



(REVIEW ARTICLE)



A review on the activity of Na⁺/K⁺-ATPase in branchial ionocytes and its role in salinity adaptation among diadromous species

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Abstract

Freshwater and saltwater environments have opposite osmotic gradients. In freshwater, organisms tend to uptake ions from the surrounding, while in saltwater, they need to excrete the excess ions. Branchial ionocytes play a significant role in euryhaline teleost species that migrate to different salinity gradients as part of their life cycle. Ionocytes are highly dependent on the activity of Na⁺/K⁺-ATPase (NKA) in salinity adaptation. Thus, this review article determines the factors affecting NKA activity in ionocytes and its role in salinity adaptation among diadromous species. Several findings suggest that NKA activity is affected by various factors such as hormones, nutrition, and gene expression. Ionocytes enriched with NKA have many functions in diadromous depending on their types, location, size, and number in branchial necessary for these organisms to carry out their biological function in ionic-regulatory processes.

Keywords: Osmoregulation; Euryhaline; Catadromous; Hypertonic; Chloride cells

1. Introduction

Several species of fish utilizing different osmotic gradient habitats for forage, reproduction, development, settlement, refuge, and others. Organisms that migrate from hypoosmotic to hyperosmotic habitats that have nothing to do with their reproduction are diadromous. These organisms can shift the environment from freshwater to marine water and vice versa without compromising their health and survival. Their ecological adaptation depends primarily on their ability to osmoregulate. The ability of their gills to excrete excess ions and uptake salts from the water column determine their adaptability behavior. These ions excretion and uptake depend on the ionocytes and its enzymes activity, such as the Na⁺/K⁺-ATPase (NKA). This enzyme has many isoforms, and their expression depends on the species and salinity.

In seawater environment, teleost fishes excrete excess sodium and chloride from the body fluid using specialized, mitochondrion-rich cells in the gill epithelium. This cell type was first identified as “chloride secreting cell” in the gills of seawater-acclimated European eel *Anguilla anguilla* [1] and then has often been referred to as “chloride cell” in many teleost species. More recently, the terms “mitochondrion-rich cell” or “ionocyte” have been preferred, because this cell type is known to be involved not only in chloride secretion in seawater, but also in multiple functions such as ion uptake in freshwater, acid-base regulation, and ammonia excretion. This review adopts “ionocyte” in place of “chloride cell” or “mitochondria-rich cell” throughout the text [2].

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The gill is the main respiratory organ in most fish but also plays an important role in ion regulation. NKA plays an important role in ion transport in teleost fish and elasmobranch [3]. This ion pump is found in the basolateral membrane of the ionocytes and can be identified with an antibody against its α -subunit. The NKA consists of three subunits; α , β , and γ . The α - subunit contains sites for cations, ATP, and ouabain. Thus it is responsible for the catalytic and ion regulatory capacity of the NKA, while the β -subunit associated with the protein maturation and anchoring of the enzyme complex in the cell membrane. The molecular weight of the catalytic α -subunit is about 100 kDa, while the smaller glycosylated β -subunit is about 55 kDa. The fish gill NKA is involved in ion regulation in both freshwater and seawater. In seawater acclimated fishes, the basolateral NKA energizes ion secretion by creating an electrochemical gradient used by the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter and apical cystic fibrosis transcellular secretion of Cl^- ; and paracellular secretion of Na^+ . In freshwater acclimated fishes, the basolateral NKA is probably also involved in driving uptake of Na^+Cl^- , possibly in conjunction with an apical V-type H^+ -ATPase, via apical Na^+ channels and $\text{Cl}^-/\text{HCO}_3^-$ exchangers. The NKA is an essential participant in maintaining ionic concentrations and body fluids within appropriate physiological limits for survival in different salinity. The activity of NKA is dependent on environmental ion concentration. In teleost fishes, the gill is a most important organ and plays a principal role in the maintenance of ion homeostasis in both freshwater and seawater acclimated fish [4].

2. Effect of salinity in branchial Na^+/K^+ -ATPase (NKA) activity

Changes in NKA activity and abundance associated with environmental salinity were observed in the gills of the Atlantic stingray *Dasyatis sabina*. The activity and abundance of NKA and the number of ionocytes decreased in the gills when freshwater stingrays were acclimated to seawater. Further reduction in branchial activity and abundance of NKA and the number of ionocytes were also observed [5]. However, higher NKA activity was reported in golden grey mullet *Liza aurata* during adaptation to different salinity levels. It showed that mullet acclimated to 36 and 46 ppt have higher NKA activity when compared to 12 ppt. This suggests the high degree of adaptability of this species to a wide range of salinity [6]. Another increasing NKA activity was also reported in spottedtail goby *Synechogobius ommaturus* when abruptly introduced from 10 ppt to higher salinity levels (40 and 50 ppt). This indicates that this species could also adapt rapidly and homeostasis in a wide range of salinities that is from freshwater to salinity 30 ppt [7]. The same result in NKA activity was also reported in Nile tilapia *Oreochromis niloticus* reared in 30 ppt [8]. While in the case of milkfish *Chanos chanos*, when transferred from a local fish farm into seawater, it exhibited the lowest NKA activity. However, the natural seawater dwelling milkfish express higher NKA activity when transferred into brackishwater and freshwater [9]. Another euryhaline species, when acclimated to different salinity, Silver moony *Monodactylus argenteus* exhibited a great number of NKA cells when acclimated to freshwater, which exceeded that of the brackishwater and seawater individuals. Moreover, the size of the NKA cells of freshwater and seawater individuals showed larger than that of brackishwater fish [10]. This different pattern of NKA activity with regards to salinity adaptation was also reported in *Oryzia* species. This result was to prove the hypothesis that the lowest level of gill NKA activity occurs in the environment with salinity close to the primary natural habitat of the studied species [11].

Other effects of salinity on the NKA activity may be indirect, such as it affects the lipid structure of an organism. This was reported in anadromous species, particularly in developing lamprey when they held in full-strength seawater. Disrupted NKA activity was observed due to the lipid composition of the gill cells' membrane. This is because the fatty acid profile of the basolateral membrane's phospholipids suffered a restructure by increasing either saturation or the ratio between oleic acid and eicosapentaenoic acid. Simultaneously, the basolateral membrane's cholesterol content revealed a positive correlation with NKA activity in a high salinity environment [12].

3. Role of Na^+/K^+ -ATPase (NKA) in seawater acclimation

During the acclimation of spottedtail goby, an increased NKA activity, serum osmolality and electrolytes concentration were observed. This phenomenon marks the first phase of acclimation to a hyperosmotic environment in which, in most cases, euryhaline underwent two phases, such as initiated by a fast increase in gill-ion fluxes followed by an increase in serum electrolytes and osmolality. This is then followed by a regulatory period in which gill NKA activity increases [7]. NKA is one of the transport proteins thought to be involved in chloride secretion in teleost fish. That is why the level of this transporter is high in saltwater-acclimated fish. This enzyme is present in ionocytes which helps for chloride secretion as observed in the case of Hawaiian goby *Stenogobius hawaiiensis*, when acclimated to 20 and 30 ppt. Following seawater acclimation, the gill tissue of Hawaiian goby increased 46% in the amount of NKA. This indicates that transporters are involved in ion uptake, NKA is known to involve in moving Na^+ from the interior of the ionocytes

into the blood, as well as providing ionic and electrical gradients used by other transporters involved in ion uptake [13]. NKA has specific isoforms that determine function distinction of ion absorptive and secretory during seawater acclimation in teleost fishes. In the model presented, a basolaterally located NKA transporter 3 Na⁺ outward in exchange for 2K⁺, creating low intracellular Na⁺ and a highly negative charge within the cell; the Na⁺ gradient is used to transport Na⁺, K⁺ and 2Cl⁻ into the cell through a basolateral Na⁺/K⁺/2Cl⁻ cotransporter 1; Cl⁻ then leaves the cells down an electrical gradient through an apical cystic fibrosis transmembrane conductance regulator (CFTR); Na⁺ is transported back outside the cells via NKA, and then leaves through a paracellular pathway between ionocytes and adjacent smaller cells known as accessory cells. The NKA enzyme is composed of two major subunits, α and β . The α -subunit is the main catalytic unit and contains all of the critical binding sites for ATP, Na⁺, K⁺. The β -subunit is a glycosylated polypeptide that assists in folding and positioning of the protein into the basolateral plasma membrane and is essential for its normal activity. A third subunit termed γ , also known as FXD, is not necessary for the catalytic function of NKA but acts to adapt the kinetic properties of sodium and potassium transport for the functions of different cell types [2].

4. Gene expression of Na⁺/K⁺-ATPase (NKA) isoforms in freshwater and seawater acclimated organisms

There are at least four α isoforms expressed in vertebrates and have different kinetic properties for Na⁺, K⁺, and ATP binding. These unique properties, along with their cell-specific distribution, suggest that the NKA α isoforms have distinct physiological function and regulation [2]. One of these physiological functions is the ionoregulation process in teleost fish. As manifested in most euryhaline species, the amount of mRNA in gill tissue increased with the level of environmental salinity. Expression of NKA α -subunit in gills of tilapia *Oreochromis mossambicus* indicates that the amount of NKA α -subunit in gills of seawater-adapted tilapia was significantly higher than that in freshwater-adapted ones. These findings indicate that the salinity dependent stimulation of mRNA of gill NKA α -subunit is associated with corresponding stimulation at the protein level. This provides direct evidence of enhanced transcription and translation of the NKA α -subunit gene upon salinity challenge [14]. The same results were also observed in the same species during the freshwater acclimation period. A significant decrease in the NKA isoform α 1-mRNA abundance was detected at 6 hrs post-transfer. This was followed by a decrease of α 1-protein amounts from 6 hrs until 24 hrs post-transfer. This rapid response as concurrent changes in branchial NKA expression is thought to improve the osmoregulatory capacity of tilapia in acclimation from hypertonic seawater to hypotonic freshwater [15]. The NKA α 1 paralogs express in Sacramento splittail *Pogonichthys macrolepidotus* is to better cope with salinity challenges [16]. Expression of NKA α -subunit isoform was also documented in gill tissue of diadromous freshwater shrimp *Macrobrachium olfersii*, following acclimated to 21 ppt and freshwater for ten days. This species is found in neotropical river systems but still dependent on brackishwater for its lengthy, larval developmental sequence [17]. Similar findings were also reported in other diadromous, freshwater palaemonid shrimps, *M. amazonicum* in which microsomal fractions possess \approx 2-fold less NKA α -subunit than *M. olfersii*, consistent with a 2.6-fold lower specific activity which suggests distinct biochemical adaptations in these related species [18]. This characteristic is also true in anadromous species such as salmon. This was found out when the NKA 1a and 1b protein isoform of Atlantic salmon *Salmo salar* were characterized. The findings suggest that the abundance of gill NKA 1a was high in freshwater and became nearly undetectable after seawater acclimation. While NKA1b was present in small amounts in freshwater and increased 13-fold after seawater acclimation. However, both NKA isoforms were detected only in ionocytes. NKA 1a was located in both filamental and lamellar ionocytes in freshwater, whereas in seawater, it was present only as a faint background in filamental ionocytes. In freshwater, NKA 1b was found in a small number of filamental ionocytes, and after seawater acclimation, it was found in all ionocytes on the filament and lamellae. Double simultaneous immunofluorescence indicated that NKA 1a and 1b are located in different ionocytes in freshwater. In many ionocytes in seawater, NKA 1b was present in greater amounts in the subapical region than elsewhere in the cell. The combined patterns in abundance and immunolocalization of these two isoforms can explain the salinity-related changes in total NKA and ionocyte abundance. This freshwater and seawater isoforms of NKA-subunit are both presents in the gills of Atlantic salmon and that they exist in distinct ionocytes [19]. The expression of two gill NKA α -subunit isoforms in relationship with salinity acclimation was again observed in a cichlid fish, Mozambique tilapia. Transfer of freshwater-acclimated fish to seawater resulted in a marked reduction in α -1a expression within 24 h and a significant increase in α -1b expression with maximum levels attained seven days after the transfer. In contrast, transfer of seawater acclimated fish to freshwater induced a marked increase in α -1a expression within two days, while α -1b expression decreased significantly after 14 days. These findings strongly suggest that α -1a is exclusively associated with branchial ion uptake in freshwater, while α -1b is involved in ion extrusion in seawater [20]. Similar results were also observed in other euryhaline species during salinity acclimation.

This showed when three-spined stickleback *Gasterosteus aculeatus* had no significant change in mRNA expression in either NKA1 A3 isoforms following freshwater or seawater acclimation. However, a significant increase in the NKA1A1 isoform was observed when acclimating to seawater, and a significant decrease was seen in the NKA1A1 isoform when acclimating to freshwater, suggesting NKA1A1 plays a role in ion secretion in marine habitats. This confirms that three-spined stickleback landlocked populations have retained the ability to acclimate to seawater [21]. These multiple NKA α -subunit isoforms expression in response to salinity acclimation was also analyzed using eels and medaka. It was found out that in eels, the commonly-reported α -1a and α -1b isoforms were absent while α -1c isoform was diversified instead (α -1c-1, α -1c-2, α -1c-3, α 2, and α 3 in eels). Phylogenetic estimation indicated that independent duplication events generated the α 1a and α 1b isoforms from salmon, tilapia, and medaka, and thus, they are paralogous isoforms. Reexamination of expression changes of known isoforms after salinity challenge revealed that the isoforms selected as predominant seawater-types varied among teleost lineages. Diversification of α 1 isoforms occurred by various types of gene duplication, or by alternative transcription among tandem genes to form chimeric transcripts, but there is no trend for more α 1 copies in euryhaline species. Recent data suggest that the isoform switching between freshwater (α 1a predominates) and seawater (α 1b predominates) that occurs in salmonids is not universal in teleosts. Instead, in eels, α 1c-1 was the major α -subunit upregulated gill in seawater. Localization of both NKA mRNA and protein showed consistent upregulation in gill in seawater eels. In medaka, α 1b was upregulated in seawater in the anterior intestine, while most other α -subunit isoforms were less responsive to salinity changes [22]. The same phenomenon was observed when freshwater spotted green pufferfish *Tetraodon nigroviridis* were transferred directly from a local aquarium to freshwater (0 ppt), brackish water (15 ppt) and seawater (35 ppt) conditions. Results in branchial NKA expression showed that the seawater-acclimated fish group was about 1.6-fold higher than freshwater-acclimated fish or 6.3-fold higher than the brackishwater-acclimated fish. The specific activity of gill NKA of fish acclimated to seawater was significantly higher than that of fish acclimated to brackishwater and freshwater. However, there was no significant difference between gill NKA activity of brackishwater and freshwater-acclimated fish. The significance of the role played by branchial NKA in ion transport. The affinities of gill NKA in euryhaline teleosts to various concentrations of ions in environments of varied salinities may thus be different and lead to changes in NKA activity. According to the current model of salt excretion in gills of seawater teleosts, the driving force for Cl^- secretion is the Na^+ electrochemical gradient established by NKA, and Na^+ secretion occurs down its electrochemical gradient via a cation-selective paracellular pathway. The highest level of NKA activity makes the pufferfish secrete excess salts efficiently and thus acclimate smoothly in seawater [23]. Adaptively differential expression of NKA- α in the gill of Chinese mitten crabs *Eriocheir japonica sinensis* was also observed. NKA had the most significant differential expression level when Chinese mitten crabs were changed in salinity. They are considered the key enzyme during the transition from the marine environment to freshwater [24].

There are five identified NKA α -isoforms in rainbow trout and characterized their expression pattern in gills following seawater transfer. Three of these isoforms were closely related to other vertebrate α 1 isoforms (designated α 1a, α 1b, and α 1c), one isoform was closely related to α 2 isoforms (designated α 2), and the fifth was closely related to α 3 isoforms (designated α 3). NKA α 1c- and α 3-isoforms were present in all tissues examined, while all others had tissue specific distributions. Four NKA α -isoforms were expressed in trout gills (α 1a, α 1b, α 1c, and α 3). NKA α 1c- and α 3-isoforms were expressed at low levels in freshwater trout gills, and their expression pattern did not change following transfer to 40% or 80% seawater. NKA α 1a and α 1b were differentially expressed following seawater transfer. Transfer from freshwater to 40% and 80% seawater decreased gill NKA α 1a mRNA, while transfer from freshwater to 80% seawater caused a transient increase in NKA α 1b mRNA. These changes in isoform distribution were accompanied by an increase in gill NKA enzyme activity by ten days after transfer to 80% seawater, though no significant change occurred following transfer to 40% seawater. Isoform switching in trout gills following salinity transfer suggests that the NKA α 1a and α 1b-isoforms play different roles in freshwater and seawater acclimation, and that assays of NKA enzyme activity may not provide a complete picture of the role of this protein in seawater transfer [25].

5. Types, location, number, and size of branchial ionocytes and its role in salinity acclimation

The number, size, and location of branchial ionocytes are known to alter with changes in environmental salinity. Transfer of euryhaline fish from freshwater to saltwater generally causes a proliferation of branchial ionocytes. There are two distinct types of ionocytes observed in filament and lamellar epithelia of several species of diadromous and euryhaline teleosts. Changes in morphology of ionocytes following transfer to saltwater and freshwater suggest that filament and lamellar ionocytes are important in saltwater and freshwater osmoregulation. The changes in ionocyte morphology of juvenile Australian snapper *Pagrus auratus* were observed, following a rapid transfer from ambient

seawater (30 ppt) to hyperosmotic (45 ppt) and diluted hyperosmotic (15 ppt) environments. It was also noted that branchial ionocytes were present in both filament and lamellar epithelia of snapper held in all salinity treatments. However, in 4 ppt, the number of filament and lamellar ionocytes did not change, but filament ionocytes were more abundant than lamellar ionocytes. In contrast, filament ionocytes had increased in size after 72 h, and by 168 h after transfer from 30 ppt were 1.4-fold larger than the initial size. In 15 ppt, the number of filament ionocytes and the size of both filament and lamellar ionocytes had decreased after 72 h. These findings demonstrate that snapper can osmoregulate in a wide range of salinity and provide indirect evidence that both filament and lamellar ionocytes are responsible for excretion of excess salt in hyperosmotic environments [26].

As reviewed, seawater-type and freshwater-type ionocytes have been identified based on their shape, location, and response to different ionic conditions. Among the freshwater-type ionocytes, subpopulations are emerging that are implicated in the uptake of Na^+ , Cl^- and Ca^{2+} , respectively, and can be distinguished by their shape of an apical crypt. The major function of the seawater ionocyte is the transcellular secretion of Cl^- , which is accomplished by four major channels and transporters: (1) Cystic fibrosis transmembrane conductance regulator Cl^- channel, (2) NKA, (3) $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter and (4) a K^+ channel. There are two types of ionocytes, and these are α and β ionocytes that differ in their location and structure of apical membranes. The β ionocytes have been postulated to be a freshwater-specific subtype. Both have an apical pit, but the freshwater-type cell has a larger apical surface with microvilli [27].

In euryhaline teleosts, ionocytes alter their morphology and ion-transporting functions to meet unexpected environmental salinity changes, whereas alteration of ionocyte functions takes place as a pre-adaptive response for the forthcoming upstream and downstream migrations in diadromous teleosts. In stenohaline teleosts inhabiting either freshwater or seawater, on the other hand, ionocytes do not possess the functional plasticity to switch the direction of ion transport, but merely adjust the degree of unidirectional ion transport. Thus, euryhalinity or stenohalinity of teleosts is primarily determined by their ability of functional alteration and plasticity of ionocytes [28].

Lee et al. [29] described three types of ionocytes in Mozambique tilapia *Oreochromis mossambicus* with various morphological features of apical surface: wavy convex of type I, shallow-basin of type II, and deep-hole of type III. These three types of ionocytes exhibited abundant sodium pumps and thus were NKA immunoreactive. In gills of seawater-adapted tilapia, sometimes more than one ionocyte was observed to share one apical crypt and formed a multicellular complex. The study revealed that there were three types of freshwater ionocytes and only one type of seawater ionocytes, those seawater ionocytes cells were larger in size than all types of freshwater ionocytes, and increased in density with elevated salinities. However, branchial ionocytes of Mozambique tilapia were found to have no significant differences in densities of apical openings between seawater- and freshwater-adapted individuals. Instead, there is an increase in size of ionocytes in seawater, which is accompanied by the expansion of the tubular system in those cells where the NKA is located. Mature seawater and freshwater-type ionocytes differed in both the morphology of the apical openings as well as the sodium pump contents per cell. Upon salinity challenge, functional ionocytes changed into the seawater type containing more NKA, thus driving a series of ion transporting systems to meet physiological requirements [30].

Changes in ionocytes abundance in the gills of golden grey mullet *Liza aurata* fry were observed during adaptation to different salinities. In freshwater, ionocytes were observed on the epithelia of filaments mainly in inter-lamellar regions and on the lamellae. Mullet from 36 ppt and 46 ppt salinity showed a high density of ionocytes on the filaments' epithelia and a few cells on the lamellae. This suggests a role of the lamellar ionocytes in ion uptake in hypo-osmotic environments. Ionocytes are normally abundant in filament epithelia of both freshwater and seawater teleost, and these are effective at secreting ions in hypertonic seawater as well as taking up ions in hypotonic freshwater [6].

The increasing size and number of ionocytes was also observed in kutum *Rutilus frisii kutum* reared from brackish (Caspian Sea) and freshwater (Khoshkrood River). It was shown that the average size and number of ionocytes in the fish from seawater were considerably larger than those from freshwater. The mean size of ionocytes was $6.89 \pm 1.16 \mu\text{m}$ in brackish water samples and $5.1 \pm 0.81 \mu\text{m}$ in river samples. The average number of ionocytes in brackish and river water samples was 16.92 and 6.57, respectively [31]. The density and size of ionocytes increased with increasing salinity was also reported in Nile tilapia *O. niloticus* [8]. A positive correlation between salinity and ionocytes size has been reported in some fish. Larger ionocytes reared in higher salinity were observed in Nile tilapia which strongly suggests

higher salt excretion [8]. Ionocytes have different functions in fish adapted to freshwater and seawater. In seawater, ionocytes are involved in the excretion of excessive ions, while in freshwater they are responsible for ion uptake to compensate for ion loss in hypoosmotic environments. The high number of gill ionocytes in specimens from the sea reflects the greater requirement of these individuals for ion transport through these cells. On the other hand, the reduction in the size and number of ionocytes in kutum following migration into freshwater represents a reduction of their requirement for the active excretion of Na^+ and Cl^- in hypoosmotic environments [31].

In cururu ray *Potamotrygon sp.*, the ionocytes were scarcely present in the lamellar epithelium but were found at high density in the interlamellar epithelium of filaments as a follicular structure with 8-12 ionocytes. The density of ionocytes differed in the lamellar among the gill arches. In *P. aiereba*, ionocytes density was significantly higher in the 4th-gill arch. Similarly, the 4th-gill arch had the highest ionocytes density in cururu ray, although the total number of ionocytes in the lamella was deficient, varying from 0 to 16 cells/ mm^2 . Relating to ion regulation, there is strong evidence that the density of ionocytes is directly related to NKA activity in *P. aiereba*. The number of ionocytes and the specific NKA activity in different gill arches of *P. aiereba* suggest that the 4th gill arch is strongly related to the ionic regulation in this species. Conversely, in *Potamotrygon sp.*, although the 4th gill arch had more ionocytes was significantly higher than that of other gill arches, the relationship between the number of ionocytes in the filament and lamella was not significant. Nevertheless, it was clear that specific NKA activity is associated with a higher number of ionocytes in *Potamotrygon sp.* The presence of follicular ionocytes in the interlamellar region of the filament epithelium in this species may be responsible for this difference as they share a single short channel that opens at the epithelial surface, creating a micro-environment inside of this channel influencing ion exchange [3].

6. Hormone modulates branchial Na^+/K^+ -ATPase (NKA) activity

In fish, prolactin controls water and electrolyte balance (especially in freshwater conditions). In waters of low salinity or freshwater environments, pituitary expression of prolactin mRNA and plasma levels of prolactin increase to produce various osmoregulatory changes. The hyperosmoregulatory role of prolactin is well established in teleosts since treatment with this hormone decreases gill NKA activity through changes in the expression of the subunits of the enzymes. However, depending on the environment, in some species, prolactin treatment increased or did not affect gill NKA activity. In some studies, prolactin treatment did not affect gill NKA activity in contrast with the decrease reported in specimens of the same species acclimated to seawater or brackish water. It is assumed that prolactin acts on the kidney producing an increase in sodium reabsorption and water excretion. However, the effects of prolactin treatment on kidney NKA are contradictory in freshwater-adapted fish. Prolactin treatment did not affect kidney NKA activity in agreement with previous studies in salmonids. Suggested that the role of prolactin on kidney function is related to water balance. Prolactin treatment increased cortisol levels in tilapia and also gilthead sea bream [32]. Prolactin plays an important role in the freshwater adaptation of most euryhaline fish by preventing the loss of ions, and uptake of water and prolactin receptors in teleost has been long recognized [27]. This was observed when prolactin replacement therapy fully restored NKA α -1a expression in freshwater-acclimated cichlid fish. The clear prolactin dependence of gill α -1a expression may partially explain the importance of this hormone to hyperosmoregulation in this species [20]. Watanabe et al. [33] also reported the role of prolactin in maintaining freshwater-type ionocytes. Prolactin mediates the maintenance of hyperosmoregulatory ionocytes in gills of Mozambique tilapia. Prolactin is known as a fundamental endocrine factor for hyper-osmoregulation in teleost fishes, acting on ionocytes in the gills to maintain ion concentrations of body fluid within narrow physiological ranges in freshwater conditions. This was also shown to maintain the density of freshwater-type ionocytes in isolated gill filaments; this effect of prolactin is not achieved by the activation of cell proliferation, but by the maintenance of existing ionocytes. Prolactin maintained relative Na^+ , Cl^- cotransporter-2, and NKA α 1a mRNA abundance [33].

Another interesting hormone that modulates NKA activity is cortisol. This is a seawater-adapting hormone in teleost. The teleost cortisol receptor was first cloned from the rainbow trout *Oncorhynchus mykiss* and shown to have a similar structure to those of the steroid/thyroid/retinoid receptor family members. The presence of the cortisol receptor in the ionocytes and undifferentiated cells has been demonstrated in chum salmon *O. keta* fry at both the protein and mRNA levels. Cortisol may be one of the important regulators of ionocytes function and differentiation [27]. Cortisol had a modest, but significant, stimulatory effect on α -1a expression in cichlid fish. Their synergistic manner was observed when used with prolactin. Their combination with prolactin resulted in a marked increase in α -1a mRNA to levels far exceeding other operated fish [20]. Prolactin and cortisol mediate the maintenance of hyperosmoregulatory ionocytes in gills of Mozambique tilapia. Increasing cortisol level was also reported in Nile tilapia reared in hyperosmotic

conditions suggesting its role in salinity adaptation [34]. Cortisol is known as an osmoregulation-related steroid in teleosts. Cortisol showed a stimulatory effect on relative Na⁺, Cl⁻ cotransporter-2, and NKAA1a mRNA levels in combination with prolactin, though cortisol alone exerted no effect on these genes. An increase in NKA α1b mRNA abundance was detected in cortisol-treated groups. This indicates that cortisol stimulates the function of ionocytes maintained by prolactin [33].

The structure and hormonal actions of the octapeptide angiotensin II have been studied in a variety of teleosts and elasmobranchs. Angiotensin II emerges as a seawater-adapting hormone in several studies in teleost. It appears to be involved in fish ion regulation through direct action on ionocytes [2]. Other studies even showed that a synthetic steroid dexamethasone affects the activity of NKA in both gill pavement and ionocytes from freshwater and seawater-adapted. Moreover, this hormone increases the NKA activity in seawater and decreases it in freshwater, in a dose-dependent manner in both environmental adaptations in European yellow eels *Anguilla anguilla* [35].

From the osmoregulatory point of view, growth hormone is known to facilitate seawater acclimation in several salmonid species, including rainbow trout. The growth hormone increases salinity tolerance through its stimulation of ionocytes number, gill NKA activity, and expression of NKA α-subunit. The growth hormone may also influence acclimation to seawater indirectly through effects on energy metabolism. Seawater acclimation is a highly energetic process, undoubtedly associated with major changes in plasma osmotic pressure and ion concentration during seawater adaptation. A better effect of growth hormone on osmoregulation of rainbow trout *Oncorhynchus mykiss* was reported. This was accompanied by the enhanced hypo-osmoregulatory capacity of ovine growth hormone fish, as suggested by the increase observed in gill NKA activity. An increase in gill NKA activity was also observed in this species treated with 2 ug growth hormone g⁻¹ bodyweight. The increased ATPase activity in this species reflects an enhanced hypo-osmoregulatory capacity elicited by growth hormone treatment in agreement with the role of this hormone during seawater adaptation [36]. The Growth hormone also facilitates seawater acclimation through ionocytes proliferation; thus, it enhances branchial NKA capacity and stimulates expression of the NKA α-subunit. Growth hormone and prolactin actions on osmoregulation and energy metabolism of gilthead sea bream *Sparus aurata* were also reported. The gilthead sea bream *S. aurata* is a euryhaline fish where prolactin and growth hormone plays a role in the adaptation to different environmental salinities. In this species, growth hormone increased branchial NKA activity and decreased sodium levels in line with its predicted hypo-osmoregulatory action [32].

7. Nutrition affects Na⁺/K⁺-ATPase (NKA) Activity

The nutritional status of fish can have a pronounced impact on performance and determine the competency to adapt to changing environments. Period of nutrient deprivation is a natural phenomenon in wild populations of fish, especially during reproduction and migration. It has already been demonstrated that energy deficiency due to feed deprivation can adversely affect numerous physiological systems such as ionic balance. One of the experiments on hypo-osmotic stress-induced physiological and ion-osmoregulatory responses in European sea bass *Dicentrarchus labrax* is modulated differently by nutritional status. This investigated the impact of nutritional status on the physiological, metabolic, and ion-osmoregulatory performance of European sea bass *D. labrax* when acclimated to seawater (32 ppt), brackishwater (20 and 10 ppt) and hyposaline water (2.5 ppt) for two weeks. Following acclimation to different salinities, fish were either fed or fasted (unfed for 14 days). Plasma osmolality, (Na⁺), (Cl⁻), and muscle water content were severely altered in fasted fish acclimated to 10 and 2.5 ppt compared to normal seawater-acclimated fish, suggesting ion regulation and acid-base balance disturbances. In contrast to feed-deprived fish, fed fish were able to avoid osmotic perturbation more effectively. This was accompanied by an increase in NKA expression and activity, transitory activation of H⁺-ATPase (only at 2.5 ppt), and down-regulation of Na⁺/K⁺/2Cl⁻ gene expression. Feed deprivation tends to reduce physiological, metabolic, ion-osmoregulatory, and molecular compensatory mechanisms limiting the fish's ability to adapt to a hypo-osmotic environment [37].

8. Ontogeny of branchial ionocytes in fish

Osmoregulation is a vital function that is essential to all vertebrates. Ionocytes are epithelial cells responsible for this function and have been extensively studied in adult teleost fish gills. The euryhaline medaka *Oryzias latipes* has recently emerged as an investigative model because of its ability to acclimatize easily to water presenting various salinities. The study on medaka has focused on the development of ionocytes in medaka embryos. The first analyses were the distribution of ionocytes in the skin and gills during development, using a specific marker of differentiated ionocytes

(the NKA pump). Strikingly, there were two ionocyte domains identified on the yolk surface ectoderm that was named the vitellin zone and the lateral zone. In zebrafish, ionocyte differentiation has been shown to be controlled by two forkhead-box genes, *foxi3a* and *foxi3b*. The cloned medaka *foxi3* ortholog, which appeared to be highly similar to *foxi3b*. Whole-mount in situ hybridizations performed on medaka embryos revealed that *O1-foxi3* is expressed in differentiated ionocytes of the pharyngeal endoderm, the branchial arches, and the yolk epidermis, as well as in epibranchial placode territories. Further focused on the expression patterns of the yolk epidermis and comparison of the expression of *O1-foxi3* with that of the non-neural progenitor marker *p63*. The evidence showed that *O1-foxi3* is expressed in progenitor cells, which are first of all located uniformly in the vitellin zone and then transitory clustered in the lateral zone. These data are taken together to contribute to a clearer understanding of osmoregulatory tissue ontogenesis in euryhaline fish [38]. Most teleost pre-larvae are able to osmoregulate at hatch, and their ability increases in later stages. Before the occurrence of gills, the pre-larval tegument where a high density of ionocytes (displaying high contents of NKA) is located appears temporarily as the main osmoregulatory site. Gills develop gradually during the pre-larval stage along with the numerous ionocytes they support. The tegument and gill NKA activity vary ontogenetically. During the larval phase, the osmoregulatory function shifts from the skin to the gills, which become the main osmoregulatory site. The drinking rate normalized to body weight tends to decrease throughout development. The kidney and urinary bladder develop progressively during ontogeny, and the capacity to produce hypotonic urine at low salinity increases accordingly. The development of the osmoregulatory functions is hormonally controlled. These events are inter-related and correlated with changes in salinity tolerance, often increasing markedly at the metamorphic transition from larva to juvenile. In summary, the ability of ontogenetical stages of fish to tolerate salinity through osmoregulation relies on integumental ionocytes, then digestive tract development and drinking rate, developing branchial chambers and urinary organs. The physiological changes leading to variations in salinity tolerance are among the main basis of the ontogenetical migrations or movements between habitats of different salinity regimes [39]. Another study supports that the presence of NKA in ionocytes in the yolk-sac epithelium of the tilapia *O. mossambicus*, provides biochemical evidence for the functions of the ionocytes in the yolk-sac epithelium of the developing larvae of tilapia *O. mossambicus*. Findings demonstrated the presence of NKA in the yolk-sac epithelium of tilapia larvae and about 1.46-fold more of the enzyme in seawater larvae than in freshwater ones. These biochemical results are further evidence that yolk-sac ionocytes are responsible for a major role in the osmoregulatory mechanism of early developmental stages before the function of gills is fully developed [40].

9. Genes regulate Na⁺/K⁺-ATPase (NKA) activity

The NKA is a ubiquitous membrane-bound protein that actively maintains the Na⁺ and K⁺ gradients between the intra- and extracellular milieu of animal cells. Long-term regulation is found to be mediated by mineralocorticoid or thyroid hormone and leads to a significant change in the total amount of NKA, whereas short-term regulation involves protein kinase and results in modulation of NKA expression in the cell membrane. Also, a novel regulatory mechanism that revealed tissue and isozyme-specific interaction of NKA with FXYD protein family members have been elucidated in mammals and elasmobranchs. The FXYD proteins, named because of their invariant extracellular motif FXYD, belong to a family with a conserved single-span transmembrane domain. These proteins are characterized by a conserved FXYD motif, two identified glycine residues, and a serine residue. In the teleost, salinity-dependent expression of FXYD protein and its interaction with NKA in gills of the euryhaline fish was first reported in pufferfish. Branchial FXYD protein expression in response to salinity change and its interaction with NKA of the euryhaline teleost *Tetraodon nigroviridis*, NKA was observed. Immunofluorescent staining of frozen sections demonstrated that pufferfish FXYD was colocalized to NKA-immunoreactive cells in the gill filaments. Besides, the interaction between pufferfish FXYD and NKA was demonstrated by co-immunoprecipitation. Salinity-dependent expression of pufferfish FXYD protein and Na⁺/K⁺-ATPase, as well as the evidence for colocalization and interaction in pufferfish gills, suggested that pufferfish FXYD regulates NKA activity in gills of euryhaline teleosts upon salinity challenge. The pufferfish FXYD protein regulation of NKA appears to exist in all vertebrates from human to fish [41]. The topic of other genes that regulates and helps the NKA activity in fish is limited due to limited studies done yet.

10. Conclusion

The branchial ionocytes is one of the major features of fish gill anatomy that is useful in salinity adaptation. Species that migrate from freshwater to marine water as part of their life cycle have a well-established ionocyte type in their gills. The activity of its enzymes governs this ionocytes for the ion uptake in freshwater and ion excrete in marine water

environment. Marine fish that enter freshwater in search of food have a much localized lamellar and interlamellar ionocytes, necessarily for the quick response and adaptation to the changing osmotic gradients. These ionocytes tend to become larger depending on the osmotic gradient experienced by the fish, especially fish from freshwater that migrates to marine environments. Aside from that, the location of ionocytes on the gill will determine its function and activities. Euryhaline species have a very well developed type of ionocytes necessary to excrete ions when they are in saltwater and uptake ions when they encounter freshwater. This physiological adaptation is essential in fish evolution, as they have a wide range of adaptability in salinity tolerance. Species that could tolerate a wide range of salinity gradients have a greater chance of survival. In aquaculture, species such as diadromous that can tolerate both freshwater and saltwater is one of the good characteristics of a promising species in the field. This allows the aquaculturist to rear both in freshwater and marine water for huge production. This is very important because of the limited space in freshwater areas, and the expansion of production towards the sea is a promising venture.

Compliance with ethical standards

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Disclosure of conflict of interest

The author declares no possible conflict of interest from any individual or group.

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