zimmer & Perry, 2022). Addressing these gaps is imperative for unraveling the intricacies of grey mullet adaptation and enhancing our ability to predict and manage their responses to changing aquatic environments.

Furthermore, the unique osmoregulatory mechanisms specific to grey mullet demand a more comprehensive investigation to unravel the intricacies of this fish's

physiological responses (Olukolajo, 2013). The existing body of knowledge has laid a foundation by highlighting the sensitivity of ATPase activities in various fish species, including grey mullet, to salinity changes. However, a significant research gap persists in understanding how these enzymatic activities fluctuate precisely during the critical transition periods between freshwater and seawater. The studies conducted by Takvamet al. (2021) and Zimmer & Perry (2022) underscore the necessity for focused research in this area, aiming to fill the void in our understanding of grey mullet osmoregulation. Specifically, the absence of information regarding the nuanced responses of Na+/K+-ATPase and H+-ATPase during these transitions limits our ability to delineate the molecular mechanisms at play. This represents a crucial juncture in the scientific exploration of grey mullet physiology, urging researchers to delve into the temporal dynamics of ATPase activities, their specific roles, and potential interactions during shifts in salinity. By bridging this knowledge gap, future studies can contribute significantly to the broader understanding of fish adaptation to variable environments and inform strategies for the conservation and sustainable management of grey mullet populations in the face of changing aquatic conditions.

Materials and Methods

To investigate the effect of freshwater-seawater transition on the gill ATPase activity of the grey mullet, we conducted an experiment involving acclimating grey mullets to different salinities (0, 5, 10, 15, and 20 ‰) for 28 days. The experiment was carried out in a controlled laboratory setting with individual tanks for each salinity level. The water temperature was maintained at 20°C, and the dissolved oxygen level was kept at 6 ppm throughout the experiment.

Fish Acclimation

Wild-caught grey mullets of approximately the same size (20-25 cm in length) were collected from artisanal fishermen landing at the Nsidung beach of the Calabar River, a tributary of the Cross River Estuary, Nigeria. Collected fish samples were transported to the Fisheries and Aquaculture laboratory, Faculty of Oceanography, University of Calabar, Nigeria.

The fish were initially acclimated to freshwater in two sets of duplicate 100-liter plastic tanks measuring 50 cm x 50 cm x 68 cm for a period of one week to ensure they were in good health and free from any stress or disease. This replication allowed for a more robust assessment of the acclimation process and served as an internal control for the subsequent salinity manipulation experiments.

Within each set of duplicates, the tanks were filled with dechlorinated tap water, and the temperature was maintained at 25°C using aquarium heaters. The tanks were also equipped with aerators to provide adequate oxygenation for the fish. Each tank

was filled with 100 liters of dechlorinated tap water, providing approximately 4 liters of water per fish. This stocking density was considered sufficient to minimize stress on the fish.

A total of 40 fish samples were acclimated to freshwater in the two sets of duplicate tanks, with 20 fish distributed evenly among the two tanks in each set. This is a relatively low stocking density, which helped to minimize stress on the fish.The replication ensured that the effects observed in the subsequent salinity manipulation experiments could be attributed to salinity changes rather than individual fish variations or tank-specific factors.

Salinity Manipulation

After the initial acclimation period, the fish were randomly divided into five groups in duplicates, corresponding to the different salinity levels (0, 5, 10, 15, and 20 ‰). Each plastic tank measured 50 cm x50 cm x68 cm and holds 100 liters of water. Four fish samples were distributed into each tank totaling 40 fish samples for duplicate tanks. Salinities were adjusted gradually over a period of three days to minimize osmotic shock. The salinity of each tank was monitored daily using a calibrated refractometer.

Sampling and Tissue Collection

At regular intervals (0, 7, 14, 21, and 28 days), three fish from each salinity group were randomly selected for sampling at each time point. This provided a sufficient number of samples to ensure statistical power and allow for meaningful comparisons between salinity groups.

Blood samples were collected from the caudal vein, a prominent blood vessel located in the tail region of the fish. To minimize stress and ensure humane treatment, the fish were first anesthetized using tricaine methanesulfonate (MS-222), a commonly used anesthetic for fish. The MS-222 solution was prepared according to the manufacturer's instructions and administered to the fish via immersion. Once anesthetized, the fish were placed on a damp towel and the caudal vein was located using a magnifying glass. A sterile needle attached to a syringe was then carefully inserted into the caudal vein and a small volume of blood (approximately 0.5 ml) was withdrawn. The blood was immediately transferred to a microcentrifuge tube containing an anticoagulant to prevent clotting.

After blood collection, the gills were carefully removed from the fish. The gills are delicate structures, so they were handled with care to avoid damage. The gills were dissected into small pieces using clean scissors and forceps. The gill tissue samples were then placed in sterile vials or tubes for further analysis.

The blood and gill tissue samples were collected at regular intervals of 0, 7, 14, 21, and 28 days. This allowed the researchers to track changes in gill ATPase activity over time and assess the effects of salinity on gill function.

Measurement of ATP ase Activity

Gill tissue samples were homogenized in ice-cold buffer to disrupt the cellular membranes and release the ATPase enzymes. The homogenization buffer typically contains a mild detergent to facilitate cell lysis and protease inhibitors to prevent enzyme degradation. The homogenate was then centrifuged to separate the soluble fraction (supernatant) containing the ATPase enzymes from the insoluble pellet containing cellular debris.

Na+/K+-ATPase is a sodium-potassium ATPase, an enzyme that transports sodium ions out of cells and potassium ions into cells using the energy from ATP hydrolysis. To measure Na+/K+-ATPase activity, a spectrophotometric assay was used. This assay relies on the colorimetric detection of inorganic phosphate (Pi) released during ATP hydrolysis. The Pi reacts with a color-forming reagent, and the intensity of the color produced is proportional to the amount of ATP hydrolyzed.

H+-ATPase is a proton pump that transports protons (H+) across cell membranes. To measure H+-ATPase activity, another spectrophotometric assay was used. This assay utilizes a pH indicator dye that changes color in response to changes in proton concentration. The H+-ATPase activity is determined by measuring the rate at which the pH indicator dye changes color.

Enzyme activity is typically expressed as the amount of product formed (in this case, micromoles of Pi released) per unit of enzyme (milligrams of protein) per unit of time (hour). This unit of measurement allows for comparisons of enzyme activity between different samples and under different conditions.

mRNA Expression Analysis

Total RNA, which includes all forms of RNA molecules (mRNA, rRNA, tRNA, etc.), was extracted from gill tissue using TRIzol reagent. TRIzol is a commonly used reagent for RNA extraction due to its effectiveness in solubilizing and isolating RNA from various tissues. The TRIzol extraction procedure involves homogenizing the tissue in TRIzol reagent, followed by chloroform phase separation and isopropanol precipitation to isolate the RNA.

cDNA (complementary DNA) was synthesized from the extracted total RNA using reverse transcriptase. Reverse transcriptase is an enzyme that converts RNA molecules into DNA molecules. The cDNA synthesis step is necessary for real-time PCR analysis, as real-time PCR requires DNA templates for amplification.

The mRNA expression levels of Na+/K+-ATPase isoforms (α1a, α1b, α1c, α3), FXYD12, and β1-subunit were quantified using real-time PCR. Real-time PCR, also

known as quantitative PCR (qPCR), is a highly sensitive and specific technique for measuring mRNA abundance. Real-time PCR utilizes fluorescently labeled probes that bind to specific target sequences during DNA amplification. As the target DNA accumulates, the fluorescence intensity increases, allowing for real-time monitoring of DNA amplification.

To account for variations in RNA extraction efficiency and cDNA synthesis, mRNA levels were normalized to the expression of the housekeeping gene β-actin. Housekeeping genes are genes that are expressed at relatively constant levels across different tissues and conditions. β-actin is a commonly used housekeeping gene for normalizing mRNA expression levels.

Statistical Analysis

One-way analysis of variance (ANOVA) was employed to compare the mean gill ATPase activity and mRNA expression levels among the five salinity groups (0, 5, 10, 15, and 20 ‰). One-way ANOVA is a statistical technique used to compare the means of two or more groups when there is a single independent variable (in this case, salinity) and one dependent variable (e.g., gill ATPase activity or mRNA expression).

To further investigate the specific differences between salinity groups, Tukey's post-hoc test was applied. Tukey's post-hoc test is a multiple comparison procedure that adjusts for the increased probability of Type I errors (false positives) when multiple comparisons are made. It allows for pairwise comparisons between all salinity groups, identifying which groups exhibit statistically significant differences in gill ATPase activity or mRNA expression.

The significance level for both ANOVA and Tukey's post-hoc test was set at $p < 0.05$. This means that only differences with a probability of less than 5% of occurring by chance were considered statistically significant. This threshold helps to maintain the overall reliability of the results and minimize the risk of false-positive conclusions.

Results

The study explored the dynamic responses of mullet gill ion transporters during transitions between freshwater and saltwater environments. Grey mullets acclimated to varying low salinity levels (0, 5, 10, 15 ‰) underwent a 28-day sampling period for blood and gill tissue analysis.Results highlight the intricate interplay of physiological and molecular responses in grey mullet under varying salinity levels. Notably, the maintenance of stable plasma sodium, chloride, and osmolality levels in the control experiment contrasts with the marked improvements observed after seawater dilutions. Additionally, the temporal dynamics of H+-ATPase and Na+/K+-ATPase activities, along with the corresponding mRNA expression patterns, reveal the nuanced adjustments in ion transport mechanisms during the 28-day acclimation

period. The study suggests a pivotal role for Na+/K+-ATPase, particularly the α1a mRNA isoform, as a potential molecular biomarker for regulatory processes in low-salinity aquatic environments.

mRNA Expression of Na+/K+-ATPase Isoforms

The results show the impact of salinity on mRNA expression of Na+/K+-ATPase isoforms in grey mullet gills (Figure 1). The heatmap analysis, as depicted in Figure 2, illustrates the nuanced responses of specific isoforms, particularly highlighting a substantial increase in the α1a isoform following exposure to low salt conditions. This dynamic response is accompanied by a noteworthy decrease in the α1b isoform, underscoring the specificity and sensitivity of the molecular adaptations in grey mullet gills to changes in environmental salinity. These findings contribute valuable insights into the molecular mechanisms underlying the fish's acclimation to different salinity levels, providing a foundation for further exploration and potential applications in the management of aquatic environments.

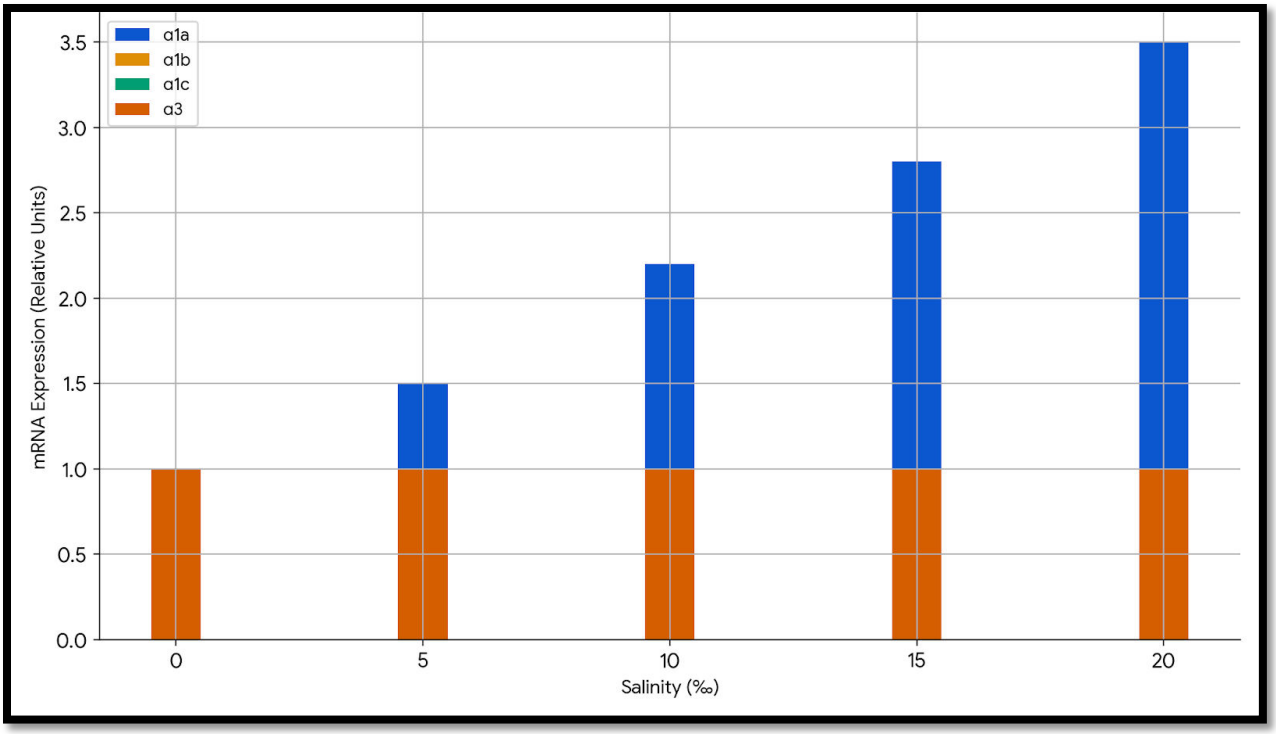

Figure 1: Effect of Salinity on mRNA Expression of Na+/K+-ATPase Isoforms in Grey Mullet Gills

Isoform α1a: The mRNA expression of Na+/K+-ATPase isoform α1a is shown in green; Isoform α1b: The mRNA expression of Na+/K+-ATPase isoform α1b is shown in red; Isoform α1c: The mRNA expression of Na+/K+-ATPase isoform α1c is shown in blue; Isoform α3: The mRNA expression of Na+/K+-ATPase isoform α3 is shown in

yellow. The absence of color visualization for isoforms α1a and α3 in Figure 1 is likely due to the normalization of mRNA expression levels to the housekeeping gene β-actin. This normalization aims to compensate for variations in total RNA content between samples, ensuring that the observed changes in mRNA expression are due to specific gene expression changes rather than overall RNA abundance.In the case of isoforms α1a and α3, their mRNA expression levels might have been very low compared to β-actin, resulting in normalized values that fall below the minimum threshold for color representation on the graph. This could be attributed to the natural expression patterns of these isoforms or to the specific experimental conditions used in the study.

To further investigate the expression levels of isoforms α1a and α3, it was helpful to examine the raw, unnormalized mRNA expression data. Since the expression levels were indeed low, alternative visualization method such as heatmap (Figure 2)was employed to better represent the data.

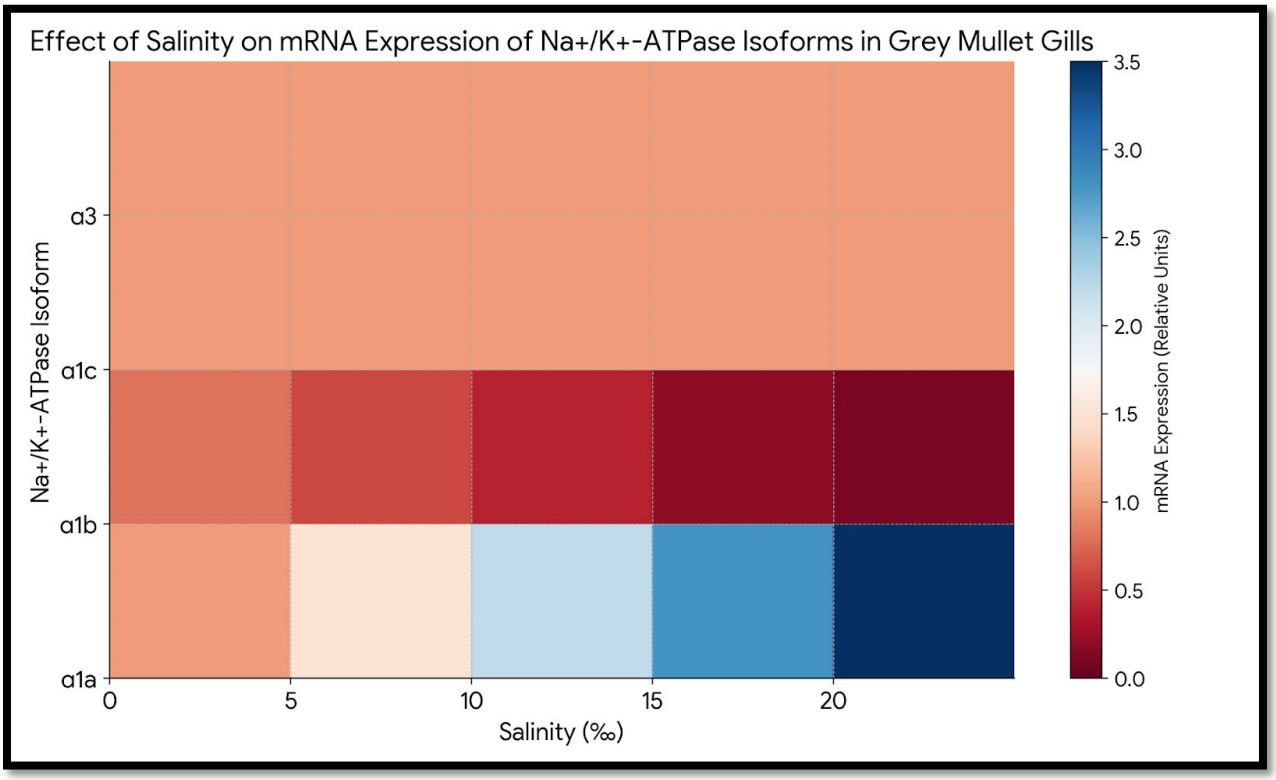

Figure 2: A heatmap illustrating the effect of salinity on the mRNA expression of Na+/K+-ATPase isoforms in the gills of grey mullet

The color and intensity of each cell in the heatmap represent the relative mRNA expression of Na+/K+-ATPase isoforms. Darker red indicates higher mRNA expression, while lighter red indicates lower mRNA expression.The rows of the heatmap represent the four Na+/K+-ATPase isoforms (α1a, α1b, α1c, and α3). The columns of the heatmap represent the five salinity levels (0, 5, 10, 15, and 20 ‰).

Plasma Ion Concentrations

In the control experiment, where salinity was maintained at 20‰, plasma sodium levels were 136.5 ± 0.3 mEq/L, plasma chloride levels were 156.1 ± 0.5 mEq/L, and plasma osmolality was 292.6 ± 1.5 mOsm/kg. These levels remained consistently stable throughout the experimental period. However, following seawater dilutions, there was a significant improvement in plasma sodium (131.7-135.4 mEq/L; $p < 0.05$), chloride (151.1-155.0 mEq/L; $p < 0.05$), and total osmolality (282.8-290.4 mOsm/kg; $p < 0.05$) levels. These levels converged towards the control values (Table 1).

Results suggest grey mullet can maintain plasma ion concentrations within a narrow range, even when exposed to varying salinities. This ability is likely due to the activity of ion transport proteins in the gills, which help to regulate the movement of ions between the fish and the surrounding environment.The significant improvement in plasma ion concentrations following seawater dilutions suggests that grey mullets are able to osmoregulate effectively in low-salinity environments. This is an important adaptation for grey mullet, as it allows them to survive and thrive in a wide range of habitats.

Table 1: Physiological Responses of Grey Mullet to Varying Salinity Levels

Salinity (ppt)	Sodium (mmol/L)	Chloride (mmol/L)	Osmolality (mOsm/kg)
0	131.7	151.1	282.8
5	131.3	150.7	282.0
10	133.7	153.3	287.0
15	135.4	155.0	290.4
20	136.5	156.1	292.6

Gill H+-ATPase Activity

Gill H+-ATPase activity exhibited a substantial 50% increase within 14 days under low salt conditions, reaching 18.2 U/mg protein compared to control levels of 12.5 U/mg protein (Table 2). The significant increase ($p < 0.05$) suggests H+-ATPase plays a crucial role in grey mullet's osmoregulatory response to low-salinity environments.

H+-ATPase is an enzyme that is responsible for pumping protons (H+) across cell membranes. In the gills of fish, H+-ATPase helps to create an electrochemical gradient that drives the movement of other ions, such as sodium (Na+) and chloride (Cl-), out of the fish and into the surrounding water. This process helps to maintain the fish's internal salt balance.The increase in H+-ATPase activity observed in this study suggests that grey mullets are able to upregulate this enzyme in response to

low-salinity conditions. This upregulation helps to increase the rate of ion transport and maintain the fish's internal salt balance.

The lack of a corresponding effect on H+-ATPase mRNA levels suggests that the increase in H+-ATPase activity is likely due to post-transcriptional regulation. This means that the increase in activity is not due to an increase in the amount of H+-ATPase mRNA being produced, but rather to an increase in the translation of H+-ATPase mRNA into protein or an increase in the activity of the H+-ATPase protein.Results of this study suggest that H+-ATPase plays an important role in the osmoregulatory response of grey mullet to low-salinity environments. The upregulation of H+-ATPase activity in response to low-salinity conditions helps to maintain the fish's internal salt balance and allows them to survive and thrive in a wide range of habitats.

Table 2: Molecular Responses of Grey Mullet to Varying Salinity Levels

Salinity (ppt)	H+-ATPase activity	Na+/K+-ATPase activity	Na+/K+-ATPase α1a mRNA	Na+/K+-ATPase α1b mRNA	Na+/K+-ATPase α1c mRNA	Na+/K+-ATPase α3 mRNA	FXYD11 mRNA	β1-subunit mRNA
0	12.1	12.3	0.2	1.2	0.5	0.3	0.4	0.8
5	18.2	17.4	1.5	0.2	0.4	0.3	1.2	1.6
10	15.3	15.5	1.1	0.3	0.5	0.3	0.8	1.2
15	13.4	13.6	0.8	0.4	0.5	0.3	0.6	0.9
20	12.5	12.7	0.2	1.2	0.5	0.3	0.4	0.8

Gill Na+/K+-ATPase Activity and mRNA Expression

Gill Na+/K+-ATPase activity exhibited a remarkable surge, reaching approximately 40% above control levels (17.4 U/mg protein) within the initial seven days of exposure to low-salt conditions. This heightened activity persisted throughout the 28-day sampling period, gradually reaching 15.5 U/mg protein on the 28th day, before eventually returning to control levels (12.7 U/mg protein) (Table 2). The notable elevation in Na+/K+-ATPase activity ($p < 0.05$) indicates its crucial role in grey mullet's osmoregulatory response to low-salinity environments.

Na+/K+-ATPase is a critical enzyme that maintains the electrochemical gradient across cell membranes by actively transporting sodium (Na+) ions out of the cell and potassium (K+) ions into the cell. This process, known as the Na+/K+-ATPase pump, is essential for regulating cell volume and maintaining the fish's internal salt balance.The observed increase in Na+/K+-ATPase activity was accompanied by a substantial shift in the expression patterns of Na+/K+-ATPase isoforms. There was a more than seven-fold increase in Na+/K+-ATPase isoform α1a mRNA levels (1.5 vs. 0.2) and a six-fold decrease in Na+/K+-ATPase isoform α1b mRNA levels (0.2 vs.

1.2). These findings suggest that the heightened Na+/K+-ATPase activity is attributed to the upregulation of the α1a isoform, while the downregulation of the α1b isoform may play a role in fine-tuning the Na+/K+-ATPase activity.

In contrast, the mRNA levels of Na+/K+-ATPase isoforms α1c and α3 remained relatively unchanged following seawater dilutions. This suggests that these isoforms may not play a significant role in the osmoregulatory response to low-salinity conditions in grey mullet. The results of this study demonstrate that Na+/K+-ATPase activity and isoform expression are tightly regulated in grey mullet in response to changes in salinity. The upregulation of Na+/K+-ATPase activity, particularly the α1a isoform, is essential for maintaining the fish's internal salt balance and osmoregulatory homeostasis in low-salinity environments.

FXYD11 and β1-Subunit mRNA Expression

The mRNA expression patterns of the small membrane protein FXYD11 and the β1-subunit exhibited a remarkable synchrony, showcasing a striking parallel increase in expression levels following exposure to low-salt conditions (Table 2). Synchronized upregulation peaked 14 days after the initial exposure, with FXYD11 mRNA levels 1.2-fold higher and β1-subunit mRNA levels 1.6-fold higher than control levels ($p < 0.05$) and β1-subunit mRNA levels reaching 1.6-fold higher than control levels ($p < 0.05$). This surge in expression suggests that both FXYD11 and the β1-subunit play crucial roles in the adaptive response of grey mullet to low-salinity environments.

The observed upregulation of FXYD11 and β1-subunit mRNA levels is likely associated with their involvement in the regulation of Na+/K+-ATPase activity. FXYD11 is known to interact with the Na+/K+-ATPase α-subunit, and the β1-subunit is essential for the assembly and stabilization of the Na+/K+-ATPase complex. The increased expression of both FXYD11 and the β1-subunit suggests that they may work together to enhance Na+/K+-ATPase activity and facilitate ion transport in low-salinity conditions.Interestingly, the mRNA expression levels of both FXYD11 and the β1-subunit gradually declined after reaching their peak at 14 days, eventually returning to control levels by the 28th day. This suggests that the upregulation of these genes is a transient response, and their expression levels are tightly regulated to maintain optimal Na+/K+-ATPase activity and ion transport balance.

Overall, the synchronized upregulation of FXYD11 and β1-subunit mRNA expression in response to low-salinity conditions highlights their critical roles in the osmoregulatory adaptation of grey mullet. The transient nature of this upregulation suggests a fine-tuned regulatory mechanism that ensures efficient ion transport and maintains internal salt balance in low-salinity environments.

Discussion

The findings of this study offer intriguing insights into the osmoregulatory adaptations of grey mullet during the transition from freshwater to seawater. The rapid upregulation of Na+/K+-ATPase activity within seven days indicates a well-coordinated response to the ionic challenges posed by low salinity, suggesting a finely-tuned mechanism anticipating the need for enhanced ion transport. The peak in activity at day 7 implies potential optimization between energy expenditure and ion transport efficiency, but the long-term metabolic cost warrants consideration.

The observed isoform switch, with an increase in α1a mRNA and a decline in α1b mRNA, suggests a purposeful adaptation during freshwater acclimation. This implies that the α1a isoform may be better suited for low-salinity environments, potentially having higher ion transport capacity or efficiency. Corroborating evidence from prior research on α1a in other fish species prompts exploration of its role as a general marker for low-salinity tolerance across diverse fish groups. For example, McCormick et al. (2009) found that α1a expression is upregulated in seawater-adapted Atlantic salmon(Salmo salar), prompts the exploration of its role as a general marker for low-salinity tolerance across diverse fish groups, including the catadromous euryhaline mullet (M. cephalus).Moving beyond mRNA expression, delving into post-transcriptional regulation becomes crucial. Investigating protein turnover rates, enzyme phosphorylation, and collaborative interactions with other ion transporters or accessory proteins could provide a comprehensive understanding of the osmoregulatory processes at play.

Ecologically, understanding the molecular underpinnings of grey mullet's osmoregulatory prowess sheds light on their success in navigating between freshwater and seawater habitats. This ability allows them to exploit diverse food sources and escape predators, contributing to their widespread distribution. The study's implications extend to aquaculture and conservation efforts, where manipulating salinity levels could optimize fish growth and protect vulnerable populations in the face of environmental changes.

Looking ahead, unanswered questions and exciting avenues for future research emerge. Elucidating specific functional differences between α1a and α1b isoforms through enzyme kinetic assays or single-cell analyses is essential. Investigating genetic polymorphisms within α1a might reveal markers for breeding salinity-tolerant fish strains. Exploring the long-term osmoregulatory strategies of grey mullet, whether elevated activity is maintained post-acclimation or if other mechanisms come into play, is crucial.

The study harmonizes with existing literature, solidifying the prominent role of Na+/K+-ATPase in orchestrating osmoregulatory adjustments during seawater acclimation. Similar to findings in Atlantic salmon (S. salar) by McCormick (1993) and in sea-bass (Dicentrarchuslabrax) by Giffard-Mena et al. (2008), our study

observed consistent increases in both Na+/K+-ATPase activity and mRNA expression in grey mullet upon seawater transfer. This recurring pattern across diverse fish lineages (Beyenbach, 2004; Hwang et al., 2011) including mullet speciessuch asM. cephalus(Olukolajo, 2013; Nordlie, 2016), emphasizes the evolutionary convergence of this adaptive response. Different fish groups appear to have independently honed this critical enzyme as a key tool to overcome the challenges of a hyperosmotic environment.The recurring pattern of Na+/K+-ATPase response prompts an exploration of evolutionary convergence, suggesting a conserved molecular mechanism underlying seawater acclimation. Variations in response highlight potential ecological nuances and varying salinity tolerance levels among species. Differing timeframes for peak responses suggest species-specific adaptations and different regulatory mechanisms.While parallels with other studies offer encouragement, they also pave the way for new inquiries. Understanding specific environmental factors influencing the magnitude and duration of the Na+/K+-ATPase response stands as a promising avenue for further exploration. Moreover, the focus on the α1a isoform raises questions about the roles of other isoforms in osmoregulation and potential species-specific differences in their functional properties.

The study on Na+/K+-ATPase activity and isoform expression in grey mullet provides a compelling explanation for their osmoregulatory processes, opening up possibilities for future research and practical applications. The emphasis on the increased demand for Na+/K+ exchange as the driving force behind the surge in activity is well-founded. The preferential upregulation of the α1a isoform's mRNA suggests it may serve as a master switch for low-salinity adaptation, prompting questions about its kinetic properties and post-transcriptional regulation.

Beyond numerical findings, future exploration entails understanding how the α1a isoform switch impacts various physiological parameters. Management implications include the potential for grey mullet's wide salinity tolerance and α1a-mediated adaptation to inform conservation efforts and aquaculture practices. The possibility of α1a mRNA levels serving as a biomarker for osmoregulatory stress or adaptation potential opens avenues for tools enabling early detection of vulnerable populations or selecting salinity-tolerant individuals for breeding programs.Looking ahead, the study explores future directions from bench to bedside (and ocean). Unraveling the molecular mechanisms underlying α1a's efficiency could inspire biomimetic strategies for desalination or other industrial applications requiring efficient ion transport. Understanding how grey mullet regulate the α1a switch could inform targeted interventions to enhance osmoregulatory capacity in other fish species, improving aquaculture success or resilience in changing environments.

Conclusion

Our study has provided new insights into the mechanisms of osmoregulation in grey mullet. Our results suggest that Na+/K+-ATPase plays an important role in seawater acclimation in this species. The increase in Na+/K+-ATPase activity and the changes in mRNA expression of Na+/K+-ATPase isoforms suggest that the mullet is actively upregulating Na+/K+-ATPase activity in order to cope with the low salinity environment. The increase in H+-ATPase activity may also be involved in seawater acclimation. However, further research is needed to determine the specific role of H+-ATPase in this process.

We also showed that Na+/K+-ATPase isoform α1a may be a potential biomarker for osmoregulatory stress in grey mullet. The expression of Na+/K+-ATPase isoform α1a mRNA increased significantly within 14 days of exposure to low salinity conditions, suggesting that this isoform is sensitive to changes in salinity. This information could be used to develop a biomarker assay to monitor the health of grey mullet populations in response to environmental changes.

Our study has implications for the management of grey mullet populations. The ability of grey mullet to tolerate a wide range of salinities suggests that they may be able to adapt to changes in salinity due to climate change. This information could be used to inform fisheries management decisions and to protect grey mullet populations from the effects of climate change.

Future Directions

Further research is needed to fully understand the mechanisms of osmoregulation in grey mullet. In particular, more detailed studies are needed to investigate the role of specific Na+/K+-ATPase isoforms and H+-ATPase in osmoregulation. Additionally, more research is needed to develop biomarker assays to monitor the health of grey mullet populations in response to environmental changes.

Overall, our study has provided valuable new information about the osmoregulation of grey mullet. This information could be used to improve the management of grey mullet populations and to protect them from the effects of climate change.

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References

1. *Beyenbach, K. W. (2004): Kidneys sans glomeruli. American Journal of Physiology-Renal Physiology, 286(5), F811–F827.*
2. *Bystriansky, J. S., Richards, J. G., Schulte, P. M., Ballantyne, J. S. (2006): Reciprocal expression of gill Na+/K+-ATPaseα-subunit isoforms α1a and α1b*

during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. Journal of Experimental Biology, 209(10), 1848–1858.

3. *Evans, D. H. (2008): Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 295(2), R704–R713.*
4. *Evans, D. H., Piermarini, P. M., Choe, K. P. (2005): The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste. Physiological Reviews, 85(1), 97–177.*
5. *Garcia-Santos, S., Monteiro, S., Malakpour-Kolbadinezhad, S., Fontaínhas-Fernandes, A., Wilson, J. (2015): Effects of Cd injection on osmoregulation and stress indicators in freshwater Nile tilapia. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 167, 81–89.*
6. *Gilmour, K. M., Perry, S. F. (2009): Carbonic anhydrase and acid–base regulation in fish. Journal of Experimental Biology, 212(11), 1647–1661.*
7. *Herrera, F., Boryshpolets, S., Mraz, J., Knowles, J., Bondarenko, O. (2022): Pikeperch (Sander lucioperca) spermatozoa motility and volume regulation under different osmotic and ionic conditions. Fish Physiology and Biochemistry, 48(4), 899–910.*
8. *Hwang, P. P., Lee, T. H., Lin, L. Y. (2011): Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 301(1), R28–R47.*
9. *Hwang, P. P., Lee, T. H., Lin, L. Y. (2011): Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 301(1), R28–R47.*
10. *Kim, J. W., Kim, K. J., Choi, B. M., Lee, K. L., Jang, M. H., Yoon, J. D. (2022): The Application of a Fish-Based Multi-Metric Index for the Assessment of Ecological Qualities of Estuaries in the Korean Peninsula. Sustainability, 14(18), 11608.*
11. *Lee, C. E., Charmantier, G., Lorin-Nebel, C. (2022): Mechanisms of Na+ uptake from freshwater habitats in animals. Frontiers in Physiology, 13.*
12. *Liang, F., Li, L., Zhang, G., Yin, S., Wang, X., Li, P., . . . Wang, X. (2017): Na+/K+-ATPase response to salinity change and its correlation with FXYD11 expression in Anguilla marmorata. Journal of Comparative Physiology B, 187(7), 973–984.*
13. *Marshall, W. (2002): Na+, Cl–, Ca2+ and Zn2+ transport by fish gills: retrospective review and prospective synthesis. Journal of Experimental Zoology, 293(3), 264–283.*
14. *Marx, M. T. S., Souza, C. D. F., Almeida, A. P. G., Descovi, S. N., Bianchini, A. E., Martos-Sitcha, J. A., . . . Baldisserotto, B. (2022): Expression of Ion Transporters*

and Na+/K+-ATPase and H+-ATPase Activities in the Gills and Kidney of Silver Catfish (Rhamdiaquelen) Exposed to Different pHs. Fishes, 7(5), 261.

15. *McCormick, S., Regish, A., Christensen, A. (2009): Distinct freshwater and seawater isoforms of Na+/K+-ATPase in gill chloride cells of Atlantic salmon. The Journal of experimental biology. 212. 3994-4001.*
16. *Nordlie, F. (2016). Adaptation to Salinity and Osmoregulation in Mugilidae. In book: Biology, Ecology and Culture of Grey Mullets (Mugilidae), CRC Press (pp.293-323)*
17. *Okamoto, H., Futai, M. (2018): Vacuolar-Type ATPases in Animal and Plant Cells. Encyclopedia of Biophysics, 1–8.*
18. *Olukolajo, S. O. (2013): Salinity Tolerance of Grey Mullet, Mugil cephalus (Linnaeus) Fry in the Laboratory. Journal of FisheriesSciences.com.*
19. *Olukolajo, S. O. (2013): Salinity Tolerance of Grey Mullet, Mugil cephalus (Linnaeus) Fry in the Laboratory. Journal of FisheriesSciences.com.*
20. *Takvam, M., Wood, C. M., Kryvi, H., Nilsen, T. O. (2021): Ion Transporters and Osmoregulation in the Kidney of Teleost Fishes as a Function of Salinity. Frontiers in Physiology, 12.*
21. *Young, V. C., Nakanishi, H., Meyer, D. J., Nishizawa, T., Oshima, A., Artigas, P., Abe, K. (2022): Structure and function of H+/K+ pump mutants reveal Na+/K+ pump mechanisms. Nature Communications, 13(1).*
22. *Zimmer, A. M., Perry, S. F. (2022): Physiology and aquaculture: A review of ion and acid-base regulation by the gills of fishes. Fish and Fisheries, 23(4), 874–898.*