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# Expression of Ion Transporters and $Na^+/K^+$-ATPase and $H^+$-ATPase Activities in the Gills and Kidney of Silver Catfish (*Rhamdia quelen*) Exposed to Different pHs

**Morgana Tais Streck Marx [1], Carine de Freitas Souza [2], Ana Paula Gottlieb Almeida [3], Sharine Nunes Descovi [4], Adriane Erbice Bianchini [3], Juan Antonio Martos-Sitcha [5], Gonzalo Martínez-Rodríguez [6], Alfredo Quites Antoniazzi [7] and Bernardo Baldisserotto [1,2,3,4,*]**

1 Post-Graduate Program in Animal Biodiversity, Federal University of Santa Maria, Santa Maria 97105-900, Brazil
2 Department of Physiology and Pharmacology, Federal University of Santa Maria, Santa Maria 97105-900, Brazil
3 Post-Graduate Program in Pharmacology, Federal University of Santa Maria, Santa Maria 97105-900, Brazil
4 Post-Graduate Program in Animal Husbandry, Federal University of Santa Maria, Santa Maria 97105-900, Brazil
5 Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI·MAR), Universidad de Cádiz, Puerto Real 11519, Cádiz, Spain
6 Institute of Marine Sciences of Andalusia, Spanish National Research Council (ICMAN-CSIC), Puerto Real 11519, Cádiz, Spain
7 Post-Graduate Program in Veterinary Medicine, Federal University of Santa Maria, Santa Maria 97105-900, Brazil
* Correspondence: bernardo.baldisserotto@ufsm.br; Tel.: +55-55-3220-9382; Fax: +55-55-3220-8142

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**Abstract:** Exposure to acidic and alkaline pHs results in an ionic imbalance. Cellular responses involved in osmoregulation in silver catfish exposed to different pHs (5.5, 7.5, and 9.0) for 24 h were evaluated. The gills and kidney were collected to measure $Na^+/K^+$-ATPase (NKA) and $H^+$-ATPase (V-ATPase) activities and to evaluate the expression of ion transporter-related genes: NKA (*atp1a1*), $H^+$-ATPases (*atp6v0a1b*, *atp6v0a2a*, *atp6v0a2b*), $Na^+/H^+$ antiporter (*slc9a3*), $K^+/Cl^-$ symporters (*slc12a4*, *slc12a6*, *slc12a7a*, *slc12a7b*), $Na^+/K^+/2Cl^-$ symporter (*slc12a2*), and ammonium transporter Rh type b (*rhbg*). The gills presented greater responses to pH changes than the kidney. The pH alterations changed the *atp1a1* gene expression and NKA activity, whereas the $H^+$-ATPase activity increased in the gills in alkaline water, probably to maintain ionic balance. The *slc9a3* and *slc12a2* genes play more prominent roles in the ion uptake at acidic pH than $H^+$-ATPase. The *slc12a7a* was the only isoform of this transporter affected by pH. The *rhbg* is apparently related to ammonia excretion through the gills and kidney (minor scale). Exposure to alkaline pH seems to be battled by impairment of NKA and $H^+$-ATPase activities in the gills, whereas the expression of some ion transporters in silver catfish changes during both acidic and alkaline pHs.

**Keywords:** acidic and alkaline pH; fish osmoregulation; ionocytes; mRNA; *Rhamdia quelen*

## 1. Introduction

In the aquatic environment, fish may encounter severe and challenging ionic/osmotic gradients to maintain internal homeostasis. The low concentration of ions in freshwater (FW) causes fish to lose ions and gain water by osmosis [1–3]. The problem of osmotic water gain is solved by low water ingestion [4], decreasing the water permeability of the integument [5] and producing large amounts of diluted urine [6]. The net ion loss is mainly solved by the combined response of a reduction in diffusive loss [5] and an increased ability to absorb ions to maintain liquid ionic status [7]. To maintain this internal balance, fish have

developed sophisticated mechanisms that are achieved by osmoregulatory organs [8]. Fish gills are the main organ for gas exchange, ion regulation, acid-base balance, and nitrogen waste excretion [3,7,9]. The gills are the main target of acidic or alkaline water because they are in direct contact with the environment [10]. The kidney, together with the gills, is essential for osmoregulatory processes in freshwater teleosts [11]. The kidney is the primary organ for the elimination of water, being particularly important for freshwater species due to the efficient ion reabsorption mechanisms to minimize ion loss [12]. Considering the importance of these two organs in the osmoregulatory process, the gills and kidney were the focus of this study.

At the cellular level, ion transport is accomplished through mitochondria-rich cells, the ionocytes, which express specific ion transporters (or enzymes) and, therefore, are responsible for ion transport (primarily $Na^+$, $Cl^-$, and $Ca^{2+}$ uptake and $H^+$, $HCO_3^-$ uptake/secretion) and $NH_3$ excretion in FW [1,2,7,13]. Different transmembrane proteins have been described as being involved in ion exchange (ion channels, co-carriers). The specificity, location (apical/basolateral), and relative abundance and expression of these proteins in ionocytes result in the uptake or excretion of ions [14].

The pH of water is a very important parameter to be considered because it has a direct effect on homeostasis, metabolism, physiological processes, and fish survival [15]. The pH of water is usually regulated by the carbonate–bicarbonate system, generally remaining between 6.0 and 8.0 in freshwater [16]. Oscillations over this range may be due to the abundance of phytoplankton or the presence of high concentrations of dissolved $HCO_3^-$ and $CO_3^{2-}$ salts (alkaline pH) or even by an excess of mineral and organic acids (acidic pH) [17]. When exposed to acidic or alkaline waters, the normal functions of the gills tend to decrease [15].

In fish exposed to low pH, acid loading through the gills is a source of acid-based disturbance, with increased $H^+$ and $NH_4^+$ excretion in the urine to compensate for this problem [18]. In addition, it increases the ion loss [19,20], which decreases plasma ion levels and the pH [18,21]. Very acidic pH levels impair branchial protein junctions, which increases paracellular ion loss [22,23]. A decrease in $Na^+$ uptake also occurs in fish exposed to acidic waters due to the inhibition of $Na^+/H^+$ or $Na^+/NH_4^+$ and $H^+$-ATPase (V-ATPase) apical transporters [23].

In alkaline waters, the main problems are related to the inhibition of ammonia excretion [18] and increased excretion of $CO_2$ [24]. Specimens of Amur ide (*Leuciscus waleckii*) that live in alkaline waters (pH 9.6) showed higher expression of some *slc12* genes ($Na^+/K^+/2Cl^-$, $K^+/Cl^-$, and $Na^+/Cl^-$ symporters), which may facilitate $Cl^-$ uptake [25,26]. In FW fish, ammonia excretion is maintained through a $Na^+/NH_4^+$ exchange complex, which consists of several membrane transporters (Rhbg, $H^+$-ATPase, $Na^+/H^+$) that together create an acid capture mechanism in the gill boundary layer [27]. At neutral pH, ammonia leaves the gills by diffusion in the form of $NH_3$ (non-ionized), which is converted to $NH_4^+$ (ionized) in water, maintaining a favorable gradient for $NH_3$ diffusion [16]. Thus, any disruption in the acidified gill microenvironment may cause impairment of the excretion mechanism and result in an internal accumulation of ammonia [28]. These physiological effects, combined with ion loss, can be considered the main mechanism associated with mortality in fish exposed to high environmental pH [29].

The silver catfish inhabits different FW environments from Central and South America [30] and has economic and ecological importance [20]. This species can survive acute changes in the 4.0 to 9.0 pH range for 96 h without significant mortality [31], but plasma and urinary $Na^+$ and $Cl^-$ levels were altered after 24 h exposure to different water pH [18]. Studies on physiology under various water pH levels are still necessary because stressful conditions of water pH may negatively affect fish performance and survival [32–34].

Considering that cellular responses to acute pH changes in this species remain uncertain and the lack of studies analyzing the activity of enzymes and expression of genes involved in FW fish osmoregulation, the aim of this study was to evaluate the activity of the enzymes involved in ion and ammonia exchange, such as the sodium potas-

sium pump ($Na^+/K^+$-ATPase, NKA), the proton pump ($H^+$-ATPase), and the expression of ion transporter-related genes: NKA (*atp1a1*), $H^+$-ATPases (*atp6v0a1b*, *atp6v0a2a*, *atp6v0a2b*), $Na^+/H^+$ antiporter (*slc9a3*), $K^+/Cl^-$ symporters (*slc12a4*, *slc12a6*, *slc12a7a*, *slc12a7b*), $Na^+/K^+/2Cl^-$ symporter (*slc12a2*), and ammonium transporter Rh type b (*rhbg*), in the gill and kidney of silver catfish exposed to acute pH changes.

## 2. Materials and Methods

### *2.1. Experimental Animals and Handling Conditions*

Silver catfish juveniles (7.32 ± 0.40 g and 11.35 ± 1.30 cm; mean ± SEM) ($n$ = 36) were obtained from a fish farm at Santa Maria, southern Brazil. These juveniles were acclimated for one week in a continuously aerated 250 L tank (two air pumps of 12 W each) at pH levels of 7.0–7.5. The animals were fed once daily to satiety with a commercial feed (32% crude protein).

### *2.2. Experimental Conditions*

After acclimation, juveniles were placed in three recirculation systems with biological filters containing four 40 L tanks each (three fish in each replicate, four replicates). Temperature and dissolved oxygen levels were determined with the oxygen meter Y5512 (YSI Inc., Yellow Springs, OH, USA). Total ammonia (Labcon Test) and water hardness (EDTA titrimetric method) levels were verified. In each system, the pH was adjusted to the following values: 5.5, 7.5, and 9.0. This acidic to alkaline pH range was chosen because it reduces silver catfish growth but does not cause mortality [35]. The pH adjustment and control were performed every hour with a DMPH-2 pH meter (Digimed, São Paulo, SP, Brazil). The water was acidified with 1 N $H_2SO_4$ and alkalinized with 1 N NaOH. The fish were transferred directly from the acclimation tanks (pH 7.0–7.5) to the experimental pH and collected after 24 h of exposure.

### *2.3. Sample Collection and Analysis*

Fish from each tank ($n$ = 12 per treatment) were sampled and collected after 24 h of exposure to the experimental pH, anesthetized with 50 µL/L eugenol [36], and then euthanized by spinal cord section to remove gills and caudal kidneys. A small portion of these organs was stored in 2 mL RNase-free tubes containing TRIzol® Reagent (Invitrogen, Waltham, MA, USA) for analysis of gene expression. The remaining collected tissues were immersed in liquid nitrogen and subsequently stored at −80 °C until the enzyme analysis could be performed.

### *2.4. Expression of Genes Related to Ion and Ammonia Transport*

In order to determine the sequence of the genes of NKA (sodium potassium-transporting ATPase subunit alpha-1, *atp1a1*), $H^+$_ATPases (v-type proton ATPase subunit a, *atp6v0a1b*, *atp6v0a2a*, *atp6v0a2b*), $Na^+/H^+$ antiporter (sodium/hydrogen exchanger, *slc9a3*), $K^+/Cl^-$ symporters (slc12a4, *slc12a6*, *slc12a7a*, *slc12a7b*), $Na^+/K^+/2Cl^-$ symporter (solute carrier family 12 member 2, *slc12a2*), and ammonium transporter Rh type B (*rhbg*) in silver catfish, in brief, samples of the head and caudal kidney, brain including the pituitary gland, and liver were mass sequenced as previously described [37]. Subsequently, other alternatives, such as the alignment of the readings against related species, were performed. Primers were then designed using the Primer Express software 3.0 (Applied Biosystems, San Francisco, CA, USA) and validated by standard curves. The genes actin beta (*actb*) and eukaryotic elongation factor 1-alpha (*eef1a*), described in [37,38], respectively, served as internal reference genes (Table 1). All of the gene names followed the ZFIN Zebrafish Nomenclature Conventions and were named after the mammalian orthologues. These genes were chosen because their complete sequences were available in the mass sequence of silver catfish and were expressed in other freshwater-adapted teleosts [1,3,8,11,39–48]. In addition, previous analysis with the respective primers tested indicated the expression of these genes in the gills and kidney of silver catfish.

**Table 1.** Primer oligonucleotide sequences used for RT-PCR analysis.

| Primer | Nucleotide Sequence (5′ → 3′) | Amplicon | Gills | | Kidney | | Accession Number |
|---|---|---|---|---|---|---|---|
| | | Size (bp) | E (%) | $R^2$ | E (%) | $R^2$ | |
| $actb_F$<br>$actb_R$ | CCACCTTCAACTCCATCATGAA<br>GCAATGCCAGGGTACATGGT | 100 | 100.0 | 0.995 | 107.6 | 0.983 | KC195970.1 |
| $atp1a1_F$<br>$atp1a1_R$ | ACCTGTGCTTTGTTGGACTCA<br>AATGGCTTTAGCTGTGATTGGA | 137 | 93.9 | 0.995 | 110.0 | 0.987 | KT005453.1 |
| $atp6v0a1b_F$<br>$atp6v0a1b_R$ | TTTCCCATTTCGTTCTTTCG<br>GTCCACGGGTAGTGAGAGGA | 117 | 96.0 | 0.980 | 108.9 | 0.985 | MZ041102 |
| $atp6v0a2b_F$<br>$atp6v0a2b_R$ | TGCAAATCATAGCGAAGTGG<br>CCAATCAGCTTCCAGACTCA | 129 | 110.0 | 0.990 | 98.4 | 0.988 | MZ041103 |
| $atp6v0a2a_F$<br>$atp6v0a2a_R$ | GCAGGTGTTTCTTCTGGTCA<br>CGCCGAACTCTTTCATAACC | 132 | 110.0 | 0.987 | 90.5 | 0.980 | MZ041104 |
| $eef1a_F$<br>$eef1a_R$ | GCTTCCTTGCTCAGGTCATC<br>CGGTCGATCTTCTCCTTGAG | 127 | 102.2 | 0.998 | 100.1 | 0.991 | MH107165 |
| $slc12a6_F$<br>$slc12a6_R$ | CTCTCTCTTTCCCGCCAAC<br>TACGCATCCCGCACTTAC | 140 | 100.5 | 0.996 | 106.3 | 0.996 | MZ041107 |
| $slc12a7a_F$<br>$slc12a7a_R$ | TCATATTACATGATTTCCAGGGC<br>CCCGCATGTTATTCAGTTG | 178 | 90.3 | 0.999 | 100.3 | 0.992 | MZ041108 |
| $slc12a7b_F$<br>$slc12a7b_R$ | TCGTATTACATGATCTCTAGGTC<br>CTCGCATGTTGTTCAGCAT | 205 | 105.3 | 0.989 | 99.6 | 0.983 | MZ041109 |
| $slc9a3_F$<br>$slc9a3_R$ | ACCAACCAACCAGGAGTGT<br>GAAGCATTGTGTCAGGTAGAGA | 142 | 109.2 | 0.999 | 98.4 | 0.984 | MZ041110 |
| $slc12a2_F$<br>$slc12a2_R$ | AATCAAAGCAACAGCAAGGA<br>CAGCAACACCCACATAGACG | 237 | 108.6 | 0.986 | 105.6 | 0.991 | MZ041111 |
| $rhbg_F$<br>$hbg_R$ | CAGCCGTAGCAAGTGATGC<br>CCATTCCAAGAGTGACAGCA | 129 | 95.6 | 0.994 | 96.4 | 0.996 | MZ041101 |
| $slc12a4_F$<br>$sc12a4_R$ | TTCCTTCTCAAACAGCACAA<br>TTCCACCACCTCCACCTCT | 150 | 100 | 0.980 | 110.0 | 0.987 | MZ041106 |

Total RNA from the gills and kidney was extracted using TRIzol®® Reagent (Thermo Fisher Scientific, São Paulo, SP, Brazil), as indicated in the manufacturer's instructions. Quantification and determination of RNA purity was performed as described previously [37]. Total RNA (500 ng) was treated with 0.1 U DNase Amplification Grade (Invitrogen) for 15 min at 27 °C, followed by DNase inactivation with 1 µL of EDTA at 65 °C for 10 min. Then, the complementary DNA (cDNA) was synthetized by reverse transcription reaction using an iScript™ cDNA Synthesis Kit (BioRad, São Paulo, SP, Brazil), according to the manufacturer's instructions. The reverse-transcription was performed using a SimpliAmp™ Thermal Cycler (Applied Biosystems™, São Paulo, SP, Brazil), and the program consisted of 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C, finishing with a hold at 4 °C. Real-time quantitative PCR (qPCR) reactions were performed with 2 µL cDNA (5 ng of cDNA in a final reaction volume of 10 µL), 1 µL of specific forward and reverse primers for each gene at a final concentration of 200 nM, 5 µL of SYBR Green (GoTaq® qPCR Master Mix, Promega, São Paulo, SP, Brazil), and 1 µL of nuclease-free water. The qPCR reactions were conducted in a CFX384 thermocycler (BioRad), and the thermal profile was as follows: initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min, melting curve from 65 to 95 °C increasing 0.5 °C every 5 s. Melting curve analyses were performed to verify that a single product was amplified and to check the absence of primer-dimer artifacts. Samples were run in duplicate and relative gene quantification was performed using the $2^{-\Delta\Delta Cq}$ method [49], corrected for efficiencies [50] and by geometric averaging of two internal reference genes [51].

The primer oligonucleotide sequences used for RT-PCR analyses are shown in Table 1. E (%) is the reaction efficiency and $R^2$ the coefficient of determination for each pair of oligonucleotides, both in gills and kidney. The genes actb and eef1a served as internal reference genes.

### *2.5. Determination of NKA and H⁺-ATPase Activities*

The branchial and kidney tissues were homogenized (1:10 *w*/*v*) in a homogenization buffer (150 mM sucrose, 50 mM imidazole, and 10 mM EDTA, pH 7.5) and centrifuged at 1000× *g* for 10 min at 4 °C, and the supernatant was stored at −80 °C until use. Branchial and renal NKA and $H^+$-ATPase activities were determined in microplates, as described by [52]. Briefly, 5 µL of homogenate and 200 µL of reaction solution (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM KCN 1 mM ATP, 0.2 mM NADH, 0.1 mM fructose 1,6 diphosphate, and 2 mM phosphoenolpyruvate) were added to each sample. Ouabain (2 mM) was used as an inhibitor. $H^+$-ATPase (HA) activity was measured in the same manner as the NKA, using Bafilomycin A1 as a specific inhibitor of the V-type $H^+$-ATPase [53] in a final concentration of 100 nM. Results were expressed as mmol ADP released/min/mg of protein.

### *2.6. Phylogenetic Trees*

Phylogenetic trees for the deduced protein sequences from genes *atp1a1*, *atp6v0a1b*, *atpv0a2a*, *atpv0a2b*, *slc9a3*, *slc12a4*, *slc12a6*, *slc12a7a*, *slc12a7b*, *slc12a2*, and *rhbg* were performed using NGPhylogeny.fr, with default parameters (PhyML), visualizing the tree with the interactive Tree of Life (iTOL; https://itol.embl.de (accessed on 1 August 2022)). The accession number of the Genbank and NCBI reference sequences are in Supplementary Figures S1–S8.

### *2.7. Statistical Analysis*

A Kolmogorov–Smirnov test was conducted to evaluate the homogeneity of the variances. The effect of tanks (replicates) within each treatment was analyzed using one-way analysis of variance (ANOVA), and no significant difference was found, so data from all replicates were pooled. The data were compared using one-way ANOVA followed by Tukey's test. All analyses were performed using the GraphPad Prism 5.0® software. The minimum level of significance was $p < 0.05$. The results for enzyme activities were expressed as the mean ± standard error of the mean (SEM), whereas for gene expression data were expressed according to BioRad CFX Maestro 2.3 software (Bio-Rad Laboratories, Hercules, CA, USA), using SEM in the error bars.

## 3. Results

Water physicochemical parameters did not differ significantly between treatments, except pH (Table 2). No fish died during the whole experimental period.

**Table 2.** Water parameters measured during the experimental period.

| pH | Dissolved Oxygen (mg $L^{-1}$) | Temperature (°C) | Total Ammonia (mg $L^{-1}$) | $Na^+$ (mg $L^{-1}$) | $Cl^-$ (mg $L^{-1}$) | Hardness (mg $CaCO_3$ $L^{-1}$) |
|---|---|---|---|---|---|---|
| 5.5 | 6.0 | 22.6 | 0.125 ± 0.01 | 5.3 ± 0.02 | 4.0 ± 0.1 | 22 ± 1.4 |
| 7.7 | 6.0 | 22.6 | 0.134 ± 0.03 | 5.9 ± 0.01 | 4.0 ± 0.1 | 22 ± 1.4 |
| 9.0 | 6.0 | 22.6 | 0.120 ± 0.005 | 7.8 ±0.02 | 4.0 ± 0.1 | 22 ± 1.4 |

### *3.1. Phylogenetic Comparisons of the Sequences*

The deduced protein sequences for *atp1a1*, *atp6v0a1b*, *atp6v0a2a*, and *atp6v0a2b* in silver catfish showed over 90% identity with the same proteins and isoforms of other Siluriformes. The Atp1a1 protein has an 88.33% identity with zebrafish, *Danio rerio*. The Atp6v0a1b protein also has an identity with zebrafish of 92.29% and 91.15% with rainbow

trout, *Oncorhynchus mykiss*. The Atpv0a2b protein showed 82.73% identity with rainbow trout and 82.45% with zebrafish. The Atpv0a2a protein also showed 82.22% identity with rainbow trout and 82.22% with zebrafish (Supplementary Figures S1 and S2). The protein sequences for Slc9a3 showed 83–87% identity with other Siluriformes and 67.74% with zebrafish (Supplementary Figure S3). The protein sequence for Slc12a4 showed 88–97% identity with other Siluriformes: 93.0% with zebrafish, and 90.59% with rainbow trout (Supplementary Figure S4). The protein sequence for Slc12a6 showed 88–97% identity with other Siluriformes (Supplementary Figure S5). The protein sequence for Slc12a7a showed 77.50% identity with rainbow trout and 74.36% with zebrafish, and Slc12a7b showed 85.66% identity with zebrafish (Supplementary Figure S6). The protein sequence for Sc21a2 showed 84.44% identity with zebrafish and 81.63% with rainbow trout (Supplementary Figure S7). The protein sequence for Rhbg showed 85–89% identity with other Siluriformes, 80.26% with rainbow trout, and 79.91% with zebrafish (Supplementary Figure S8).

### 3.2. Gene Expression

Three different $H^+$-ATPase isoforms were expressed in the gills and kidney of silver catfish: *atp6v0a1b*, *atp6v0a2a*, and *atp6v0a2b*. The treatments did not significantly affect their expression in the gills and kidney (Figures 1a–c and 2a–c). Silver catfish that were exposed to pH levels of 5.5 and 9.0 showed significantly higher *atp1a1* expression in gills when compared to those kept at a pH of 7.5 (Figure 1d). The contrary occurred in the kidney, where there was lower expression in fish exposed to pH levels of 5.5 and 9.0, when compared to a pH of 7.5 (Figure 2d).

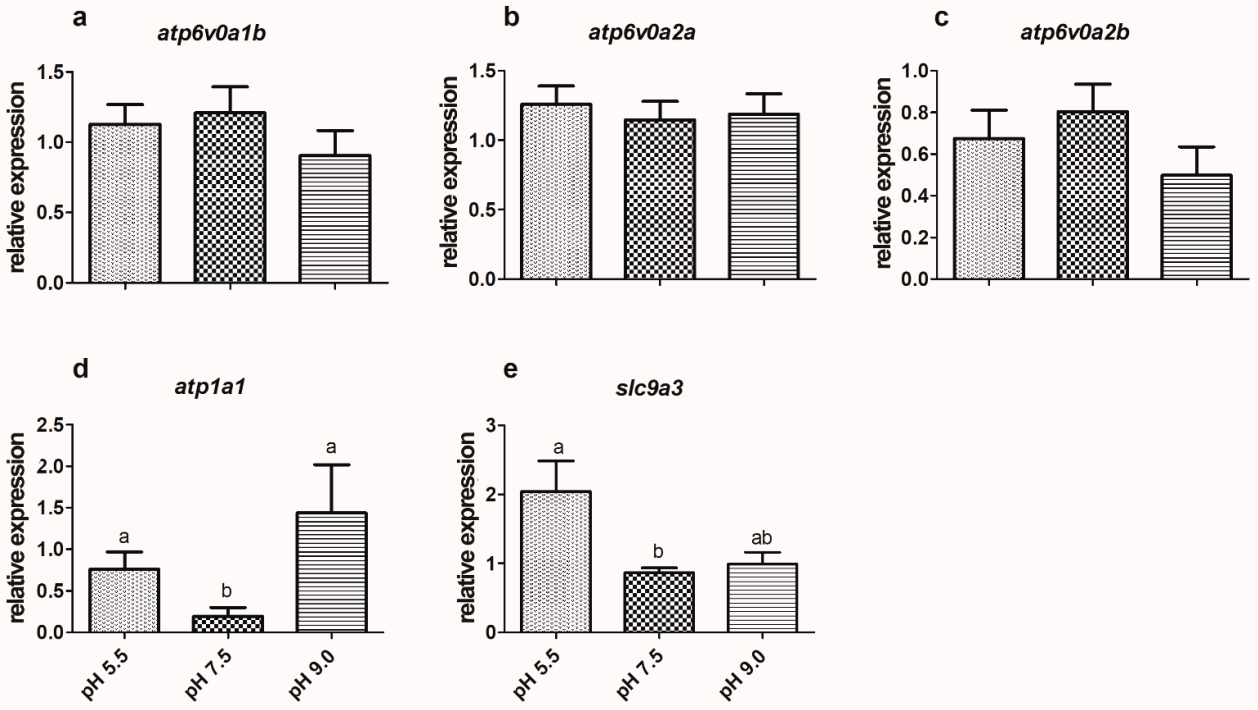


**Figure 1.** Expression of $H^+$-ATPase isoforms (**a**—*atp6v0a1b*, **b**—*atp6v0a2a*, **c**—*atp6v0a2b*), **d**—$Na^+/K^+$-ATPase (*atp1a1*), and **e**—$Na^+/H^+$ antiporter (*slc9a3*) in the gills of silver catfish (*Rhamdia quelen*, $n = 12$) exposed to different water pHs. Different letters indicate significant differences between treatments. One-way ANOVA and Tukey's test were used to determine statistical significance ($p < 0.05$).

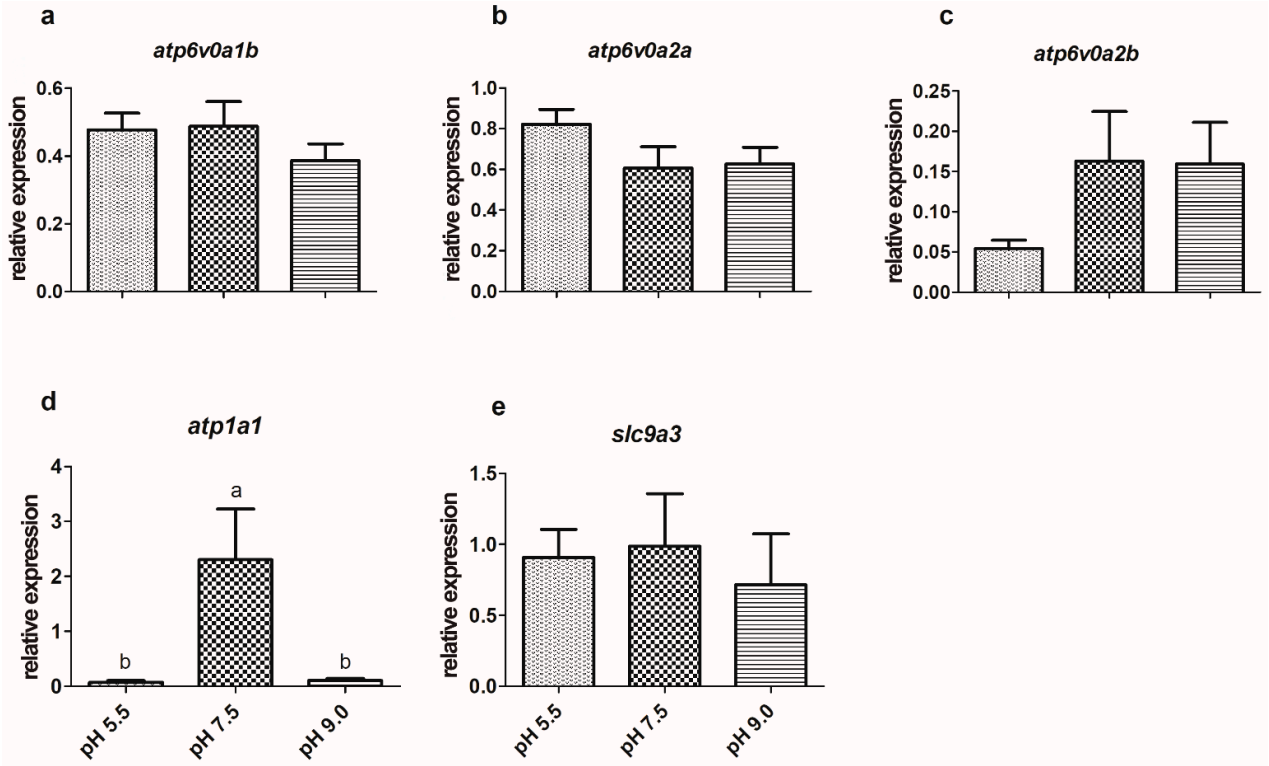


**Figure 2.** Expression of $H^+$-ATPase (**a**—*atp6v0a1b*, **b**—*atp6v0a2b*, **c**—*atp6v0a2a*), **d**—$Na^+/K^+$-ATPase (*atp1a*), and **e**—$Na^+/H^+$ antiporter (*slc9a3*) in the kidney of silver catfish (*Rhamdia quelen*, $n = 12$) exposed to different water pHs. Different letters indicate significant differences between treatments. One-way ANOVA and Tukey's test were used to determine statistical significance ($p < 0.05$).

The expression of *slc9a3* in the gills of silver catfish exposed to a pH of 5.5 was higher than those exposed to a pH of 7.5, but there was no difference in relation to a pH of 9.0 (Figure 1e), as well as in the expression of this gene in the kidney among the three experimental conditions (Figure 2e). The treatments did not significantly affect the expression of the *slc12a6* and *slc12a7b* genes (Figures 3a,c and 4a,c) in the gills and kidney. Fish exposed to acidic pH levels (5.5) presented higher *slc12a7a* expression in the gills than fish maintained at a pH of 9.0, but those kept at a pH of 7.5 did not show significant differences from those exposed to acidic and alkaline pH levels (Figure 3b). There was no significant difference in the expression of this gene in the kidney (Figure 4b).

Silver catfish exposed to a pH of 9.0 showed a higher expression of *slc12a4* in the kidney, compared to those at a pH of 5.5 (Figure 4d), but in the gills, there was no significant differences between fish from different pHs (Figure 3d). The highest expression of branchial *slc12a2* was observed at a pH of 5.5 (Figure 3e), and there were no changes in the renal expression of this gene (Figure 4e). The expression of branchial *rhbg* was higher at acidic pH (5.5) than at neutral pH (Figure 3f); whereas in the kidney, its expression was lower at a pH of 5.5 and 9.0 than at a pH of 7.5 (Figure 4f).

### *3.3. NKA and $H^+$-ATPase Activities*

The activity of NKA in the gills was significantly higher in silver catfish exposed to a pH of 9.0 than in those exposed to 5.5 (Figure 5A). The highest activity of the $H^+$-ATPase in the gills was observed in fish exposed to a pH of 9.0 (Figure 5B). Exposure to acidic and alkaline pHs did not significantly change the activities of these enzymes in the kidney (Figure 5C,D).

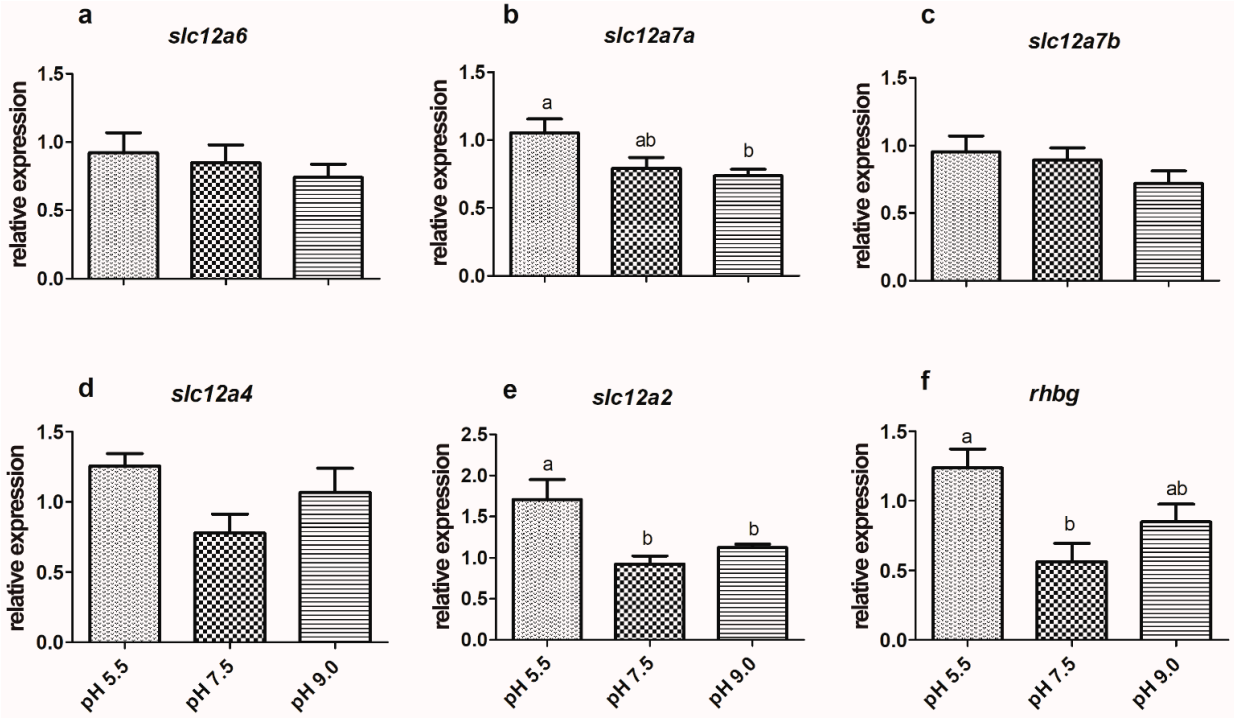


**Figure 3.** Expression of $K^+/Cl^-$ cotransporters (**a**—*slc12a6*, **b**—*slc12a7a*, **c**—*slc12a7b*, **d**—*slc12a4*), **e**—$Na^+/K^+/2Cl^-$ cotransporter (*slc12a2*), and **f**—ammonium transporter Rh type C-like, glycoprotein b (*rhbg*) in the gills of silver catfish (*Rhamdia quelen*, $n = 12$) exposed to different water pHs. Different letters indicate significant differences between treatments. One-way ANOVA and Tukey's test were used to determine statistical significance ($p < 0.05$).

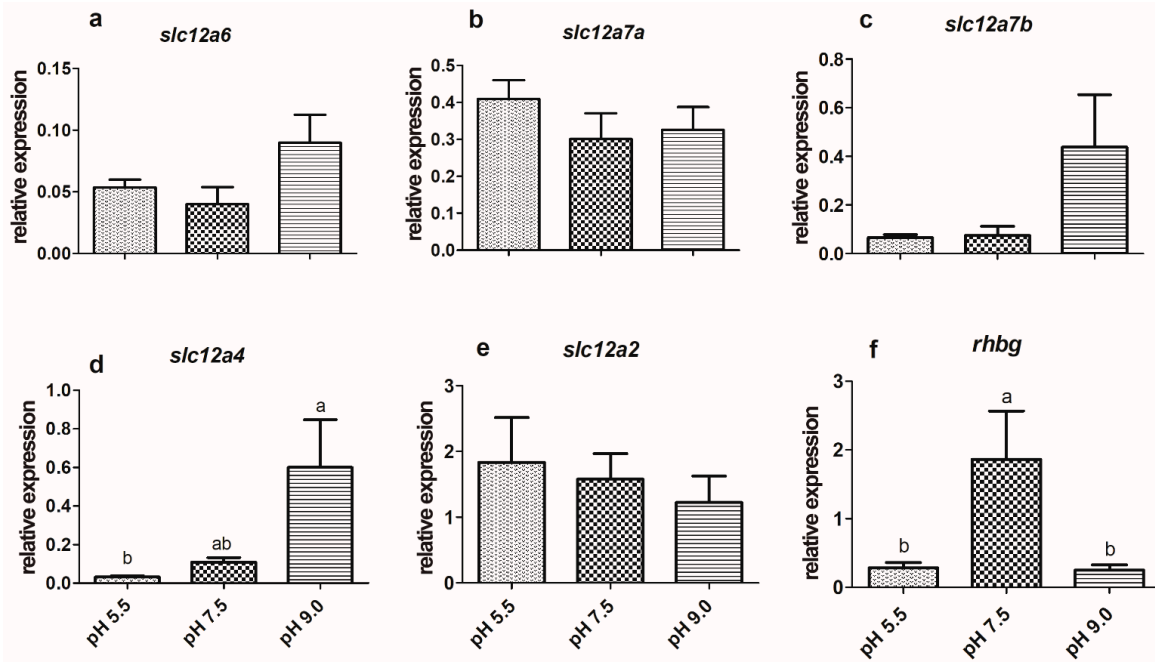


**Figure 4.** Expression of $K^+/Cl^-$ cotransporters (**a**—*slc12a6*, **b**—*slc12a7a*, **c**—*slc12a7b*, **d**—*slc12a4*), **e**—$Na^+/K^+/2Cl^-$ cotransporter (*slc12a2*), and **f**—ammonium transporter Rh type C-like, glycoprotein b (*rhbg*) in the kidney of silver catfish (*Rhamdia quelen*, $n = 12$) exposed to different water pHs. Different letters indicate significant differences between treatments. One-way ANOVA and Tukey's test were used to determine statistical significance ($p < 0.05$).

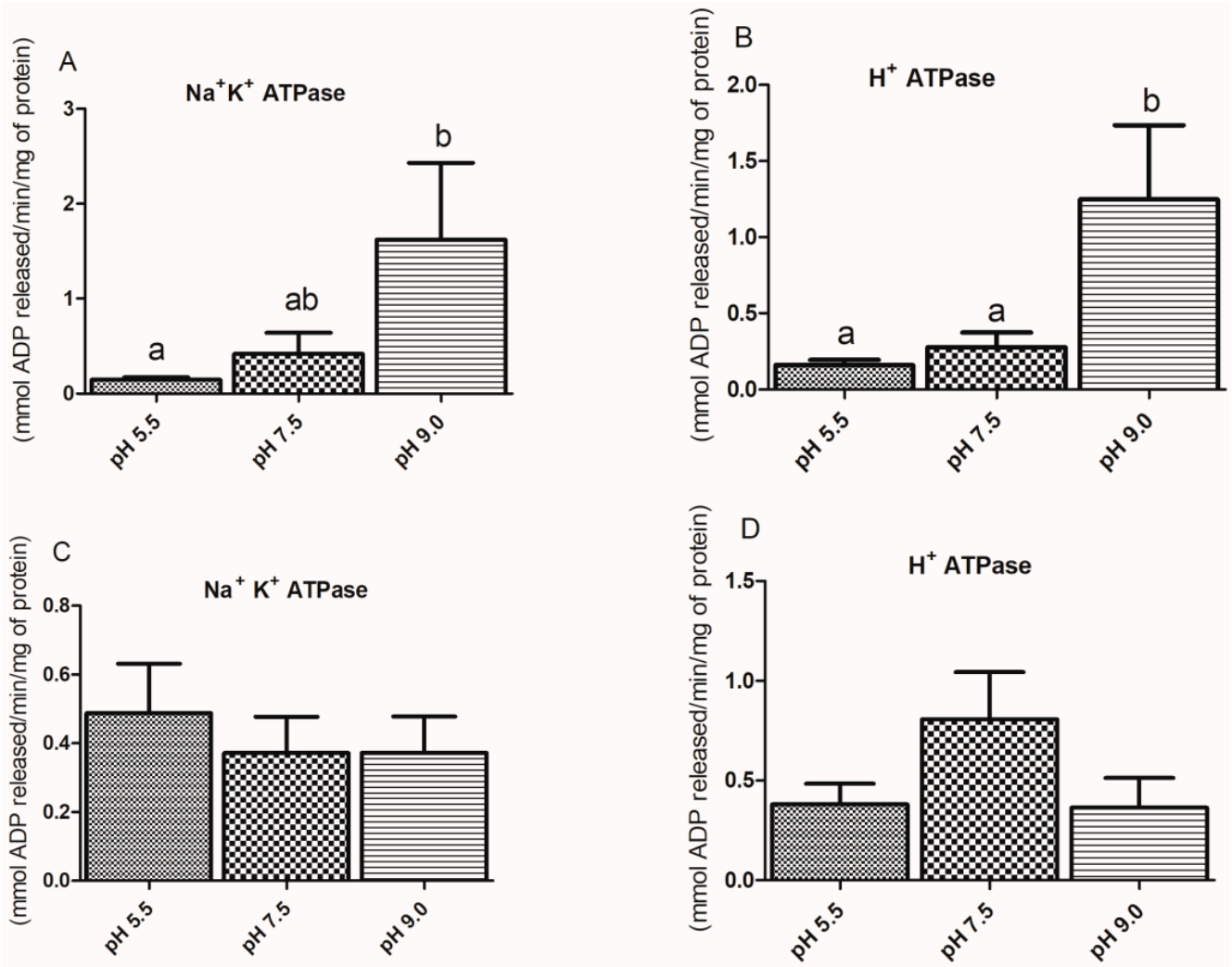


**Figure 5.** Activities from $Na^+/K^+$-ATPase and $H^+$-ATPase in gill (**A**,**B**) and kidney (**C**,**D**) of silver catfish (*Rhamdia quelen*, $n = 12$) exposed to different water pHs. Different letters indicate significant differences between treatments. One-way ANOVA and Tukey's test were used to determine statistical significance ($p < 0.05$).

## 4. Discussion

In the current study, we identified and analyzed some transporters located in the gills and kidney ionocytes of silver catfish exposed to different pHs. The silver catfish expresses in the gills and kidney several transporters from genes, namely *atp1a1*, *atp6v0a1b*, *atp6v0a2a*, *atp6v0a2b*, *slc9a3*, *slc12a4*, *slc12a6*, *slc12a7a*, *slc12a7b*, *slc12a2*, and *rhbg*.

To compensate the ion loss due to diffusion, FW fish use ionocytes [5,6]. Furthermore, ionocytes also act to regulate the release of $H^+$ or $HCO_{3-}$ to water [54,55] to achieve blood pH homeostasis. Freshwater fish went through multiple adaptations and re-adaptations during evolution, and there are probably distinct molecular strategies for obtaining ions and acid-base homeostasis in different species. There are several proposed names for ionocyte subtypes, as well as different patterns in their physiology and morphology, each unique to the species studied [1].

Several transporters, such as *atp1a1*, *slc12a4*, *slc9a3*, and *slc12a2*, were also expressed in the gills of freshwater-adapted Mozambique tilapia (*Oreochromis mossambicus*), as well as *slc12a10.2* (*ncc*) [8,39–44]. In the gills of zebrafish, the expression of the transporters *atp1a1a* (*nka1a*), *atp2b2* (*pmca2*), *atp6v1aa rhcg1*, *slc4a4b* (*nbc*), *slc12a10.2* (*ncc*), *slc12a2* (*nkcc1*) (low expression), *slc8a* (*ncx*), and *slc9a3* (*nhe3*) has been identified [45,46]. The genes *slc9a2* (*nhe2*) and *slc9a3* (*nhe3*) were cloned in rainbow trout and are present in ionocytes [47].

Other studies showed the possible involvement of some genes of the same transporters in the gills for the ionoregulation of killifish (*Fundulus heteroclitus*), such as *slc12a10* (*ncc2*), *slc12a1* (*nkcc2*), and *slc12a2* (*nkcc1*) [11,39,44,48]. Changes in the activity of proteins and in the expression of the genes involved in the osmoregulation of freshwater teleosts illustrate some of the possible responses that animals may exhibit in the face of osmotic alterations.

NKA plays a very important role in the kidney and gills of teleosts, positively regulating their activity in response to environmental changes [13,56,57]. In the present study, there was an increase in $Na^+/K^+$-ATPase activity in the gills of silver catfish exposed to alkaline water, compared to those exposed to acidic pH, but there was no difference compared to those at neutral pH. There was no change in renal NKA activity, probably due to the time of exposure. The gills and kidney of fish tend to have normal functioning under exposure to very acidic or alkaline waters [15,18,58].

Alkaline water causes transient decreases in the capacity of the ion transport system by directly acting on $Cl^-$ and $Na^+$ transport sites in the gills [17]. Gill NKA activity increased in Amur ide exposed to alkaline water for 5–7 days [26]. Silver catfish exposed to alkaline pH for 24 h showed higher NKA activity in the gills and kidney [59]. Different from enzyme activity, the expression of the NKA transporter (*atp1a1*) increased at acidic (5.5) and alkaline (9.0) pHs in the gills, suggesting that acute exposure is likely to alter the gene expression differently from the activity of the enzyme. In agreement with this hypothesis, gill NKA activity was higher in pacu (*Piaractus mesopotamicus*) exposed for 1 day to pH 9.0, but after 15 days it was higher in those exposed to a pH of 5.5 [34]. The expression of *atp1a1* was lower in the kidney of silver catfish exposed to a pH of 5.5 and 9.0 than the control (pH 7.5). The opposite was observed in the kidney of common carp (*Cyprinus carpio*) exposed to a pH of 4.0 for three days [60].

Vacuolar $H^+$-ATPase (V-ATPase), which also participates in ion regulation and acid-base balance in fish [61,62], secretes $H^+$ by the apical membrane in the $H^+$-ATPase-rich ionocyte of zebrafish gills [46]. In zebrafish larvae exposed to a pH of 4.0 for 4 days, there was an increase in the expression of *atp6v1aa* in the gills [63]. In killifish, $H^+$-ATPase is expressed in the basolateral membranes of cuboidal cells, the cell type responsible for $Na^+$ uptake, and according to [64], $Na^+$ uptake is most likely not coupled with active proton excretion in this species. In the present study, $H^+$-ATPase has higher activity in the gills of silver catfish exposed to alkaline water compared to the other treatments, but there was no significant difference in the activity of this enzyme in the kidney. The same results were observed in the gills and kidney of pacu exposed to a pH of 9.0 for 1 day [34]. However, the expression of the genes of all three different $H^+$-ATPase isoforms expressed in the gills and kidney of silver catfish were not affected by water pH, which could suggest a clear species-specific regulation of this physiological mechanism.

$Na^+/H^+$ exchangers (Slc9a family) are considered to be the main actors in apical $Na^+$ uptake and acid excretion, and can be functionally coupled to ammonia excretion in fish gill ionocytes [3,65]. Slc9a3 (Nhe3) is the major isoform for apical $Na^+/H^+$ exchangers in the gill ionocytes of several FW teleosts (killifish, Mozambique tilapia, medaka—*Oryzias latipes*, and zebrafish, among others) [40,56,66–69]. However, the presence of the Nhe2 protein (*slc9a2*) has been shown in cuboidal cells of killifish [70], whereas *slc9a2* (*nhe2*) and *slc9a3* (*nhe3*) have also been identified in the peanut lectin agglutinin ($PNA^+$) ionocytes of rainbow trout [1,71,72]. In [65], *slc9a3* (*nhe3*) was expressed in gills and kidneys of FW- and seawater-adapted (SW) sea bass (*Dicentrarchus labrax*), with a higher expression in FW-adapted fish.

In zebrafish, the expression of *slc9a3.2* (*slc9a3 tandem repeat 2*) decreased and *atp6v0ca* increased in the gills after a 7-day acclimation to a pH of 4, suggesting that $H^+$-ATPase most likely plays a more prominent role in the uptake of $Na^+$ in acidic water [67]. However, in silver catfish gills, we observed that there was no significant difference in the expression of the different isoforms and activity of $H^+$-ATPase, but there was an increase in the expression of *slc9a3* (*nhe3b*) after exposure to acidic pH. Consequently, it seems that, in this species, *slc9a3* plays a more prominent role in the uptake of $Na^+$ at acidic pHs. Likewise, [66]

showed that exposure to acidic pHs led to a significant increase in the expression of gill *slc9a3* (*nhe3*), but only a slight increase in the β-subunit of $H^+$-ATPase in Osorezan dace, *Tribolodon hakonensis*, suggesting that *slc9a3* (*nhe3*) may play a role in acid secretion, as well as $Na^+$ absorption.

The ammonia transporters (Rhcg1 and/or Rhcg2) present in fish gills [73] form a functional metabolon with Slc9a2 (Nhe2)/Slc9a3 (Nhe3) at acidic pHs [74]. In silver catfish, it was possible to observe a similar response pattern in the *slc9a3* and *rhbg* genes, given there was a significant increase in their expression in the gills of fish exposed to acidic compared to neutral pHs.

The *slc12a7a* and *slc12a4* are the two isoforms tested in silver catfish of this transporter whose expressions are affected by pH because the other two related genes (*slc12a6*, *slc12a7b*) did not change. Mozambique tilapia showed no difference in the expression of *slc12a7b* (*kcc4*) and *slc12a4* (*kcc1*) between fresh water and fresh water with high potassium concentration [53], which is consistent with the results of SW-acclimated tilapia gills [75]. However, *slc12a7a* was highly expressed in the kidney compared to the gills, both in the control and in exposure with a high concentration of $K^+$, suggesting a significant renal role of the $K^+/Cl^-$ symporter [53]. The *slc12a4* gene was expressed in the gills and kidneys of silver catfish, but it showed a significant difference only in the kidney, in which fish exposed to a pH of 9.0 showed higher expression compared to a pH of 5.5.

The expression of *slc12a2* in the gills of silver catfish was higher at pH 5.5 compared to the other treatments, and its expression in the kidney was not significantly affected by the pH change. The occurrence of the $Na^+/K^+/2Cl^-$ symporter has been demonstrated, not only in the branchial and opercular epithelia [44,48,76–78], but also in the renal [79] and intestinal epithelia [80,81]. Bumetamide, an inhibitor of this symporter, did not affect $Na^+$ influx in native cardinal tetra (*Paracheirodon axelrodi*), hemigrammus tetra (*Hemigrammus rhodostomus*), and moenkhausia tetra (*Moenkhausia diktyota*) from the Rio Negro, an acidic, ion-poor, and black water river, but these species have different $Na^+$ uptake mechanisms from the "standard" FW teleosts [82]. This symporter is considered to be mainly related to SW adaptation [8,48,78], and the expression of its gene decreased in the opercular epithelium of killifish one day after transference from seawater to freshwater, but three days later its expression was not significantly different between these two waters [11], demonstrating a high-performance plasticity of this gene/protein to acquire its homeostatic load. The same authors verified that killifish maintained for one year in freshwater presented the same expression of this gene in the opercular epithelium than seawater killifish. The freshwater-adapted Mozambique tilapia presents this symporter in the basolateral membrane of gill ionocytes type III [1]. As of yet, the relationship between the $Na^+/K^+/2Cl^-$ symporter and ion uptake in freshwater has not been explained [3], and consequently, it is not clear if the increased expression of the *slc12a2* gene in the gills of silver catfish exposed to acidic pH might be related to a mechanism for compensating the ion loss by diffusion.

Most teleost species presented ammonia as the main nitrogenous excretory product [59,83]. Non-ionized ammonia ($NH_3$) enters the ionocytes with the aid of the Rhbg glycoprotein, while the Rhcg1 and/or Rhcg2 glycoproteins transport ammonia out of ionocytes [74], and the formation of the $NH_4^+$ boundary layer from the reaction of $NH_3$ and $H^+$ (from function Nhe) would locally increase pH and decrease $NH_3$, facilitating NHE activity in apparently thermodynamically unfavorable low pH environments [1]. In the current study, the expression of *rhbg* was higher in the gills of silver catfish exposed to acidic pH (5.5) than in neutral pH (7.5), which could be related to increased ammonia excretion at acidic pHs, but additional studies investigating expression of the *rhcg1* and *rhcg2* genes must be performed to confirm this hypothesis and to verify if this could be a cause or a consequence of pH and ammonia balance. The increase in ammonia excretion at acidic pHs may be a strategy to facilitate $Na^+$ uptake, as observed in the larvae of *D. rerio* exposed to acidic pHs [84]. However, in the kidney, there was lower *rhbg* expression at a pH of 5.5 and 9.0 compared to a neutral pH, suggesting a lower contribution (if any) of this

transporter at the renal level during acute pH variations. Lower renal *rhbg* expression was also observed in common carp exposed for three days to a pH of 4.0 [60].

The genes *slc12a7b* and *slc121a6* did not show significant differences between treatments in the gills and kidney. Overall, the gills of silver catfish exposed to acidic and alkaline pHs showed more significant alterations in the gene expression of ion transporters, probably because this structure is the primary site for ion regulation, acid-base balance, and nitrogen waste excretion [3,7,9]. Longer exposure to acidic and alkaline pHs may yield different results for the enzyme activities and the expression of transporters in the gills and kidney of silver catfish.

In conclusion, silver catfish expressed several transporters in the gills and kidneys that are involved with osmoregulation. Acute pH alterations (24 h) change the *atp1a1* gene expression differently from the activity of the enzyme, and the increase in the $H^+$-ATPase activity in the gills in alkaline water seems to be an important strategy to maintain ionic balance. The *slc9a3* gene seems to play a more prominent role in the uptake of $Na^+$ at acidic pHs than $H^+$-ATPase in silver catfish. It was possible to observe a response pattern in the *slc9a3* and *rhbg* genes in fish exposed to acidic pH. The *slc12a7a* and *slc12a4* seem to be affected by pH because the other two related genes (*slc12a6*, *slc12a7b*) did not change their expression levels. The results of the expression of *slc12a4* suggest that it may be involved in the adaptation to alkaline pHs. The *slc12a2* may be related to a mechanism to increase ion uptake at acidic pHs to compensate for the loss of ions by diffusion. As expected, *rhbg* most likely participates in the excretion of ammonia in the gills, and it does not contribute to change renal activity during acute pH variations.

Finally, as the gills are the main target of acidic or alkaline waters and are in close contact with the environment, they showed greater responses than the kidneys to changes in pH. Changes in the activity of proteins and in the expression of the genes involved in the osmoregulation of freshwater teleosts illustrate some of the responses that animals may exhibit in the face of osmotic alteration.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7050261/s1, Supplementary Figures S1–S8.

**Author Contributions:** Conceptualization, M.T.S.M., B.B., G.M.-R.; methodology: B.B., G.M.-R., J.A.M.-S., C.d.F.S., A.P.G.A., A.Q.A.; validation: G.M.-R., J.A.M.-S., C.d.F.S., A.P.G.A., A.Q.A.; formal analysis, M.T.S.M., B.B., G.M.-R., J.A.M.-S., C.d.F.S., A.P.G.A., A.Q.A.; investigation, M.T.S.M., C.d.F.S., A.P.G.A., S.N.D., A.E.B.; resources, B.B., G.M.-R., J.A.M.-S., A.Q.A.; data curation, M.T.S.M., C.d.F.S., A.P.G.A., S.N.D., A.E.B.; writing—original draft preparation, M.T.S.M., C.d.F.S., A.P.G.A., S.N.D., A.E.B., B.B., G.M.-R., J.A.M.-S., A.Q.A.; writing—review and editing, M.T.S.M., A.P.G.A., B.B., G.M.-R., J.A.M.-S.; visualization, M.T.S.M., A.P.G.A., B.B., G.M.-R., J.A.M.-S.; supervision, B.B., G.M.-R., J.A.M.-S.; project administration, B.B., G.M.-R., J.A.M.-S.; funding acquisition, B.B., G.M.-R., J.A.M.-S., A.Q.A. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Acronyms

| | |
|---|---|
| atp1a1 | sodium/potassium-transporting ATPase subunit alpha-1 |
| Atp1a1 | Sodium/potassium-transporting ATPase |
| *atp2b2* | *Plasma membrane $Ca^{2+}$ transporting ATPase 2* (previously known as *pmca2*) |
| Atp2b2 | Plasma membrane $Ca^{2+}$ transporting ATPase 2 |
| *atp6v1aa* | ATPase $H^+$ transporting V1 domain |
| Atp6v1aa | V-type proton ATPase V1 |
| *atp6v0a1b* | ATPase $H^+$ transporting V0 subunit a1b |
| Atp6v0a1b | V-type proton ATPase subunit a1 isoform b |
| *atp6v0a2a* | ATPase $H^+$ transporting V0 subunit a2a |
| Atp6v0a2a | V-type proton ATPase subunit a2 isoform a |
| *atp6v0a2b* | ATPase $H^+$ transporting V0 subunit a2b |
| Atp6v0a2b | V-type proton ATPase subunit a2 isoform b |
| *atp6v0ca* | ATPase $H^+$ transporting V0 subunit c |
| Atp6v0ca | V-type proton ATPase proteolipid subunit |
| *rhbg* | Rh family B glycoprotein |
| Rhbg | Ammonium transporter Rh type B |
| *rhcg1* | Rh family C glycoprotein 1 |
| Rhcg1 | Ammonium transporter Rh type C-like 1 |
| *rhcg2* | Rh family type C glycoprotein 2 |
| Rhcg2 | Ammonium transporter Rh type C 2 |
| *slc4a4b* | solute carrier family 4 member 4b (previously known as nbc) |
| Slc4a4b | solute carrier family 4 member 4b ($Na\text{-}HCO_3$ cotransporter) |
| *slc8a* | solute carrier family 8 (previously known as ncx) |
| Slc8a | solute carrier family 8 (Na/Ca exchanger) |
| *slc9a2* | solute carrier family 9 member 2 (previously known as nhe2) |
| Slc9a2 | solute carrier family 9 member 2 (sodium/hydrogen exchanger) |
| *slc9a3* | solute carrier family 9 member 3 |
| Slc9a3 | Sodium/hydrogen exchanger (Previously known as Nhe3b) |
| *slc12a2* | solute carrier family 12 member 2 (previously known as *nkcc1*) |
| Slc12a2 | Solute carrier family 12 member 2 (previously known as Na-K-Cl cotransporter 1) |
| *slc12a4* | solute carrier family 12 member 4 |
| Slc12a4 | Solute carrier family 12 member 4 (Potassium/chloride transporter) |
| *slc12a6* | solute carrier family 12 member 6 |
| Slc12a6 | Solute carrier family 12 member 6 (Potassium/chloride transporter) (previously known as Electroneutral potassium-chloride cotransporter 3, or K-Cl cotransporter 3, Kcc3) |
| *slc12a7a* | solute carrier family 12 member 7a |
| Slc12a7a | Solute carrier family 12 member 7a (Potassium/chloride transporter) (previously known as Electroneutral potassium-chloride cotransporter 4, K-Cl cotransporter 4, Kcc4) |
| *slc12a7b* | solute carrier family 12 member 7b |
| Slc12a7b | Solute carrier family 12 member 7b (Potassium/chloride transporter) (previously known as Electroneutral potassium-chloride cotransporter 4, K-Cl cotransporter 4, Kcc4) |
| *slc12a10* | solute carrier family 12 member 10 |
| Slc12a10 | Solute carrier family 12 member 10 (previously known as Ncc) |

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