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Insights in aquatic physiology: 2021

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Editorial: Insights in aquatic physiology: 2021

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Editorial on the Research Topic
Insights in aquatic physiology: 2021

In recent years, scientists have made exceptional achievements that have led to major advancements in the fast-growing field of Aquatic Physiology. This editorial initiative, led by Prof. Andreas Fahlman, the previous Specialty Chief Editor of the Aquatic Physiology Section, is focused on the new insights, novel developments, current challenges, latest discoveries, and future perspectives in the field of Aquatic Physiology. Distinguished and active researchers have been invited to identify the greatest challenges in the sub-disciplines of Aquatic Physiology and offer solutions to address those challenges. This article Research Topic covers the basic mechanisms of osmoregulation, acid-base regulation, respiration, the nervous system, endocrines, behaviors, and digestion at the molecular, cellular, and biochemical levels, and further extends the discussion to aspects of evolutionary physiology. These articles will inspire, inform, and provide new direction to researchers in the field.

Aquatic animals must maintain ionic and acid-base homeostasis in their body fluids to ensure the normal operations of cellular activities and physiological processes. This mechanism is a basic platform for studying environmental adaptation, pollution toxicology, stress biology, and evolutionary physiology of aquatic animals, and further provides important information to improve management of aquaculture practice. Life in fresh water (FW) is osmotically and energetically challenging for living animals because they need to continuously take up Na^+ (and other ions) from a salt-dilute environment. Our understanding of Na^+ uptake mechanisms in invertebrates is much poorer than that in fishes. To this end, Lee et al. proposed the Na^+/H^+ antiporter (NHA) as a possible candidate for the Wieczorek Exchanger in many crustaceans and insects, which is hypothetically driven by apical proton pump V-type H^+ ATPase (VHA). More studies are awaited to explore the Na^+ uptake of NHA and other potential Wieczorek Exchangers. In the case of teleosts, which are the most studied fish group, Shih et al. reviewed the recent progress and clarified the related debates in their Na^+ uptake mechanisms. FW teleosts absorb Na^+ from the environment mainly via Na^+/H^+ exchanger 3 (NHE3)-mediated and Na^+-Cl^- co-transporter (NCC) mediated mechanisms in gill/skin ionocytes, and they rely on NHE for a majority of Na^+ uptake, probably due to a favorable NH_4^+ gradient that efficiently drives NHE. NHEs are considered the major acid-secreting transporters in seawater (SW) fishes as well as in FW ones. NHE3 is the most studied NHE isoform, while NHE2 and its function has never been clarified. According to Liu et al., NHE2 is localized at the basolateral membrane of fish ionocytes and appeared to facilitate acid and salt secretion in sea water (SW) medaka, providing a new insight into the role of NHE2 in SW osmoregulation. Gill (or skin) ionocytes are important sites for fish

iono- and osmo-regulation. Different types of ionocytes expressing distinct sets of ion transporters were identified by Inokuchi and her colleagues (Inokuchi et al.) in tilapia (*Oreochromis mossambicus*) gills, providing a platform to explore the plasticity in ion transport functions and ionocyte morphologies in fish when coping with salinity challenges.

In addition to the gills, the kidney is an essential organ participating in fish osmoregulation. Recent progress in the identification and functional analyses of relevant ion transporters in the kidney of FW and SW teleosts were reviewed by Kato et al. These transporters are involved in the secretion of sulfate and magnesium ions (by the proximal tubules in SW), the reduction of urine volume (by the collecting ducts in SW), and the excretion of water through hypotonic urine (by the distal tubules and collecting ducts in FW). The kidney also plays a major role in osmoregulation of the few euryhaline elasmobranchs. For examples, in the euryhaline stingray that has acclimated to FW, the kidney, rather than gills, is the major organ responsible for NaCl reabsorption and dilute urine production. Aburatani et al. explored the roles of Na^+-K^+-Cl^- cotransporter 2 (NKCC2) and Na^+/K^+-ATPase (NKA) in NaCl and urea reabsorption (associated with dilute urine production) in the early distal tubule in the bundle zone of the Japanese red stingray (*Hemitrygon akajei*) kidney. Upregulation of the genes encoding NKCC2 and NAK found in FW-acclimated individuals is proposed as an advantageous feature that facilitate acclimation to a wide range of salinities, which might have allowed the batoids to expand their distribution towards low salinity habitats. The gastro-intestinal tract is the major organ for water drinking and absorption in fishes acclimation to SW. Grosell et al. compared different methods, whole-animal and isolated-tissue oxygen consumptions and the estimates of ATP consumption, to determine the metabolic cost of osmoregulation in the marine fish (*Opsanus beta*). The theoretical estimates of esophageal and intestinal osmoregulatory costs were in close agreement with direct measurements on isolated tissues, suggesting that these tissues amount to ~2.5% of standard metabolic rates. The whole animal measurements were variable between fish and is not suited to detect the relatively modest metabolic cost of osmoregulation. The antennal gland is also an important osmoregulatory organ, particularly in terrestrial crabs. In the review by Tseng et al., species with higher NKA activity in the antennal gland showed a lower urine-hemolymph ratio for Na^+ concentration under hypo-osmotic stress, suggesting a correlation between the structural and functional differences in gills and lung-like structure among crabs. This finding provides new insights into the evolutionary physiology of crab osmoregulation.

Secreting the excess acid that results from metabolic acidosis or an acidified environment is another essential task for aquatic animals to maintain their body fluid homeostasis. Acid secretion mechanism in aquatic animals is known to interact with the mechanisms involved in ion transport and ammonia excretion as well as to affect energy metabolism. It has been proposed that acid secretion mechanisms in fishes, as well as in mammals, is tightly controlled by hormones. Lin et al. reported a novel action of vitamin D (VD), a well-known calciotropic hormone, on zebrafish (*Danio rerio*) acid secretion. Signaling of VD and VD receptors upregulated acid secretion through stimulating the related acid-secreting transporters. Meanwhile, cephalopods live a lifestyle with high energy expenditure, which is fueled exclusively by protein diets, thus resulting in high production of ammonia as metabolic waste. According to Lin et al., benthic octopuses with lower aerobic respiration rates utilize both active and Na^+-driven secondary transport mechanisms, while pelagic squids, with higher aerobic respiration rates, prefer active NH_4^+ and H^+ transport mechanisms that consume ATP intensively. Therefore, diverse strategies were adopted by cephalopods to fit their different lifestyles.

It is critically important for fish to regulate and control their ventilation and maintain adequate water flow to match branchial gas transfer with their metabolic needs under different environmental or physiological situations. Perry et al. reviewed the control and consequences of ventilatory adjustments, the chemoreceptor cells and the mechanisms used to sense O_2 and/or CO_2 in zebrafish larvae. This review on zebrafish larvae paves the way to study fish respiratory physiology using advanced molecular physiological approaches that have been recently established in the species. A further study on fish respiratory physiology was done by Reed and Jonz. All the receptors implicated in the neurotransmission or neuromodulation associated with O_2 sensing in the gills, including serotonergic, cholinergic, purinergic, and dopaminergic signaling, were summarized, but the receptor characterization at the cellular level remains to be done.

Behaviors are the outcomes of physiological performance that are integrated by the nervous system. Fu et al. summarized recent studies regarding the effects of internal and external stimuli on fish behaviors and proposed that studying behaviors can be an alternative way to conduct environmental and physiological assessments. Investigations into fish stress coping strategies, which are involved in endocrine control, physiological regulation, and behavioral response, are important because the related knowledge can improve fish welfare and promote the sustainable development of aquaculture. In the study by Zeng et al., active coping flounder increased prolactin-mediated ionocyte differentiation to deal with low-salinity, while passive coping flounder employed a passive strategy of reducing gill ionocytes number. Finally, the study by Moffatt et al. was extended to understand the mechanisms underpinning fish digestion and growth performance. The experiments with omeprazole, a gastric proton pump inhibitor, highlighted the importance of stomach acidification in digestion and growth and present a novel way of determining the cost of gastric digestion.

This Research Topic provides some new insights into our understanding of the basic physiology in aquatic animals and further suggests new directions for future studies.

Author contributions

All authors contributed to the article and approved the submitted version.

Conflict of interest

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Role of the Basolateral Na^+/H^+ Exchanger-2 (NHE2) in Ionocytes of Seawater- Acclimated Medaka (*Oryzias latipes*)

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Ionocytes in the skin and gills of seawater (SW) fishes are responsible for acid-base regulation and salt secretion. Na^+/H^+ exchangers (NHEs) are considered the major acid (H^+)-secreting transporters in ionocytes of SW fishes. However, the subcellular localization and function of a specific NHE isoform (NHE2) have never clearly been revealed. In this study, we cloned and sequenced NHE2 from an SW-acclimated medaka (*Oryzias latipes*) and examined its functions in medaka embryos. A phylogenetic analysis showed that the evolutionary relationships of mammalian NHE2 and NHE4 are close to those of fish NHE2. A gene structure analysis showed that tetrapod NHE4 might be a tandem duplication of fish NHE2. Immunohistochemistry with a medaka-specific antibody localized NHE2 to the basolateral membrane of ionocytes. Lost-of-function experiments with photo-activated morpholino oligonucleotides showed that both H^+ and Cl^- secretion by ionocytes were suppressed in NHE2-knockdown embryos, suggesting that the basolateral NHE2 facilitates acid and salt secretion by ionocytes of medaka in seawater.

Keywords: salt secretion, acid-base balance, medaka, ionocyte, Na^+/H^+ exchange

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INTRODUCTION

Acidification of both marine and fresh water is a major environmental problem, and it poses a global threat to aquatic animals including fishes. Understanding the molecular and cellular mechanisms for acid-base regulation in fishes can establish the fundamental knowledge to evaluate the impacts of environmental acidification on fishes. Acid-base regulation in fishes is mainly achieved by gills and skin (Evans et al., 2005; Hwang et al., 2011). In gill and skin epithelium, ionocytes (also called mitochondria-rich cells or chloride cells) play major roles in ion and acid-base regulation (Yan and Hwang, 2019). It is generally accepted that sodium (Na^+) and hydrogen (H^+) exchangers (NHEs) are the major transporters of acid (H^+) secretion by ionocytes (Hwang and Chou, 2013; Liu et al., 2013). Several NHE isoforms (NHE1, NHE2, NHE3, NHE5, NHE6, NHE7, and NHE8) were found in fishes, (Hu et al., 2013; Yan and Hwang, 2019); and NHE1, NHE2, and NHE3 were found in the gills of fishes (Claiborne et al., 1999, 2002; Edwards et al., 2005). In freshwater (FW) fishes, NHE3 at the apical membrane was demonstrated to be the major isoform for acid secretion by ionocytes (Yan et al., 2007; Inokuchi et al., 2008; Wu et al., 2010).

In seawater (SW) fishes, two NHE isoforms, NHE2 and NHE3, were identified in ionocytes of gills. In Japanese eel (*Anguilla japonica*) and mangrove rivulus (*Kryptolebias marmoratus*), NHE3 was localized to the apical membrane of ionocytes (Cooper et al., 2013; Seo et al., 2013). In medaka (*Oryzias latipes*) acclimated to SW, NHE3 was localized to apical membranes of ionocytes and demonstrated to be involved in acid secretion (Liu et al., 2013, 2016). Compared to NHE3, however, the localization and function of NHE2 in ionocytes of SW fish are still uncertain. Several studies showed that messenger (m)RNA and protein expressions of NHE2 were stimulated by acidic water or infusion of HCl, suggesting that NHE2 is involved in acid secretion by gills (Claiborne et al., 1999; Tresguerres et al., 2005; Catches et al., 2006).

Medaka is highly adaptable to different salinities, is amenable to genetic manipulation, and is suitable for studies of organogenesis (Wittbrodt et al., 2002; Takeda and Shimada, 2010). Our previous studies demonstrated that medaka embryos are an ideal model for the functional study of ion transport and acid-base regulation by skin ionocytes (Wu et al., 2010; Shen et al., 2011; Liu et al., 2013, 2016). Previously, we found that both NHE2 transcripts and NHE3 proteins were localized at the same ionocytes in SW-acclimated medaka embryos (Liu et al., 2013), and demonstrated that H^+ secretion by ionocytes was linked to Cl^- secretion (Liu et al., 2016). However, the protein localization and function of NHE2 in SW-type ionocytes are still unclear. Is NHE2 also located on the apical membrane of ionocytes? If so, what is the functional difference between NHE2 and NHE3 in acid secretion? We attempted to answer these questions in this study. First, we cloned and sequenced NHE2 (*slc9a2*) from gills of medaka, and analyzed the evolutionary relationships among fish and mammalian NHE paralogs (NHE1∼9) with a phylogenic tree analysis. We analyzed the gene structures of NHE2 and NHE4 orthologs to clarify evolutionary relations between NHE2 and NHE4. In addition, we also collected the deduced amino acid sequences of NHE2 and compared differences between fishes and mammals. Second, a specific antibody was generated to localize NHE2 in ionocytes. Finally, we knock down the gene expression of NHE2 in medaka embryos by using photo-activated morpholino oligonucleotides (photo-MOs), which can suppress the gene expression at a specific stage of embryos (Tallafuss et al., 2012). Functional changes (H^+ and Cl^- secretion) were analyzed *in vivo* with an electrophysiological technique following our previous studies (Wu et al., 2010; Shen et al., 2011; Liu et al., 2016).

MATERIALS AND METHODS

Medaka Husbandry and Seawater Acclimation

The animal husbandry and experimental protocols of the present study were reviewed and approved by the Animal Care and Utilization Committee of National Taiwan Normal University. Adult medaka (*Oryzias latipes*) were reared in 1200 (length) × 450 (width) × 600 (height)-mm FW tanks equipped with water filters. We refreshed a quarter of the tank water every week. The water temperature of the tanks was controlled to 28°C by a thermal controller and the photoperiod of the animal room was controlled 14 h of light and 10 h of the dark. Fish were fed at least twice a day. In the morning of ten to ten-thirty, the fertilized eggs were collected from the belly of the female medaka. For SW acclimation, the collected eggs were directly transferred to Petri dishes containing 30 ppt SW and incubated at 28°C. We added the appropriate amounts of sea salt (Instant Ocean, Aquarium System, Mentor, OH, United States) to FW to prepare SW. SW-acclimated adult medaka were obtained by transferring adult fish to 30 ppt SW tanks for 2 weeks.

Phylogenetic Tree and Genomic Sequences Analysis

Coding sequences of known and predicted medaka NHE homologs were obtained from GenBank and Ensembl databases. For the phylogenetic analysis of NHEs candidates, the multiple sequence alignment program "ClustalW2" (European Molecular Biology Laboratory, Heidelberg, Germany) was used to compare the deduced amino acid sequence of medaka NHE homologs with all known data available in the European Institute of Bioinformatics (EBI) database and were subjected to phylogenetic inferences using the Neighbor-joining (NJ) method. The MEGA 6.0 (Molecular Evolutionary Genetics Analysis Version 6.0) software was used to perform 1,000 bootstrap replicate analyses. Based on the assemblies of the Ensembl Genome Browser, the physical gene map of the verified *slc9a2* (NHE2) and *slc9a4* (NHE4) loci was scaled. Genes located up-and downstream of *slc9a2* (NHE2) and *slc9a4* (NHE4) genes in these loci were compared to genomes of fish, amphibians, reptilian and mammalian to determine the highest score.

Preparation of RNA and Reverse-Transcription Polymerase Chain Reaction

mRNA levels of NHE2 in different tissues (brain, gills, eyes, heart, liver, intestines, spleen, kidneys, muscles, and fins) of SW-acclimated adult medaka were determined using an RT-PCR. Tissues isolated from three to seven individuals were pooled as one sample. We used Trizol reagent (Invitrogen, Carlsbad, CA, United States) to homogenize all samples according to a volume ratio of 1:10 (sample: Trizol). After homogenizing, 0.1 ml of 1-bromo-3-chloropropane (BCP) solution (Sigma-Aldrich, St. Louis, MO, United States) was added to each sample. After centrifugation at 4°C and 14,000 rpm for 30 min, the supernatant was extracted and taken into the same volume of 100% isopropanol for RNA precipitation. Subsequently, total RNA was purified and DNA contamination was removed by a MasterPure^{TM} RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, United States). After purifying total RNA, the amount and the quality of total RNA were determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, United States). The integrity of total RNA was checked by electrophoresis through an RNA-denaturing gel.

For the reverse-transcription reaction, 3 μg of total RNA was added into a final volume of 13 μl containing 1 μl of 10 mM

dNTPs, 1 μl of 50 mM oligo(dT)$_{20}$ at 65°C for 5 min, and then the samples immediately moved onto the ice for at least 1 min. After that, each sample was supplemented to a final volume of 20 μl containing 5 mM dithiothreitol (DTT), 40 units of an RNase inhibitor (RNAaseOUT) and 200 units of SuperScript IV (SSIV) (Invitrogen, Carlsbad, CA, United States), and then incubated at 55°C for 15 min. For PCR amplification, 0.5 μl of complementary (c)DNA (dilute 1:200 by diethylpyrocarbonate water) from various tissues, including the brain, gills, eyes, heart, liver, intestines, spleen, kidneys, muscles and fins, were used as a template in a 25-μl final reaction volume containing 1 μl of 10 mM dNTP, 1 μl of 40 units of Gen-Taq polymerase (Genemark, Taipei, Taiwan), and 1 μl of 10 mM of NHE2 primers (forward: 5′-ATCGTCTGTTGTGCCCTC-3′; reverse: 5′-CAGTTCCACTCGTGCTCT-3′).

Morpholino Oligonucleotides Design and Microinjection

An antisense morpholino-modified oligonucleotides (MOs; Open Biosystems, Huntsville, AL) with the sequence 5′-TTCTCGTGGACCGAGTACATGATGC-3′ was used to target −6 to + 19 of the coding region of medaka NHE2; a sense photo-activated (photo)-MOs (Open Biosystems, Huntsville, AL), 5′-GCATCATGTACT-photo-GGTCCAC-3′, was used to target normal MOs with the middle of the photo-sensitive subunit. Standard control oligonucleotides (5′-ATCCATCTTGTGTGTTAGAAAACTG-3′; Gene Tools, Philomath, OR, United States) were used as the control MO, based on its no target and no significant biological activity.

The MOs were resuspended in 1X Danieau's solution, stored at −20°C as a stock solution. Before use, we diluted the stock to desired concentrations (0.5, 1.0, 2.0, or 4.0 ng/embryo). In the present study, both MOs (0.5 ng/embryo) and photo-MOs (0.6 ng/embryo) were incubated at 50°C for 10 mins. Subsequently, both MOs and photo-MOs were co-injected into embryos at one to the two-cell stage by a gas-driven microinjection apparatus (ASI, Eugene, OR). After injection with both NHE2 MOs and photo-MOs, 5-day post-fertilization (dpf) embryos were stimulated by UV light (365 nm) for 1 min from UV Lightbox (Gene Tools, Philomath, OR, United States), and then 8-dpf embryos were sampled to measure proteins expression. The 5-dpf embryos not stimulated with UV light (365 nm) served as the control photo-MOs.

Immunohistochemistry

We collected 8-dpf embryos and fixed them in 4% paraformaldehyde (PFA) at 4°C overnight. The protocol for NHE2 IHC followed by that of a previous report (Inoue and Wittbrodt, 2011). For the immunoreaction, a polyclonal antibody against the domain (VAYPGRRSRFGNRSS; Genomics, Taipei, Taiwan) of medaka NHE2 (diluted 1: 300) and goat anti-rabbit immunoglobulin G (IgG) conjugated with Alexa Fluor 488 (Molecular Probes, Carlsbad, CA) (diluted 1: 500) were, respectively, used as the primary and secondary antibody, respectively. For Na^+/K^+-ATPase (NKA) staining, an α5-monoclonal antibody against the α-subunit of the avian NKA (Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA) was used to detect NKA. The protocol was conducted as described in previous reports (Wu et al., 2010; Liu et al., 2013). Finally, images were captured by using a fluorescence microscope (Axioplan 2 Imaging, Carl Zeiss Oberkochen, Germany).

Western Blotting

Ten 8-dpf embryos were collected as one sample and washed twice with phosphate-buffered saline (PBS). Afterward, homogenization buffer (100 mM imidazole, 5 mM EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate) (with the pH adjusted to 7.6) with 10 units of protease inhibitors (Roche, Indianapolis, IN, United States) was used to homogenize all samples. After centrifuging at 4°C and 10,000 rpm for 10 min, the supernatant was extracted for analysis by using BCA protein assay reagents (Pierce Chemical Company, Rockford, IL, United States). Each sample (equivalent to a volume of 20 μg protein) was incubated at 95°C for 10 min for denaturing and then subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was transferred to polyvinylidene difluoride membranes (Millipore, Billerica, CA, United States). For the blocking reaction, the membranes were transferred to 5% non-fat milk at room temperature for 2 h. Afterward, blots were incubated with a rabbit anti-medaka NHE2 polyclonal antibody (diluted 1:1000) and a rabbit anti-human β-actin (Abcam, Tokyo, Japan, diluted 1:1000) at 4°C overnight. Signals of blots were enhanced with a chemiluminescence system (Millipore, Billerica, CA, United States). The membranes were incubated with goat anti-rabbit IgG (H + L) HRP (dilute 1:10000; Invitrogen, Carlsbad, CA, United States). The ImageQuant 4000 system (GE Healthcare, Buckinghamshire, United Kingdom) was used to capture the clear images for analysis. Finally, we quantitated the intensities of immunoreactive bands using ImageJ software[1].

Measurement of H^+ Flux and Cl^- Flux at Ionocytes

The scanning ion-selective electrode technique (SIET; also called the non-invasive micro-test technique, NMT) was used to measure H^+ and Cl^- secretion by skin ionocytes of medaka larvae. H^+-selective and Cl^--selective microelectrodes were prepared as described in previous studies (Shen et al., 2011; Liu et al., 2016). The signal of the microelectrode was connected to the main amplifier through an Ag/AgCl wire electrode holder and preamplifier (Younger United States, Amherst, MA, United States). The movement and positioning of the microelectrodes were performed with a three-dimensional (3D) positioner (Younger United States) driven by a step motor. Imfluxes V2.0 software (youngerusa.com; xuyue.net) was used to control the system and calculate ion fluxes.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Student's unpaired *t*-test (two-tailed) was used for

[1]http://rsbweb.nih.gov/ij/download.html

comparisons of the means of two groups. The significance level was set to $p < 0.05$.

RESULTS

Phylogenetic Analysis, Sequence Identity, and Sequence Analysis of Medaka NHE2

The sequence of medaka NHE2 (ENSORLG00000012399) was predicted using the Ensembl database and was further cloned and sequenced. The NJ method was used to generate the phylogenetic tree of protein sequences of NHEs from human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), medaka (*Oryzias. latipes*), zebrafish (*Danio rerio*), tilapia (*Oreochromis niloticus*), stickleback (*Gasterosteus aculeatus*), and pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*). The phylogenetic analysis demonstrated that the eight members of medaka NHEs (NHE1, NHE2, NHE3, NHE5, NHE6, NHE7, NHE8, and NHE9) were classified into different groups and clustered with orthologues from mammals (**Figure 1**). According to the phylogenetic analysis, the fish NHE2 clade was most closely related to the mammalian NHE2 and NHE4 clades. Further, gene arrangements in the genomic regions encompassing NHE2 and NHE4 were compared. Results showed that NHE2, TMEM182, and MFSD9 were evolutionarily located on the same chromosome from fish to mammals except for INPP4AA (**Figure 2**). The *NHE4* gene occurred beside *NHE2* on the same chromosome of amphibians, reptiles, birds, and mammals but not in fishes, suggesting that *NHE2* duplication had occurred in the chromosome of amphibians and that had formed *NHE4*.

The putative transmembrane (TM) protein prediction of medaka NHE2 was predicted using TMHMM (server v. 2.0)[2] (**Figure 3**). Results showed that 12 TMs (black line) were found in medaka NHE2. In addition, compared to amino acid sequences of mammalian NHE2, we found that fish NHE2 lacked the two proline-rich sequences (Pro-1 and Pro-2) (dashed line) (**Figure 3**). It was demonstrated that Pro-1 and Pro-2 are necessary for appropriate subcellular targeting of rat NHE2 (Chow et al., 1999).

NHE2 (*slc9a2*) mRNA Expression in Various Medaka Tissues

To examine the distribution of *slc9a2* in medaka tissues, mRNA expression of *slc9a2* in various tissues including the brain, gills, eyes, heart, liver, intestines, spleen, kidneys, muscles, and fins, were examined by an RT-PCR. The result showed that *slc9a2* was abundantly expressed in the gills, intestines, and muscles (**Figure 4**). In contrast, *slc9a2* expression was quite weak in the liver, spleen, and kidneys (**Figure 4**). In this study, *rpl7* mRNA was used as an internal control.

[2]http://www.cbs.dtu.dk/services/TMHMM/

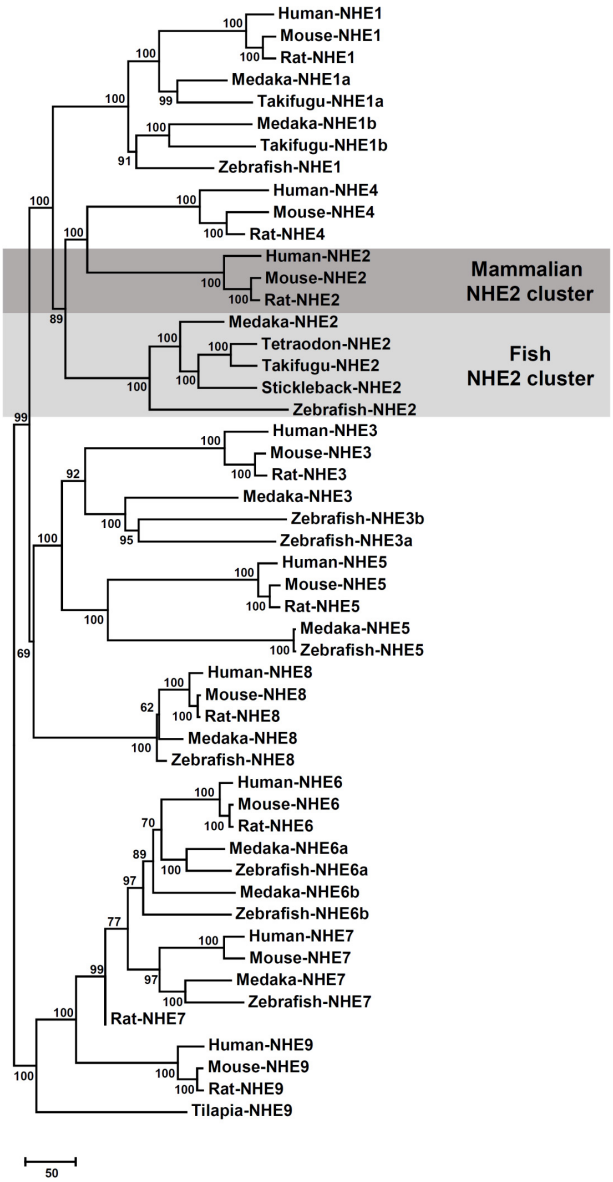

FIGURE 1 | Phylogenetic tree based on comparisons of Na^+/H^+ exchanger (NHE) sequences. Putative sequences of amino acids in medaka (*Oryzias latipes*) and other species were collected from NCBI and Ensembl databases (listed in **Supplementary Table 1**). The Neighbor-joining method was used to build the phylogenetic trees of NHEs and confirmed by 1000 bootstraps. The unit of the scale bar represents the number of substitutions per site.

Immunolocalization of NHE2 in Embryonic Ionocytes

IHC was applied with a medaka NHE2-specific antibody to localize NHE2 protein in ionocytes of SW acclimated embryos (**Figure 5**). NKA immunostaining was used as a marker of ionocytes. Confocal images showed that NHE2 signals (green; **Figures 5A,C**) were colocalized with NKA signals in the basolateral membrane of ionocytes (red; **Figures 5B,C**). The basolateral localization of

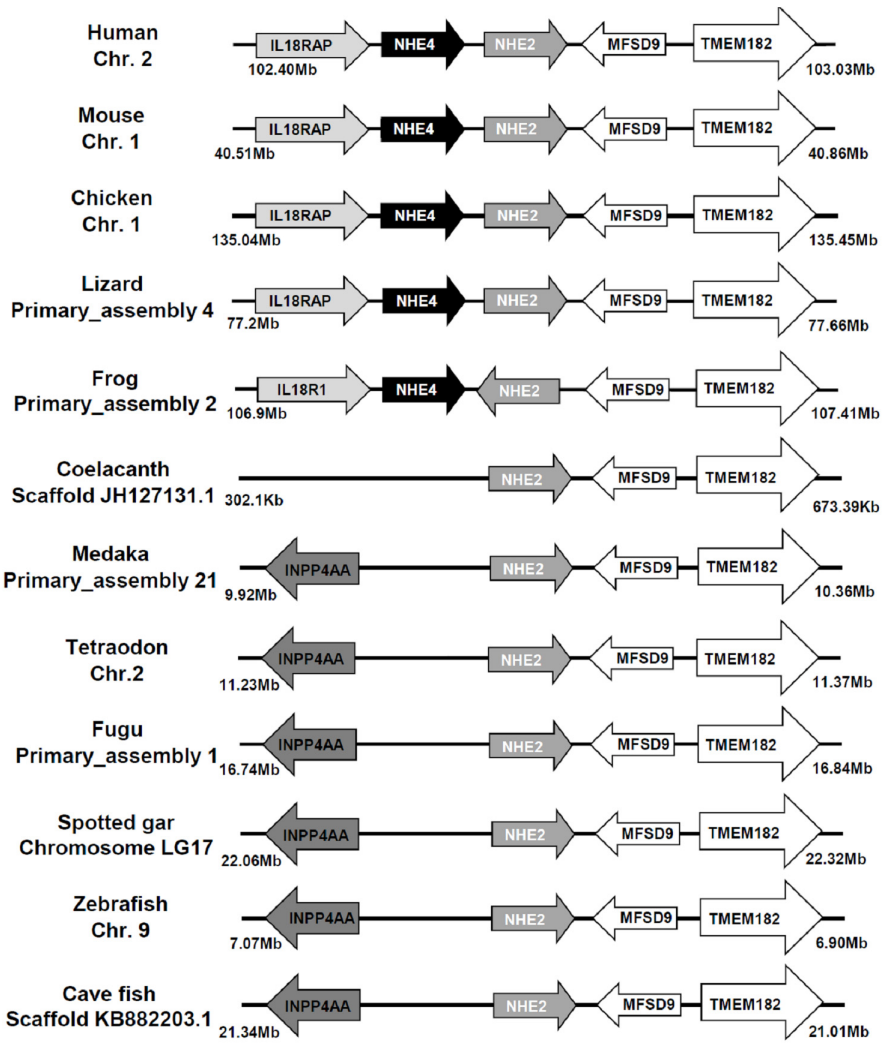

FIGURE 2 | Gene structures of Na^+/H^+ exchanger 2 (NHE2) and NHE4 orthologs. The distance of the genomic region is presented on both sides. The arrow indicates the direction of the gene. Both NHE2 and NHE4 orthologs and their neighboring transcripts were obtained from NCBI and Ensembl databases (as described in **Supplementary Table 1**). Chr., chromosome; IL18RAP (IL18R1), interleukin-18 receptor accessory protein; INPP4AA, inositol polyphosphate-4-phosphatase type I; MFSD9, major facilitator superfamily domain containing 9; TMEM182, transmembrane protein 182.

NHE2 was also confirmed by the confocal z-scanning images (**Figure 5D**).

Mortality Rate and Morphology of Embryos Injected With Traditional or Photo-Activated Morpholino Oligonucleotides

To knockdown NHE2 protein expression, traditional MOs were injected into embryos at the 2-cell stage. Injection with a low dose of NHE2-MOs (0.5 ng/embryo) dramatically increased the mortality rate to 83% at 5 dpf which reached 90% at 8 dpf (**Figure 6A**), while control-MOs showed only 12% mortality at 8 dpf. We also observed severe deformation in embryos, including edema and tail bending (**Figure 6B**), suggesting that NHE2 might be required for early embryo development. To reduce the impact on early development, a photo-MO technique was applied to knock-down NHE2 expression at a later stage (5 dpf). Results showed that the mortality of NHE2-photo-MOs was about 35% at 5 dpf and gradually increased to 55% at 8 dpf, while control-photo-MOs only caused 35% mortality at 8 dpf. Morphological defects were barely observed in NHE2-photo-MOs, with only slight edema or tail bending exhibited (**Figure 6A**).

An immunocytochemical analysis was used to compare NHE2 protein expression in 8-dpf embryos with or without NHE2-knockdown (KD). In the control-photo-MO group, green spots (NHE2 immunosignals) were observed on the yolk sac (**Figure 7A**), while those spots were not evident in the NHE2-photo-MO group. In addition, we also used a Western blot analysis to confirm NHE2-KD. Results showed a predicted band

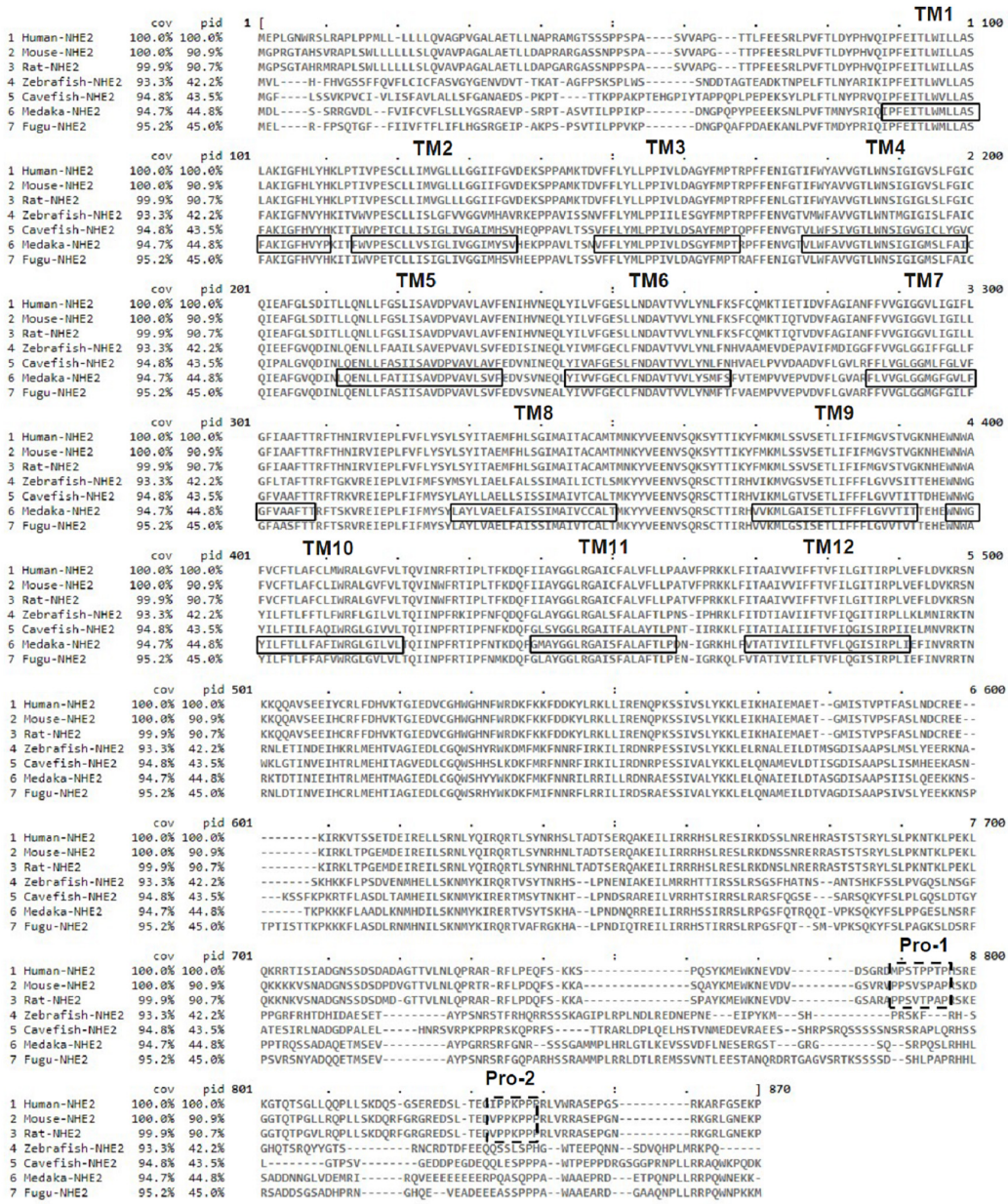

FIGURE 3 | Alignment of the deduced amino acid sequences of Na^+/H^+ exchanger 2 (NHE2) of medaka and other species. The solid line and dotted lines, respectively, mark the postulated transmembrane (TM) domains of NHE2 and proline-rich sequences (Pro-1 and Pro-2).

at 91 kDa in the control-photo-MO group, while the band was very faint in the NHE2-photo-MO group (**Figure 7B**).

Effects of NHE2 Knockdown on the H^+ Flux and Cl^- Flux of Ionocytes in Seawater-Acclimated Medaka Embryos

To analyze the effects of NHE2 knockdown on H^+ and Cl^- secretion by ionocytes, the SIET was used to measure H^+ and Cl^- effluxes at individual ionocytes on the yolk sac skin of medaka. Results showed that the H^+ efflux and Cl^- efflux significantly decreased by 60 and 34%, respectively, following NHE2 knockdown (**Figures 8A,B**).

DISCUSSION

According to the phylogenetic analysis (**Figure 1**), the fish NHE2 clade clustered with mammalian NHE2 and NHE4. This finding is consistent with previous findings that NHE2 of mummichog (*Fundulus heteroclitus*), dogfish (*Squalus acanthias*), and sculpin (*Myoxocephalus octodecemspinosus*)

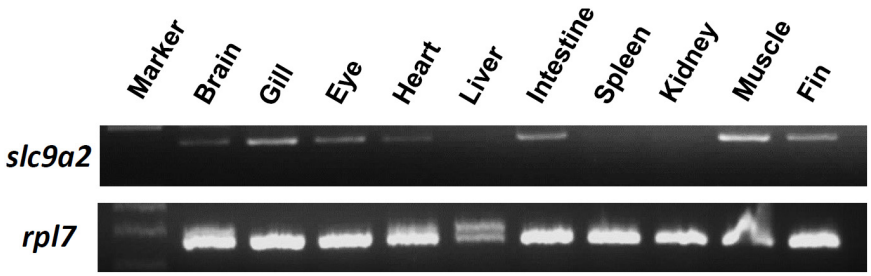

FIGURE 4 | Distribution of *slc9a2* in various tissues of medaka. *slc9a2* transcripts were detected in adult medaka by an RT-PCR. *rpl7* was used as an internal control.

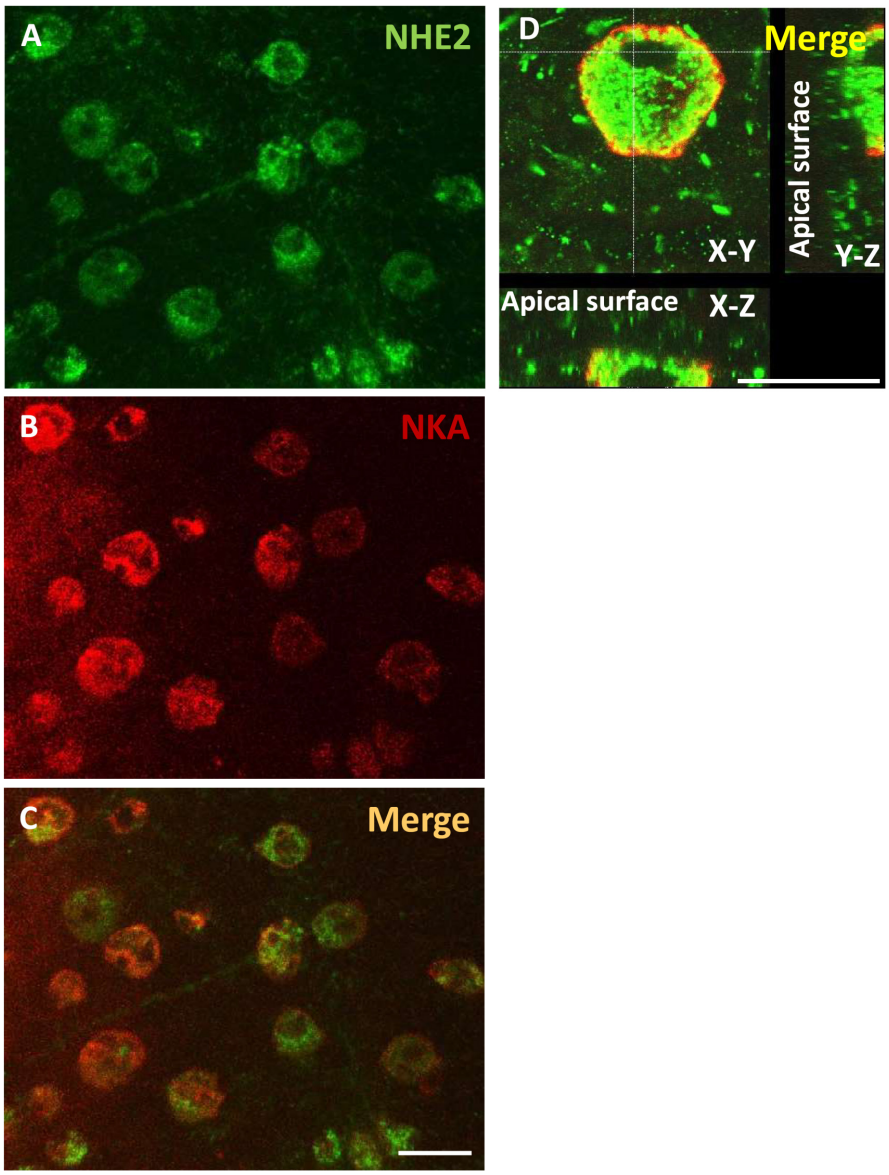

FIGURE 5 | Localization of Na^+/H^+ exchanger 2 (NHE2) in the skin of seawater (SW)-acclimated medaka embryos. Immunocytochemistry of NHE2 **(A)** and Na^+-K^+-ATPase (NKA) **(B)** in 8-day post-fertilization (dpf) medaka embryos, as shown as merged images in **(C,D)**. Scale bar is 10 μm.

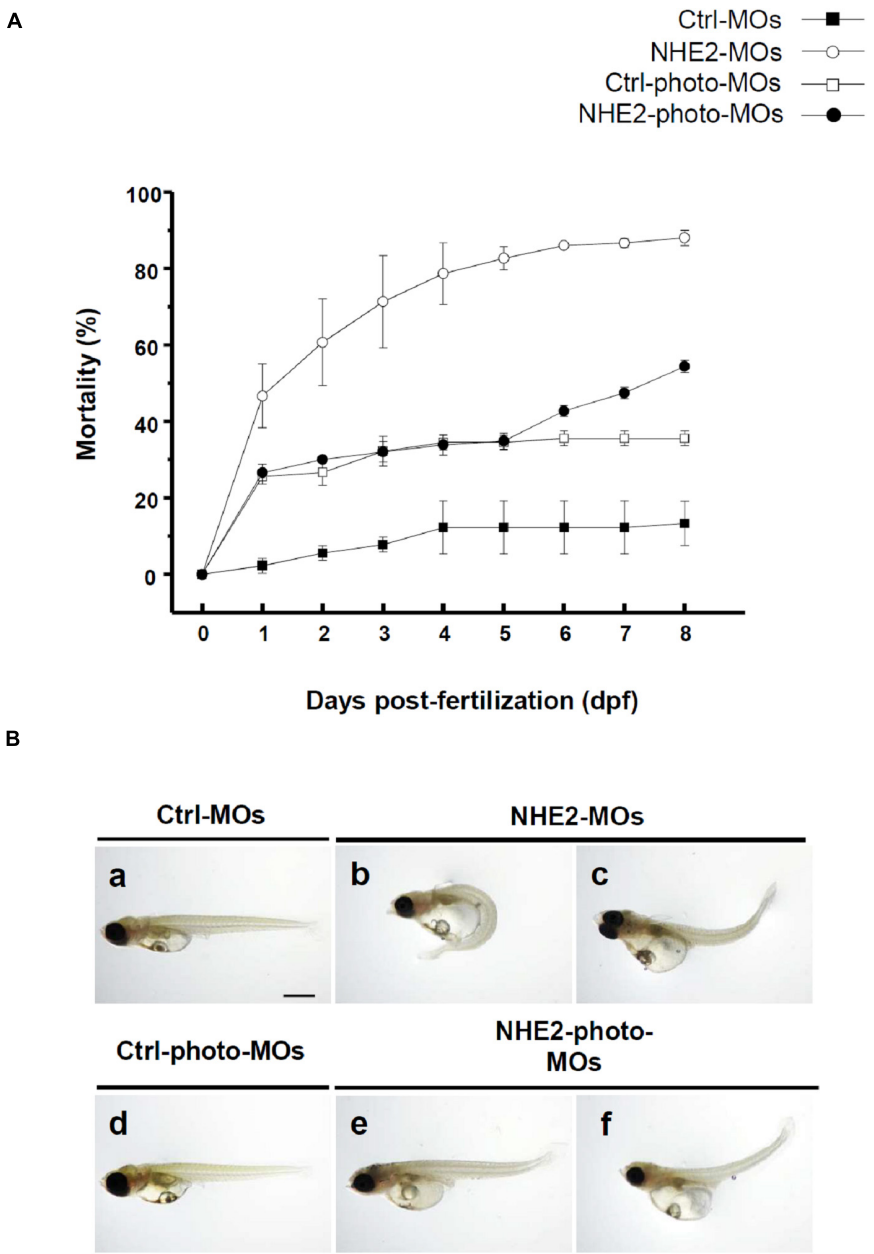

FIGURE 6 | Effects of Na^+/H^+ exchanger 2 (NHE2)-knockdown on mortality and phenotype in seawater (SW)-acclimated embryos. Mortality of control morpholino oligonucleotides (Ctrl-MOs), NHE2-MOs, Ctrl-photoactivated (photo)-MOs, and NHE2-photo-MOs at 0-day post-fertilization (dpf) to 8-dpf SW-acclimated embryos **(A)**. Phenotypes of Ctrl-MOs, NHE2-MOs, Ctrl-photo-MOs, and NHE2-photo-MOs of 8-dpf SW-acclimated embryos **(B)**. Scale bar is 200 μm.

were similar to rat NHE2 and NHE4 (Claiborne et al., 2002). Furthermore, genomic loci of the physical distance (according to information collected from the Ensembl database) showed that *NHE2* and its adjacent genes, including *TMEM182* and *MFSD9*, were evolutionarily located on the same chromosome from fish to mammals. The *NHE4* gene appeared beside *NHE2* on the same chromosome of amphibians, reptiles, birds, and mammals but not in that of fishes, suggesting that *NHE4* might have been tandem-duplicated in amphibians from the proto *NHE2* gene (**Figure 2**).

Moreover, the full-length amino acid identities between the fish NHE2 clade and mammalian NHE2 clade were about 40.1∼44.7%, while those of the fish NHE2 clade and mammalian NHE4 clade were about 37.4∼41.9% (**Supplementary Table 2**), suggesting that the properties of fish NHE2 might be similar to those of both mammalian NHE2 and NHE4. In mammalian kidneys, NHE2 is mainly expressed in the apical membrane of the thick ascending limb (TAL), distal convoluted tubules (DCTs) and connecting tubules (CTs), while NHE4 is expressed in the basolateral membrane of the TAL and DCTs

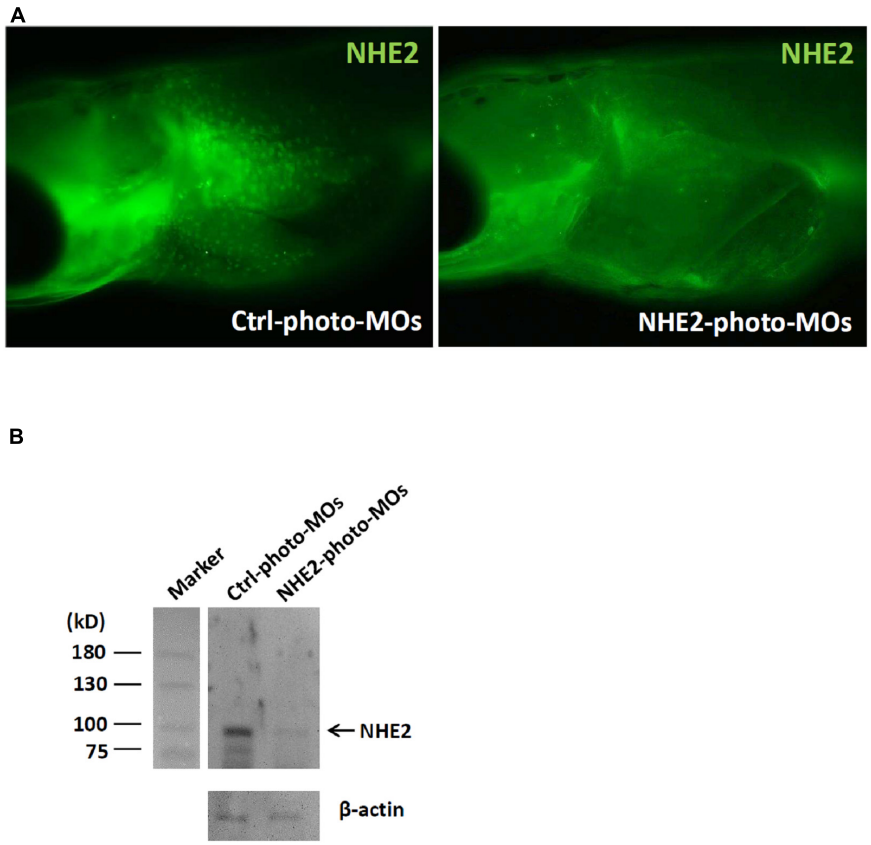

FIGURE 7 | Na^+/H^+ exchanger 2 (NHE2) protein expression in seawater (SW)-acclimated medaka embryos. **(A)** Immunocytochemistry of NHE2 in 8-day post-fertilization (dpf) medaka embryos in the control (Ctrl) photoactivated morpholino oligonucleotide (photo-MO) and NHE2 photo-MO groups. **(B)** Western blot analysis of NHE2 in the Ctrl photo-MO and NHE2 photo-MO groups. β-Actin was used as an internal control.

(Chambrey et al., 1998, 2001; Houillier and Bourgeois, 2012). The basolateral localization of medaka NHE2 is similar to that of mammalian NHE4. Chow and colleagues (Chow, 1999; Chow et al., 1999) identified an apical targeting signal in a 45-residue region of the cytosolic domain of mammalian NHE2. Deletion of the targeting region of NHE2 caused a miss-targeting of NHE2 to the basolateral membrane of renal cells. In the deduced protein sequences alignment of fishes to mammals, the apical targeting sequence of mammals was not found in fish NHE2 (**Figure 3**), suggesting that fish NHE2 is probably not an apical protein.

It is generally accepted that SW fishes excrete metabolic acid *via* NHEs, including NHE2 and NHE3, in the apical membrane of ionocytes (Evans et al., 2005; Hwang et al., 2011). However, previous studies did not provide convincing data to demonstrate the subcellular localization (apical or basolateral side) of NHE2 in SW fishes. Using heterologous antibodies (against mammalian NHE2), Edwards and colleagues localized NHE2 to ionocytes in gills of elasmobranches (Edwards et al., 2002). However, the subcellular localization of NHE2 was uncertain because granular signals were found in the cytoplasm of ionocytes (Edwards et al., 2002). In longhorn sculpin (*Myoxocephalus octodecemspinosus*), a homologous antibody was applied to localize NHE2 in ionocytes (Catches et al., 2006). They found only partial NHE2 signals in the apical membrane but most signals in the cytoplasm of ionocytes. Our previous study showed that the NHE2 transcript and NHE3 protein were expressed in the same ionocytes in SW-acclimated medaka (Liu et al., 2013). However, the subcellular localization of NHE2 in medaka ionocytes was unclear. In the present study, we generated an isoform-specific antibody to demonstrate the basolateral localization of NHE2 in medaka. To our knowledge, this is the first study to reveal the basolateral localization of NHE2 in fish ionocytes and the first study to examine its function with a gene-knockdown technique.

To investigate the function of NHE2 in medaka, a loss-of-function approach with MOs knockdown was conducted. With a traditional MOs injection, we found that the mortality rate was very high (∼90%), indicating that NHE2 might play a critical role in early embryonic development. To prevent the lethal effects in early development, a photo-MOs technique (Tallafuss et al., 2012) was used to knock down NHE2 expression at a later developmental stage. With the photo-MOs technique, we activated antisense MOs with UV light to knock down NHE2 expression at 5 dpf, and the mortality rate of embryos at 8 dpf was relatively lower (55%). In addition, the NHE2 protein abundance was successfully suppressed demonstrating the feasibility of the photo-MOs technique in medaka embryos. Using the SIET to

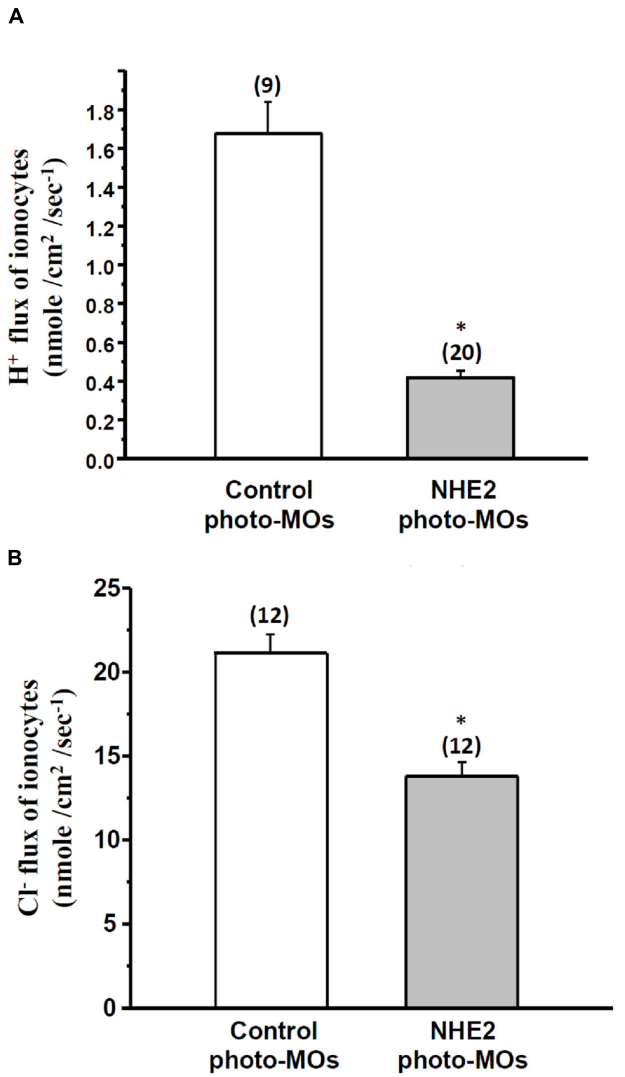

FIGURE 8 | Effects of Na^+/H^+ exchanger 2 (NHE2)-knockdown on H^+ flux and Cl^- flux of ionocytes in seawater (SW)-acclimated medaka embryos. Effects of control photoactivated morpholino oligonucleotides (Ctrl-photo-MOs) and NHE2 photo-MOs on H^+ flux **(A)** and Cl^- flux **(B)** of ionocytes in medaka embryos acclimated to SW. Data are presented as the mean ± SE. The number of cells or larvae is shown in parentheses. * Indicates a significant difference (unpaired two-tailed Student's *t-test*, $p < 0.05$).

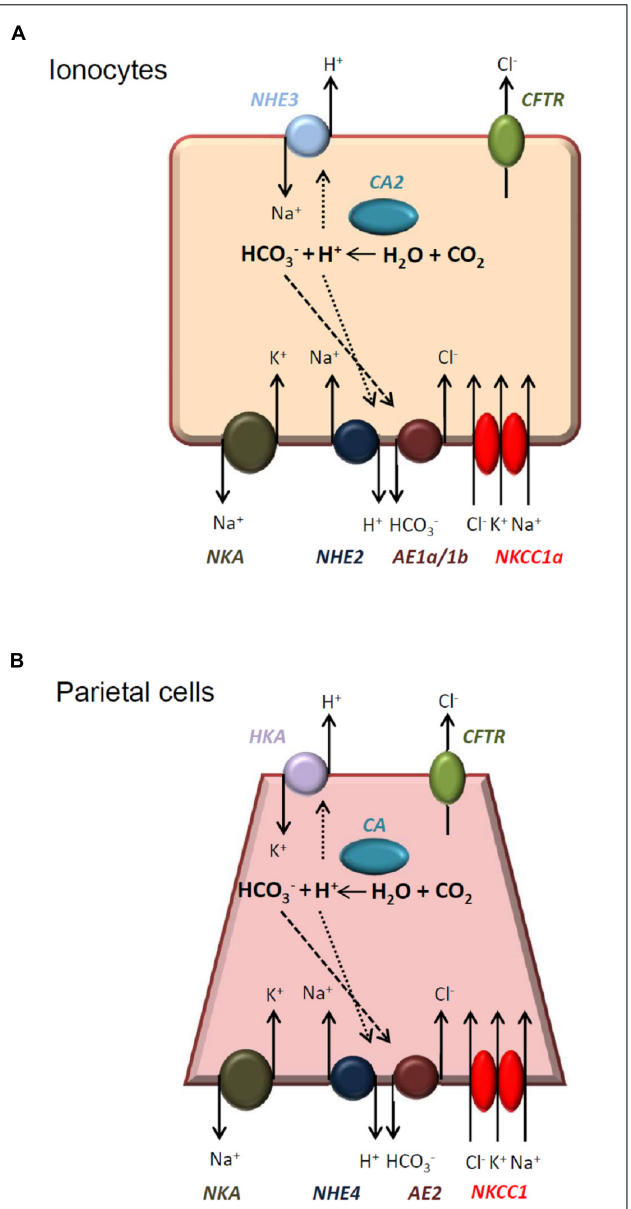

FIGURE 9 | Illustrated models of the acid-secretion by medaka ionocytes and mammalian parietal cells. Medaka ionocytes **(A)** and mammalian parietal cells **(B)** are both acid-secreting cells and have similar transport mechanisms. See "Discussion" for a detailed comparison. NKA, Na^+-K^+-ATPase; NHE, Na^+/H^+ exchanger; AE, anion exchanger; NKCC, Na^+-K^+-Cl^- cotransporter; CA, carbonic anhydrase; HKA, H^+/K^+-ATPase; CFTR, cystic fibrosis transmembrane conductance regulator.

analyze functional changes of ionocytes in NHE2 knockdown embryos, we found that both H^+ and Cl^- excretion by ionocytes were suppressed (**Figures 8A,B**), suggesting that basolateral NHE2 plays a role in the H^+ and Cl^- secretion.

Our previous study (Liu et al., 2013) compared the mRNA level of NHE3 and NHE2 in medaka acclimated to FW and SW. We found that NHE3 level was 4.5-fold higher in FW than in SW, suggesting that apical NHE3 is probably required for acid secretion in both conditions (the driving force for NHE is higher in SW). In contrast, NHE2 level was much lower in FW than in SW(19-fold lower), suggesting that basolateral NHE2 is specific for SW acclimation. The presence of NHE2 in SW ionocytes might be tightly associated with C1$^-$ secretion which is specific for SW acclimation.

Our previous study demonstrated that ionocytes in the skin of SW-acclimated medaka are responsible for Cl^- and H^+ secretion and proposed a mechanism for the linkage of Cl^- and H^+ secretion (Liu et al., 2016). The basolateral localized NHE2 was probably involved in the mechanism and thus both Cl^- and H^+ secretions were suppressed in the NHE2 knockdown

embryos. Herein we proposed a modified model (**Figure 9**) for the possible role of NHE2 in ionocytes of medaka. In this model, anion exchanger 1(AE1), carbonic anhydrase 2 (CA2) and NHE2 might form a metabolon for HCO_3^- reclaimation and Cl^- secretion by ionocytes. Cytosolic CA2 catalyzes CO_2 hydration and produces H^+ and HCO_3^- intracellularly, and H^+ and HCO_3^- are, respectively, transported out of ionocytes *via* apical NHE3, basolateral NHE2 and basolateral AE1. In the basolateral membrane, an Na^+ gradient provides the driving force for NHE2 to transport H^+ out of the cell, while the H^+ gradient facilitates Cl^-/HCO_3^- exchange by AE1. This proposed model is similar to the model (**Figure 9**) of gastric H^+ secretion by mammalian parietal cells of gastric glands (Muallem and Sachs, 1985; Muallem et al., 1988; Gawenis et al., 2004). In parietal cells, basolateral NHE4 was suggested to be functionally associated with AE2 to promote Cl^- and HCO_3^- exchange (Gawenis et al., 2005).

In conclusion, the present study demonstrated that NHE2 is expressed in the basolateral membrane of ionocytes in SW-acclimated medaka. Lost-of-function experiments with photo-activated morpholino oligonucleotides suggested that NHE2 is involved in H^+ and Cl^- secretions by ionocytes. NHE2 and other basolateral transporters (such as AE1) might form a metabolon for HCO_3^- reclaimation and Cl^- secretion by ionocytes of SW fishes. In the future, it is necessary to examine if the presented model (**Figure 9**) can be applied to other SW teleosts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Utilization Committee of National Taiwan Normal University.

AUTHOR CONTRIBUTIONS

S-TL: conceptualization, methodology, writing—original draft preparation, investigation, and data curation. J-LH: conceptualization, methodology, formal analysis, and writing—reviewing and editing. L-YL: conceptualization, methodology, formal analysis, writing—reviewing and editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.870967/full#supplementary-material

REFERENCES

Catches, J. S., Burns, J. M., Edwards, S. L., and Claiborne, J. B. (2006). Na^+/H^+ antiporter, V-H^+-ATPase and Na^+/K^+-ATPase immunolocalization in a marine teleost (*Myoxocephalus octodecemspinosus*). *J. Exp. Biol.* 209, 3440–3447. doi: 10.1242/jeb.02384

Chambrey, R., St John, P. L., Eladari, D., Quentin, F., Warnock, D. G., Abrahamson, D. R., et al. (2001). Localization and functional characterization of Na^+/H^+ exchanger isoform NHE4 in rat thick ascending limbs. *Am. J. Physiol. Renal Physiol.* 281, F707–F717. doi: 10.1152/ajprenal.2001.281.4.F707

Chambrey, R., Warnock, D. G., Podevin, R. A., Bruneval, P., Mandet, C., Bélair, M. F., et al. (1998). Immunolocalization of the Na^+/H^+ exchanger isoform NHE2 in rat kidney. *Am. J. Physiol. Physiol.* 275, F379–F386. doi: 10.1152/ajprenal.1998.275.3.F379

Chow, C. W. (1999). Regulation and intracellular localization of the epithelial isoforms of the Na^+/H^+ exchangers NHE2 and NHE3. *Clin. Invest. Med.* 22, 195–206. doi: 10.1016/S0016-5085(98)81606-5

Chow, C. W., Woodside, M., Demaurex, N., Yu, F. H., Plant, P., Rotin, D., et al. (1999). Proline-rich motifs of the Na^+/H^+ exchanger 2 isoform. Binding of Src homology domain 3 and role in apical targeting in epithelia. *J. Biol. Chem.* 274, 10481–10488. doi: 10.1074/jbc.274.15.10481

Claiborne, J. B., Blackston, C. R., Choe, K. P., Dawson, D. C., Harris, S. P., Mackenzie, L. A., et al. (1999). A mechanism for branchial acid excretion in marine fish: identification of multiple Na^+/H^+ antiporter (NHE) isoforms in gills of two seawater teleosts. *J. Exp. Biol.* 202, 315–324. doi: 10.1111/j.1095-8649.2009.02534.x

Claiborne, J. B., Edwards, S. L., and Morrison-Shetlar, A. I. (2002). Acid-base regulation in fishes: cellular and molecular mechanisms. *J. Exp. Zool.* 293, 302–319. doi: 10.1002/jez.10125

Cooper, C. A., Wilson, J. M., and Wright, P. A. (2013). Marine, freshwater and aerially acclimated mangrove rivulus (*Kryptolebias marmoratus*) use different strategies for cutaneous ammonia excretion. *Am. J Physiol. Regul. Integr. Comp. Physiol.* 304, R599–R612. doi: 10.1152/ajpregu.00228.2012

Edwards, S. L., Donald, J. A., Toop, T., Donowitz, M., and Tse, C. M. (2002). Immunolocalisation of sodium/proton exchanger-like proteins in the gills of elasmobranchs. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 131, 257–265. doi: 10.1016/S1095-6433(01)00449-4

Edwards, S. L., Wall, B. P., Morrison-Shetlar, A., Sligh, S., Weakley, J. C., and Claiborne, J. B. (2005). The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). *J. Exp. Zool. A Comp. Exp. Biol.* 303, 464–475. doi: 10.1002/jez.a.175

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97–177. doi: 10.1152/physrev.00050.2003

Gawenis, L. R., Gawenis, L. R., Greeb, J. M., Prasad, V., Grisham, C., Sanford, L. P., et al. (2005). Impaired gastric acid secretion in mice with a targeted disruption of the NHE4 Na^+/H^+ exchanger. *J. Biol. Chem.* 280, 12781–12789. doi: 10.1074/jbc.M414118200

Gawenis, L. R., Hut, H., Bot, A. G., Shull, G. E., de Jonge, H. R., Stien, X., et al. (2004). Electroneutral sodium absorption and electrogenic anion

secretion across murine small intestine are regulated in parallel. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287, G1140–G1149. doi: 10.1152/ajpgi.00177.2004

Houillier, P., and Bourgeois, S. (2012). More actors in ammonia absorption by the thick ascending limb. *Am. J. Physiol. Renal Physiol.* 302, F293–F297. doi: 10.1152/ajprenal.00307.2011

Hu, M. Y., Lee, J. R., Lin, L. Y., Shih, T. H., Stumpp, M., Lee, M. F., et al. (2013). Development in a naturally acidified environment: Na^+/H^+-exchanger 3-based proton secretion leads to CO_2 tolerance in cephalopod embryos. *Front. Zool.* 10:51. doi: 10.1186/1742-9994-10-51

Hwang, P. P., and Chou, M. Y. (2013). Zebrafish as an animal model to study ion homeostasis. *Pflügers Arch.* 465, 1233–1247. doi: 10.1007/s00424-013-1269-1

Hwang, P. P., Lee, T. H., and Lin, L. Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R28–R47. doi: 10.1152/ajpregu.00047.2011

Inokuchi, M., Hiroi, J., Watanabe, S., Lee, K. M., and Kaneko, T. (2008). Gene expression and morphological localization of NHE3, NCC and NKCC1a in branchial mitochondria-rich cells of *Mozambique tilapia* (*Oreochromis mossambicus*) acclimated to a wide range of salinities. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 151, 151–158. doi: 10.1016/j.cbpa.2008.06.012

Inoue, D., and Wittbrodt, J. (2011). One for all–a highly efficient and versatile method for fluorescent immunostaining in fish embryos. *PLoS One* 6:e19713. doi: 10.1371/journal.pone.0019713

Liu, S. T., Horng, J. L., Chen, P. Y., Hwang, P. P., and Lin, L. Y. (2016). Salt secretion is linked to acid-base regulation of ionocytes in seawater-acclimated medaka: new insights into the salt-secreting mechanism. *Sci. Rep.* 6:31433. doi: 10.1038/srep31433

Liu, S. T., Tsung, L., Horng, J. L., and Lin, L. Y. (2013). Proton-facilitated ammonia excretion by ionocytes of medaka (*Oryzias latipes*) acclimated to seawater. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 305, R242–R251. doi: 10.1152/ajpregu.00047.2013

Muallem, S., and Sachs, G. (1985). Ca^{2+} metabolism during cholinergic stimulation of acid secretion. *Am. J. Physiol.* 248, G216–G228. doi: 10.1152/ajpgi.1985.248.2.G216

Muallem, S., Blissard, D., Cragoe, E. J. Jr., and Sachs, G. (1988). Activation of the Na^+/H^+ and Cl^-/HCO_3- exchange by stimulation of acid secretion in the parietal cell. *J. Biol. Chem.* 263, 14703–14711.

Seo, M. Y., Mekuchi, M., Teranishi, K., and Kaneko, T. (2013). Expression of ion transporters in gill mitochondrion-rich cells in Japanese eel acclimated to a wide range of environmental salinity. *Am. J Physiol. Regul. Integr. Comp. Physiol.* 166, 323–332.

Shen, W. P., Horng, J. L., and Lin, L. Y. (2011). Functional plasticity of mitochondrion-rich cells in the skin of euryhaline medaka larvae (*Oryzias latipes*) subjected to salinity changes. *Am. J Physiol. Regul. Integr. Comp. Physiol.* 300, R858–R868. doi: 10.1152/ajpregu.00705.2010

Takeda, H., and Shimada, A. (2010). The art of medaka genetics and genomics: what makes them so unique ? *Annu. Rev. Genet.* 44, 217–241. doi: 10.30047/JGMB.199103.0005

Tallafuss, A., Gibson, D., Morcos, P., Li, Y., Seredick, S., Eisen, J., et al. (2012). Turning gene function ON and OFF using sense and antisense photomorpholinos in zebrafish. *Development* 139, 1691–1699. doi: 10.1242/dev.072702

Tresguerres, M., Katoh, F., Fenton, H., Jasinska, E., and Goss, G. G. (2005). Regulation of branchial V-H^+-ATPase, Na^+/K^+-ATPase and NHE2 in response to acid and base infusions in the Pacific spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.* 208, 345–354. doi: 10.1242/jeb.01382

Wittbrodt, J., Shima, A., and Schartl, M. (2002). Medaka–a model organism from the far East. *Nat. Rev. Genet.* 3, 53–64. doi: 10.1038/nrg704

Wu, S. C., Horng, J. L., Liu, S. T., Hwang, P. P., Wen, Z. H., Lin, C. S., et al. (2010). Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Am. J. Physiol. Cell Physiol.* 298, C237–C250. doi: 10.1152/ajpcell.00373.2009

Yan, J. J., Chou, M. Y., Kaneko, T., and Hwang, P. P. (2007). Gene expression of Na^+/H^+ exchanger in zebrafish H^+ -ATPase-rich cells during acclimation to low-Na^+ and acidic environments. *Am. J. Physiol. Cell Physiol.* 293, C1814–C1823. doi: 10.1152/ajpcell.00358.2007

Yan, J. J., and Hwang, P. P. (2019). Novel discoveries in acid-base regulation and osmoregulation: a review of selected hormonal actions in zebrafish and medaka. *Gen. Comp. Endocrinol.* 277, 20–29. doi: 10.1016/j.ygcen.2019.03.007

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Why can Mozambique Tilapia Acclimate to Both Freshwater and Seawater? Insights From the Plasticity of Ionocyte Functions in the Euryhaline Teleost

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In teleost fishes, ionocytes in the gills are important osmoregulatory sites in maintaining ionic balance. During the embryonic stages before the formation of the gills, ionocytes are located in the yolk-sac membrane and body skin. In Mozambique tilapia embryos, quintuple-color immunofluorescence staining allowed us to classify ionocytes into four types: type I, showing only basolateral Na^+/K^+-ATPase (NKA) staining; type II, basolateral NKA and apical Na^+, Cl^- cotransporter 2; type III, basolateral NKA, basolateral Na^+, K^+, $2Cl^-$ cotransporter 1a (NKCC1a) and apical Na^+/H^+ exchanger 3; and type IV, basolateral NKA, basolateral NKCC1a and apical cystic fibrosis transmembrane conductance regulator Cl^- channel. The ionocyte population consisted mostly of type I, type II and type III in freshwater, while type I and IV dominated in seawater. In adult tilapia, dual observations of whole-mount immunocytochemistry and scanning electron microscopy showed morphofunctional alterations in ionocytes. After transfer from freshwater to seawater, while type-II ionocytes closed their apical openings to suspend ion absorption, type-III ionocytes with a concave surface were transformed into type IV with a pit *via* a transitory surface. The proposed model of functional classification of ionocytes can account not only for ion uptake in freshwater and ion secretion in seawater, but also for plasticity in ion-transporting functions of ionocytes in tilapia.

Keywords: ionocyte, tilapia, osmoregulation, classification, plasticity

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INTRODUCTION

Osmoregulation is a physiological mechanism to maintain a stable internal environment which is required for optimal cell function. In teleost fishes, although they inhabit various osmotic environments, their plasma osmolality is maintained within narrow physiological ranges, equivalent to about one-third seawater (SW) osmolality. Osmoregulation in adult teleosts largely consists of integrated ion and water transport activities of the gills, kidney and intestine. In particular, ionocytes, previously referred to as chloride cells or mitochondrion-rich cells, in the gills are important osmoregulatory sites in maintaining ionic balance (Hwang and Lin, 2013; Kültz and Gilmour, 2020). Ionocytes are responsible for ion uptake in freshwater (FW) and ion secretion in SW. Ionocytes in the gills are also known as a multifunctional cell, which is the dominant site for acid-base

regulation and nitrogenous waste excretion in addition to osmoregulation. As is the case in adult fish, teleost embryos and larvae are also able to maintain the osmotic balance of their body fluid (Guggino 1980; Kaneko et al., 1995; Varsamos et al., 2005; Brauner and Rombough 2012). During the early life stages of fishes, a rich population of ionocytes are present in the yolk sac membrane and body skin as a main osmoregulatory site (Lasker and Threadgold 1968; Guggino 1980; Hwang and Hirano 1985; Hwang et al., 1999). In early studies on ionocytes, electron microscopic observations and molecular identification of ion transporters have demonstrated the difference in morphology and function between FW and SW. However, it had not been elucidated how multiple ion transporters cooperate in ionocytes and how the cells switch their function following environmental salinity change. In this review, we describe the ionocyte classification and their functional plasticity between FW and SW during early life and adult stages of Mozambique tilapia (*Oreochromis mossambicus*).

Tilapia, the genus *Oreochromis*, are widely distributed in tropical areas in the wild and cultivated in fish farms owing to their hardy nature, rapid growth rates, and tolerance to varied environmental salinity (Pullin 1991). Among those tilapia species, Mozambique tilapia *O. mossambicus* is one of the most suitable fish species for studies on osmoregulation, because this euryhaline tilapia is tolerant not only to a wide range of salinity from FW to SW but also to extremely low-ion water and even to double-strength SW (Stickney, 1986; Uchida et al., 2000; Fiess et al., 2007; Inokuchi et al., 2008). Mozambique tilapia is also known as a maternal mouth brooder, in which the female incubates the fertilized eggs in her mouth, and its embryos are available all year around. Furthermore, they can breed either in FW or in SW, and embryos develop normally in respective media. The tilapia embryos are also able to survive direct transfer from FW to SW and *vice versa*. Their strong euryhalinity during both early life and adult stages intrigued us to explore the mechanisms of osmoregulation and salinity tolerance.

IONOCYTE CLASSIFICATION OF YOLK-SAC MEMBRANE AND BODY SKIN IN TILAPIA EMBRYOS

During the embryonic stages before the formation of the functional gills and kidney, ionocytes have been detected in the epithelia covering the yolk and body in several teleost species (Kaneko et al., 2008). The yolk-sac membrane and body skin of embryos and larvae are structurally simple, and those ionocytes would be observable more easily than branchial ionocytes. Thus, those cutaneous ionocytes could serve as an excellent model to investigate the functions and morphology of ionocytes. Hwang and Hirano (1985) found notable differences in the intercellular organization and tight-junction structure of ionocytes in the yolk-sac membrane between FW- and SW-acclimated teleost embryos. It was also reported that, after direct transfer of tilapia larvae from FW to SW, the ionocytes markedly increased their cell size, being accompanied by accessory cells (Ayson et al., 1994; Shiraishi et al., 1997). On the other hand, ionocyte density in the yolk-sac membrane did not vary appreciably between FW and SW larvae (Ayson et al., 1994).

These morphological observations suggest the occurrence of distinct FW- and SW-type ionocytes in the yolk-sac membrane of tilapia embryos and larvae, as is the case with gill ionocytes in adult fish. However, it was not elucidated whether FW-type ionocytes are replaced by newly-differentiated SW-type ionocytes after transfer from FW to SW, or whether preexisting FW ionocytes are transformed into SW ionocytes.

By observing *in vivo* sequential changes in individual ionocytes in the yolk-sac membrane of tilapia embryos and larvae, Hiroi et al. (1999) succeeded in following morphological changes in ionocytes during acclimation to different salinities. In this study, ionocytes were vitally stained with DASPEI, a fluorescent dye specific for mitochondria, and each individual ionocyte was sequentially observed for 4 days under a confocal laser scanning microscope. The sequential observation revealed that 75% of skin ionocytes survived for 96 h after transfer from FW to SW. Moreover, the ionocytes showed a remarkable increase in size after transfer, while the size did not change in embryos and larvae kept in FW. These findings suggest that FW-type small ionocytes possess the ability to survive after direct transfer to SW and to be transformed into SW-type large ionocytes (Hiroi et al., 1999).

In addition to the crude classification of ionocytes into FW and SW types, Hiroi et al. (2008) have proposed a more detailed functional classification of ionocytes. Ion-transporting functions of ionocytes are defined by various ion transporters located at either basolateral or apical membranes. Since Na^+ and Cl^- are the major electrolytes in plasma, occupying more than 90% of inorganic electrolytes, they focused on transporters in charge of Na^+ and Cl^- transport among various transporters expressed in ionocytes. Among those ion transporters, Na^+/K^+-ATPase (NKA) located at the basolateral membrane is universally present in ionocytes, providing the driving force for ion transport (Hwang and Lin, 2013; Kültz and Gilmour, 2020). The well accepted model of ion secretion in SW-type ionocytes is mediated by NKA and Na^+, K^+, $2Cl^-$ cotransporter 1a (NKCC1a) in the basolateral membrane, and by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in the apical membrane (Marshall, 2011; Kültz and Gilmour, 2020). By contrast, the mechanisms for ion uptake in ionocytes of FW-acclimated teleosts vary according to species (Dymowska et al., 2012). Therefore, the research on detailed molecular mechanisms of FW ionocytes was required across a wide range of teleost species.

To detect NKCC in ionocytes, mouse monoclonal antibody against human colonic NKCC1 (named T4; available from the Developmental Studies Hybridoma Bank; Lytle et al., 1995) has been widely and repeatedly used in various teleost species. However, the immunoreaction with T4 antibody was unexpectedly detected in the apical and basolateral membranes of FW- and SW-type ionocytes, respectively, in Mozambique tilapia (Wu et al., 2003; Hiroi et al., 2005), mummichog (Katoh et al., 2008), Japanese medaka (Hsu et al., 2014), European sea bass (Lorin-Nebel et al., 2006) and Japanese sea bass (Inokuchi

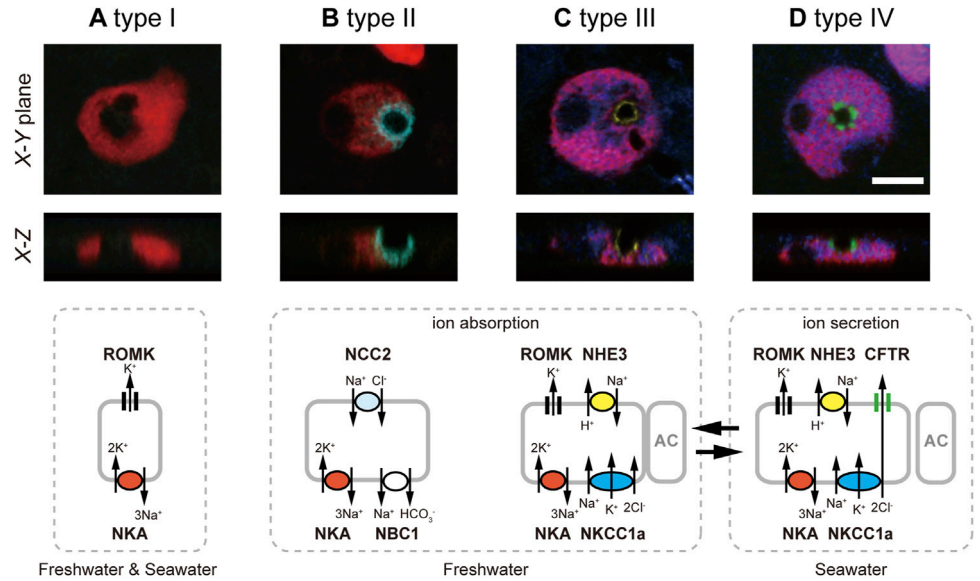

FIGURE 1 | Four types of ionocytes in the yolk-sac membrane of Mozambique tilapia embryos, by means of quintuple-color immunofluorescence staining: type-I **(A)**, type-II **(B)**, type-III **(C)** and type-IV **(D)**. Five images for NKA (red), NKCC1a (blue), NCC2 (cyan), NHE3 (yellow) and CFTR (green) are merged and shown in X–Y and X–Z planes. Type-I ionocytes show only basolateral NKA. Type-II ionocytes possess basolateral NKA and distinct apical NCC2. Type-III ionocytes are defined by basolateral NKA, basolateral NKCC1a (red for NKA and blue for NKCC1a are merged into magenta), and apical NHE3. Type-IV ionocytes are provided with the three major ion-transporting proteins for salt secretion, basolateral NKA, basolateral NKCC1a and apical CFTR. Scale bar, 10 μm. Schematic diagrams of the 4 cell types are presented in the bottom row, showing the apical or basolateral localization patterns of NKA (red), NKCC1a (blue), NCC2 (cyan), NHE3 (yellow) CFTR (green), NBC1 and ROMK. Modified from Hiroi et al. (2008).

et al., 2017). This phenomenon implied that two different cation–chloride cotransporters existed in ionocytes: apically-located, ion-absorptive "FW-type" and basolaterally-located, ion-secretory "SW-type". By immunoscreening of a cDNA expression library with T4 antibody, Hiroi et al. (2008) obtained cDNA of tilapia Na^+, Cl^- cotransporter 2 (NCC2) in addition to NKCC1a, NKCC1b and NKCC2. Among the four cation-chloride cotransporter candidates, the mRNA of NKCC1a was highly expressed in the yolk-sac membrane and the gills of SW-acclimated fish, whereas the NCC2 expression was restricted to those of FW fish.

Generating antibodies specific for tilapia NKCC1a and NCC2, Hiroi et al. (2008) conducted whole-mount immunofluorescence staining for NKCC1a and NCC2, together with NKA, CFTR and Na^+/H^+ exchanger 3 (NHE3), on the yolk-sac membrane of tilapia embryos acclimated to FW or SW. The powerful quintuple-color immunofluorescence staining allowed them to successfully classify ionocytes into four distinct types: type I, showing only basolateral NKA staining; type II, basolateral NKA and apical NCC2; type III, basolateral NKA, basolateral NKCC1a and apical NHE3; and type IV, basolateral NKA, basolateral NKCC1a and apical CFTR (**Figure 1**). The type-I ionocyte is relatively small in size, showing only basolateral NKA staining. Therefore, it was first suggested as an immature ionocyte (Hiroi et al., 2005). However, this type was later considered to be an independent functional cell type (Hiroi et al., 2008), and one possible function is K^+ secretion through renal outer medullary K^+ channel (ROMK) found at the apical membrane (Furukawa et al., 2014). The type-II ionocyte possessing basolateral NKA and apical NCC2 is specific to FW, and the apical NCC2 is considered as a pathway to absorb Na^+ and Cl^- (Hiroi et al., 2008). Type-III ionocyte defined by basolateral NKA and basolateral NKCC1a and apical NHE3 rarely appeared in SW, rapidly increased in number following transfer from SW to FW and disappeared following transfer from FW to SW. Type IV represents a typical distributional pattern of ion transporters for NaCl-secretory ionocytes in SW teleost fish. This type possesses basolateral NKA and basolateral NKCC1a similar to type-III ionocytes, but CFTR is located in the apical membrane instead of NHE3. Furthermore, the type-IV ionocyte was not observed in FW fish, but rapidly increased in number after SW transfer and disappeared after transfer back into FW (Hiroi et al., 2008). This inverse relationship between type III and IV suggests that those ionocytes have the same cell linage but transform reversibly into each other during environmental salinity changes. The proposed model of functional classification of ionocytes can account not only for ion uptake in FW and ion secretion in SW, but also for plasticity in ion-transporting functions of ionocytes in the euryhaline tilapia.

BRANCHIAL IONOCYTES IN ADULT TILAPIA

The classification of ionocytes proposed in embryos turned out to be applicable to that in adult fish. Subsequent studies in adult gills showed that Na^+, HCO_3^- cotransporter 1 (NBC1) and ROMK were also localized in branchial ionocytes of Mozambique tilapia

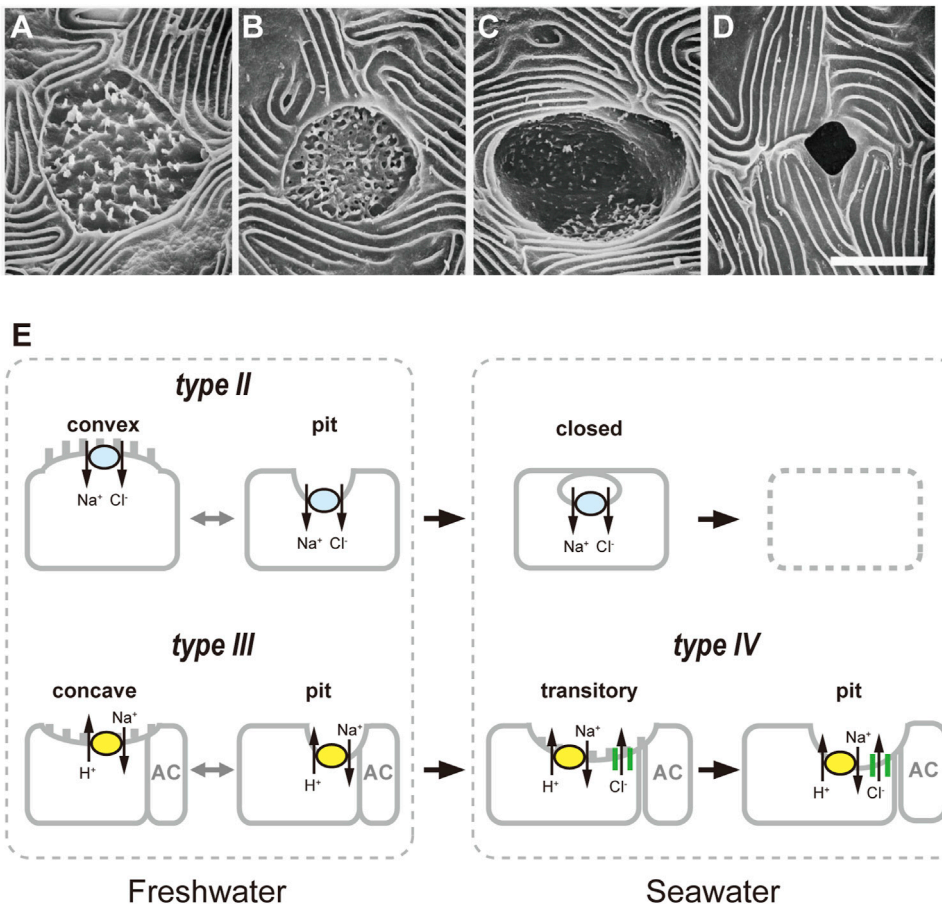

FIGURE 2 | Classification of apical openings of ionocytes in the gills of Mozambique tilapia. Four types of apical openings, identified by scanning electron microscopy: a convex apical surface **(A)**, a concave apical surface **(B)**, a transitory apical surface **(C)** and an apical pit **(D)**. Scale bar, 5 μm. **(E)**, Schematic diagrams of relationship between apical opening structure and ionocyte types. The type-II ionocytes with convex apical surfaces or apical pits suspend ion-absorptive function by closing the apical openings as a quick response after transfer from freshwater to 70% seawater, followed by cell disappearance. After transfer, concave apical surfaces or small apical pits typically seen in type-III ionocytes are transformed into enlarged apical pits in type-IV ionocytes *via* the transitory apical surfaces. Modified from Choi et al. (2011).

(Furukawa et al., 2011; Furukawa et al., 2014, **Figure 1**). The basolateral NBC1 of type-II ionocytes is likely to function as exit of Na^+ absorbed by apical NCC2 (Furukawa et al., 2011). On the other hand, ROMK was detected at the apical membrane of types I, III and IV (Furukawa et al., 2014). As ROMK was upregulated after transfer to high-K^+ FW or SW, ROMK seems to play an important role in K^+ excretion in those ionocytes (Furukawa et al., 2014).

In earlier studies, scanning (SEM) and transmission (TEM) electron microscopy has been used to identify and quantify functional ionocytes including FW and SW subtypes in tilapia as in other teleost species (Pisam et al., 1995; Lee et al., 1996; van der Heijden et al., 1999). While the apical membrane of SW ionocytes typically forms a pit structure, the apical surface of FW ionocytes often appears as a flat or slightly projecting disk (Lee et al., 1996). In SEM observations, three subtypes of ionocytes were exhibited in FW tilapia: wavy convex, shallow-basin, and deep-hole (Lee et al., 1996). The apical features of deep-hole ionocytes were narrow, deep, round-to-oval pores in which little or no internal structure was visible by SEM. The wavy-convex ionocytes are characterized by a wide apical opening and a rough surface appearance. The ovoid apertures of shallow-basin ionocytes were occasionally ornamented with short microvilli. Following transfer from artificial high-Cl^- to low-Cl^- media, the wavy-convex cells were rapidly increased, while in turn the deep-hole ionocytes disappeared (Chang et al., 2003). In contrast, low-Ca^{2+} fish developed more shallow-basin ionocytes in the gills and a higher Ca^{2+} influx than those acclimated to other media (Chang et al., 2001). These findings indicated that the shallow-basin and wavy-convex ionocytes are mainly responsible for the uptake of Ca^{2+} and Cl^-, respectively. Following transfer of tilapia from FW to brackish water (20 ppt), the wavy-convex ionocytes disappeared within 3 h, but deep-hole ones increased from 48 h (Wang et al., 2009). The rapid disappearance of wavy convex ionocytes might be due to the internalization of the apical membrane (Lin and Hwang, 2004). The morphological changes in apical surface post transfer indicates that different phenotypes play different roles in ion regulation. However, the

evident relationship between the variable morphology of apical surfaces and the localization pattern of ion-transporting proteins was not clarified.

In tilapia acclimated to hypoosmotic environments, a direct comparison between SEM image and the distribution of ion transporters demonstrated the relationship between the apical membrane structures and cellular function of ionocytes (Inokuchi et al., 2009). While both NHE3 (type-III) and NCC2 (type-II) ionocytes possess small apical openings in normalized artificial FW, the NHE3 and NCC2 were confined to concave and convex apical surfaces, respectively, in lower ion conditions. The ionocytes with apical NHE3 enlarged their concave apical surface in low Na^+ media to facilitate Na^+ absorption. In contrast, under low Cl^- condition, apical NCC2 cells developed a large apical convex surface to activate Na^+ and Cl^- uptake (Inokuchi et al., 2009; Choi et al., 2010).

In order to further clarify the functional and morphological response of ionocytes to acute salinity change, Choi et al. (2011) developed a new technique, dual observations of whole-mount immunocytochemistry and SEM. In this method, the gill filaments were first subjected to triple immunofluorescent staining for NKA, NHE3 and NCC2/NKCC1 (T4 antibody) to identify the types of ionocytes, followed by SEM observation on the apical structures of ionocytes in the same specimens. The comparison of the fluorescent and SEM images enables to link the apical structure to a certain ionocyte type. To examine the short-term responses of ionocytes during acclimation to different salinity environments, tilapia were directly transferred from a hypoosmotic environment (FW) to a hyperosmotic environment (70% SW). In addition to a pit surface (deep-hole type), a convex apical surface (wavy-convex type) and a concave apical surface (shallow-basin type), the SEM observation identified a transitory apical surface between the pit and concave structures (**Figures 2A–D**). In FW-acclimated tilapia gills, as previously reported, three types of apical openings were observed; that is, pit, convex and concave surfaces. At 6 h after transfer to 70% SW, there appeared the transitory type that was not observed in FW fish. The occurrence of the transitory apical surface that shared morphological characteristics of both a concave apical surface and an apical pit suggests a possible transformation from a concave apical surface to an apical pit. The dual observations of whole-mount immunocytochemistry and SEM then revealed that concave apical surfaces typically seen in type-III ionocytes were transformed into enlarged apical pits in type-IV (SW-type) ionocytes *via* the transitory apical surfaces. It should be noted that this dual-observation technique also revealed the occurrence of ionocytes with a closed apical surface, which was completely covered with adjacent pavement cells. The type-II ionocytes, the other FW-type cells with apical NCC2, possess a small apical pit or a convex apical opening in FW. After transfer of tilapia to 70% SW, type-II ionocytes suspended ion-absorptive function by closing the apical surfaces as a quick response to the salinity change. Interestingly, whereas type-III and type-IV ionocytes show functional plasticity to switch ion-transporting functions between FW and SW, type-II ionocytes are specific for FW acclimation (**Figure 2E**).

The functional plasticity of ionocytes has also been reported in other teleost species. The scanning ion-selective electrode technique (SIET) demonstrated the functional change from ion uptake to ion secretion in individual ionocytes in the skin of medaka larvae during acute salinity change (Shen et al., 2011). In Japanese sea bass, the immunocytochemical observation revealed the occurrence of intermediate-type between type-III and type-IV ionocytes at 1 day after transfer from SW to hypoosmotic environment (10% SW) (Inokuchi et al., 2017). The intermediate-type ionocytes showed apical CFTR and NHE3 immunoreactions, which are characteristic of type-IV cells; however, their apical region showing a convex appearance is more like type-III ionocytes.

CONCLUSION

In this review, in order to address the mechanism of functional switching between hyper- and hypo-osmoregulation in euryhaline teleosts, we describe the ionocyte classification of Mozambique tilapia and their functional plasticity between FW and SW. In tilapia embryos, quintuple-color immunofluorescence staining allowed us to classify ionocytes into four types (Hiroi et al., 2008). In adult tilapia, dual observations of whole-mount immunocytochemistry and SEM showed morphofunctional alterations in ionocytes (Choi et al., 2011). Our findings indicate functional plasticity of type-III/IV ionocytes to switch their ion-transporting functions, whereas type-II ionocytes are considered to be specific for FW acclimation. Our morphological and functional analyses revealed how ionocytes switch their function between FW and SW, but the biological pathway to regulate functions of ionocytes is yet to be elucidated. Further studies are required, focusing not only on the ion-transporting function of ionocytes, but also on the molecular pathway regulating the functional plasticity of ionocytes. For example, the utilization of the state-of-the-art molecular approaches, such as the single-cell RNA sequencing, may provide a better understanding of this issue.

Salinity is regarded as one of the most important physical characteristics of the aquatic environment that govern the distribution of species in nature. Mozambique tilapia is a euryhaline teleost with a worldwide tropical distribution, originating from estuaries and near-shore rivers from the lower Zambezi River to the southeast coast of South Africa (Trewevas, 1983). The capacity of ionocytes to alter their morphology and ion-transporting function, or the morphofunctional plasticity of ionocytes, account at least in part for their strong salinity tolerance.

AUTHOR CONTRIBUTIONS

MI, JH, and TK contributed to conception and design of the review. MI wrote the first draft of the manuscript. JH and TK wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

Ayson, F. G., Kaneko, T., Hasegawa, S., and Hirano, T. (1994). Development of Mitochondrion-Rich Cells in the Yolk-Sac Membrane of Embryos and Larvae of tilapia,*Oreochromis mossambicus*, in Fresh Water and Seawater. *J. Exp. Zool.* 270, 129–135. doi:10.1002/jez.1402700202

Brauner, C. J., and Rombough, P. J. (2012). Ontogeny and Paleophysiology of the Gill: New Insights from Larval and Air-Breathing Fish. *Respir. Physiology Neurobiol.* 184, 293–300. doi:10.1016/j.resp.2012.07.011

Chang, I. C., Lee, T. H., Yang, C. H., Wei, Y. Y., Chou, F. I., and Hwang, P. P. (2001). Morphology and Function of Gill Mitochondria-Rich Cells in Fish Acclimated to Different Environments. *Physiological Biochem. Zoology* 74, 111–119. doi:10.1086/319304

Chang, I. C., Wei, Y. Y., Chou, F. I., and Hwang, P. P. (2003). Stimulation of Cl−Uptake and Morphological Changes in Gill Mitochondria-Rich Cells in Freshwater Tilapia (*Oreochromis mossambicus*). *Physiological Biochem. Zoology* 76, 544–552. doi:10.1086/375438

Choi, J. H., Lee, K. M., Inokuchi, M., and Kaneko, T. (2011). Morphofunctional Modifications in Gill Mitochondria-Rich Cells of Mozambique tilapia Transferred from Freshwater to 70% Seawater, Detected by Dual Observations of Whole-Mount Immunocytochemistry and Scanning Electron Microscopy. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 158, 132–142. doi:10.1016/j.cbpa.2010.09.019

Choi, J. H., Lee, K. M., Inokuchi, M., and Kaneko, T. (2010). Acute Responses of Gill Mitochondria-Rich Cells in Mozambique tilapia *Oreochromis mossambicus* Following Transfer from Normal Freshwater to Deionized Freshwater. *Fish. Sci.* 76, 101–109. doi:10.1007/s12562-009-0195-9

Dymowska, A. K., Hwang, P.-P., and Goss, G. G. (2012). Structure and Function of Ionocytes in the Freshwater Fish Gill. *Respir. Physiology Neurobiol.* 184, 282–292. doi:10.1016/j.resp.2012.08.025

Fiess, J. C., Kunkel-Patterson, A., Mathias, L., Riley, L. G., Yancey, P. H., Hirano, T., et al. (2007). Effects of Environmental Salinity and Temperature on Osmoregulatory Ability, Organic Osmolytes, and Plasma Hormone Profiles in the Mozambique tilapia (*Oreochromis mossambicus*). *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 146, 252–264. doi:10.1016/j.cbpa.2006.10.027

Furukawa, F., Watanabe, S., Inokuchi, M., and Kaneko, T. (2011). Responses of Gill Mitochondria-Rich Cells in Mozambique tilapia Exposed to Acidic Environments (pH 4.0) in Combination with Different Salinities. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 158, 468–476. doi:10.1016/j.cbpa.2010.12.003

Furukawa, F., Watanabe, S., Kakumura, K., Hiroi, J., and Kaneko, T. (2014). Gene Expression and Cellular Localization of ROMKs in the Gills and Kidney of Mozambique tilapia Acclimated to Fresh Water with High Potassium Concentration. *Am. J. Physiology-Regulatory, Integr. Comp. Physiology* 307, R1303–R1312. doi:10.1152/ajpregu.00071.2014

Guggino, W. B. (1980). Salt Balance in Embryos of *Fundulus heteroclitus* and F. Bermudae Adapted to Seawater. *Am. J. Physiology-Regulatory, Integr. Comp. Physiology* 238, R42–R49. doi:10.1152/ajpregu.1980.238.1.r42

Hiroi, J., Kaneko, T., and Tanaka, M. (1999). *In Vivo* sequential Changes in Chloride Cell Morphology in the Yolk-Sac Membrane of mozambique tilapia (*Oreochromis mossambicus*) Embryos and Larvae during Seawater Adaptation. *J. Exp. Biol.* 202, 3485–3495. doi:10.1242/jeb.202.24.3485

Hiroi, J., McCormick, S. D., Ohtani-Kaneko, R., and Kaneko, T. (2005). Functional Classification of Mitochondrion-Rich Cells in Euryhaline Mozambique tilapia (*Oreochromis mossambicus*) Embryos, by Means of Triple Immunofluorescence Staining for Na+/K+-ATPase,Na+/K+/2Cl- Cotransporter and CFTR Anion Channel. *J. Exp. Biol.* 208, 2023–2036. doi:10.1242/jeb.01611

Hiroi, J., Yasumasu, S., McCormick, S. D., Hwang, P.-P., and Kaneko, T. (2008). Evidence for an Apical Na-Cl Cotransporter Involved in Ion Uptake in a Teleost Fish. *J. Exp. Biol.* 211, 2584–2599. doi:10.1242/jeb.018663

Hsu, H.-H., Lin, L.-Y., Tseng, Y.-C., Horng, J.-L., and Hwang, P.-P. (2014). A New Model for Fish Ion Regulation: Identification of Ionocytes in Freshwater- and Seawater-Acclimated Medaka (*Oryzias latipes*). *Cell Tissue Res.* 357, 225–243. doi:10.1007/s00441-014-1883-z

Hwang, P. P., and Hirano, R. (1985). Effects of Environmental Salinity on Intercellular Organization and Junctional Structure of Chloride Cells in Early Stages of Teleost Development. *J. Exp. Zool.* 236, 115–126. doi:10.1002/jez.1402360202

Hwang, P. P., Lee, T. H., Weng, C. F., Fang, M. J., and Cho, G. Y. (1999). Presence of Na-K-ATPase in Mitochondria-Rich Cells in the Yolk-Sac Epithelium of Larvae of the TeleostOreochromis Mossambicus. *Physiological Biochem. ZoologyPhysiol. Biochem. Zool.* 72, 138–144. doi:10.1086/316660

Hwang, P. P., and Lin, L. Y. (2013). "Gill Ionic Transport, Acid-Base Regulation, and Nitrogen Excretion," in *The Physiology of Fishes 4*. Editors D. H. Evans and J. B. Claiborne (Boca Raton, FL: CRC Press), 205–233.

Inokuchi, M., Nakamura, M., Miyanishi, H., Hiroi, J., and Kaneko, T. (2017). Functional Classification of Gill Ionocytes and Spatiotemporal Changes in Their Distribution after Transfer from Seawater to Freshwater in Japanese Seabass. *J. Exp. Biol.* 220, 4720–4732. doi:10.1242/jeb.167320

Inokuchi, M., Hiroi, J., Watanabe, S., Hwang, P.-P., and Kaneko, T. (2009). Morphological and Functional Classification of Ion-Absorbing Mitochondria-Rich Cells in the Gills of Mozambique tilapia. *J. Exp. Biol.* 212, 1003–1010. doi:10.1242/jeb.025957

Inokuchi, M., Hiroi, J., Watanabe, S., Lee, K. M., and Kaneko, T. (2008). Gene Expression and Morphological Localization of NHE3, NCC and NKCC1a in Branchial Mitochondria-Rich Cells of Mozambique tilapia (*Oreochromis mossambicus*) Acclimated to a Wide Range of Salinities. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 151, 151–158. doi:10.1016/j.cbpa.2008.06.012

Kaneko, T., Hasegawa, S., Takagi, Y., Tagawa, M., and Hirano, T. (1995). Hypoosmoregulatory Ability of Eyed-Stage Embryos of Chum Salmon. *Mar. Biol.* 122, 165–170. doi:10.1007/bf00349290

Kaneko, T., Watanabe, S., and Lee, K. M. (2008). Functional Morphology of Mitochondrion-Rich Cells in Euryhaline and Stenohaline Teleosts. *AquaBioSci. Monogr. (ABSM)* 1, 1–62. doi:10.5047/absm.2008.00101.0001

Katoh, F., Cozzi, R. R. F., Marshall, W. S., and Goss, G. G. (2008). Distinct Na+/K+/2Cl- Cotransporter Localization in Kidneys and Gills of Two Euryhaline Species, Rainbow Trout and Killifish. *Cell Tissue Res.* 334, 265–281. doi:10.1007/s00441-008-0679-4

Kültz, D., and Gilmour, K. M. (2020). "Iono-and Osmoregulation," in *The Physiology of Fishes" in the Physiology of Fishes 5*. Editors S. Currie and D. H. Evans (Boca Raton, FL: CRC Press), 63–78.

Lasker, R., and Threadgold, L. T. (1968). "Chloride Cells" in the Skin of the Larval Sardine. *Exp. Cell Res.* 52, 582–590. doi:10.1016/0014-4827(68)90498-9

Lee, T.-H., Hwang, P.-P., Lin, H.-C., and Huang, F.-L. (1996). Mitochondria-rich Cells in the Branchial Epithelium of the teleost,*Oreochromis mossambicus*, Acclimated to Various Hypotonic Environments. *Fish. Physiol. Biochem.* 15, 513–523. doi:10.1007/bf01874924

Lin, L.-Y., and Hwang, P.-P. (2004). Mitochondria-rich Cell Activity in the Yolk-Sac Membrane of tilapia(*Oreochromis mossambicus*) Larvae Acclimatized to Different Ambient Chloride Levels. *J. Exp. Bio.* 207, 1335–1344. doi:10.1242/jeb.00869

Lorin-Nebel, C., Boulo, V., Bodinier, C., and Charmantier, G. (2006). The Na+/K+/2Cl- Cotransporter in the Sea bassDicentrarchus Labraxduring Ontogeny: Involvement in Osmoregulation. *J. Exp. Biol.* 209, 4908–4922. doi:10.1242/jeb.02591

Lytle, C., Xu, J. C., Biemesderfer, D., and Forbush, B., 3rd (1995). Distribution and Diversity of Na-K-Cl Cotransport Proteins: a Study with Monoclonal Antibodies. *Am. J. Physiology-Cell Physiology* 269, C1496–C1505. doi:10.1152/ajpcell.1995.269.6.c1496

Marshall, W. S. (2011). Mechanosensitive Signalling in Fish Gill and Other Ion Transporting Epithelia. *Acta. Physiol. (Oxf)* 202, 487–499. doi:10.1111/j.1748-1716.2010.02189.x

Pisam, M., Le Moal, C., Auperin, B., Prunet, P., and Rambourg, A. (1995). Apical Structures of ?mitochondria-Rich? ? and ? Cells in Euryhaline Fish Gill: Their Behaviour in Various Living Conditions. *Anat. Rec.* 241, 13–24. doi:10.1002/ar.1092410104

Pullin, R. S. V. (1991). "Cichlids in Aquaculture," in *Cichlid Fishes: Behaviour, Ecology and Evolution*. Editor M. A. Keenleyside (London): Chapman and Hall), 280–299.

Shen, W.-P., Horng, J.-L., and Lin, L.-Y. (2011). Functional Plasticity of Mitochondrion-Rich Cells in the Skin of Euryhaline Medaka Larvae (*Oryzias latipes*) Subjected to Salinity Changes. *Am. J. Physiology-Regulatory, Integr. Comp. Physiology* 300, R858–R868. doi:10.1152/ajpregu.00705.2010

Shiraishi, K., Kaneko, T., Hasegawa, S., and Hirano, T. (1997). Development of Multicellular Complexes of Chloride Cells in the Yolk-Sac Membrane of tilapia (*Oreochromis mossambicus*) Embryos and Larvae in Seawater. *Cell Tissue Res.* 288, 583–590. doi:10.1007/s004410050844

Stickney, R. R. (1986). Tilapia Tolerance of Saline Waters: a Review. *Progressive Fish-Culturist* 48, 161–167. doi:10.1577/1548-8640(1986)48<161:ttosw>2.0.co;2

Trewevas (1983). *Tilapiine Fishes of the Genera Sarotherodon, Oreochromis and Danakilia*. Ithaca, New York: Comstock Publishing Associates.

Uchida, K., Kaneko, T., Miyazaki, H., Hasegawa, S., and Hirano, T. (2000). Excellent Salinity Tolerance of Mozambique tilapia (*Oreochromis mossambicus*): Elevated Chloride Cell Activity in the Branchial and Opercular Epithelia of the Fish Adapted to Concentrated Seawater. *Zoological Sci.* 17, 149–160. doi:10.2108/zsj.17.149

van der Heijden, A. J. H., Van der Meij, J. C. A., Flik, G., and Wendelaar Bonga, S. E. (1999). Ultrastructure and Distribution Dynamics of Chloride Cells in tilapia Larvae in Fresh Water and Sea Water. *Cell Tissue Res.* 297, 119–130. doi:10.1007/s004410051339

Varsamos, S., Nebel, C., and Charmantier, G. (2005). Ontogeny of Osmoregulation in Postembryonic Fish: a Review. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 141, 401–429. doi:10.1016/j.cbpb.2005.01.013

Wang, P.-J., Lin, C.-H., Hwang, L.-Y., Huang, C.-L., Lee, T.-H., and Hwang, P.-P. (2009). Differential Responses in Gills of Euryhaline tilapia, *Oreochromis mossambicus*, to Various Hyperosmotic Shocks. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 152, 544–551. doi:10.1016/j.cbpa.2008.12.012

Wu, Y. C., Lin, L. Y., and Lee, T. H. (2003). Na^+, K^+, $2Cl^-$-cotransporter: a novel marker for identifying freshwater-and seawater-type mitochondria-rich cells in gills of the euryhaline tilapia, *Oreochromis mossambicus*. *Zool. Stud.* 42, 186–192.

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REVIEW
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A Multi-Species Comparison and Evolutionary Perspectives on Ion Regulation in the Antennal Gland of Brachyurans

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Brachyurans inhabit a variety of habitats and have evolved diverse osmoregulatory patterns. Gills, antennal glands and a lung-like structure are important organs of crabs that maintain their homeostasis in different habitats. Species use different processes to regulate ions in the antennal gland, especially those with high terrestriality such as Grapsoidea and Ocypodoidea. Our phylogenetic generalized least square (PGLS) result also suggested that there is a correlation between antennal gland NKA activity and urine-hemolymph ratio for Na^+ concentration in hypo-osmotic environments among crabs. Species with higher antennal gland NKA activity showed a lower urine-hemolymph ratio for Na^+ concentration under hypo-osmotic stress. These phenomenon may correlate to the structural and functional differences in gills and lung-like structure among crabs. However, a limited number of studies have focused on the structural and functional differences in the antennal gland among brachyurans. Integrative and systemic methods like next generation sequencing and proteomics method can be useful for investigating the differences in multi-gene expression and sequences among species. These perspectives can be combined to further elucidate the phylogenetic history of crab antennal glands.

Keywords: evolutionary physiology, brachyurans, antennal gland, NKA activity, ion regulation

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INTRODUCTION

Evolutionary physiology uses phylogenetics to describe physiological patterns (Felsenstein, 1985; Garland et al., 1992; Garland and Carter, 1994). When we compare the physiological patterns among species, it is important to acknowledge that species are not independent to one another, and that an underlying phylogenetic relationship connects them all (Felsenstein, 1985). Directly comparing the differences among species is inadequate because being independent and identically distributed (IID) is a prior assumption for parametric statistical analysis (Garland et al., 1993; Gotelli and Ellison, 2004). Previous studies used 1) the topology of phylogenetic trees to adjust the variance between variables and 2) degrees of freedom to remove the non-independent effect and more accurately compare features among species (Felsenstein, 1985; Garland et al., 1992; Garland et al., 1993). For example, Garland et al. (1993) used the Monte Carlo method and gave tree topologies to estimate the 95th percentile of *F* value distribution for the null hypotheses of ANOVA and ANCOVA, then used this *F* value as a threshold for hypothesis testing. This is known as the phylogenetic ANOVA and ANCOVA. In addition, studies used methods such as Moran's I autocorrelation, which originated from spatial analysis, to justify whether the similarity of traits corresponds with the phylogenetic

distance among species (Gittleman and Kot, 1990; Faria et al., 2017). Evolutionary physiology was further extended to quantitative genetics and genome studies to recognize the differences in gene sequences and expressions among species based on phylogenetics (Storz et al., 2015). In their pioneer study, McNamara and Faria (2012) used Pearson's correlation analysis with phylogenetically independent contrasts (PIC) correction to analyze the relationship between habitat and hemolymph osmolality among palaemonid shrimp. Result showed that type I error can be reduced when the phylogenetic structure has been taken into consideration (McNamara and Faria, 2012). Their result indicated that performing a physiological comparison from a phylogenetic perspective can reduce statistical errors when performing multiple species investigations and connecting the physiological features of species to their phylogenetic history.

Brachyura is a very diverse group found across the world. It is made up of at least 7,250 species in a wide range of habitats, including marine, intertidal, terrestrial and freshwater zones (Péqueux, 1995; Ng et al., 2008; Davie et al., 2015). Several ion regulatory patterns have been reported in this clade (Henry et al., 2012; McNamara and Faria, 2012; Allen and Weihrauch, 2021). Two important ion regulatory organs—gills and antennal glands—have different functional patterns in different species. Additionally, some species have well-developed lung-like structure which can facilitate the oxygen exchange in terrestrial areas (Greenaway and Farrelly, 1984; Innes and Taylor, 1986; Tsai and Lin, 2012). Along with organ morphology and function, two previous studies outlined the phylogenetic relationships among brachyuran species (Tsang et al., 2014; Ma et al., 2019). This gives us the opportunity to analyze and describe the ion regulatory patterns among crabs from a phylogenetic perspective. Previous reviews and studies focused efforts on identifying the function and structure of gills in crabs (Kirschner, 2004; Weihrauch et al., 2004; Freire et al., 2008; Charmantier et al., 2009; Henry et al., 2012; McNamara and Faria, 2012; Weihrauch et al., 2017; Weihrauch and Allen, 2018; Allen and Weihrauch, 2021) and several important reviews have laid out more details on the structure and ion regulation mechanism of antennal gland of brachyurans (Weihrauch et al., 2004; Freire et al., 2008; Charmantier et al., 2009). In this review, we follow these studies and focus on comparing the structure and ion regulation in the antennal gland among crab species in different clades and habitats. First, we will briefly introduce the habitats and phylogeny of brachyurans, then discuss the differences in the ion regulatory patterns of species' antennal glands and give an integrated perspectives and introduce questions about the ion regulatory patterns in the antennal gland among brachyurans.

HABITATS AND PHYLOGENETIC RELATIONSHIPS AMONG BRACHYURAN SPECIES

The phylogenetic relationships and habitat diversity of brachyuran crabs interweave to form a wide spectrum of osmoregulatory and ion regulatory patterns among different species. Here we introduce what is currently understood of the habitat properties and phylogenetic relationships among brachyurans. Tsang et al. (2014) included 142 crab species in 58 families in an investigation on the phylogeny of brachyurans using six chromosomal genes and two mitochondrial genes. Four sections belonged to brachyuran—Dromiacea, Raninoida, Cyclodorippoida and Eubrachyura—and there were two monophyletic subsections in Eubrachyura—Heterotremata and Thoracotremata (Ng et al., 2008; Tsang et al., 2014; Davie et al., 2015). The tree topology of superfamilies in these sections/subsections was complex; some parts of superfamilies were monophyletic—e.g., Majoidea, Portunoidea and Xanthoidea—and some were polyphyletic—e.g., Grapsoidea and Ocypodoidea (Tsang et al., 2014; Ma et al., 2019).

The habitats of different clades are diverse and include marine, intertidal, terrestrial and freshwater zones. Environmental factors in marine and subtidal zones—such as salinity (33–35 ppt), water content and temperature—yield no or slight fluctuations in status (Barnes, 1974; Little, 1990). Hyper-osmotic environment will cause the ion influxes and water loss stresses in organism (McNamara and Faria, 2012). Species in superfamilies such as Calappoidea, Dromioidea, Majoidea, Corystoidea, Goneplacoidea, Carpilioidea, Dorippoidea, Leucosioidea, Portunoidea, Pilumnoidea and Xanthoidea (belonging to Dromiacea and Heterotremata) are distributed in these habitats (Ng et al., 2008; Tsang et al., 2014; Davie et al., 2015; Naderllo, 2017). Crabs in Portunoidea, Pilumnoidea, Xanthoidea, Eriphioidae, Leucosioidea, Trapezioidea, Grapsoidea and Ocypodoidea (in clades Thoracotremata and Heterotremata) inhabit the intertidal zone (Takeda et al., 1996; Ng et al., 2008; Tsang et al., 2014; Shin et al., 2015; Naderllo, 2017), where there is a salinity gradient from marine to estuarine (about 5 ppt) and water content that fluctuates based on the daily tidal cycles (Barnes, 1974). Ion regulatory functions help the individual overcome dramatic salinity and water content changes over a short period (McNamara and Faria, 2012).

Species in terrestrial habitats showed a different way to maintain their water balance, gas exchange and ammonia excretion compared to the aquatic one (Little, 1990; Weihrauch et al., 2004). Terrestriality among crabs is categorized into five grades (grades of terrestriality; *T*-grades) (Hartnoll, 1988). Species in grade T3 and above are considered terrestrial (Li and Chiu, 2019); this includes Gecarcinidae (Grapsoidea); Ocypodidae (Ocypodoidea); and Potamoidea, Gecarcinucoidea and Eriphioidea (Thoracotremata and Heterotremata) (Ng et al., 2008; Davie et al., 2015; Shin et al., 2015; Naderllo, 2017; Li and Chiu, 2019). With the exception of primary freshwater crabs, terrestrial species migrate to coastal and intertidal areas during the breeding season to release their zoea (Saigusa, 1980). Potamoidea, Gecarcinucoidea, Pseudothelphusoidea and Trichodactyloidea (Heterotremata) invaded fully freshwater habitats, which have a low ionic concentration, on two occasions (Barnes, 1974; Ng et al., 2008; Lee et al., 2011; Tsang et al., 2014; Davie et al., 2015; Ma et al., 2019). Some freshwater species, for example, *Austrothelphusa transversa* which be called desert crab in Gecarcinucoidea, showed high terrestriality which inhabited in arid burrow

during dry period (Taylor and Greenaway, 1979; Greenaway, 1984a). Diadromous species such as Varunidae (Grapsoidea) inhabit freshwater, but they migrate to estuaries through streams or rivers during the breeding season (Kobayashi, 1998; Ng et al., 2008; Tsang et al., 2014). Crabs can also inhabit extreme conditions. For example, species of Xenograpsidae (Grapsoidea) and Bythogaeidae (Bythograeoidea) live around hydrothermal vents, which have a low pH and high sulfide concentrations (Martinez et al., 2001; Hu et al., 2016; Allen et al., 2020).

BRIEF INTRODUCTION TO OSMOREGULATORY PATTERNS IN BRACHYURANS

Species in various environments have different osmoregulatory and ion regulatory patterns. They exhibit variation between being an osmo-conformer and an osmoregulator (Péqueux, 1995; Charmantier et al., 2009). The hemolymph osmolality of osmo-conformers changes following the isosmotic line between hemolymph and environment, but they can still regulate their cell volume and be an ion-regulator which modify their hemolymph ion composition (McNamara and Faria, 2012). Most of the marine (deep sea) crustaceans are considered osmo-conformers (McNamara and Faria, 2012). The osmoregulators regulate their hemolymph osmolality by actively absorbing or secreting ions. This group can be further divided into two types: the hyper-osmoregulators and the hyper-hypo-osmoregulators (Péqueux, 1995; Charmantier et al., 2009). Hyper-osmoregulators can sustain a higher hemolymph osmolality than that of their environment up to the isosmotic point, but they will become an osmo-conformer in hyper-osmotic mediums, such as freshwater species and some intertidal species (Shaw, 1958; Gross, 1964; Chung and Lin, 2006; Charmantier et al., 2009). The hyper-/hypo-osmoregulators can keep their hemolymph osmolality within a limited range, regardless of the environmental osmolality; for example, *Minuca* and *Leptuca* species can maintain their hemolymph osmotic concentration lower and higher than the isosmotic line between hemolymph and environment in hyper-osmotic and hypo-osmotic environments, respectively (Thurman, 2005; Faria et al., 2017).

The underlying mechanism of different osmoregulatory patterns among crabs are promoting by two important active transporters—Na^+, K^+-ATPase (NKA) and V-type H^+-ATPase (VHA)—which are the crucial driving forces behind the ion regulatory process (Brown and Breton, 1996; Kirschner, 2004). NKA, a P-type ATPase, is an important enzyme that transports three Na^+ ions into the hemolymph and brings two K^+ ions into the cytoplasm to generate a Na^+ and K^+ concentration gradient and trans-membrane potential difference (Lodish et al., 2000; Jorgensen et al., 2003). NKA is composed of α, β, and γ subunits; the α subunit—the largest one—was the site ion transport process occurs (Jorgensen et al., 2003; Sáez et al., 2009). Protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase (CaMK) can inhibit the gill NKA activity of *Ucides cordatus* by phosphorylation (Leone et al., 2020). β and γ subunits interacted with α subunit to stable α subunit structure (Jorgensen et al., 2003; Sáez et al., 2009) or interacted with other proteins, such as FXYD, to regulate the activity of NKA (Jorgensen et al., 2003). For example, the gill NKA activity of *U. cordatus* increased about 1.8 fold in low salinity condition when exogenous FXYD2 was present (Leone et al., 2020). NKA will prompt the Na^+ and Cl^- absorption mechanism in gills of crabs during hypo-osmotic stress with other ion transporters. Na^+, K^+, $2Cl^-$, symporter (NKCC) transport Na^+ and Cl^- by electrochemical gradient generate by basolateral NKA, Cl^- channel, K^+ channel and apical K^+ channel; Na^+, H^+, exchanger (NHE) exchanges Na^+ by Na^+ gradient produce by NKA and H^+ gradient by carbonic anhydrase (CA)—can catalyze the H_2O and CO_2 to H^+ and HCO_3^- and vice versa—in an osmo-conformer or weak hyper-osmoregulator (Kirschner, 2004; Freire et al., 2008; Henry et al., 2012; McNamara and Faria, 2012; Allen and Weihrauch, 2021). In addition, Cl^- absorption will also be executed by Cl^-, HCO_3^-, exchanger (AE), that is, driven by the HCO_3^- gradient which is produced by CA (Kirschner, 2004; Freire et al., 2008; Henry et al., 2012; McNamara and Faria, 2012; Allen and Weihrauch, 2021). On the other hand, NKA, basolateral NKCC and apical Cl^- channel involved in the Na^+ and Cl^- secretion in gills when crabs were subjected to hyper-osmotic condition (Freire et al., 2008; Henry et al., 2012; McNamara and Faria, 2012).

VHA, considered an acid-base regulatory enzyme, also involved in the ion regulatory process (Klein, 1992; Onken and Putzenlechner, 1995; Beyenbach and Wieczorek, 2006). VHA in the intercalated cells (ICs) of the rat kidney and in the Madin-Darby Canine Kidney cell line (MDCK cell) participates in ion regulation (Feifel et al., 1997; Silver et al., 2000). VHA is also believed to participate in ion regulation in the Malpighian tubules and the gut goblet cells of insects, frog skin, crustacean gill and fish gill (Klein, 1992; Maddrell and O'Donnell, 1992; Ehrenfeld and Klein, 1997; Wieczorek et al., 1999; Jensen et al., 2002). In brachyuran, *Carcinus maenas*, a weak hyper-osmoregulators, VHA distributed in cytoplasm of gills and the transepithelial potential was not affected by bafilomycin, a VHA inhibitor (Weihrauch et al., 2001). In addition, VHA mRNA expression was higher in the anterior gills than the posterior ones in hypo- and hyper-osmotic conditions (Weihrauch et al., 2001). These evidences indicated that VHA in gills of *C. maenas* might majorly involves in the organelle acidification but not in Na^+ or Cl^- absorption (Weihrauch et al., 2001). In contrast, VHA involves in ion regulation of strong hyper-osmoregulators in hypo-osmotic condition. The short-circuit current or transepithelial potential difference in gills of *Eriocheir sinensis* and *Chasmagnathus granulatus* were reduced when the bafilomycin was present in the apical sides of epithelium (Onken and Putzenlechner, 1995; GenoveseOrtiz et al., 2005). This indicated that the VHA in gills of these two species was involved in Cl^- absorption (Onken and Putzenlechner, 1995; GenoveseOrtiz et al., 2005). In addition, the gill VHA mRNA expression of freshwater species, *Dilocarcinus pagei*, decreased when species was subjected to the hyper-osmotic stress for reducing the V-ATPase-dependent ions absorption (Mantovani and McNamara, 2021). A hypothesis was proposed that the apically located VHA is for excreting H^+

directly in acid-base regulation or generating the electric gradient for ion regulation; while the cytoplasmic one is for organelle acidification that can further secrete the proton or produce "acid-trapping" to transport the ammonia into vesicle and secrete from apical membrane of gills by exocytosis (Wieczorek et al., 1999; Weihrauch et al., 2001; Jensen et al., 2002; Kirschner, 2004; Weihrauch et al., 2004; Hu et al., 2016).

Crab osmoregulatory patterns are supported by the distinct arrangement of proteins in ion regulatory organs, such as gills and antennal glands. But even species with similar osmoregulatory patterns and habitats can also have completely different strategies to sustain their homeostasis. Physiological plasticity is an important mechanism for some species to at least temporarily maintain their homeostasis in multiple habitat types (Henry, 1994). Local adaptation may occur when populations of the same species invade to the different habitat types with different environmental stress (Kawecki and Ebert, 2004). This phenomenon may be one of a possible process for speciation (Savolainen et al., 2013). Studies and reviews showed that the structure and ion regulatory functions of gills differ among crabs in different habitats and lineages (Takeda et al., 1996; Tsai and Lin, 2007; Freire et al., 2008; Charmantier et al., 2009; Henry et al., 2012; McNamara and Faria, 2012; Allen and Weihrauch, 2021). This phenomenon may correlate with ion regulation in the brachyuran antennal gland; we will summarize the evidence for this in later sections.

COMPARISON OF STRUCTURE AND ION REGULATION IN THE ANTENNAL GLAND AMONG CRABS

The antennal gland—which is functionally similar to the kidney of vertebrates and the Malpighian tubule of insects—is believed to be the excretory organ that plays an important role in the volume and ion composition of hemolymph regulation (Feifel et al., 1997; Lin et al., 2000; Freire et al., 2008; Brown et al., 2009). The water turnover of an individual can be regulated by different urine production rates in crabs among various environments. Researchers used the clearance rate of isotope material, such as inulin with ^{14}C or EDTA with ^{51}Cr, to estimate the urine production rate of crabs in different mediums (Riegel and Lockwood, 1961; Binns, 1969; Kormanik and Harris, 1981; Morris and Ahern, 2003). For example, the intertidal species *Carcinus maenas* and shore crab *Pachygrapsus crassipes* can increase their urine production rate when salinity decreases (Gross and Marshall, 1960; Binns, 1969). The urine flow and EDTA clearance of the land crab *Gecarcoidea natalis* during the wet season was higher than that during the dry season (Morris and Ahern, 2003). *Gecarcinus lateralis* showed a higher urine production rate when it came in contact with the moist sand compared to the dry sand (Harris, 1977). Most brachyuran species can only produce isosmotic urine, but some of them can modify the ion composition of their urine to distinguish it from hemolymph (Gross, 1964; Kormanik and Harris, 1981; Weihrauch et al., 2004; Tseng et al., 2020). The intertidal species *Tubuca arcuata* produces an isosmotic urine in 5 and 35 PSU seawater, but its urine-hemolymph ratio (U/B) for Na^+ and Cl^- is lower and higher than 1, respectively. These results indicated that *T. arcuata* could reabsorb Na^+ and excrete Cl^- from urine in different environments (Tseng et al., 2020).

Structure and Distribution of Ion Regulatory Proteins in the Antennal Gland

The structure and distribution of ion regulatory proteins in the antennal gland of crayfish are well-documented and reviewed (Peterson and Loizzi, 1974a; Roldan and Shivers, 1987; Wheatly and Henry, 1987; Sarver et al., 1994; Wheatly and Gannon, 1995; Ueno and Inoue, 1996; Khodabandeh et al., 2005; Freire et al., 2008; Charmantier et al., 2009). The antennal gland of crayfish contains three parts: the coelomosac, labyrinth and nephridial canal (Peterson and Loizzi, 1973; Peterson and Loizzi, 1974a; Freire et al., 2008; Charmantier et al., 2009). The coelomosac is composed of the coelomic cells connected to adjacent cells by foot processes similar to those of the podocytes. It is considered the place to produce primary urine (Peterson and Loizzi, 1973; Peterson and Loizzi, 1974a; Ueno and Inoue, 1996). The labyrinth contains cells with apical microvilli, membrane folding, and mitochondria and it is analogous to the proximal tubule in the vertebrate kidney (Peterson and Loizzi, 1973; Peterson and Loizzi, 1974a; Roldan and Shivers, 1987). Finally, the nephridial canal—which has thicker cells and higher levels of Mg^{2+}-dependent ATPase than other parts of the antennal gland—is functionally similar to the distal tubule (Peterson and Loizzi, 1973; Peterson and Loizzi, 1974a). Peterson and Loizzi (1974b) found that the labyrinth and nephridial canal of *Procambarus* species had a higher level of NKA activity and distribution than the coelomosac. Khodabandeh et al. (2005) also found a similar result in the antennal gland in embryos and juveniles of the crayfish *Astacus leptodactylus*. In addition, the urine chloride concentration and osmotic pressures were lower in the distal part of nephridial canal of *Austropotamobius pallipes* than in the coelomosac and labyrinth (Riegel, 1963). These evidences may indicate that expression level and activity of NKA were correlated to the ionic and osmotic regulation ability and the distal part of nephridial canal with higher level of NKA activity and distribution was a presumed major site for ion regulation in antennal gland of crayfish (Charmantier et al., 2009).

In contrast to crayfish, studies on the structure and distribution of ion regulatory proteins in the antennal gland of brachyuran crabs are limited to the genera *Uca*, *Ocypode* and *Callinectes* (Schmidt-Nielsen et al., 1968; Johnson, 1980; Tsai and Lin, 2014). Two major cell types, coelomic and labyrinthine cells, were discovered in the antennal gland of crabs (Schmidt-Nielsen et al., 1968; Johnson, 1980; Tsai and Lin, 2014) (**Figure 1A**). McGaw (2005) used a perfusion method to construct the cardiovascular system of *Cancer* species. The study showed that the coelomosac artery (CCA) extends into the antennal gland and becomes branches that form circular structures; the empty space in the circle is considered to be where the coelomosac and labyrinth originally resided (McGaw, 2005). The coelomosac is surrounded by the labyrinth, and

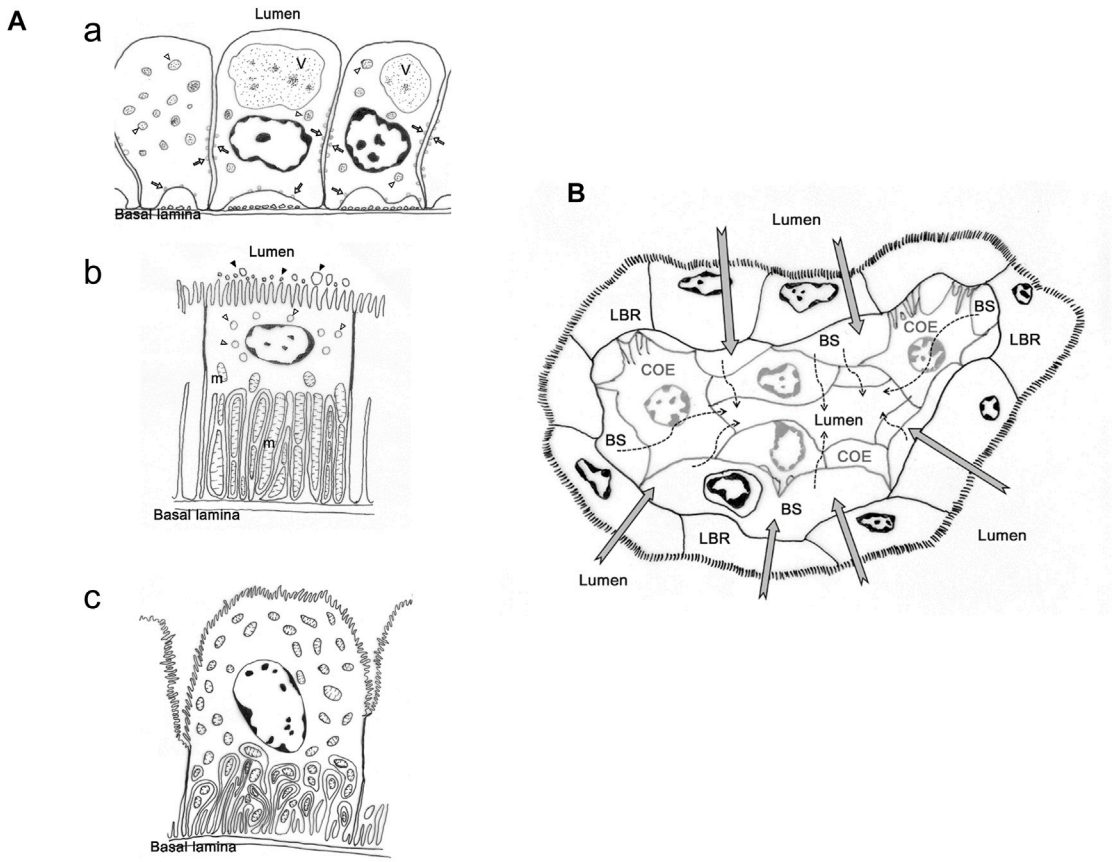

FIGURE 1 | The cell types and presumed urine production and re-absorption process in antennal gland of *Ocypode stimpsoni*. **(A)** Three types of cell in antennal gland of *O. stimpsoni*. Coelomic cells with different size of vesicle (open arrow and triangle) and endosome (V), **(a)**. Labyrinthine cells have a great of mitochondria (m) in basal folding, vesicles (open triangle) in sub-apical region and aposomes (black triangle) around apical microvilli, **(b)**. End-labyrinthine cells, another type of labyrinthine cells, showed a different shape of apical membrane compared to labyrinthine cells. In addition, mitochondria not only distributed in basal folding, but also in other regions of end-labyrinthine cells, **(c)**. **(B)** Hemolymph will be filtrated through coelomic cells (dash arrow), and the ions and other substances will be re-absorbed into hemolymph by labyrinthine cells (grey arrow). COE, coelomic cell. hs, haemolymph sinus. LBR, labyrinthine cell. The figure comes from Tsai and Lin, (2014).

hemolymph sinus is between these two structures (Schmidt-Nielsen et al., 1968; Johnson, 1980). Based on the cardiovascular path and ultrastructure of the antennal gland, Tsai and Lin (2014) suggested that primary urine is produced by the foot processes of the coelomosac connected with the capillaries from CCA—urine flows into the lumen, where ions are reabsorbed by the labyrinth back into the capillaries (**Figure 1B**).

However, there were several different features in ultrastructure among labyrinthine cells in different part of antennal gland (Schmidt-Nielsen et al., 1968; Tsai and Lin, 2014). Schmidt-Nielsen et al. (1968) found at least two types of cells in the labyrinth, one with more mitochondria and basolateral infolding, but these structures were fewer in other cell type in *M. mordax*. In *O. stimpsoni*, the thickness and length of apical microvilli in labyrinthine cells are different between proximal and distal tubular regions in the head part of the antennal gland. In addition, the so-called end-labyrinthine cell (ELBR) in the tail part of the antennal gland is even thicker and the cell's apical microvilli are even longer than in the head part of the antennal gland (Tsai and Lin, 2014) (**Figure 1A**).

McNamara et al. (2015) compared the density of apical microvilli and the basal invaginations of the antennal gland among six palaemonid shrimps from intertidal to freshwater zones in *Macrobrachium* and *Palaemon*. The species in *Macrobrachium* in the freshwater habitat mainly had higher apical microvilli and basal invagination densities in the antennal gland; this lineage also showed a higher gill apical evaginations density. None of this was the case for *Palaemon* in the intertidal zone. (McNamara et al., 2015). In addition, the osmotic gradient between hemolymph and environment was greater in *Macrobrachium* species. The authors indicated that the extension of surface area in the gills and antennal gland of *Macrobrachium* species can facilitate the ion and water regulation in freshwater environment. This phenomenon may be caused by natural selection in these species (McNamara et al., 2015). However, more data on the structure of the antennal gland are needed among crabs to do meta-analyses for comparing the

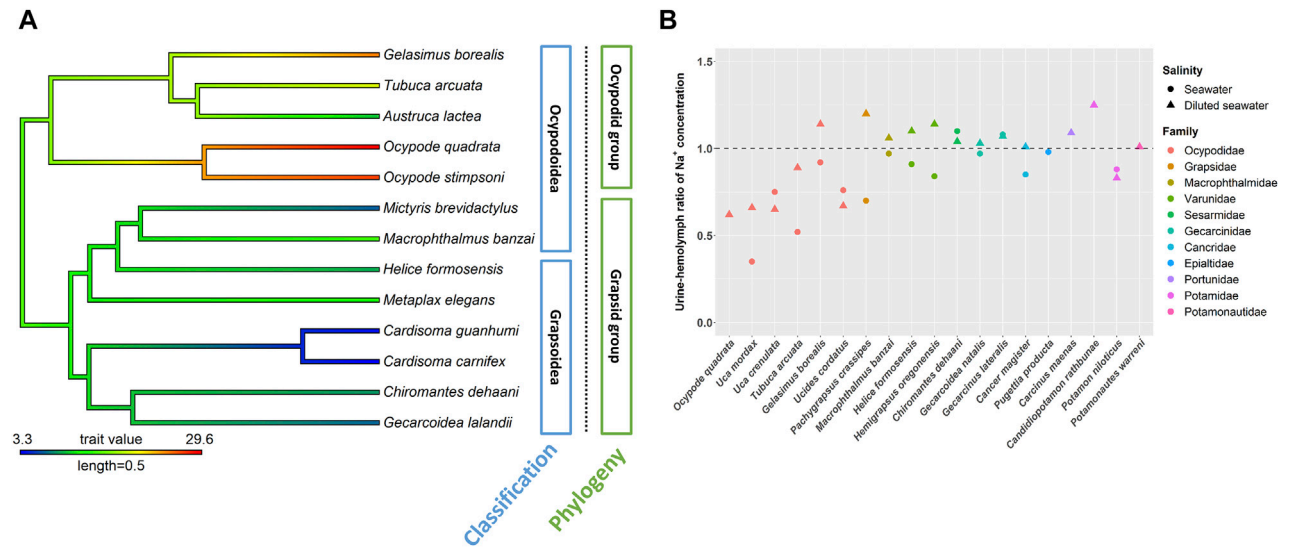

FIGURE 2 | The NKA activity and urine and hemolymph ratio (U/H) for Na^+ in the antennal glands of different crab species. The phylogenetic tree was generated by Bayesian inference with COI and 16S rDNA. The antennal gland NKA activity of crabs in 5 ppt was mapped on the phylogenetic tree and the ancestor status was predicted by the phylogenetic structure. Labels in blue boxes were the superfamilies of brachyuran. Labels in green boxes were the groups separated by the clades in phylogenetic trees. **(A)** Antennal gland NKA activity in the ocypodid group was higher than that in the grapsid group. **(B)** Ocypodidae species had a lower U/H for Na^+ than did species in other families. The figure comes from Tseng et al. (2020).

structural differences among species in different habitats and phylogenetic clades.

Furthermore, the arrangement and distribution level of ion regulatory proteins in the antennal gland are also different among coelomic, labyrinthine and end-labyrinthine cells (Tsai and Lin, 2014). NKA distributed in the basolateral regions of coelomic and two types of labyrinthine cells—VHA and NKCC—were found in the vesicles and apical region of the coelomic and labyrinthine cells, respectively. The intensity of the immunocytochemical stain in NKA is highest in ELBR, and coelomic cell only showed a slight NKA distribution (Tsai and Lin, 2014). This result is similar to the NKA distribution in the antennal gland of crayfish (Peterson and Loizzi, 1973; Khodabandeh et al., 2005). The labyrinthine cells showed various ultrastructures and ion regulatory protein distribution in different parts of the antennal gland in *Minuca mordax* and *Ocypode stimpsoni*, but the linkage between the structure and functions of labyrinthine cells needs further investigation.

Previous research proposed a possible pathway by which various ion regulatory protein arrangements in gills evolved among brachyurans based on phylogenetic relationships and ion regulatory patterns (McNarmara and Faria, 2012). The presumed ancestral status is similar to osmo-conformers in marine species; the apical NHE losses and VHA are independent in strong hyper-osmoregulators in freshwater and diadromous clades; and apical and the basal NKCC, basal K^+ channel and basal Cl^- channel are found in weak hyper-osmoregulators in brackish water, as well as semi-terrestrial and diadromous species (McNarmara and Faria, 2012). If the evolution of ion regulatory protein arrangements in the antennal gland can be further investigated, the possible process that how the evolution and environment shape the function of antennal gland among brachyuran may also be speculated.

Differences in Ion Regulatory Functions of Antennal Glands Among Crabs

The involvement of ion regulatory functions in the antennal gland varied among crab species (Gross, 1964; Bliss, 1968; Harris and Santos, 1993; DeVries et al., 1994; Morris, 2001; Weihrauch et al., 2004; Freire et al., 2008; Charmantier et al., 2009; Tseng et al., 2020) (**Figure 2**). Among Ocypodidae species, *Ocypode quadrata* had high terrestriality and showed an ability to reabsorb Na^+ and excrete NH_4^+ from urine during hypo-osmotic stress (DeVries et al., 1994). This phenomenon also occurred in some *Uca* and Gelasiminae species. *Minuca pugnax* can reabsorb Na^+ and excrete NH_4^+ from urine in 100 and 175% seawater (Green et al., 1959). The Na^+ concentration in the urine of the semi-terrestrial species *Lepuca crenulata* was lower than that in the hemolymph in 50, 100 and 150% seawater (Gross, 1964). The semi-terrestrial species *Minuca mordax* and bimodal/intertidal species *Tubuca arcuata* can reabsorb Na^+ from urine in both hypo-osmotic and hyper-osmotic mediums (Schmidt-Nielsen et al., 1968; Tseng et al., 2020). In addition, the bimodal/intertidal species *Ucides cordatus* has a relatively low urine to hemolymph ratio (U/H) with Na^+ concentration in diluted seawater (Harris and Santos, 1993). On the other hand, the following only showed a limited ability to reabsorb Na^+ from urine in hypo-osmotic mediums: the terrestrial species *Gecarcoidea natalis* and *G. lateralis* (Gecarcinidae) (Gross, 1964; Taylor and Greenaway, 2002); semi-terrestrial/intertidal crabs *Chiromantes dehaani* (Sesarmidae), *Helice formosensis* and

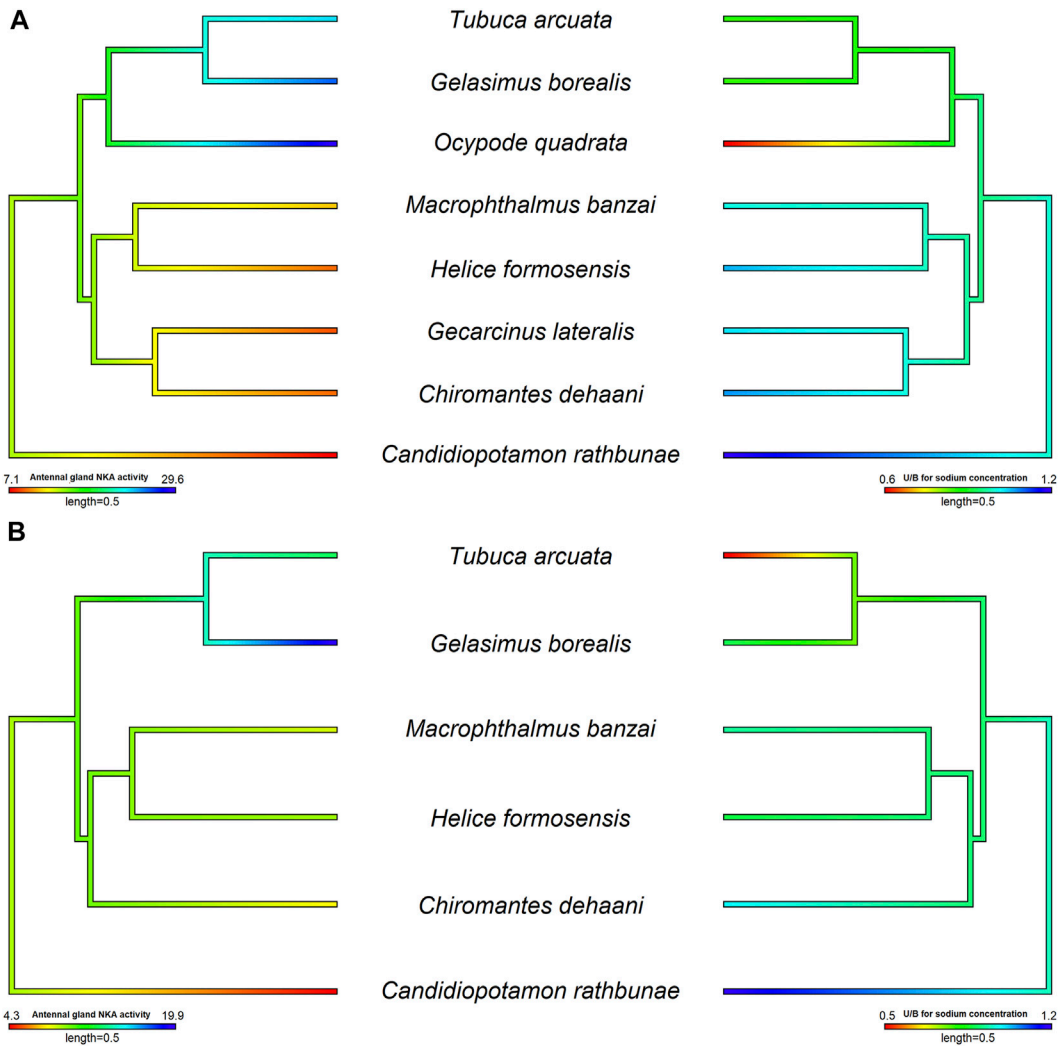

FIGURE 3 | The relationship between antennal gland NKA activity and U/H for Na^+ concentration in the antennal gland of different crab species in hypo- and hyper-osmotic environments. The phylogenetic tree was generated by Bayesian inference with COI, 12S and 16S rDNA. The antennal gland NKA activity and U/H for Na^+ concentration of crabs in 5 or 35 ppt were mapped on the phylogenetic tree and the ancestor status was predicted by the phylogenetic structure. The correlation between antennal gland NKA activity and U/H for Na^+ concentration was analyzed by PGLS. Left pictures were the results of antennal gland NKA activity, and right sides were U/H for Na^+ concentration. **(A)** The antennal gland NKA activity was correlated with the U/H for Na^+ concentration in the antennal gland when species were transferred into hypo-osmotic environments (t value = –5.7963, df = 8, $p = 0.0012$, $R^2 = 0.88$). **(B)** There was no correlation between antennal gland NKA activity and the U/H for Na^+ concentration in the antennal gland when species were transferred into hyper-osmotic mediums (t value = –0.1627, df = 6, $p = 0.8786$, $R^2 < 0.001$). Data of *Ocypode quadrata* are from DeVries et al. (1994). Data of *Gecarcinus lateralis* are from Gross (1964) and DeVries et al. (1994). Data of *Candidiopotamon rathbunae* are from Wang and Lin (2011).

Hemigrapsus oregoncnsis (Varunidae) (Gross, 1964; Tseng et al., 2020; Allen and Weihrauch, 2021); intertidal/marine species *Cancer antennarius* and *Cancer magister* (Cancridae) (Gross, 1964; Hunter and Rudy, 1975), *Carcinus maenas* and *Callinectes sapidus* (Portunidae) (Carmeron and Batterton, 1978; Harris and Santos, 1993) and *Macrophthalmus banzai* (Macrophthalmidae) (Tseng et al., 2020); and freshwater species *Candidiopotamon rathbunae* (Wang and Lin, 2011) and *Potamonautes warreni* (Morris and Van Aardt, 1998) (**Figure 2B**). Two Varunidae species, *Pachygrapsus crassipes* (Grapsidae) and two Cancridae species can reabsorb Na^+ from urine to some degree in hyper-osmotic environments (Prosser et al., 1955; Gross, 1964; Hunter and Rudy, 1975; Harris and Santos, 1993; Tseng et al., 2020; Allen Weihrauch, 2021). Although crabs showed different degrees of magnesium excretion from urine, species of Cancridae, Gecarcinidae, Grapsidae, Ocypodidae, Varunidae and Grapsidae can excrete/lose Mg^{2+} from urine in hypo- and/or hyper-osmotic environments (Gross, 1964; Hunter and Rudy, 1975; DeVries et al., 1994; Taylor and Greenaway, 2002; Allen and Weihrauch, 2021).

The Na^+, K^+-ATPase (NKA) activity in the antennal gland showed a similar pattern to the U/H for Na^+ concentration among crabs in hypo-osmotic environments (DeVries et al.,

1994, Weihrauch et al., 2004; Tsai and Lin, 2014; Tseng et al., 2020) (**Figures 2**, **3**). *Ocypode stimpsoni*, *O. quadrata* and two Gelasiminae species had a relatively higher antennal gland NKA activity in hypo-osmotic environments (DeVries et al., 1994; Tsai and Lin, 2014; Tseng et al., 2020). In contrast, *Austruca lactea*, *Cardisoma carnifex*, *Cardisoma guanhumi*, *Chiromantes dehaani*, *Gecarcoidea lalandii*, *Helice formosensis*, *Macrophthalmus banzai*, *Metaplax elegans*, *Mictyris brevidactylus*, *Scylla paramamosain* and *Ucides cordatus* exhibited a lower antennal gland NKA activity in diluted mediums (Schmidt-Nielsen et al., 1968; Towle, 1981; Harris and Santos, 1993; DeVries et al., 1994; Chung and Lin, 2006; Tseng et al., 2020). Based on this, habitat type, which is considered an important factor controlling the diverse physiological patterns of crabs, seems to not be the only factor driving the evolution of this distinct ion regulation mechanism in the antennal gland.

Tsang et al. (2014) indicated that, in Ocypodoidea and Grapsoidea, some of the families in one superfamily are more closely related to a family in the other—e.g., Macrophthalmidae and Mictyridae (Ocypodoidea) are more closely related to families in Grapsoidea—such as Varunidae—than the other Ocypodidae in the same superfamily. This finding implies an underlying possibility that phylogeny affects the physiological patterns of the antennal gland among crabs. Tseng et al. (2020) compared the antennal gland NKA activity among Ocypodoidea and Grapsoidea in a hypo-osmotic environment by phylogenetic ANOVA and Moran's *I* auto-correlation. The results showed that the antennal gland NKA activity of crabs is phylogenetically correlated and significantly different between ocypodid and grapsid groups (Tseng et al., 2020) (**Figure 2A**). In the present review, we further used phylogenetic generalized least square (PGLS) to analyze whether there is a correlation between antennal gland NKA activity and U/B for Na^+ concentration in hypo-osmotic environments among crabs. The result showed that these two features were correlated (t value = -5.7963, df = 8, p = 0.0012, R^2 = 0.88). Species with higher antennal gland NKA activity showed a lower U/B for Na^+ concentration under hypo-osmotic stress (**Figure 3A**). However, *Ocypode* species showed a greater antennal gland NKA activity and Na^+ reabsorption capacity than did Gelasiminae and *Uca* species. This is an indication that the terrestriality of crabs still plays an important role in the ion regulatory functions of the antennal gland in Ocypodidae species (DeVries et al., 1994; Tsai and Lin, 2014; Tseng et al., 2020).

On the other hand, crabs' capacities to reabsorb Na^+ from urine under hyper-osmotic environments had a similar pattern to the capacities under hypo-osmotic stress, Ocypodidae species showed a higher Na^+ reabsorption ability from urine (**Figure 2B**). However, the antennal gland NKA activity of *Tubuca arcuata*—which contained a low U/H for Na^+ concentration—did not differ from those of *Helice formosensis* and *Macrophthalmus banzai*—which rarely or slightly reabsorb Na^+ from urine—in a 100% seawater environment (Tseng et al., 2020) (**Figure 3B**). Based on the PGLS analysis, Na^+ reabsorption from urine did not correlate to the antennal gland NKA activity among crabs in hyper-osmotic mediums (t value = -0.1627, df = 6, p = 0.8786, R^2 < 0.001). On the other hand, the antennal gland is also involved in Mg^{2+} regulation in brachyurans. Although the trend in U/H for magnesium (Mg^{2+}) concentration among crabs corresponds to a trend in Na^+ reabsorption capacity from urine, the U/H for Mg^{2+} concentration of Cancridae, Gecarcinidae, Grapsidae, Ocypodidae, Varunidae and Grapsidae species was >1 in hypo- and/or hyper-osmotic environments (Green et al., 1959; Gross, 1964; Taylor and Greenaway, 2002). Green et al. (1959) indicated that Na^+ reabsorption and Mg^{2+} excretion from urine may be correlated; for example, *P. crassipes*, *L. crenulata*, *L. pugilator*, *M. pugnax* and *H. oregonensis* had a U/H for Na^+ concentration < 1 and a U/H for Mg^{2+} concentration >1 in seawater (Prosser et al., 1955; Green et al., 1959; Gross, 1964). In addition, the U/H for the NH_4^+, SO_4^{2-} and Ca^{2+} concentrations of *L. crenulata* and *L. pugilator* were >1 in 100 and 175% seawater (Green et al., 1959). However, in *G. lateralis*, *G. natalis* and *C. antennarius*, the U/H for Na^+ concentration was close to one and the Mg^{2+} concentration in urine was higher than that in the hemolymph (Gross, 1964; Taylor and Greenaway, 2002). The relationship between Na^+, NH_4^+ and divalent ion regulation in hyper-osmotic environments and which ion regulatory proteins are involved in these mechanisms in antennal glands need further investigation.

One reason why ion regulation in the antennal gland differs among crabs may be correlated with the differences in the morphology and function of gills and lung-like structures in different brachyuran clades. Takeda et al. (1996) indicated that land crabs in Ocypodoidea and Grapsoidea invaded terrestrial habitats through different routes: Ocypodid species directly from intertidal/supratidal zones and Grapsid species through estuaries and rivers/streams. The terrestrial species *Gecarcinus lateralis* in Gecarcinidae and *Chiromantes dehaani* in Sesarmidae have eight or nine pairs of gills, similar to marine, intertidal and bimodal species such as *Macrophthalmus banzai* in Macrophthalmidae, *Scylla paramamosain* in Portunidae and *Helice formosensis* and *Metaplax elegans* in Varunidae, which also had eight pairs of gills (Copeland and Fitzjarrell, 1968; Takeda et al., 1996; Tseng et al., 2020). However, Ocypodidae species—such as the semi-terrestrial species *Ocypode stimpsoni*; intertidal species *Austruca lactea*; and intertidal/bimodal species *Gelasimus borealis*, *Tubuca arcuata*, and *Xeruca formosensis*—have an inconsistent reduced number of pairs of gills (Takeda et al., 1996; Lin et al., 2002; Tsai and Lin, 2012; Tseng et al., 2020).

In addition, the lung types and properties were also different among crabs and the degree of complexity was higher in Ocypodidae than in Grapsidae. The lung-like structure was specialized from the branchiostegite and formed a complex folding with branching hemolymph vessels in the inner side of the branchial chamber (Greenaway, 1984b; Farrelly and Greenaway, 1987; 1993; Greenaway and Farrelly, 1990; Greenaway, 1999). *Ocypode* species had a highly compact and evaginated lung; in contrast, Gecarcinidae, Grapsidae and Varunidae species showed expanded and smooth lungs (Tsai and Lin, 2012) (**Table 1**). Investigations suggested the lung can increase the efficiency of gas exchange when it is active on land (Taylor and Greenaway, 1979; Innes and Taylor, 1986).

The development of the lung from the branchiostegite may cause a functional shift in gas exchange from the gill to lung; in

TABLE 1 | Lung types and habitats of crabs among families.

Superfamily	Family	Habitat†	Species	Lung type‡			References
				Cp/Ex	Sm/Ev/Iv/Un	2D/3D	
Ocypodoidea	Ocypodidae	T	*Ocypode ceratophthalmus*	Cp	Ev	3D	(3)
		T	*Ocypode cordimanus*	Cp	Ev	3D	(1)
		T	*Ocypode stimpsoni*	Cp	Ev	3D	(5)
		UT	*Tubuca coarctata*	Cp	Sm	2D	(3)
	Mictyridae	IT	*Mictyris longicarpus*	Cp	Iv	3D	(1), (3)
Grapsoidea	Grapsidae	UT	*Hemigrapsus nudus*	Ex	Sm	2D	(2)
		T	*Geograpsus grayi*	Ex	Sm	2D	(3)
	Gecarcinidae	T	*Cardisoma hirtipes*	Ex	Sm	2D	(3)
		T	*Cardisoma carnifex*	Ex	Sm	2D	(3)
		T	*Gecarcoidea natalis*	Ex	Sm	2D	(2)
	Varunidae	U	*Neohelice granulatus*	Ex	Sm	2D	(4)
Gecarcinucoidea	Gecarcinucidae	Fw/T	*Austrothelphusa transversa*	--	Sm	--	(2), (3)

†*Habitat: T, terrestrial; IT, intertidal; UT, upper intertidal; Fw, freshwater.*

‡*Lung type: Cp, compact; Ex, expanded; Sm, smooth; Ev, evaginated; Iv, invaginated; Un, unclear lung type, indicating that no high quality data was obtained by paraffin* ***section 2D****: the lung was extended through a 2-dimensional direction. 3D, the lung was extended through a 3-dimensional direction. --, data was not available.*

References: (1) Farrelly and Greenaway, 1987. (2) Greenaway and Farrelly, 1990. (3) Farrelly and Greenaway, 1993. (4) Halperin et al., 2000. (5) Tsai and Lin, 2012. Table was modified from Tsai and Lin, 2012.

addition, the principal site for gas exchange—the anterior gills—becomes involved in ion regulation (Innes and Taylor, 1986; Santos et al., 1987; Greenaway and Farrelly, 1990; Tsai and Lin, 2012). *Tubuca arcuata*, *Austruca annulipes*, *Tubuca urvillei*, *Gelasimus tetragonon* and *Xeruca formosensis* have reduced pairs of gills and an osmoregulatory lamella in their anterior gills (Takeda et al., 1996; Lin et al., 2002). The NKA activity of the anterior gills in *Ocypode stimpsoni* significantly increased 4 days after being transferred into 5 ppt seawater (Tsai and Lin, 2012). In these species, *Ocypode* spp. and *Tubuca arcuata* also showed a relatively high NKA activity in the antennal gland and Na^+ absorption ability from urine (DeVries et al., 1994; Tsai and Lin, 2014; Tseng et al., 2020) (**Figure 3A**). The osmoregulatory functional shifts may not only occur in anterior gills, but also to the antennal gland (Tsai and Lin, 2012; Tsai and Lin, 2014). This phenomenon may lead to differences among species in ion regulation in the gills and antennal gland. However, more studies are needed to fill the missing data in other phylogenetic clades of brachyurans. This will lead to a more comprehensive phylogeny of ion regulatory functions in brachyurans.

MULTI-OMICS METHODS FOR ION REGULATION STUDIES IN CRABS

Integrating other physiological features to describe the ion regulatory functions in organisms can let us compare the ion regulatory difference among species in a comprehensive way. Next generation sequencing (NGS) generates a great deal of sequencing data in a short time; these data can then connect the physiological studies to multi-gene or genome level perspectives. Over the past decade, this technique has emerged as a mature method for facilitating the development of a wide range of disciplines (Schuster, 2008; Shendure and Ji, 2008; Liu et al., 2012; Goodwin et al., 2016). RNA sequencing by NGS (RNAseq) can simultaneously detect the expression level and number of functional genes to analyze the relationships between genes and physiological pathways in different environments (Wang et al., 2009). Proteomics method is another powerful tool to detect the expression of several proteins or discover novel proteins involve in different physiological pathways (Aslam et al., 2017; Suhre et al., 2020).

Researchers have used RNAseq to investigate the transcriptomes of organs in marine and freshwater crabs, most of them focusing on ion-regulatory gills (Lv et al., 2013; Li et al., 2014; Yang et al., 2019; Niu et al., 2020; Malik and Kim, 2021). Their results identified not only the routine responses of ion regulatory proteins, but other important physiological pathways, such as metabolism, signal transduction and anti-oxidation up-/down- regulation during salinity stresses in posterior gills (Lv et al., 2013; Li et al., 2014; Havird et al., 2016; Yang et al., 2019; Niu et al., 2020; Malik and Kim, 2021). For example, NKA and VHA was found to be downregulated and NKCC upregulated in *E. sinensis* in a seawater treatment; in addition, the expression of Cu^{2+}/Zn^{2+} superoxide dismutase in the antioxidant activity pathway increased about 6.5 fold in a 25 ppt treatment (Yang et al., 2019). VHA and CA in ion regulation and Acetyl-CoA acetyltransferase in the metabolism pathway and signaling mucin HKR1 in the signal transduction pathway increased when *S. paramamosain* was transferred into a 5 ppt medium (Niu et al., 2020). These important physiological pathways can help individuals resist stress and maintain an energy homeostasis when salinity changes (Li et al., 2014; Yang et al., 2019; Niu et al., 2020).

Proteomics level changes of gills among salinity was also investigated in marine species (Wang et al., 2018; Niu et al., 2020). Wang et al. (2018) used SDS-PAGE and HPLC-MS to detect the proteome in gills of *Scylla paramamosain* under

hypo-osmotic condition. KEGG pathway analysis results showed that proteins in amino acid metabolism and NKA regulation upregulated in 3 ppt seawater (Wang et al., 2018). These physiological pathways were considered as important mechanisms for osmotic and ionic regulation of brachyurans (Wang et al., 2018). In addition, Niu et al. (2020) showed that VHA subunit B and CA2 in gills of *S. paramamosain* not only upregulated in gene level, but also increased in protein level under hypo-osmotic stress. The protein expression of Cl^- channel, Rh protein for ion regulation and NADH dehydrogenase in energy metabolism of gills also increased in 5 ppt seawater (Niu et al., 2020). As the number of transcriptomic and proteomics studies increase and are applied to other brachyurans in intertidal or terrestrial habitats and different phylogenetic clades, we will become better equipped to compare the physiological responses of ion regulation.

On the other hand, Havird et al. (2016) used RNAseq to show that the gene expression levels between anterior and posterior gills differed in *Callinects sapidus* under different salinity stresses. The expressions of the NKA, NHE and Na^+ channels in posterior gills were higher than in anterior ones at 35 ppt (Havird et al., 2016). And NKA expression in the posterior gills was also higher than in the anterior gills in the 5 ppt treatment (Havird et al., 2016). Furthermore, genes expression in the metabolism pathway also increase in the posterior gills under hypo-osmotic stress (Havird et al., 2016). Moshtaghi et al. (2016) analyzed the gene expressions in gills, antennal gland and hepatopancreas of the freshwater prawn *Macrobrachium australiense*. Results indicated that the gill expresses arginine kinase, NKA, VHA and CA more highly than the antennal gland and hepatopancreas (Moshtaghi et al., 2016). If we compare the gene and protein expression levels of gills and antennal glands between Ocypodoidea and other superfamilies species, we will realize how the antennal gland supports homeostasis and what the differences are in ion regulatory mechanisms between gills and the antennal gland among different species in various environments.

CONCLUSION

We can broaden our understanding of diverse ion regulatory patterns among brachyuran when we consider the combined effects of phylogenetic relationships and environmental properties. The complex interactions among the antennal gland, gill and lung-like structure and can somehow be inferred using crab phylogenies and habitats. Statistical methods with phylogenetic correction, including PGLS and Moran's *I*, can give us a more precise results in multi-species comparison. PGLS analysis in present review showed that the NKA activity in antennal gland is correlated to the urine-hemolymph ratio for Na^+ in crabs under hypo-osmotic stress. Only a few studies address the structure and the distribution of ion regulatory proteins in the antennal gland among brachyurans, and there are still gaps in the linkage between structural and functional differences in crab antennal glands. It is possible to conduct the trait evolution analysis of ion regulation in a more integrative way by including a number of ion regulatory proteins in different organs among species—for example, using RNA sequencing or proteomics method to detect the expression difference of ion regulatory proteins and analyzing the patterns in the phylogenetic tree of crabs.

AUTHOR CONTRIBUTIONS

H-CL, K-YT, and J-RT contributed to conception and design of the study. K-YT and J-RT conducted the experiments. K-YT and J-RY wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

Allen, G. J. P., Kuan, P.-L., Tseng, Y.-C., Hwang, P.-P., Quijada-Rodriguez, A. R., and Weihrauch, D. (2020). Specialized Adaptations Allow Vent-Endemic Crabs (*Xenograpsus Testudinatus*) to Thrive under Extreme Environmental Hypercapnia. *Sci. Rep.* 10, 11720. doi:10.1038/s41598-020-68656-1

Allen, G. J. P., and Weihrauch, D. (2021). Exploring the Versatility of the Perfused Crustacean Gill as a Model for Transbranchial Transport Processes. *Comp. Biochem. Physiology Part B Biochem. Mol. Biol.* 254, 110572. doi:10.1016/j.cbpb.2021.110572

Aslam, B., Basit, M., Nisar, M. A., Khurshid, M., and Rasool, M. H. (2017). Proteomics: Technologies and Their Applications. *J. Chromatogr. Sci.* 55, 182–196. doi:10.1093/chromsci/bmw167

Barnes, R. S. K. (1974). *Estuarine Biology*. London: Edward Arnold.

Beyenbach, K. W., and Wieczorek, H. (2006). The V-type H+ ATPase: Molecular Structure and Function,physiological Roles and Regulation. *J. Exp. Biol.* 209, 577–589. doi:10.1242/jeb.02014

Binns, R. (1969). The Physiology of the Antennal Gland of Carcinus Maenas (L.). *J. Exp. Biol.* 51, 11–16. doi:10.1242/jeb.51.1.11

Bliss, D. E. (1968). Transition from Water to Land in Decapod Crustaceans. *Amer. Zool.* 8, 355–392. doi:10.1093/icb/8.3.673

Brown, D., and Breton, S. (1996). Mitochondria-rich, Proton-Secreting Epithelial Cells. *J. Exp. Biol.* 199, 2345–2358. doi:10.1242/jeb.199.11.2345

Brown, D., Paunescu, T. G., Breton, S., and Marshansky, V. (2009). Regulation of the V-ATPase in Kidney Epithelial Cells: Dual Role in Acid-Base Homeostasis and Vesicle Trafficking. *J. Exp. Biol.* 212, 1762–1772. doi:10.1242/jeb.028803

Carmeron, J. N., and Batterton, C. V. (1978). Antennal Gland Function in the Freshwater Blue Crab, *Callinectes sapidus*: Water, Electrolyte, Acid-base and Ammonia Excretion. *J. Comp. Physiol.* 123, 143–148.

Charmantier, G., Charmantier-Daures, M., and Towle, D. (2009). "Osmotic and Ionic Regulation in Aquatic Arthropods," in *Osmotic and Ionic Regulation: Cells and Animals*. Editor D. H. Evans (Boca Raton: CRC Press), 165–230.

Chung, K.-F., and Lin, H.-C. (2006). Osmoregulation and Na,K-ATPase Expression in Osmoregulatory Organs of Scylla Paramamosain. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 144, 48–57. doi:10.1016/j.cbpa.2006.02.003

Copeland, D. E., and Fitzjarrell, A. T. (1968). The Salt Absorbing Cells in the Gills of the Blue Crab (*Callinectes sapidus Rathbun*) with Notes on Modified Mitochondria. *Z. Zellforsch Mikrosk Anat.* 92, 1–22. doi:10.1007/bf00339398

Davie, P. J. F., Guinot, D., and Ng, P. K. L. (2015). "Systematics and Classification of Brachyuran," in *Decapoda: Brachyura (Part 2). Treatise on Zoology – Anatomy, Taxonomy, Biology. The Crustacea Vol. 9C-II*. Editors P. Castro, P. J. F. Davie, D. Guinot, F. R. Schram, and J. C. Von Vaupel Klein (Leiden and Boston: Brill), 1049–1130.

De Vries, M. C., Wolcott, D. L., and Holliday, C. W. (1994). High Ammonia and Low pH in the Urine of the Ghost Crab, *Ocypode Quadrata*. *Biol. Bull.* 186, 342–348. doi:10.2307/1542280

Ehrenfeld, J., and Klein, U. (1997). The Key Role of the H+ V-ATPase in Acid-Base Balance and Na+ Transport Processes in Frog Skin. *J. Exp. Biol.* 200, 247–256. doi:10.1242/jeb.200.2.247

Faria, S. C., Provete, D. B., Thurman, C. L., and McNamara, J. C. (2017). Phylogenetic Patterns and the Adaptive Evolution of Osmoregulation in Fiddler Crabs (Brachyura, *Uca*). *PLoS One* 12, e0171870. doi:10.1371/journal.pone.0171870

Farrelly, C. A., and Greenaway, P. (1993). Land Crabs with Smooth Lungs: Grapsidae, Gecarcinidae, and Sundathelphusidae Ultrastructure and Vasculature. *J. Morphol.* 215, 245–260. doi:10.1002/jmor.1052150306

Farrelly, C., and Greenaway, P. (1987). The Morphology and Vasculature of the Lungs and Gills of the Soldier crab,Mictyris Longicarpus. *J. Morphol.* 193, 285–304. doi:10.1002/jmor.1051930307

Feifel, E., Krall, M., Geibel, J. P., and Pfaller, W. (1997). Differential Activities of H+ Extrusion Systems in MDCK Cells Due to Extracellular Osmolality and pH. *Am. J. Physiol.* 273, F499–F506. doi:10.1152/ajprenal.1997.273.4.F499

Felsenstein, J. (1985). Phylogenies and the Comparative Method. *Am. Nat.* 125, 1–15. doi:10.1086/284325

Freire, C. A., Onken, H., and McNamara, J. C. (2008). A Structure-Function Analysis of Ion Transport in Crustacean Gills and Excretory Organs. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 151, 272–304. doi:10.1016/j.cbpa.2007.05.008

Garland, T., JR., and Carter, P. A. (1994). Evolutionary Physiology. *Annu. Rev. Physiol.* 56, 579–621. doi:10.1146/annurev.ph.56.030194.003051

Garland, T., JR., Dickerman, A. W., Janis, C. M., and Jones, J. A. (1993). Phylogenetic Analysis of Covariance by Computer Simulation. *Syst. Biol.* 42, 265–292. doi:10.1093/sysbio/42.3.265

Garland, T., JR., Harvey, P. H., and Ives, A. R. (1992). Procedures for the Analysis of Comparative Data Using Phylogenetically Independent Contrasts. *Syst. Biol.* 41 (1), 18–32. doi:10.1093/sysbio/41.1.18

Genovese, G., Ortiz, N., Ortiz, N., Urcola, M. R., and Luquet, C. M. (2005). Possible Role of Carbonic Anhydrase, V-H+-ATPase, and Cl–/HCO3– Exchanger in Electrogenic Ion Transport across the Gills of the Euryhaline Crab Chasmagnathus Granulatus. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 142, 362–369. doi:10.1016/j.cbpa.2005.08.024

Gittleman, J. L., and Kot, M. (1990). Adaptation: Statistics and a Null Model for Estimating Phylogenetic Effects. *Syst. Zool.* 39 (3), 227–241. doi:10.2307/2992183

Goodwin, S., McPherson, J. D., and McCombie, W. R. (2016). Coming of Age: Ten Years of Next-Generation Sequencing Technologies. *Nat. Rev. Genet.* 17, 333–351. doi:10.1038/nrg.2016.49

Gotelli, N. J., and Ellison, A. M. (2004). *A Primer of Ecological Statistics*. 2nd ed. England: Oxford University Press.

Green, J. W., Harsch, M., Barr, L., and Prosser, C. L. (1959). The Regulation of Water and Salt by the Fiddler Crabs, *Uca Pugnax* and *Uca Pugilator*. *Biol. Bull.* 116, 76–87. doi:10.2307/1539157

Greenaway, P., and Farrelly, C. A. (1984). The Venous System of the Terrestrial crabOcypode Cordimanus (Desmarest 1825) with Particular Reference to the Vasculature of the Lungs. *J. Morphol.* 181, 133–142. doi:10.1002/jmor.1051810202

Greenaway, P., and Farrelly, C. (1990). Vasculature of the Gas-Exchange Organs in Air-Breathing Brachyurans. *Physiol. Zool.* 63 (1), 117–139. doi:10.1086/physzool.63.1.30158157

Greenaway, P. (1999). "Physiological Diversity and the Colonization of Land," in *Proceedings of the Fourth International Crustacean*. Editors F. R. Schram and J. C. von Vaupel Klein (Florida, US: CRC Press), 823–842.

Greenaway, P. (1984a). "Survival Strategies in Desert Crabs," in *Arid Australia*. Editors H. G. Cogger and E. E. Cameron (Sydney: Australian Museum), 145–152.

Greenaway, P. (1984b). The Relative Importance of the Gills and Lungs in the Gas Exchange of Amphibious Crabs of the Genus *Holthuisana*. *Aust. J. Zool.* 32, 1–6. doi:10.1071/zo9840001

Gross, W. J., and Marshall, L. A. (1960). The Influence of Salinity on the Magnesium and Water Fluxes of a Crab. *Biol. Bull.* 119 (3), 440–453. doi:10.2307/1539261

Gross, W. J. (1964). Trends in Water and Salt Regulation Among Aquatic and Amphibious Crabs. *Biol. Bull.* 127, 447–466. doi:10.2307/1539247

Halperin, J., Ansaldo, M., Pellerano, G. N., and Luquet, C. M. (2000). Bimodal Breathing in the Estuarine Crab Chasmagnathus Granulatus Dana 1851 - Physiological and Morphological Studies. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 126, 341–349. doi:10.1016/s1095-6433(00)00216-6

Harris, R. R., and Santos, M. C. F. (1993). Ionoregulatory and Urinary Responses to Emersion in the Mangrove Crab *Ucides Cordatus* and the Intertidal Crab *Carcinus maenas*. *J. Comp. Physiol. B* 163, 18–27. doi:10.1007/bf00309661

Harris, R. R. (1977). Urine Production Rate and Water Balance in the Terrestrial Crabs *Gecarcinus lateralis* and *Cardisoma Guanhumi*. *J. Exp. Biol.* 68, 57–67. doi:10.1242/jeb.68.1.57

Hartnoll, R. G. (1988). "Evolution, Systematics, and Geographical Distribution," in *Biology of the Land Crabs*. Editors W. W. Burggren and B. R. McMahon (Cambridge, England: Cambridge University Press), 6–54. doi:10.1017/cbo9780511753428.003

Havird, J. C., Mitchell, R. T., Henry, R. P., and Santos, S. R. (2016). Salinity-induced Changes in Gene Expression from Anterior and Posterior Gills of *Callinectes sapidus* (Crustacea: Portunidae) with Implications for Crustacean Ecological Genomics. *Comp. Biochem. Physiology Part D Genomics Proteomics* 19, 34–44. doi:10.1016/j.cbd.2016.06.002

Henry, R. P. (1994). Morphological, Behavioral, and Physiological Characterization of Bimodal Breathing Crustaceans. *Am. Zool.* 34, 205–215. doi:10.1093/icb/34.2.205

Henry, R. P., Lucu, Č., Onken, H., and Weihrauch, D. (2012). Multiple Functions of the Crustacean Gill: Osmotic/Ionic Regulation, Acid-Base Balance, Ammonia Excretion, and Bioaccumulation of Toxic Metals. *Front. Physio.* 3, 1–33.

Hu, M. Y., Guh, Y. J., Shao, Y. T., Kuan, P. L., Chen, G. L., Lee, J. R., et al. (2016). Strong Ion Regulatory Abilities Enable the Crab *Xenograpsus Testudinatus* to Inhabit Highly Acidified Marine Vent Systems. *Front. Physiol.* 7, 14–11. doi:10.3389/fphys.2016.00014

Hunter, K. C., and Rudy, P. P. (1975). Osmotic and Ionic Regulation in the Dungeness Crab, *Cancer Magister Dana*. *Comp. Biochem. Physiology Part A Physiology* 51, 439–447. doi:10.1016/0300-9629(75)90392-8

Innes, A. J., and Taylor, E. W. (1986). The Evolution of Air-Breathing in Crustaceans: A Functional Analysis of Branchial, Cutaneous and Pulmonary Gas Exchange. *Comp. Biochem. Physiology Part A Physiology* 85, 621–637. doi:10.1016/0300-9629(86)90271-9

Jensen, L. J., Willumsen, N. J., and Larsen, E. H. (2002). Proton Pump Activity Is Required for Active Uptake of Chloride in Isolated Amphibian Skin Exposed to Freshwater. *J. Comp. Physiol. B* 172, 503–511. doi:10.1007/s00360-002-0276-x

Johnson, P. T. (1980). *Histology of the Blue Crab,* Callinectes sapidus *A Model for the Decapoda*. New York: Praeger Publishers.

Jorgensen, P. L., Håkansson, K. O., and Karlish, S. J. D. (2003). Structure and Mechanism of Na,K-ATPase: Functional Sites and Their Interactions. *Annu. Rev. Physiol.* 65, 817–849. doi:10.1146/annurev.physiol.65.092101.142558

Kawecki, T. J., and Ebert, D. (2004). Conceptual Issues in Local Adaptation. *Ecol. Lett.* 7, 1225–1241. doi:10.1111/j.1461-0248.2004.00684.x

Khodabandeh, S., Kutnik, M., Aujoulat, F., Charmantier, G., and Charmantier-Daures, M. (2005). Ontogeny of the Antennal Glands in the Crayfish Astacus leptodactylus (Crustacea, Decapoda): Immunolocalization of Na+,K+-ATPase. *Cell Tissue Res.* 319, 167–174. doi:10.1007/s00441-004-0970-y

Kirschner, L. B. (2004). The Mechanism of Sodium Chloride Uptake in Hyperregulating Aquatic Animals. *J. Exp. Biol.* 207, 1439–1452. doi:10.1242/jeb.00907

Klein, U. (1992). The Insect V-ATPase, a Plasma Membrane Proton Pump Energizing Secondary Active Transport: Immunological Evidence for the Occurrence of a V-ATPase in Insect Ion-Transporting Epithelia. *J. Exp. Biol.* 172, 345–354. doi:10.1242/jeb.172.1.345

Kobayashi, S. (1998). Settlement and upstream migration of the Japanese mitten crab *Eriocheir japonica* (de Haan). *Ecol. Civ. Eng.* 1 (1), 21–31. doi:10.3825/ece.1.21

Kormanik, G. A., and Harris, R. R. (1981). Salt and Water Balance and Antennal Gland Function in Three Pacific Species of Terrestrial Crab (Gecarcoidea lalandii,Cardisoma carnifex,Birgus Latro). I. Urine Production and Salt Exchanges in Hydrated Crabs. *J. Exp. Zool.* 218, 97–105. doi:10.1002/jez.1402180112

Lee, C. E., Kiergaard, M., Gelembiuk, G. W., Eads, B. D., and Posavi, M. (2011). Pumping Ions: Rapid Parallel Evolution of Ionic Regulation Following Habitat Invasions. *Evolution* 65, 2229–2244. doi:10.1111/j.1558-5646.2011.01308.x

Leone, F. A., Lucena, M. N., Fabri, L. M., Garçon, D. P., Fontes, C. F. L., Faleiros, R. O., et al. (2020). Osmotic and Ionic Regulation, and Modulation by Protein Kinases, FXYD2 Peptide and ATP of Gill (Na+, K+)-ATPase Activity, in the Swamp Ghost Crab Ucides Cordatus (Brachyura, Ocypodidae). *Comp. Biochem. Physiology Part B Biochem. Mol. Biol.* 250, 110507. doi:10.1016/j.cbpb.2020.110507

Li, E., Wang, S., Li, C., Wang, X., Chen, K., and Chen, L. (2014). Transcriptome Sequencing Revealed the Genes and Pathways Involved in Salinity Stress of Chinese Mitten crab,Eriocheir Sinensis. *Physiol. Genomics* 46, 177–190. doi:10.1152/physiolgenomics.00191.2013

Li, J. J., and Chiu, Y. W. (2019). *An Atlas of Land Crab of Hengchun Peninsula 2.0*. 2nd ed, Pingtung: National Museum of Marine Biology and Aquarium.

Lin, H.-C., Su, Y.-C., and Su, S.-H. (2002). A Comparative Study of Osmoregulation in Four Fiddler Crabs (Ocypodidae:Uca). *Zoological Sci.* 19, 643–650. doi:10.2108/zsj.19.643

Lin, S.-C., Liou, C.-H., and Cheng, J.-H. (2000). The Role of the Antennal Glands in Ion and Body Volume Regulation of Cannulated *Penaeus monodon* Reared in Various Salinity Conditions. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 127 (2), 121–129. doi:10.1016/s1095-6433(00)00245-2

Little, C. (1990). *The Terrestrial Invasion. An Ecophysiological Approach to the Origins of Land Animal*. England: Cambridge University Press.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., et al. (20122012). Comparison of Next-Generation Sequencing Systems. *J. Biomed. Biotechnol.* 2012, 251364. doi:10.1155/2012/251364

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., and Darnell, J. (2000). *Molecular Cell Biology*. 4th ed. New York: W. H. Freeman and Company.

Lv, J., Liu, P., Wang, Y., Gao, B., Chen, P., and Li, J. (2013). Transcriptome Analysis of *Portunus Trituberculatus* in Response to Salinity Stress Provides Insights into the Molecular Basis of Osmoregulation. *PLoS One* 8, e82155. doi:10.1371/journal.pone.0082155

Ma, K. Y., Qin, J., Lin, C.-W., Chan, T.-Y., Ng, P. K. L., Chu, K. H., et al. (2019). Phylogenomic Analyses of Brachyuran Crabs Support Early Divergence of Primary Freshwater Crabs. *Mol. Phylogenetics Evol.* 135, 62–66. doi:10.1016/j.ympev.2019.02.001

Maddrell, S., and O'Donnell, M. (1992). Insect Malpighian Tubules: V-ATPase Action in Ion and Fluid Transport. *J. Exp. Biol.* 172, 417–429. doi:10.1242/jeb.172.1.417

Mantovani, M., and McNamara, J. C. (2021). Contrasting Strategies of Osmotic and Ionic Regulation in Freshwater Crabs and Shrimps: Gene Expression of Gill Ion Transporters. *J. Exp. Biol.* 224, jeb233890. doi:10.1242/jeb.233890

Martinez, A.-S., Toullec, J.-Y., Shillito, B., Charmantier-Daures, M., and Charmantier, G. (2001). Hydromineral Regulation in the Hydrothermal Vent CrabBythograea Thermydron. *Biol. Bull.* 201, 167–174. doi:10.2307/1543331

McGaw, I. J. (2005). The Decapod Crustacean Circulatory System: A Case that Is Neither Open Nor Closed. *Microsc. Microanal.* 11, 18–36. doi:10.1017/s1431927605050026

McNamara, J. C., and Faria, S. C. (2012). Evolution of Osmoregulatory Patterns and Gill Ion Transport Mechanisms in the Decapod Crustacea: a Review. *J. Comp. Physiol. B* 182, 997–1014. doi:10.1007/s00360-012-0665-8

McNamara, J. C., Freire, C. A., Torres, A. H., and Faria, S. C. (2015). The Conquest of Fresh Water by the Palaemonid Shrimps: an Evolutionary History Scripted in the Osmoregulatory Epithelia of the Gills and Antennal Glands. *Biol. J. Linn. Soc.* 114, 637–688. doi:10.1111/bij.12443

Malik, A., and Kim, C. B. (2021). Role of transportome in the gills of Chinese mitten crabs in response to salinity change: A meta-analysis of RNA-Seq datasets. *Biology* 10, 39.

Morris, S., and Ahern, M. D. (2003). Regulation of Urine Reprocessing in the Maintenance of Sodium and Water Balance in the Terrestrial Christmas Island Red crabGecarcoidea Natalisinvestigated under Field Conditions. *J. Exp. Biol.* 206, 2869–2881. doi:10.1242/jeb.00499

Morris, S. (2001). Neuroendocrine Regulation of Osmoregulation and the Evolution of Air-Breathing in Decapod Crustaceans. *J. Exp. Biol.* 204, 979–989. doi:10.1242/jeb.204.5.979

Morris, S., and Van Aardt, W. J. (1998). Salt and Water Relations, and Nitrogen Excretion, in the Amphibious African Freshwater Crab Potamonautes Warreni in Water and in Air. *J. Exp. Biol.* 201, 883–893. doi:10.1242/jeb.201.6.883

Moshtaghi, A., Rahi, M. L., Nguyen, V. T., Mather, P. B., and Hurwood, D. A. (2016). A Transcriptomic Scan for Potential Candidate Genes Involved in Osmoregulation in an Obligate Freshwater Palaemonid Prawn (Macrobrachium Australiense). *PeerJ* 4, e2520. doi:10.7717/peerj.2520

Naderloo, R. (2017). *Atlas of Crabs of the Persian Gulf*. Cham, Switzerland: Springer Nature.

Ng, P. K. L., Guinot, D., and Davie, P. J. F. (2008). Systema Brachyurorum: Part I. An Annotated Checklist of Extant Brachyuran Crabs of the World. *Raffles Bull. Zool.* 17, 1–286.

Niu, J., Hu, X. L., Ip, J. C. H., Ma, K. Y., Tang, Y., Wang, Y., et al. (2020). Multi-omic Approach Provides Insights into Osmoregulation and Osmoconformation of the Crab *Scylla Paramamosain*. *Sci. Rep.* 10, 21771. doi:10.1038/s41598-020-78351-w

Onken, H., and Putzenlechner, M. (1995). A V-ATPase Drives Active, Electrogenic and Na+-independent Cl- Absorption across the Gills of Eriocheir Sinensis. *J. Exp. Biol.* 198, 767–774. doi:10.1242/jeb.198.3.767

Péqueux, A. (1995). Osmotic Regulation in Crustaceans. *J. Crustacean Biol.* 15, 1–60. doi:10.2307/1549010

Peterson, D. R., and Loizzi, R. F. (1974b). Biochemical and Cytochemical Investigations of (Na+-K+)-ATPase in the Crayfish Kidney. *Comp. Biochem. Physiology Part A Physiology* 49, 763–773. doi:10.1016/0300-9629(74)90903-7

Peterson, D. R., and Loizzi, R. F. (1973). Regional Cytology and Cytochemistry of the Crayfish Kidney Tubule. *J. Morphol.* 141, 133–145. doi:10.1002/jmor.1051410202

Peterson, D. R., and Loizzi, R. F. (1974a). Ultrastructure of the Crayfish Kidney? coelomosac, Labyrinth, Nephridial Canal. *J. Morphol.* 142, 241–263. doi:10.1002/jmor.1051420302

Prosser, C. L., Green, J. W., and Chow, T. J. (1955). Ionic and Osmotic Concentrations in Blood and Urine of Pachygrapsus Crassipes Acclimated to Different Salinities. *Biol. Bull.* 109, 99–107. doi:10.2307/1538662

Riegel, J. A., and Lockwood, A. P. M. (1961). The Role of the Antennal Gland in the Osmotic and Ionic Regulation of Carinus Maenas. *J. Exp. Biol.* 38, 491–499. doi:10.1242/jeb.38.2.491

Riegel, J. A. (1963). Micropuncture Studies of Chloride Concentration and Osmotic Pressure in the Crayfish Antennal Gland. *J. Exp. Biol.* 40, 487–492. doi:10.1242/jeb.40.3.487

Roldan, B. M., and Shivers, R. R. (1987). The Uptake and Storage of Iron and Lead in Cells of the Crayfish Orconectes Propinquus) Hepatopancreas and Antennal Gland. *Comp. Biochem. Physiology Part C Comp. Pharmacol.* 86, 201–214. doi:10.1016/0742-8413(87)90165-4

Sáez, A. G., Lozano, E., and Zaldívar-Riverón, A. (2009). Evolutionary History of Na,K-ATPases and Their Osmoregulatory Role. *Genetica* 136, 479–490. doi:10.1007/s10709-009-9356-0

Saigusa, M. (1980). Entrainment of a Semilunar Rhythm by a Simulated Moonlight Cycle in the Terrestrial Crab, Sesarma Haematocheir. *Oecologia* 46, 38–44. doi:10.1007/bf00346963

Santos, E. A., Baldisseroto, B., Blanchini, A., Colares, E. P., Nery, L. E. M., and Manzoni, G. C. (1987). Respiratory Mechanisms and Metabolic Adaptations of an Intertidal Crab, Chasmagnathus Granulata (Dana, 1851). *Comp. Biochem. Physiology Part A Physiology* 88, 21–25. doi:10.1016/0300-9629(87)90092-2

Sarver, R. G., Flynn, M. A., and Holliday, C. W. (1994). Renal Na,K-ATPase and Osmoregulation in the Crayfish, *Procambarus clarkii*. *Comp. Biochem. Physiology Part A Physiology* 107, 349–356. doi:10.1016/0300-9629(94)90392-1

Savolainen, O., Lascoux, M., and Merilä, J. (2013). Ecological Genomics of Local Adaptation. *Nat. Rev. Genet.* 14, 807–820. doi:10.1038/nrg3522

Schendure, J., and Ji, H. (2008). Next-generation DNA Sequencing. *Nat. Biotechnol.* 26, 1135–1145.

Schmidt-Nielsen, B., Gertz, K. H., and Davis, L. E. (1968). Excretion and Ultrastructure of the Antennal Gland of the Fiddler crabUca Mordax. *J. Morphol.* 125, 473–495. doi:10.1002/jmor.1051250406

Schuster, S. C. (2008). Next-generation Sequencing Transforms Today's Biology. *Nat. Methods* 5, 16–18. doi:10.1038/nmeth1156

Shaw, J. (1958). Salt and Water Balance in the East African Fresh-Water Crab, *Potamon Niloticus*. *J. Exp. Biol.* 36 (1), 157–176.

Shin, H. T., Chan, B. K. K., Teng, S. J., and Wong, K. J. H. (2015). *Crustacean Fauna of Taiwan: Brachyuran Crabs*. Taichung: OcypodoideaNational Chung Hsing University.

Silver, R. B., Breton, S., and Brown, D. (2000). Potassium Depletion Increases Proton Pump (H(+)-ATPase) Activity in Intercalated Cells of Cortical Collecting Duct. *Am. J. Physiol. Ren. Physiol.* 279 (1), F195–F202. doi:10.1152/ajprenal.2000.279.1.F195

Storz, J. F., Bridgham, J. T., Kelly, S. A., and Garland, T., JR. (2015). Genetic Approaches in Comparative and Evolutionary Physiology. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 309, R197–R214. doi:10.1152/ajpregu.00100.2015

Suhre, K., McCarthy, M. I., and Schwenk, J. M. (2020). Genetics Meets Proteomics: Perspectives for Large Population-Based Studies. *Nat. Rev. Genet.* 22, 19–37. doi:10.1038/s41576-020-0268-2

Takeda, S., Matsumasa, M., Kikuchi, S., Poovachiranon, S., and Murai, M. (1996). Variation in the Branchial Formula of Semiterrestrial Crabs (Decapoda: Brachyura: Grapsidae and Ocypodidae) in Relation to Physiological Adaptations to the Environment. *J. Crustacean Biol.* 16, 472–486. doi:10.2307/1548737

Taylor, H. H., and Greenaway, P. (2002). Osmoregulation in the Terrestrial Christmas Island Red Crab Gecarcoidea Natalis (Brachyura: Gecarcinidae): Modulation of Branchial Chloride Uptake from the Urine. *J. Exp. Biol.* 205, 3251–3260. doi:10.1242/jeb.205.20.3251

Taylor, H. H., and Greenaway, P. (1979). The Structure of the Gills and Lungs of the Arid-zone Crab, Holthuisana (Austrothelphusa) Transversa (Brachyura: Sundathelphusidae) Including Observations on Arterial Vessels within the Gills. *J. Zoology* 189 (3), 359–384. doi:10.1111/j.1469-7998.1979.tb03969.x

Thurman, C. L. (2005). A Comparison of Osmoregulation Among Subtropical Fiddler Crabs (*Uca*) from Southern Florida and California. *Bull. Mar. Sci.* 77, 83–100.

Towle, D. W. (1981). Transport-related ATPases as Probes of Tissue Function in Three Terrestrial Crabs of Palau. *J. Exp. Zool.* 218, 89–95. doi:10.1002/jez.1402180111

Tsai, J.-R., and Lin, H.-C. (2014). Functional Anatomy and Ion Regulatory Mechanisms of the Antennal Gland in a Semi-terrestrial Crab, *Ocypode Stimpsoni*. *Biol. Open.* 3, 409–417. doi:10.1242/bio.20147336

Tsai, J.-R., and Lin, H.-C. (2007). V-type H+-ATPase and Na+,K+-ATPase in the Gills of 13 Euryhaline Crabs during Salinity Acclimation. *J. Exp. Biol.* 210, 620–627. doi:10.1242/jeb.02684

Tsai, J. R., and Lin, H. C. (2012). A Shift to an Ion Regulatory Role by Gills of a Semi-terrestrial Crab, *Ocypode Stimpsoni*. *Zool. Stud.* 51, 606–618.

Tsang, L. M., Schubart, C. D., Ahyong, S. T., Lai, J. C. Y., Au, E. Y. C., Chan, T.-Y., et al. (2014). Evolutionary History of True Crabs (Crustacea: Decapoda: Brachyura) and the Origin of Freshwater Crabs. *Mol. Biol. Evol.* 31 (5), 1173–1187. doi:10.1093/molbev/msu068

Tseng, K.-Y., Tsai, J.-R., and Lin, H.-C. (2020). Ion Regulation in the Antennal Glands Differs Among Ocypodoidea and Grapsoidea Crab Species. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 248, 110753. doi:10.1016/j.cbpa.2020.110753

Ueno, M., and Inoue, Y. (1996). The Fine Structure of Podocytes in Crayfish Antennal Glands. *J. Electron Microsc.* 45, 395–400. doi:10.1093/oxfordjournals.jmicro.a023457

Wang, C. W., and Lin, H. C. (2011). *Functional Study on Gills and Antennal Glands of the Freshwater Crab,* Candidiopotamon Rethbunae. Master's thesis. Taichung: Tunghai University.

Wang, H., Wei, H., Tang, L., Lu, J., Mu, C., and Wang, C. (2018). A Proteomics of Gills Approach to Understanding Salinity Adaptation of *Scylla Paramamosain*. *Gene* 677, 119–131. doi:10.1016/j.gene.2018.07.059

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-seq: a Revolutionary Tool for Transcriptomics. *Nat. Rev. Genet.* 10, 57–63. doi:10.1038/nrg2484

Weihrauch, D., Joseph, G., and Allen, G. J. P. (2018). Ammonia Excretion in Aquatic Invertebrates: New Insights and Questions. *J. Exp. Biol.* 221, 1–11. doi:10.1242/jeb.169219

Weihrauch, D., Fehsenfeld, S., and Quijada-Rodriguez, A. (2017). "Nitrogen Excretion in Aquatic Crustaceans," in *Acid-base Balance and Nitrogen Excretion in Invertebrates*. Editors D. Weihrauch and M. O'Donnell (Cham, Switzerland: Springer), 1–24. doi:10.1007/978-3-319-39617-0_1

Weihrauch, D., Morris, S., and Towle, D. W. (2004). Ammonia Excretion in Aquatic and Terrestrial Crabs. *J. Exp. Biol.* 207, 4491–4504. doi:10.1242/jeb.01308

Weihrauch, D., Ziegler, A., Siebers, D., and Towle, D. W. (2001). Molecular Characterization of V-type H(+)-ATPase (B-Subunit) in Gills of Euryhaline Crabs and its Physiological Role in Osmoregulatory Ion Uptake. *J. Exp. Biol.* 204, 25–37. doi:10.1242/jeb.204.1.25

Wheatly, M. G., and Gannon, A. T. (1995). Ion Regulation in Crayfish: Freshwater Adaptations and the Problem of Molting. *Am. Zool.* 35, 49–59. doi:10.1093/icb/35.1.49

Wheatly, M. G., and Henry, R. P. (1987). Branchial and Antennal Gland Na+/K+-Dependent ATPase and Carbonic Anhydrase Activity during Salinity Acclimation of the Euryhaline Crayfish Pacifastacus Leniusculus. *J. Exp. Biol.* 133, 73–86. doi:10.1242/jeb.133.1.73

Wieczorek, H., Brown, D., Grinstein, S., Ehrenfeld, J., and Harvey, W. R. (1999). Animal Plasma Membrane Energization by Proton-Motive V-ATPases. *Bioessays* 21, 637–648. doi:10.1002/(sici)1521-1878(199908)21:8<637:aid-bies3>3.0.co;2-w

Yang, Z., Zhou, J., Wei, B., Cheng, Y., Zhang, L., and Zhen, X. (2019). Comparative Transcriptome Analysis Reveals Osmotic-Regulated Genes in the Gill of Chinese Mitten Crab (*Eriocheir Sinensis*). *PLoS One* 14 (1), e0210469. doi:10.1371/journal.pone.0210469

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Membrane Transport Proteins Expressed in the Renal Tubular Epithelial Cells of Seawater and Freshwater Teleost Fishes

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The kidney is an important organ that maintains body fluid homeostasis in seawater and freshwater teleost fishes. Seawater teleosts excrete sulfate and magnesium in small amounts of isotonic urine, and freshwater teleosts excrete water in large amounts of hypo-osmotic urine. The volume, osmolality, and ionic compositions of the urine are regulated mainly by membrane transport proteins expressed in the renal tubular epithelial cells. Gene expression, immunohistochemical, and functional analyses of the fish kidney identified membrane transport proteins involved in the secretion of sulfate and magnesium ions by the proximal tubules and reduction of urine volume by the collecting ducts in seawater teleosts, and excretion of water as hypotonic urine by the distal tubules and collecting ducts in freshwater teleosts. These studies promote an understanding of how the kidney contributes to the seawater and freshwater acclimation of teleosts at the molecular level.

Keywords: fish physiology, seawater acclimation, freshwater acclimation, renal tubule, membrane transport protein, osmoregulation, sulfate homeostasis, magnesium homeostasis

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1 INTRODUCTION

The ionic compositions and osmolarity of body fluids in seawater and freshwater teleost fishes are similar to those in humans and other mammals. Freshwater teleosts live in environments with considerably lower osmolarity than their body fluids. To balance water entry and loss of salts in freshwater environments, the kidneys actively produce a hypotonic urine with salt concentrations that is 1/10–1/20 of that of the body fluid, and the branchial ionocytes (Hwang et al., 2011), mitochondrion-rich cells scattered along the lamellae, and intestinal epithelia absorb salts from the environmental water and food, respectively (**Figure 1A**). Conversely, seawater teleosts live in environments that have approximately three-fold higher osmolarity than their body fluids. To balance salt entry and loss of water in seawater environments, seawater is ingested, salt and water are absorbed from the ingested seawater via the intestinal epithelial cells, Cl^-, Na^+, and K^+ are excreted from the branchial ionocytes, and Mg^{2+} and SO_4^{2-} are excreted in the isotonic urine (**Figure 1B**). The mechanism of body fluid homeostasis in freshwater and seawater teleosts can be understood as the balance of the epithelial transport of ions and water by the branchial ionotyes, renal tubules, and intestinal epithelial cells. The molecular mechanisms of these processes can be explained by the membrane transport proteins (channels, transporters, and pumps) expressed in the plasma membrane of these epithelial cells. Here, we focus on the functional differences in the kidney of

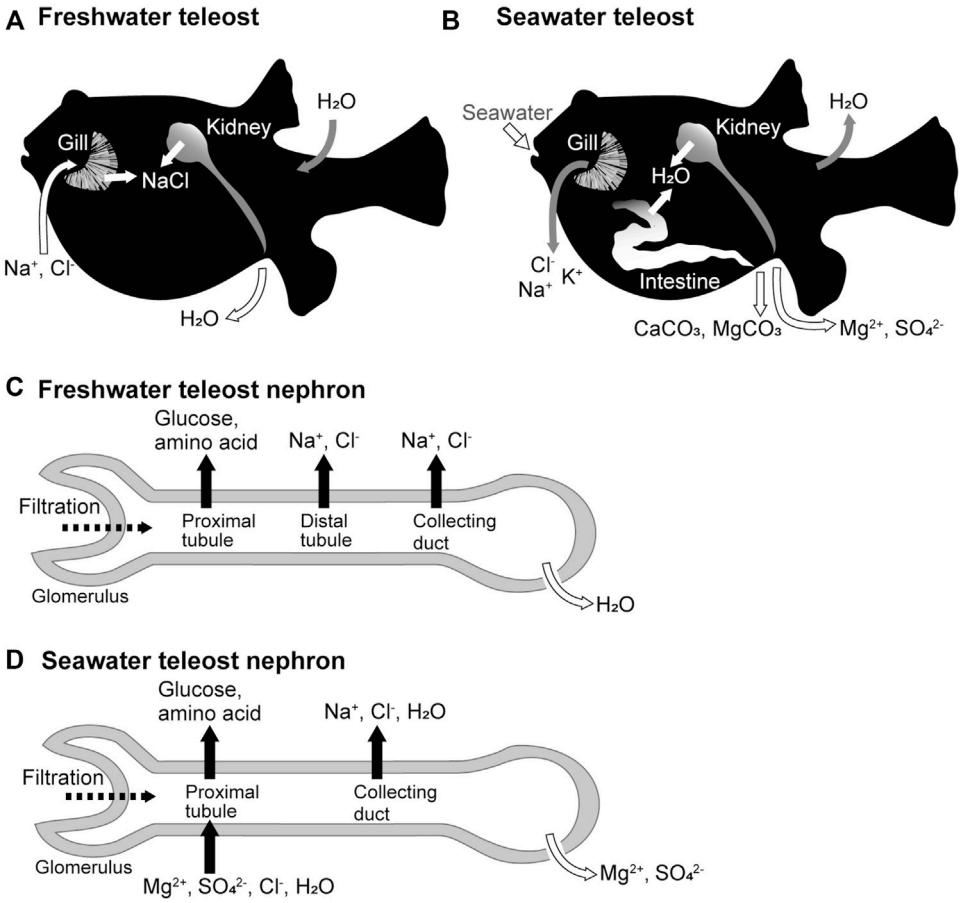

FIGURE 1 | Freshwater and seawater acclimations of teleost fishes and organ functions. **(A,B)** Movement of ions and water across the epithelia of the gill, kidney, and intestine of freshwater and seawater teleosts. **(C,D)** Ion and water movement in the nephrons of freshwater and seawater teleosts.

seawater and freshwater teleosts, and review the membrane transport proteins that are expressed in the renal tubular epithelial cells of teleost fishes.

2 FUNCTIONAL DIFFERENCES IN THE KIDNEYS OF FRESHWATER AND SEAWATER TELEOSTS

The kidney is the only organ in vertebrates that can produce urine (Schmidt-Nielsen, 1997). Urine is produced by blood filtration, followed by ion and water secretion and reabsorption by nephrons. The glomeruli are responsible for filtration, while the tubules are responsible for secretion and reabsorption. Urine volume is controlled balancing the amount of water filtered by the glomerulus and the amount of water secreted and reabsorbed by the tubules. The urine composition is also regulated by the secretion and reabsorption of selective ions by the tubules.

In freshwater fish, the kidney functions as the organ responsible for water excretion (Hickman and Trump, 1969; Marshall and Grosell, 2006). The glomeruli of freshwater teleosts filter more blood and produce more primary urine than those of seawater teleosts (Fleming and Stanley, 1965; Nishimura and Imai, 1982). The proximal tubules, which are the renal tubule segments closest to the glomerulus, reabsorb nutrients such as glucose and amino acids from the primary urine (Dickman and Renfro, 1986). The distal tubules and collecting ducts actively reabsorb Na^+ and Cl^- from the primary urine and are called diluting segments because they do not permeate much water. As a result, the freshwater fish kidney produces hypotonic urine resulting in net water excretion (Nishimura et al., 1983) (**Figure 1C**).

In seawater fish, the kidney functions as an organ responsible for the excretion of divalent ions (Mg^{2+}, SO_4^{2-}, and so on) (Hickman and Trump, 1969; Marshall and Grosell, 2006). The proximal tubules of seawater fish actively secrete fluid containing Mg^{2+}, SO_4^{2-}, and Cl^- into the tubular lumen (forming urine), a function not observed in the kidneys of freshwater fish or terrestrial animals (Beyenbach et al., 1986; Beyenbach, 2004). The collecting ducts may then actively reabsorb water along with Na^+ and Cl^- to reduce the urine volume, producing a relatively small volume of isotonic urine with high concentrations of Mg^{2+} and SO_4^{2-} (**Figure 1D**).

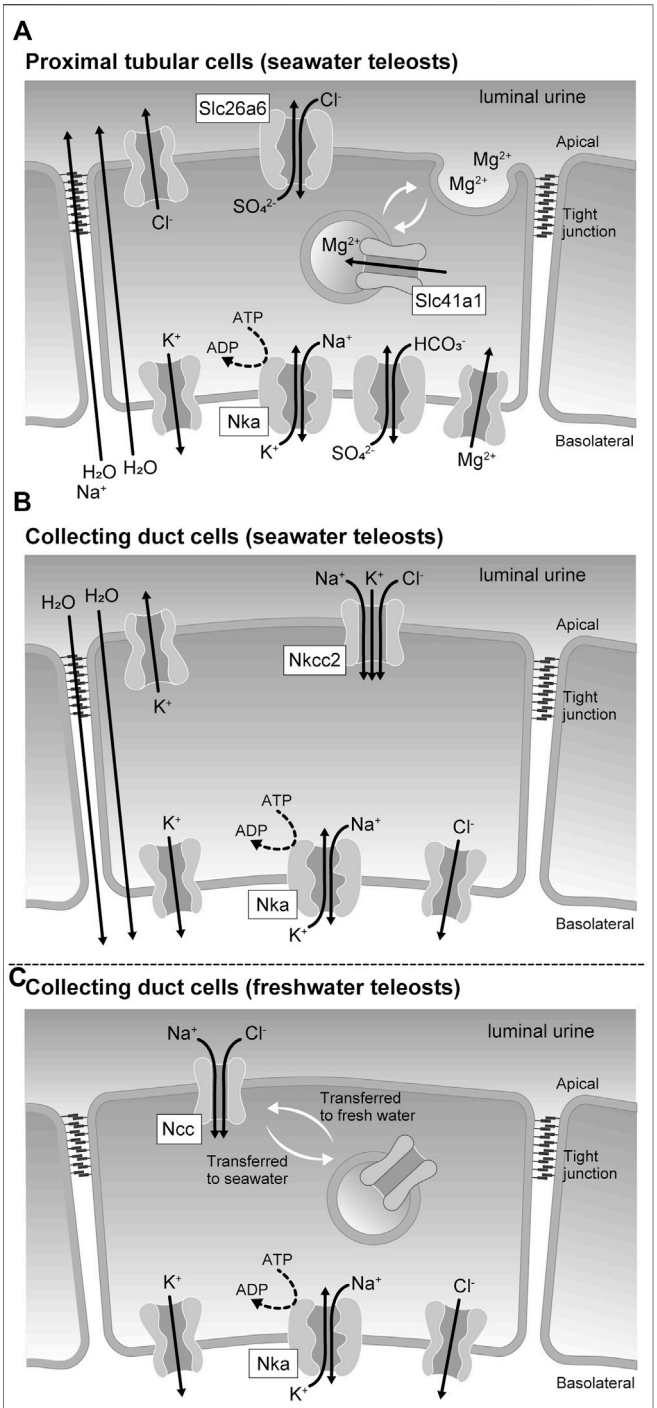

FIGURE 2 | Membrane transport proteins expressed in the renal tubular epithelial cells of seawater and freshwater teleost fishes. **(A)** Membrane transporters involved in the sulfate and magnesium ion secretion mechanism by proximal tubular cells of seawater teleosts. **(B)** Membrane transporters involved in the urinary volume reduction mechanism by the collecting duct cells of seawater teleosts. **(C)** Membrane transporters responsible for the urine dilution by the collecting duct cells of freshwater teleosts.

3 MOLECULAR MECHANISMS UNDERLYING REABSORPTION AND SECRETION BY THE RENAL TUBULAR CELLS

Reabsorption and secretion in the renal tubules are mediated by a single layer of renal tubular epithelial cells. Cells are directly connected to each other by cell-cell adhesions, including tight junctions, which function as barriers separating the primary urine from the tissue fluid. Cell-cell adhesions are also responsible for transporting various substances during renal tubular reabsorption and secretion, and the tight junction protein claudins regulate the paracellular permeability (Tsukita et al., 2019). The tight junction also separates the apical and basolateral membrane domains in epithelial cells (**Figure 2**). Epithelial transport can occur through the transcellular pathway via the apical membrane, cytoplasm, and basolateral membrane of epithelial cells and through the paracellular pathway via the intercellular spaces between epithelial cells. The basolateral membrane of tubular cells, containing sodium pumps (Nkas, Na^+/K^+-ATPases), potassium channels, and chloride channels, maintains a low Na^+, low Cl^-, and high K^+ intracellular environment (**Figure 2**) and generates inside negative membrane potentials. The high Na^+ and Cl^- contents of the extracellular fluids create an ionic gradient between the inside and the outside of the cell. These chemical gradient and membrane potentials (electrical gradients) are used as driving forces for secondary active transport through various cotransporters and exchangers.

The use of fish genome data has drastically accelerated the research in identifying membrane transport proteins in the renal tubular epithelial cells responsible for seawater and freshwater acclimation of fishes. The genomes of many fish species have recently been sequenced by the development of next-generation sequencers. However, we have focused on two closely related fish species, the euryhaline species river pufferfish (*Takifugu obscurus*) and the marine species Japanese pufferfish (*Takifugu rubripes*) (Kato et al., 2005) because the whole genome data of Japanese pufferfish was published in 2002 (Aparicio et al., 2002). Comparing the expression levels of membrane transport protein families in the kidney of seawater-, brackish water-, and freshwater-acclimated *Takifugu* species identified some of the molecular mechanisms involved in seawater and freshwater acclimation of teleosts as described below.

4 MEMBRANE TRANSPORT PROTEINS INVOLVED IN THE DIVALENT ION SECRETION BY THE PROXIMAL TUBULAR CELLS OF SEAWATER TELEOSTS

The solute carrier (Slc) 26 is a family of proteins with anion-exchange export activity (Mount and Romero, 2004; Alper and

Sharma, 2013). Slc26 is mainly found in the plasma membrane and mediates the influx of Cl^- and efflux of various anions, such as HCO_3^-, SO_4^{2-}, and oxalate^{2-}. A member of this family, Slc26a6, is expressed in the kidney and intestine of river pufferfish and the Japanese eel (*Anguilla japonica*), and its expression increases during seawater acclimation (Kato et al., 2009; Watanabe and Takei, 2011). Electrophysiological analysis of pufferfish Slc26a6 expressed in *Xenopus laevis* oocytes showed that it has a very highly electrogenic Cl^-/SO_4^{2-} exchange activity. In the kidney of seawater-acclimated river pufferfish and Japanese eel, Slc26a6 localizes to the brush border of the apical membrane of the proximal tubule. These results are consistent with studies that showed the Cl^-/SO_4^{2-} exchange activity of brush border membrane vesicles isolated from the kidney of the seawater teleost southern flounder (*Paralichthys lethostigma*) (Renfro and Pritchard, 1983), and suggest that Slc26a6 is at least one of the major pathways of apical SO_4^{2-} secretion in the proximal tubule of seawater teleosts (**Figure 2A**). Slc26a1, another SO_4^{2-} transporter of the Slc26 family, localizes to the basolateral membrane of the proximal tubule in the eel kidney (Nakada et al., 2005; Watanabe and Takei, 2011). When expressed in *Xenopus* oocytes, fish Slc26a1 shows robust SO_4^{2-} transport activity (Nakada et al., 2005), but the mode of this transport activity has not yet been established. Analysis of human Slc26a1 expressed in *Xenopus* oocytes indicated that Slc26a1 has Cl^--dependent pH-sensitive HCO_3^-/SO_4^{2-} exchange activity (Wu et al., 2016). These results suggest that Slc26a1 mediates basolateral SO_4^{2-} uptake for luminal SO_4^{2-} secretion (**Figure 2A**).

Renal SO_4^{2-} excretion has also been observed in seawater elasmobranch species. In elephant fish (*Callorhinchus milii*), Slc26a6 and Slc26a1 are expressed in the apical and basolateral membranes, respectively, of the renal proximal tubule II segments and exhibit SO_4^{2-} transport activity when expressed in *Xenopus* oocytes (Hasegawa et al., 2016). These results suggest that the membrane transport proteins involved in renal tubular SO_4^{2-} secretion are conserved between seawater elasmobranches and teleosts.

A family of proteins homologous to the bacterial Mg^{2+} transporter MgtE was found in vertebrates and named the Slc41 (Sahni and Scharenberg, 2013). Slc41a1 is expressed in the pufferfish kidney, and its expression levels increase during seawater acclimation (Islam et al., 2013). Increased salinity also stimulates the renal expression of Slc41a1 in the euryhaline glomerular fish Atlantic salmon (*Salmo salar*) and the aglomerular marine gulf toadfish (*Opsanus beta*) (Madsen et al., 2020; Hansen et al., 2021). While the activity of fish Slc41a1 has not been successfully determined, human Slc41a1 has been reported to possess Na^+/Mg^{2+} exchange activity (Kolisek et al., 2012). Immunohistochemical analysis at the light and electron microscopic levels showed that Slc41a1 was localized to vacuoles in the apical cytoplasm of the proximal tubules in seawater-acclimated river pufferfish (Islam et al., 2013). The proximal tubules of seawater fish kidneys have intracellular granules containing high Mg^{2+} concentrations and are the site of active secretion of Mg^{2+} into the lumen (Chandra et al., 1997; Beyenbach, 2000). These results suggest that Slc41a1 is involved in a pathway that concentrates Mg^{2+} in intracellular granules that are then secreted into the luminal fluid by exocytosis (**Figure 2A**).

Another family of proteins that are homologous to the bacterial magnesium transporter CorC was found in vertebrates and named Cnnm (cyclin M or cyclin and CBS domain divalent metal cation transport mediator) family (Funato and Miki, 2019). Again, the activity of fish Cnnm has not been successfully analyzed, however, mammalian Cnnm is known to mediate plasma membrane Mg^{2+} efflux (Hirata et al., 2014). In the kidneys of river pufferfish, the expression of Cnnm3 is upregulated in seawater, while that of Cnnm2 is increased in freshwater, and Cnnm3 is expressed in the lateral membrane of proximal tubular cells (Islam et al., 2014). The role of Cnnm3 in Mg^{2+} secretion remains unclear, however, these results suggest that Cnnm3 is involved in paracellular Mg^{2+} secretion.

5 MEMBRANE TRANSPORT PROTEINS INVOLVED IN DIVALENT ION CONCENTRATION AND VOLUME REDUCTION OF URINE BY THE COLLECTING DUCT CELLS OF SEAWATER TELEOSTS

In the kidney of seawater teleosts, the renal collecting ducts and urinary bladder reabsorb Na^+, Cl^-, and water to reduce the volume of urine. As a result, Mg^{2+} and SO_4^{2-} are highly concentrated in the iso-osmotic urine, which is finally excreted from the urinary bladder (Hickman and Trump, 1969; Marshall and Grosell, 2006). Slc12a1, also called $Na^+/K^+/2Cl^-$ cotransporter 2 (Nkcc2), is highly expressed in the collecting ducts of marine pufferfish (Kato et al., 2011). In the mammalian kidney, Nkcc2 and K^+ recycling channels in the apical membrane mediate Na^+ and Cl^- reabsorption (Arroyo et al., 2013). In the collecting ducts of marine pufferfish, Nkcc2 may promote the reabsorption of Na^+ and Cl^-, which could be a driving force for water reabsorption and urinary volume reduction (**Figure 2B**).

6 MEMBRANE TRANSPORT PROTEINS IN THE DISTAL TUBULE AND COLLECTING DUCT CELLS OF FRESHWATER TELEOSTS INVOLVED IN URINE DILUTION

In general, both freshwater and euryhaline teleosts that can live in freshwater have freshwater fish type nephrons (Hickman and Trump, 1969; Kato et al., 2011). In the kidneys of freshwater teleosts and freshwater-acclimated euryhaline teleosts, the distal tubules and collecting ducts reabsorb Na^+ and Cl^-, but not water, thereby producing a large amount of hypotonic urine. Therefore, these tubules act as diluting segments in the kidney of teleosts. Both Slc12a1 (Nkcc2) and Slc12a3 (Ncc, Na^+/Cl^- cotransporter) are highly expressed in the kidneys of freshwater and euryhaline fishes (Katoh et al., 2008; Kato et al., 2011). This is in contrast to the stenohaline seawater fish that expresses Nkcc2 but does not or scarcely expresses Ncc in the kidney (Kato et al., 2011). Nkcc2 and Ncc are localized in the distal tubules and collecting ducts,

respectively. In freshwater pufferfish collecting ducts, Ncc is localized to the luminal side of the plasma membrane, whereas in seawater pufferfish collecting ducts, Ncc on the plasma membrane is incorporated into intracellular granules and is downregulated (**Figure 2C**). These data suggest that Ncc-mediated Na^+ and Cl^- reabsorption is particularly important for freshwater acclimation of teleosts.

7 CONCLUSION

The proximal tubule is an important secretory pathway for divalent ions in seawater teleosts. The proximal tubules of seawater fish kidneys secrete SO_4^{2-} directly into the lumen via membrane transporters on the plasma membrane, whereas Mg^{2+} is stored in intracellular granules and excreted into the lumen. It is interesting to understand why these differences in secretion mechanisms are necessary. The collecting duct is important for freshwater teleosts as the site of water elimination (production of hypotonic urine) and is important for seawater teleosts as a site of urine volume reduction. This difference can be explained by the differential water permeability of the collecting ducts between seawater and freshwater teleosts, and the mechanisms controlling these differences are expected to be elucidated in the future. Na^+ and Cl^- reabsorptive activity of the distal nephrons is important in both seawater and freshwater teleosts, but freshwater fish utilize both Nkcc2 and Ncc whereas seawater fish utilize only Nkcc2. Further studies will be needed to elucidate why Ncc is necessary for the functioning of the kidneys of freshwater teleosts.

AUTHOR CONTRIBUTIONS

AK, KH, and MR conceived the topic for the mini review, AK, AN, and KH drafted the manuscript, AK and AN prepared the figures, AK and MR edited the manuscript, AK, AN, KH, and MR approved final version of the manuscript.

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REFERENCES

Alper, S. L., and Sharma, A. K. (2013). The SLC26 Gene Family of Anion Transporters and Channels. *Mol. Aspects Med.* 34, 494–515. doi:10.1016/j.mam.2012.07.009

Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J.-M., Dehal, P., et al. (2002). Whole-genome Shotgun Assembly and Analysis of the Genome of *Fugu Rubripes*. *Science* 297, 1301–1310. doi:10.1126/science.1072104

Arroyo, J. P., Kahle, K. T., and Gamba, G. (2013). The SLC12 Family of Electroneutral Cation-Coupled Chloride Cotransporters. *Mol. Aspects Med.* 34, 288–298. doi:10.1016/j.mam.2012.05.002

Beyenbach, K. W. (2000). Renal Handling of Magnesium in Fish: from Whole Animal to Brush Border Membrane Vesicles. *Front. Biosci.* 5, D712–D719. doi:10.2741/beyenbach

Beyenbach, K. W. (2004). Kidneys Sans Glomeruli. *Am. J. Physiol. Renal Physiol.* 286, F811–F827. doi:10.1152/ajprenal.00351.2003

Beyenbach, K. W., Petzel, D. H., and Cliff, W. H. (1986). Renal Proximal Tubule of Flounder. I. Physiological Properties. *Am. J. Physiol.* 250, R608–R615. doi:10.1152/ajpregu.1986.250.4.r608

Chandra, S., Morrison, G. H., and Beyenbach, K. W. (1997). Identification of Mg-Transporting Renal Tubules and Cells by Ion Microscopy Imaging of Stable Isotopes. *Am. J. Physiol.* 273, F939–F948. doi:10.1152/ajprenal.1997.273.6.f939

Dickman, K. G., and Renfro, J. L. (1986). Primary Culture of Flounder Renal Tubule Cells: Transepithelial Transport. *Am. J. Physiol.* 251, F424–F432. doi:10.1152/ajprenal.1986.251.3.f424

Fleming, W. R., and Stanley, J. G. (1965). Effects of Rapid Changes in Salinity on the Renal Function of a Euryhaline Teleost. *Am. J. Physiol.* 209, 1025–1030. doi:10.1152/ajplegacy.1965.209.5.1025

Funato, Y., and Miki, H. (2019). Molecular Function and Biological Importance of CNNM Family Mg^{2+} Transporters. *J. Biochem.* 165, 219–225. doi:10.1093/jb/mvy095

Hansen, N. G. W., Madsen, S. S., Brauckhoff, M., Heuer, R. M., Schlenker, L. S., Engelund, M. B., et al. (2021). Magnesium Transport in the Aglomerular Kidney of the Gulf Toadfish (*Opsanus beta*). *J. Comp. Physiol. B* 191, 865–880. doi:10.1007/s00360-021-01392-8

Hasegawa, K., Kato, A., Watanabe, T., Takagi, W., Romero, M. F., Bell, J. D., et al. (2016). Sulfate Transporters Involved in Sulfate Secretion in the Kidney Are Localized in the Renal Proximal Tubule II of the Elephant Fish (*Callorhinchus milii*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 311, R66–R78. doi:10.1152/ajpregu.00477.2015

Hickman, C. P., Jr, and Trump, B. F. (1969). "The Kidney," in *Fish Physiology*. Editors W. S. Hoar and D. J. Randall (New York, NY: Academic Press), 91–239. doi:10.1016/s1546-5098(08)60083-7

Hirata, Y., Funato, Y., Takano, Y., and Miki, H. (2014). Mg^{2+}-dependent Interactions of ATP with the Cystathionine-Beta-Synthase (CBS) Domains of a Magnesium Transporter. *J. Biol. Chem.* 289, 14731–14739. doi:10.1074/jbc.m114.551176

Hwang, P.-P., Lee, T.-H., and Lin, L.-Y. (2011). Ion Regulation in Fish Gills: Recent Progress in the Cellular and Molecular Mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R28–R47. doi:10.1152/ajpregu.00047.2011

Islam, Z., Hayashi, N., Inoue, H., Umezawa, T., Kimura, Y., Doi, H., et al. (2014). Identification and Lateral Membrane Localization of Cyclin M3, Likely to Be Involved in Renal Mg^{2+} Handling in Seawater Fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307, R525–R537. doi:10.1152/ajpregu.00032.2014

Islam, Z., Hayashi, N., Yamamoto, Y., Doi, H., Romero, M. F., Hirose, S., et al. (2013). Identification and Proximal Tubular Localization of the Mg^{2+} Transporter, Slc41a1, in a Seawater Fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 305, R385–R396. doi:10.1152/ajpregu.00507.2012

Kato, A., Chang, M.-H., Kurita, Y., Nakada, T., Ogoshi, M., Nakazato, T., et al. (2009). Identification of Renal Transporters Involved in Sulfate Excretion in Marine Teleost Fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297, R1647–R1659. doi:10.1152/ajpregu.00228.2009

Kato, A., Doi, H., Nakada, T., Sakai, H., and Hirose, S. (2005). *Takifugu obscurus* Is a Euryhaline Fugu Species Very Close to *Takifugu rubripes* and Suitable for Studying Osmoregulation. *BMC Physiol.* 5, 18. doi:10.1186/1472-6793-5-18

Kato, A., Muro, T., Kimura, Y., Li, S., Islam, Z., Ogoshi, M., et al. (2011). Differential Expression of Na^+-Cl^- Cotransporter and Na^+-K^+-Cl^- Cotransporter 2 in the Distal Nephrons of Euryhaline and Seawater Pufferfishes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 300, R284–R297. doi:10.1152/ajpregu.00725.2009

Katoh, F., Cozzi, R. R. F., Marshall, W. S., and Goss, G. G. (2008). Distinct $Na^+/K^+/2Cl^-$ Cotransporter Localization in Kidneys and Gills of Two Euryhaline

Species, Rainbow Trout and Killifish. *Cell Tissue Res.* 334, 265–281. doi:10.1007/s00441-008-0679-4

Kolisek, M., Nestler, A., Vormann, J., and Schweigel-Röntgen, M. (2012). Human Gene *SLC41A1* Encodes for the Na^+/Mg^{2+} Exchanger. *Am. J. Physiol. Cell Physiol.* 302, C318–C326. doi:10.1152/ajpcell.00289.2011

Madsen, S. S., Bollinger, R. J., Brauckhoff, M., and Engelund, M. B. (2020). Gene Expression Profiling of Proximal and Distal Renal Tubules in Atlantic Salmon (*Salmo salar*) Acclimated to Fresh Water and Seawater. *Am. J. Physiol. Renal Physiol.* 319, F380–F393. doi:10.1152/ajprenal.00557.2019

Marshall, W., and Grosell, M. (2006). "Ion Transport, Osmoregulation, and Acid-Base Balance," in *The Physiology of Fishes*. Editors D. H. Evans and J. B. Claiborne (New York, NY: CRC Press), 177–230.

Mount, D. B., and Romero, M. F. (2004). The SLC26 Gene Family of Multifunctional Anion Exchangers. *Pflugers Archiv Eur. J. Physiology* 447, 710–721. doi:10.1007/s00424-003-1090-3

Nakada, T., Zandi-Nejad, K., Kurita, Y., Kudo, H., Broumand, V., Kwon, C. Y., et al. (2005). Roles of Slc13a1 and Slc26a1 Sulfate Transporters of Eel Kidney in Sulfate Homeostasis and Osmoregulation in Freshwater. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289, R575–R585. doi:10.1152/ajpregu.00725.2004

Nishimura, H., and Imai, M. (1982). Control of Renal Function in Freshwater and Marine Teleosts. *Fed. Proc.* 41, 2355–2360.

Nishimura, H., Imai, M., and Ogawa, M. (1983). Sodium Chloride and Water Transport in the Renal Distal Tubule of the Rainbow Trout. *Am. J. Physiol.* 244, F247–F254. doi:10.1152/ajprenal.1983.244.3.f247

Renfro, J. L., and Pritchard, J. B. (1983). Sulfate Transport by Flounder Renal Tubule Brush Border: Presence of Anion Exchange. *Am. J. Physiol.* 244, F488–F496. doi:10.1152/ajprenal.1983.244.5.f488

Sahni, J., and Scharenberg, A. M. (2013). The SLC41 Family of MgtE-like Magnesium Transporters. *Mol. Aspects Med.* 34, 620–628. doi:10.1016/j.mam.2012.05.012

Schmidt-Nielsen, K. (1997). *Animal Physiology: Adaptation and Environment*. Cambridge, UK: Cambridge University Press.

Tsukita, S., Tanaka, H., and Tamura, A. (2019). The Claudins: From Tight Junctions to Biological Systems. *Trends Biochem. Sci.* 44, 141–152. doi:10.1016/j.tibs.2018.09.008

Watanabe, T., and Takei, Y. (2011). Molecular Physiology and Functional Morphology of SO_4^{2-} Excretion by the Kidney of Seawater-Adapted Eels. *J. Exp. Biol.* 214, 1783–1790. doi:10.1242/jeb.051789

Wu, M., Heneghan, J. F., Vandorpe, D. H., Escobar, L. I., Wu, B.-L., and Alper, S. L. (2016). Extracellular Cl^- Regulates Human SO_4^{2-} anion Exchanger SLC26A1 by Altering pH Sensitivity of Anion Transport. *Pflugers Arch.* 468, 1311–1332. doi:10.1007/s00424-016-1823-8

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Differential Branchial Response of Low Salinity Challenge Induced Prolactin in Active and Passive Coping Style Olive Flounder

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Stress coping styles are very common in fish, and investigations into this area can greatly improve fish welfare and promote the sustainable development of aquaculture. Although most studies have focused on the behavioral and physiological differences of these fishes, the endocrine response of different coping styles fish when undergoing salinity challenge is still unclear. We examined the physiological response in olive flounder with active coping (AC) style and passive coping (PC) style after transferred from seawater (SW) to freshwater for 0, 2, 5, 8, and 14 days. The results showed that: 1) the plasma prolactin level of FW-acclimated AC flounder was substantially higher than that of FW-acclimated PC flounder at 5, 8, and 14 days, and the branchial gene expression of prolactin receptor (PRLR) in AC flounder was slightly higher than PC flounder after transfer. While there was no remarkable difference observed in cortisol (COR) levels between AC and PC flounder. After transfer, glucocorticoid receptor (GR) expression in AC flounder was significantly higher compared with PC flounder at 8 days. 2) Branchial NKA-IR ionocytes numbers were reduced in PC flounder after transfer, while ionocytes number remain stable in AC flounder. 3) The branchial stem cell transcription factor *foxi1* gene expression of AC flounder was significantly higher than PC flounder at 2, 5, and 14 days after transfer, while branchial stem cell transcription factor *p63* gene expression of FW-acclimated AC flounder was only substantially higher than that of PC flounder at 5 days. 4) As an apoptosis upstream initiator, the branchial gene expression of *caspase-9* in PC flounder was considerably higher than in AC flounder after transfer at 8 days. This study revealed that olive flounder with active and passive coping styles have different endocrine coping strategies after facing the low-salinity challenge. AC flounder adopt an active endocrine strategy by increasing ionocyte differentiation and prolactin secretion significantly. In contrast, PC flounder employ a passive strategy of reducing ionocytes differentiation and retaining prolactin content at a low level to reduce branchial ionocytes number.

Keywords: salinity challenge, olive flounder, coping strategy, prolactin, ionocyte

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INTRODUCTION

Coping style can be defined as a coherent set of behavioral and physiological stress responses that are consistent over time and are characteristic of a certain group of individuals (Koolhaas et al., 1999). Active coping (AC) individuals and passive coping (PC) individuals are observed in olive flounder (*Paralichthys olivaceus*) (Rupia et al., 2016; Zeng et al., 2019). Compared to PC flounder, AC flounder show differences in response to simulated capture, feed propensity and metabolic rate (Rupia et al., 2016). For example, during acute stress, passive coping individuals adopt a passive "freeze-hide" strategy by reducing their oxygen consumption rates and remaining immobile, whereas active coping individuals adopt an active "fight-flight" defence strategy by increasing their rates of respiration and activity levels (Rupia et al., 2016). The majority of studies focused on the behavioral characteristics of different coping style individuals (Overli et al., 2006; Silva et al., 2010; Martins et al., 2011), and there are also some research investigating the physiological characteristics of individuals with different coping styles, such as hypothalamus-pituitary-interrenal (HPI) activity (Castanheira et al., 2013), immunity (Kittilsen et al., 2012) and hormonal modulation (LeBlanc et al., 2012). However, only a few studies elucidate the osmoregulatory coping differences between these two coping styles individuals (Sloman et al., 2003; Sloman et al., 2004), especially under salinity challenge (Zeng et al., 2019).

Salinity challenge is a common situation for fishes: with the increasingly climate change, resultant increase of extreme events, like hurricanes, heavy rainfall, and flooding, considerably fluctuated the water salinity (Bailey and Secor, 2016; Williams et al., 2017). Olive flounder, as a coastal habitat euryhaline fish, bears the brunt of salinity challenge (Yuan et al., 2017). Flounders also have a sophisticated osmoregulatory system in which the gills, the most important organ, take on the 90% function of osmoregulation that is mainly achieved by the ionoregulatory cells i.e., ionocytes (Evans et al., 2005). Ionocyes, formerly called chloride cells or mitochondrion-rich cells, are located at the base of the gill filament and among lamella. Fishes respond to salinity challenges through the function of ionocytes.

Body fluid homeostasis is vital for the survival of organisms and includes cellular activities and many physiological processes. Fish have developed a sophisticated endocrine system, mainly through hormones, to regulate body fluid homeostasis to meet environmental stressors such as salinity challenge. Hormones regulate body fluid homeostasis by controlling epithelial transporters. Several hormones have been demonstrated to positively or negatively regulate ion transport through specific receptors at transcriptional, translational or posttranslational levels, and at different stages of ionocyte development (i.e., proliferation or differentiation) (Hwang and Chou, 2013; Guh et al., 2015; Lin et al., 2016; Guh and Hwang, 2017; Lewis and Kwong, 2018). Cortisol plays many roles in physiological processes and is involved in regulating hydromineral balance in FW and SW teleosts (Guh et al., 2015). Cortisol also maintains ion transporters and ionocytes to promote both hyper- and hypo-osmoregulation mechanisms in the gill of fish (Evans et al., 2005). In teleosts, zebrafish are model organisms for studying regulatory pathways, which is of great significance to the study of osmotic pressure, and cortisol regulates Na^+ uptake through glucocorticoid receptors (*GR*) in zebrafish (Kumai et al., 2012). Indeed, accumulated evidence has demonstrated a predominant role for cortisol-*GR* signaling in fish iono/osmoregulation (Takahashi and Sakamoto, 2013). Prolactin has long been known as an FW adapting hormone, it inhibits the formation of SW ionocytes and promotes the development of FW ionoocytes (Evans et al., 2005). Furthermore, prolactin has been linked to the regulation of Na^+-Cl^-cotransporter (NCC) expression and ionocyte differentiation (Guh et al., 2015). Prolactin receptors (*PRLR*) are also crucial in the osmoregulation of fish (Guh and Hwang, 2017), the expression level of *PRLR* in teleost gill is very high (Prunet et al., 2000; Tse et al., 2000; Santos et al., 2001).

Foxi1, as a forkhead transcription factor, plays a vital role in the mechanism of α-intercalated cells (IC) differentiation in the mammalian inner ear and kidney (Blomqvist et al., 2004). Previous research in teleost fish, however, revealed that *Foxi1* indirectly regulates the differentiation of "HR-type" ionocytes (Esaki et al., 2009). The transcription factor *p63* is a marker of stem cells in the mammalian epidermis (Horng et al., 2009). In zebrafish (*Danio rerio*), an isoform of *p63* promotes the proliferation of epidermal cells, and then epithelial stem cells differentiate into skin ionocytes (Horng et al., 2009). Salinity challenge can induce apoptosis of fish branchial epithelial cells (Ching et al., 2013). *Caspase-9* is an initiator caspase involved in the induced apoptosis of macrophages and neutrophils, an activity involving the intrinsic pathway (Awoyemi et al., 2019).

With the rapid development of the world's aquaculture industry, total fish production has almost doubled during the last 20 years in Asia. Since the mid-2000s, Asia has accounted for two-thirds of global inland production (data from "The State of World Fisheries and Aquaculture 2020″). Indeed, annual flounder production has been roughly estimated to be 28,000 metric tons since the 2000s in Japan, Korea, and China, and therefore, flounder culture has become an important economic source of aquaculture in these areas (Seikai, 2002). Studies on the coping styles of farmed fish can provide knowledge for sustainable aquaculture, provide advantages in the culture system, and could be utilized to establish selection-based breeding programs to improve domestication and production (Ibarra-Zatarain et al., 2016; Fatsini et al., 2020).

Previous studies have shown that olive flounder with AC and PC styles have different coping strategies of behavioral and physiological response during FW acclimation (Zeng et al., 2019). However, the response of endocrine system of olive flounder with different coping styles to salinity challenge are still unclear. To fill this gap, therefore, our study measured the following parameters of olive flounder with AC and PC styles after transferred from SW to FW: 1) the plasma cortisol and prolactin concentrations and gene expression of their corresponding receptors (i.e., *GR* and *PRLR*) in fish gills, 2) the density of branchial ionocytes, and 3) the gene expression of branchial *foxi1*, *p63* and *caspase-9*.

MATERIALS AND METHODS

Experimental Fish

The gynogenetic olive flounder were cultured separately in SW recirculating aquaculture systems at the Central Experimental Station of Chinese Academy of Fisheries Sciences in November 2018 (Beidaihe, Hebei, China). According to the previous method, the fish (body weights: 500 ± 50 g) were screened through behavior tests which including air exposure and feeding propensity and then divided into AC groups and PC groups, the specific experimental method has been elaborated in the previous article (Rupia et al., 2016; Zeng et al., 2019). Olive flounder with confirmed active coping style (AC, n = 48) and confirmed passive coping style (PC, n = 48) were distributed evenly into 16 holding tanks (water capacity = 500 L; n = 6 for each coping style in each tank) and maintained for 2 weeks with filtered flow-through SW at a salinity level of 30‰. Water temperature was maintained at 18 ± 1°C, with a constant 12 h light/12 h dark cycle (8:00 a.m.—20:00 p.m.). The mean light intensity, measured centrally at the bottom of each tank, was approximately 40 lux (Zeng et al., 2019). Before the experiment, the fish were fed to satiation twice daily (8:00 a.m. and 18:00 p.m.) with commercial fish pellets ("Wenger" High-Grade Aquatic Feed 6 mm; Wengzhuang, Beijing, China). After that, the fish were fasted throughout the experiment to ensure that differential rates of feeding did not influence the results of the study (Yuan et al., 2017).

The Gill Characteristics of AC and PC Coping Styles to Salinity Challenge

Olive flounder with active coping style (AC, n = 48) and passive coping style (PC, n = 48) were netted and divided into four seawater (SW, 30‰, control group) tanks and four freshwater (FW, 30‰) tanks on the basis of corresponding coping styles (n = 6 for each coping style in each tank). The SW-acclimated (n = 3 in each tank) and FW-transferred (n = 3 in each tank) fish were then sampled during daylight hours 0, 2, 5, 8, and 14 days after initial transfer. At each sampling point, fish were rapidly netted and anesthetized with 2-phenoxyethanol (0.2 ml/L, Sigma, St. Louis, MO), and blood samples (3–5 ml) were collected within 90 s using a heparinized needle and syringe (200 U/ml heparin, Sigma) by caudal venipuncture. Blood was then aliquoted into ammonium-heparinized tubes (200 U/ml, Sigma) and plasma was separated by centrifugation for 5 min at 13,000 rpm and stored at −80°C for the subsequent measurement of plasma hormone concentrations. The fish were then sacrificed humanely by severing the spinal cord and destruction of the brain. A part of the gill was removed from the first gill arch and stored at −80°C for later analysis of branchial gene expression. A part of the gill was also removed and fixed in 4% paraformaldehyde (PFA) for 24 h for immunohistochemistry.

Plasma Measurements

Plasma levels of cortisol (COR) and prolactin (PRL) were quantified by ELISA commercially (Qiyi Biotechnology Co., LTD., Shanghai, China). According to the manufacturer's instructions, the circulating level range of COR was detected between 4 and 15 ng/ml, PRL was detected between 500 and 3,000 ng/ml. In detail, −80°C stored plasma supernatant fractions were naturally warmed in an icebox. Samples were then diluted to the appropriate concentration. Commercially available ELISA kits (Qiyi biotechnology Co., LTD., Shanghai, China) were subsequently used to measure serum COR and PRL levels in duplicate as per manufacturer instructions (Mechesso, 2019).

Antibody

Rabbit polyclonal anti-alpha 1 subunit of Na^+/K^+-ATPase (Abcam, Cambridge, United Kingdom) was used to detect the NKA immunoreactive (NKA-IR) ionocytes in the gill. The commercial polyclonal NKA-α1 antibody used in present study was specialized to zebrafish's NKA-α1 (Hiroi and McCormick, 2012). We blast the protein and nucleotide sequence of the zebrafish's NKA-α1 with that of olive flounder, and the results showed highly homologous of 92% and 84%, respectively. The secondary antibody for immunohistochemistry was goat anti-mouse IgG (Thermo Fisher Scientific, United States). Na^+/K^+-ATPase NKA, as the main Na^+ transporter, has been localized to the basolateral region of ionocytes. These NKA antibodies serve as a highly-specific marker for ionocytes in fixed gill tissues (Hiroi and McCormick, 2012).

Immunohistochemical Detection and Analysis of NKA Immunoreactive Cells

The Paraformaldehyde-fixed gills were dehydrated in ethanol, cleared in xylene and embedded in paraffin. A cross section of the fish gills was cut at 6 μm thickness on a microtome and mounted on glass slides, which were dealt with positive charge. During the experiment, tissue sections were dewaxed in xylene and rehydrated in gradient alcohol. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol before slides were placed in 0.01 M citrate buffer and heated in a water bath for 20 min at 95°C. Sections were rinsed in PBS after cooling. Sections were treated with fetal bovine serum (FBS) blocking solution (1% blocking, dissolved in MABT, and 5% FBS in PBST, PBS with 0.1% Triton X-100) for 1 h at room temperature (RT) to reduce nonspecific staining and incubated with 1:500 rabbit polyclonal anti-alpha 1 subunit of Na^+/K^+-ATPase diluted with PBS in a moist chamber at 4°C overnight. Next day, the moist chamber was transferred into air oven at 37°C for 45 min, then washed six times at RT in PBST for 15 min each time and incubated with 1:500 goat anti-rabbit IgG secondary antibody. Then the sections were washed thrice in PBST. Control experiments were carried out by omission of the primary antibody and pre-absorption of the antibody with an excess of antigenic peptide.

Quantification of Branchial Ionocytes

The number of NKA immunoreactive (NKA-IR) ionocytes were counted and obtained within each gill in every five sections (five adjacent lamellas were set as one section) from all sections sampled at ×200 magnification with a microscope (Nikon ECLIPSE 55i, Nikon Corporation, Japan). Size and luminosity

TABLE 1 | Primer sequences used for real-time PCR amplifications.

Genes	Primer (5′–3′)	Gene bank	Product length (bp)
β-actin	GGAAATCGTGCGTGACATTAAG CCTCTGGACAACGGAACCTCT	HQ386788.1	161
GR	ACCAGTTTAACGCGGTTTGC ACGCCACATTTATCCATCCTTG	XM_020103452.1	261
PRLR	CCCGACTATTTCCATCGGGAG AGTTGGACGATGTGATCGGG	XM_020081654.1	247
Foxi1	TGACCCAGGAAAGGGCAACTA CAAGAAGCTGCTCAGACACG	XM_020086875.1	262
P63	CTCCCCAGGTTGGAACAGAA CGTTTCGTACCCTCACTGCT	XM_020080690.1	253
caspase-9	AAGACACTGACTGACTCCGC GTCTGGTCCCAGAGCTCTTAAT	XM_020089046.1	257

of the figures were modified with NIS-Elements Version 4.0 (Nikon, Japan), as well as the drawings.

The Branchial Gene Expression Response of AC and PC Coping Styles to Salinity Challenge

Extracted Total RNA from the samples of gills using RNAiso Plus (Takara, Japan) and then reverse transcribed the total RNA into cDNA using PrimeScript™ RT reagent (Takara, Dalian, China) according to the standard method. Relative quantification of the target gene transcripts was analyzed using *β-actin* gene expression as the reference gene (Zeng et al., 2019). Branchial *foxi1*, *p63*, *caspase-9*, *GR* and *PRLR* gene expressions were determined using ABI 7500 (Applied Biosystems, Carlsbad, CA) with SYBR Premix Ex Taq™ (Takara, Dalian, China) and the following PCR conditions were used: 10 min at 95°C, followed by 36 cycles of 95°C for 10 s, 30 s at 60°C. Gene sequences were obtained from NCBI and all the primers were designed by NCBI primer blast. The forward and reverse primers span an exon-exon junction. All the primers were tested and shown to be viable and specific. The primer sets for the quantitative RT-PCR are shown in **Table 1**.

Statistical Analysis

The $2^{-\Delta\Delta Ct}$ method was used to analyze the quantitative RT-PCR data, and the expression level in all plots of each gene is presented as the relative change in values from SW-acclimated AC at 14 days, which was set at 1 (Zeng et al., 2019). The normality and homogeneity of variance of data were tested using Shapiro-Wilk's test and Levene's test, respectively. Significant analyse of plasma hormone was assessed using nonparametric test with salinity (or coping style) and time as independent variables, followed by Mann-Whitney U test. Significant analyses of branchial inocytes numbers and gene expressions were assessed using two-way ANOVA with salinity (or coping style) and time as independent variables, followed by Tukey's multiple comparison test. Data are expressed as means ± SEM throughout and significance was accepted at $p < 0.05$ for all analyses. All statistical analysis was carried out using the SPSS Statistics 20 or GraphPad Prism 5.0 (San Diego, CA, United States).

RESULT

Plasma Hormone Level and Its Branchial Receptor Gene Expression

There was no interaction between coping style and time on plasma cortisol level (ANOVA, $F_{(4,20)} = 0.1487$, $p = 0.9614$, **Figure 1A**). While the plasma cortisol levels of PC and AC flounder increased from 0 to 2 days and decreased from 2 to 14 days during FW acclimation ($p < 0.05$; **Figure 1A**). Significant interactions between coping style and time were detected for plasma prolactin level (ANOVA, F (4,20) = 58.95, $p < 0.0001$, **Figure 1B**), indicating that prolactin response were different among AC and PC at different time points. Significant effects of treatment and time on AC flounder plasma PRL were observed, with a significant increase from 2 to 5 days ($p < 0.05$, **Figure 1B**), and significantly decreased thereafter ($p < 0.05$, **Figure 1B**). While PC flounder were significantly increased from 0 to 2 days ($p < 0.05$, **Figure 1B**), significantly decreased from 2 to 5 days ($p < 0.05$, **Figure 1B**), and then remained stable. After FW transfer, the PRL levels of AC flounder were significantly lower than PC flounder at 2 days ($p < 0.05$, **Figure 1B**), but significantly higher than PC flounder at 5, 8 and 14 days ($p < 0.05$, **Figure 1B**).

When acclimated to SW, there was no difference in *GR* and *PRLR* gene expression between olive flounder with both active and passive coping style (**Figures 2A,C**). After transferring to FW, the *GR* expression of AC flounder significantly increased up to 8 days (2 days vs. 8 days, $p = 0.0039$), and slightly decreased at 14 days, and was significantly higher than that of PC flounder at 8 days ($p = 0.0467$, **Figure 2B**). While FW-acclimated PC flounder showed no remarked changes in *GR* gene expression (**Figure 2B**). The *PRLR* expression in FW-acclimated AC flounder tented to be slightly higher than that of PC flounder from 2 to 14 days, but without significant difference (**Figure 2D**).

Branchial Ionocytes Number of AC and PC Flounder

Significant interactions between salinity and time were observed for the branchial ionocytes number of PC and AC (ANOVA, $F_{(3,32)} = 7.934$, $p = 0.0004$, **Figure 3A**; ANOVA, F (3,32) = 5.952, $p = 0.0024$, **Figure 3B**). When acclimated to SW, the branchial ionocytes

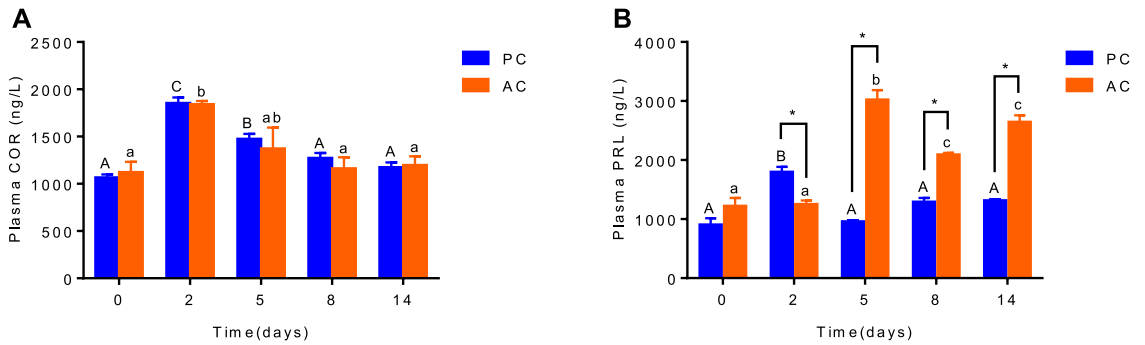

FIGURE 1 | The plasma cortisol **(A)** and prolactin **(B)** level in olive flounder with passive coping style (PC) and active coping style (AC) after transfer to fresh water. Data are mean ± SEM (n = 3). An asterisk signifies a significant difference between the PC and AC groups at each time point ($p < 0.05$). Different letters denote a significant difference across time within the same coping style group ($p < 0.05$).

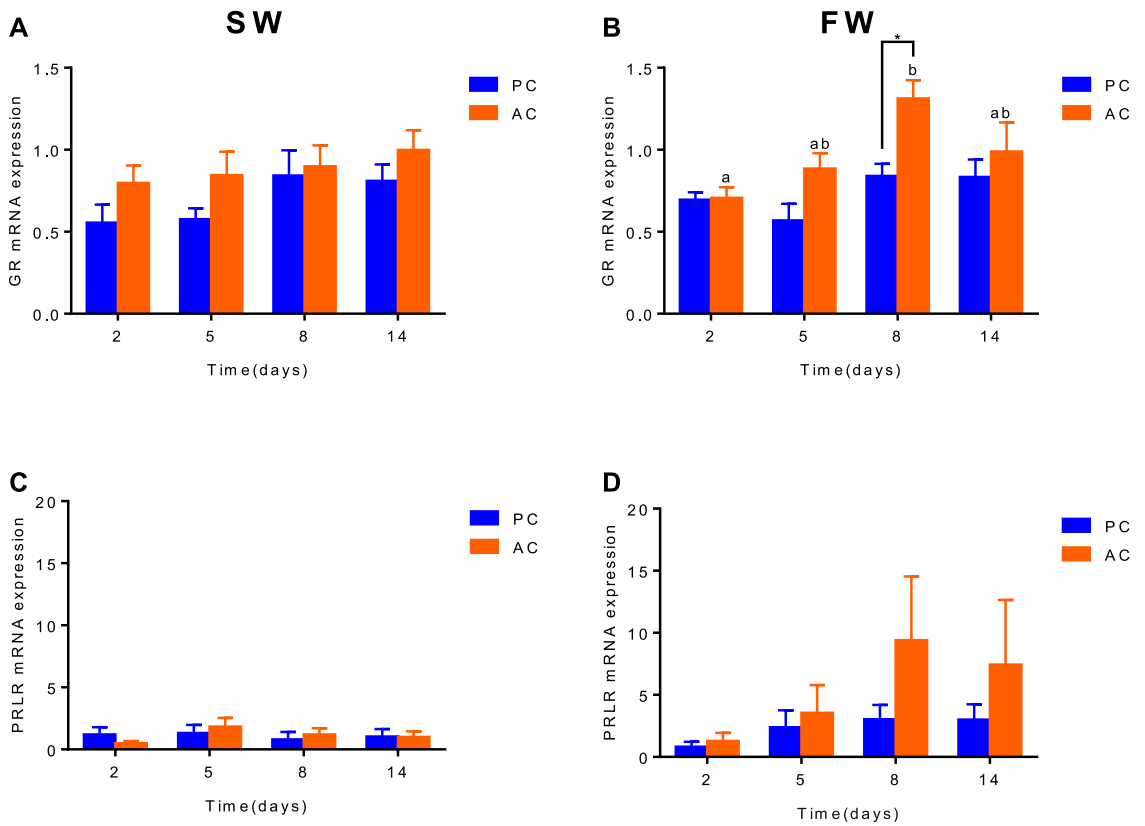

FIGURE 2 | The branchial gene expression of *GR* **(A,B)** and *PRLR* **(C,D)** in olive flounder with passive coping style (PC) and active coping style (AC) either in seawater (SW, **A,C**), or after transfer to freshwater (FW, **C,D**). Data are mean ± SEM (n = 6). An asterisk signifies a significant difference between the PC and AC groups at each time point ($p < 0.05$). Different letters denote a significant difference across time within the same coping style group ($p < 0.05$).

numbers of PC siginificantly increased from 2 to 5 days (2 days vs. 5 days, p = 0.0003, **Figure 3A**) and then remained unchanged. No significant differences were found in FW-acclimated AC and PC (**Figure 3B**). Moreover, we observed that compared with the SW control group, the number of NKA-IR ionocytes in fish gills was counted and showed dramatic decrease in FW-acclimated PC individuals from 2 to 14 days ($p < 0.05$, **Figure 3C**). In addition, the cell number of AC individuals remained unchanged regardless of whether it was acclimated to SW or FW (**Figure 3D**). The immunocytochemistry for ionocytes in gills of AC (**Figures 3F,H**) and PC (**Figures 3E,G**) flounder transferred from SW (**Figures 3E,F**) to FW (**Figures 3G,H**) also showed this phenomenon.

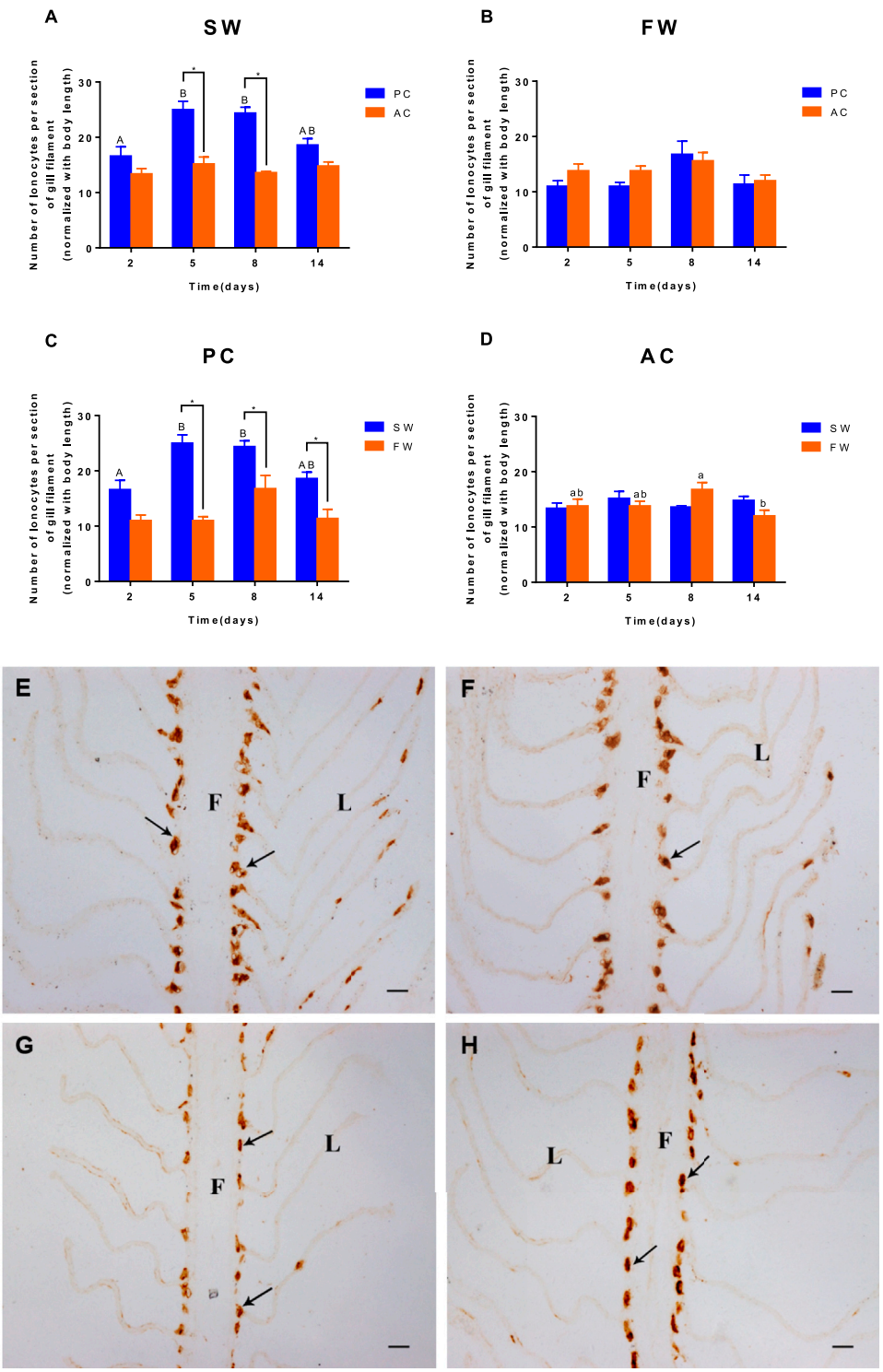

FIGURE 3 | The number of ionocytes per section of gill from olive flounder with passive coping style [PC, **(C)**] and active coping style [AC, **(D)**] after transfer from seawater [SW, **(A)**] to freshwater [FW, **(B)**]. Five adjacent lamellas were set as one section. Each gill of a fish chose five sections. Data are mean ± SEM ($n = 6$). An asterisk signifies a significant difference between the PC and AC groups at each time point ($p < 0.05$). Different letters denote a significant difference across time within each PC or AC group ($p < 0.05$). The immunocytochemistry for ionocytes in gills from olive flounder with passive coping style [PC, **(E,G)**] and active coping style [AC, **(F,H)**] from seawater [SW, **(E,F)**] to freshwater [FW, **(G,H)**] for 5 days. Scope time (×200). F, filament; L, lamella; black arrow, and ionocytes. Bar = 20 μm.

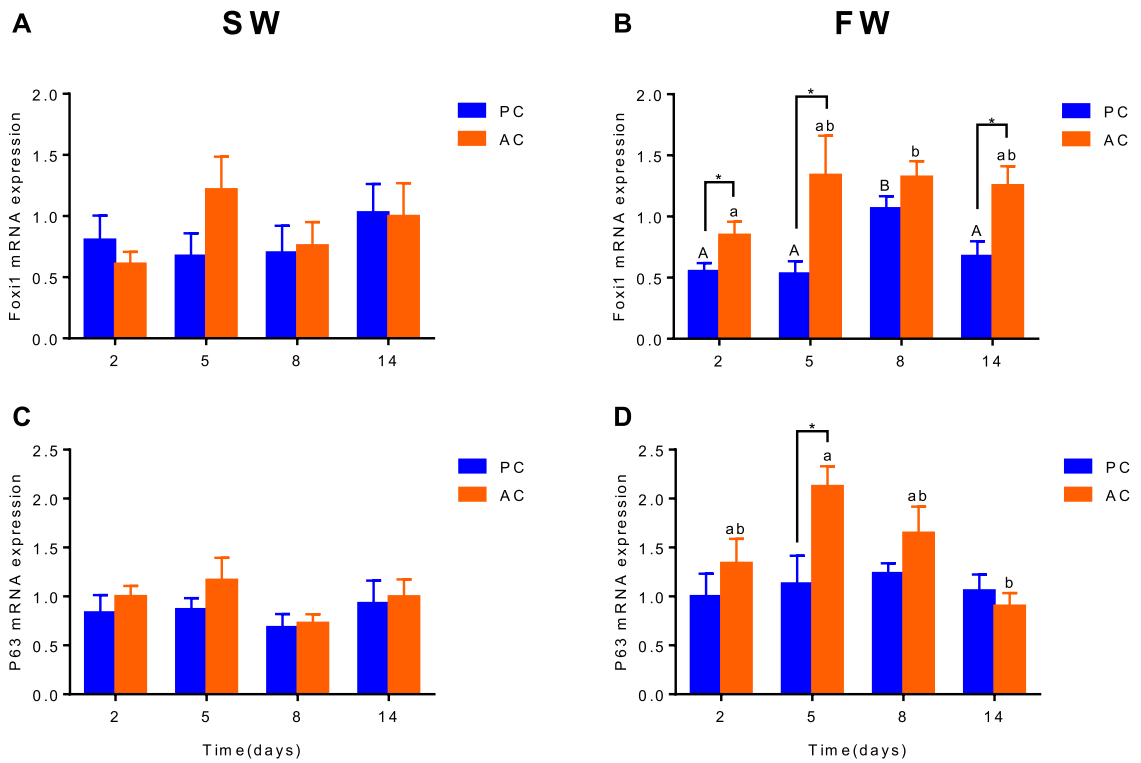

FIGURE 4 | The branchial gene expression of *Foxi1* **(A,B)** and *P63* **(C,D)** in olive flounder with passive coping style (PC) and active coping style (AC) either in seawater [SW, **(A,C)**], or after transfer to freshwater [FW, **(C,D)**]. Data are mean ± SEM ($n = 6$). An asterisk signifies a significant difference between the PC and AC groups at each time point ($p < 0.05$). Different letters denote a significant difference across time within the same coping style group ($p < 0.05$).

Gene Expression of Branchial *foxi1* and *p63*

Branchial gene expression of *foxi1* and *p63* of SW-acclimated PC flounder were similar to that of AC flounder, each group did not show significant differences between PC and AC flounder at different time points. Nevertheless, main effect of coping style was observed for *foxi1* (ANOVA, $F_{(1,40)} = 19.57$, $p < 0.0001$, **Figure 4B**) and *p63* (ANOVA, F (1,40) = 7.108, **Figure 4D**) gene expression in FW-acclimated groups. When acclimated to freshwater, *foxi1* gene expression of AC was significant increased at 2 days ($p = 0.038$, **Figure 4B**), 5 days ($p = 0.0368$, **Figure 4B**) and 14 days ($p = 0.01443$, **Figure 4B**) compared with PC, both PC and AC flounder had peak expression of *foxi1* at 8 days (2 days vs. 8 days: PC, $p = 0.0015$; AC, $p = 0.0173$; **Figure 4B**). While *p63* gene expression of FW-acclimated PC was only significantly different from that of AC at 5 days ($p = 0.035$, **Figure 4D**). FW-acclimated AC also reach the peak expression of *p63* at 5 days, and then dropped significantly at 14 days (5 days vs. 14 days, $p = 0.0044$, **Figure 4D**).

Gene Expression of Branchial Apoptosis Factor *Caspase-9*

Significant interactive effect between coping style and time were observed for *caspase-9* expression in SW (ANOVA, $F_{(3,40)} = 2.882$, $p = 0.0476$, **Figure 5A**) and FW (ANOVA, $F_{(3,40)} = 13.35$, $p < 0.0001$, **Figure 5B**), indicating that gene expression of *caspase-9* in flounder was different among coping style at different time points. There was a significant difference of *caspase-9* expression between SW-acclimated AC and PC at 5 days ($p = 0.002$, **Figure 5A**). And this gene expression of SW-acclimated AC flounder increased from 2 to 5 days ($p = 0.0063$, **Figure 5A**). After transferred to FW, significant differences of *caspase-9* expression were also observed between AC and PC flounder at 2 days ($p < 0.0001$, **Figure 5B**) and 8 days ($p = 0.005$, **Figure 5B**), respectively. FW-acclimated PC flounder increased gene expression from 2 days, and reached peak expression at 8 days (2 days vs. 8 days, $p = 0.009$, **Figure 5B**), declined thereafter (8 days vs. 14 days, $p = 0.0453$, **Figure 5B**). While FW-acclimated AC flounder expressed highest gene expression at 2 days (2 days vs. 5 days, $p = 0.0025$, **Figure 5B**) and declined thereafter (**Figure 5B**).

DISCUSSION

Our study demonstrates marked differences in the hormone and ionocyte regulation strategies of PC and AC olive flounders in response to salinity challenge. PC flounder reduced branchial ionocytes density while AC flounder remain stable during hypoosmotic challenge. Also, FW-acclimated AC flounder have higher ultimate plasma prolactin levels, slightly higher branchial gene expression of hormone receptors (i.e., *PRLR*

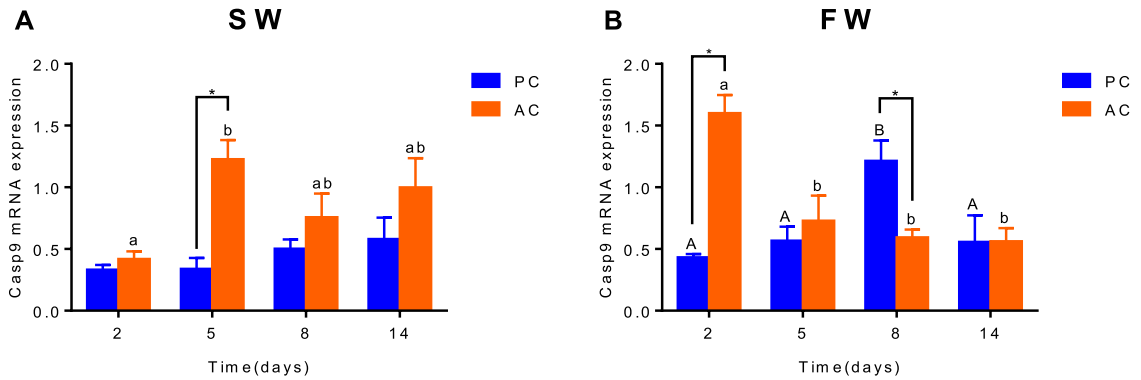

FIGURE 5 | The branchial gene expression of *Caspase-9* in olive flounder with passive coping style (PC) and active coping style (AC) after transfer from seawater to seawater [SW, **(A)**] and freshwater [FW, **(B)**]. Data are mean ± SEM ($n = 6$). An asterisk signifies a significant difference between the PC and AC groups at each time point ($p < 0.05$). Different letters denote a significant difference across time within the same coping style group ($p < 0.05$).

and *GR*), and higher ionocyte differentiation-related genes (i.e., *foxi1* and *p63*) than PC flounder. This is therefore the first study showing that ionocyte coping strategies influence the way fish deal with salinity challenge.

Many works had shown that cortisol not only function at SW-adaption but also important in FW-osmoregulation (Pelis and McCormick, 2001; Evans, 2002). Previous studies have also shown that cortisol treatment increases the densities of progenitor and mature ionocytes in larvae or isolated adult gills (Cruz et al., 2013a; Cruz et al., 2013b; Guh and Hwang, 2017). The pathway through which cortisol acts via the glucocorticoid receptor (*GR*) has been demonstrated to play a major role in fish osmoregulation (Takahashi and Sakamoto, 2013) and mediates the development of epidermal ionocytes in zebrafish (Cruz et al., 2013b). In the present study, the plasma cortisol level of both PC and AC increased from 0 to 2 days and gradually decreased from 2 to 14 days after transfer to FW, similar to previous studies of this species (Bolasina et al., 2007). Such results may be due to the rapid osmoregulatory action of cortisol in the low salinity challenge (Cruz et al., 2013a). However, after transfer to FW, AC flounder significantly increased *GR* expression, whereas no changes were found in PC. Study in zebrafish demonstrated cortisol-*GR* favors differentiation of ionocyte progenitors, thereby facilitating proliferation of mature ionocytes (Cruz et al., 2013a). Considering that the *GR* expression of AC flounder is higher than that of PC flounder after transfer to FW, there is such a possibility that AC flounder may have a higher rate of ionocytes development than PC during FW acclimation.

Prolactin, as a major "FW-adaption" hormone, promotes the development of "FW-type" ionocytes (Evans et al., 2005). Previous studies have shown that the plasma prolactin levels of FW-acclimated olive flounder were increased and remained stable from 2 to 14 days (Higashimoto et al., 2001; Yuan et al., 2017). Similarly, in our study, AC flounder also increased plasma prolactin concentration after transfer to FW from 2 to 5 days and restored stability at 14 days. However, PC flounder only showed an initial increase at 2 days and then kept to a lower level. Such results indicated a difference in hormone regulation between two coping style populations. Previous studies showed that FW treatment could increase branchial *PRLR* expression in olive flounder (Higashimoto et al., 2001; Yuan et al., 2017). Similar results were found in the present study, suggesting the role of *PRLR* in the osmoregulation process. Interestingly, during FW adaptation, both populations showed similar trends of the *PRLR* expression during acclimation to FW. However, the AC flounder *PRLR* expression was slightly higher than the PC flounder. Previous studies showed that AC individuals adopt an "active resistance" defense strategy to retain the plasma composition during hypoosmotic challenge, which was underpinned by a gradually increasing level of osmoregulatory gene expression in the gills for hypoosmotic adaptation; PC individuals employed a "passive tolerance" strategy to decrease the plasma osmolality and ionic content, which was underpinned by a sharp increase in the expression of branchial osmoregulatory gene, to conserve energy with the osmoregulatory process (Zeng et al., 2019). Moreover, the hormone synthesis is an energetically demanding bioprocess (Tseng and Hwang, 2008), Since the AC had higher metabolic space than PC flounder (Rupia et al., 2016; Zeng et al., 2019), the AC group possibly produce more hormone, which might explain the difference of prolactin level.

During adaptation to FW, NKA-immunoreactive (NKA-IR) ionocytes are mainly located on the filaments of AC and PC flounder. In this study, PC flounder reduced gill ionocytes density after transfer to FW, while that of the AC flounder remained unchanged, which means different ionocytes coping strategies between the two populations. Previous studies have found that, most euryhaline teleost fish, including chum salmon (*Oncorhynchus keta*) and Mozambique tilapia (*Oreochromis mossambicus*), as PC flounder in this study, showed significantly higher numbers of branchial NKA-IR ionocytes in SW than FW (Katoh and Kaneko, 2003; Hiroi and McCormick, 2012; Maugars et al., 2018). And tiger puffer (*Takifugu rubripes*), a stenohaline marine fish that has no change in the gill ionocytes activity after being transferred from a hypertonic to a hypotonic environment (Motohashi et al., 2009) which can also be seen in AC flounder. Similarly, our previous study found that the pattern of plasma parameters in FW-acclimated PC flounder showed a

significant decrease and is similar to that seen in other euryhaline teleosts. In contrast, the pattern of plasma parameters in FW-acclimated AC flounder showed a fluctuation that is similar to the response seen in stenohaline marine fish (Zeng et al., 2019). Consequently, such results further confirm our former conclusion that markedly different hypoosmotic-regulation processes (i.e., ionocytes and osmoregulatory coping strategies) exist between AC and PC flounders. In addition, on one hand, a previous study found that AC flounder have a higher metabolic space than PC flounder (Rupia et al., 2016), meaning that AC flounder are accessible to spending more metabolic costs on osmoregulation, which is a much more energetically demanding process (Zeng et al., 2019). Therefore, AC individuals could cater to the salinity challenge to remain branchial ionocytes while PC flounders have to reduce cell numbers due to lower metabolic space. On the other hand, previous studies have also shown that PRL-deficient zebrafish down-regulate the density of "NCC-type" ionocytes (i.e., a type of NKA-IR ionocytes) and are unable to survive in FW (Shu et al., 2016). Thus, higher prolactin levels in AC flounder are beneficial for maintaining branchial "NCC-type" ionocytes density, and lower prolactin levels will assist in reducing the density in PC flounder. Prolactin plays its role by binding to the extracellular domain of the high-affinity receptors (i.e., *PRLR*), which are anchored on the membrane of the target cells (Bole-Feysot et al., 1998). Hence, due to the higher prolactin level and *PRLR* expression in AC, AC flounder may have a higher ionocytes development rate than PC flounder when responding low salinity challenge, maintaining ionocytes density as a consequence. Notably, when flounder maintained in SW, we observed that the branchial ionocytes number in PC was higher than AC. Previous studies indicated that the plasma cortisol level of SW-adaption PC flounder was higher than that of AC flounder (Zou et al., 2019). And cortisol was originally known as a SW-adaption hormone and promoted the differentiation of SW-type ionocyte (Evans et al., 2005). Therefore, such phenomena might be related to the higher plasma cortisol levels in PC than AC in SW.

Foxi1-deficient mouse mutants showed a defect in IC (α-intercalated cells) differentiation, demonstrating an important role for *foxi1* in establishing the IC lineage (Chang and Hwang, 2011). In zebrafish, *foxi1* could indirectly regulate the differentiation of epidermal stem cells into ionocytes (Esaki et al., 2009; Horng et al., 2009; Guh and Hwang, 2017). In the present study, after transfer to FW, *foxi1* gene expression of AC flounder was significantly higher than PC flounder at 2, 5, and 14 days, which may have resulted in a different cell differentiation process between AC and PC flounder. Ionocytes progenitors are also specified from *p63* positive epidermal stem cells and blocking ΔN*p63* (an isoform of *p63*) function in zebrafish embryos stopped the proliferation of epidermal cells (Lee and Kimelman, 2002). In this study, the gene expression of *p63* in FW-acclimated AC flounder showed a continuous increase and was significantly different compared with FW-acclimated PC flounder, indicated that AC flounder may induce epidermal stem cells at a higher proliferative rate than PC flounder when in response to low salinity challenge. So far, such a model is clearly constructed in zebrafish embryos: epidermal stem cells that express *p63* and *foxi1* give rise to ionocyte progenitors, which subsequently differentiate into ionocytes (Breves et al., 2014; Guh and Hwang, 2017). Previous studies suggest that prolactin may act on ionocyte progenitors to drive differentiation of ionoctes (Breves et al., 2014). Thus, we propose such a hypothesis that as the higher expression of ionocyte differentiation-related genes (i.e., *p63* and *foxi1*) in AC flounder stimulates the proliferation of epidermal stem cells, they give rise to ionocyte progenitors, accompanied by the higher prolactin level in AC flounder. Therefore promoting the differentiation of ionocyte progenitors into ionocytes, maintaining branchial ionocytes density.

Salinity stress may induce the proapoptotic caspase system (including *Casp-9* up-stream and *Casp-3* downstream) which executes apoptosis in oliver flounder (Lee et al., 2022). The apoptotic pathways are mediated by caspases, and as such, cells dying without the participation of caspases do not display the typical morphological characteristics of apoptosis. *Capsase-9* is an initiator of caspase family of cysteine proteases that have been implicated in apoptosis and cytokine processing (Takle and Andersen, 2007). Previous studies showed *caspase-9* is apparently involved in the induced apoptosis of macrophages and neutrophils, an activity involving the intrinsic pathway (Reis et al., 2007; Awoyemi et al., 2019). Salinity challenge could induce branchial cell apoptosis in fish (Li et al., 2019), as did low-temperature stress (Chou et al., 2008). In this study, FW acclimation led to a significant increase in branchial *caspase-9* gene expression in PC flounder, suggesting that the intrinsic pathways of apoptosis have been activated (Ching et al., 2013), and that the apoptosis rate of AC flounder may be different from that of PC flounder. The notable difference between AC and PC in 5 days after transfer to SW may be explained by the different coping style strategies between two groups. AC individuals always performed active response than PC in SW, therefore could initiate many complicated cellular mechanisms. The reason for this may be due to more intense energy consumption and physiological metabolism in AC individuals. The lower apoptosis rate in AC flounder may explain the distinct ionocytes density compared with PC flounder, which supports our hypothesis described above.

CONCLUSION

Olive flounder with active coping style or passive coping style have markedly different endocrine coping strategies. When acclimated to FW, PC flounder reduced branchial ionocyte density while AC flounder remained stable. Also, compared with FW-acclimated PC flounder, that of AC flounder have higher branchial gene expression of ionocyte differentiation-related genes (i.e., *foxi1* and *p63*) and hormone receptors (i.e., *GR* and *PRLR*), higher plasma prolactin levels, and lower apoptosis upstream initiator *caspase-9* gene expression. As such, we suppose that AC flounder may have a higher epidermal stem cell proliferation rate, ionocyte progenitor differentiation rate, and ionocytes development rate than PC flounder when facing the low salinity challenge. These clearly show the different endocrine regulatory strategies between AC and PC.

PERSPECTIVE

The endocrine system is the major signaling pathway for environmental osmotic stress. When the animal is in an osmotically extreme environment, environmental osmotic stress may cause differences in endocrine response and control even among intraspecies (McCormick and Bradshaw, 2006). Our study has shown that olive flounder with different coping styles have different endocrine responses when acclimated to FW. However, a previous study showed that SW-type and FW-type ionocytes in fish gills could rapidly and reversely transform to accommodate salinity change (Hwang and Lee, 2007). Moreover, gills may sustain their functions by producing mature ionocytes from pre-existing undifferentiated progenitors in low-temperature environments (Chou et al., 2008). Therefore, further research is needed on the cell types and dynamic changes of ionocytes in flounder gill in response to a low salinity challenge.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Ethics Committee of Shanghai Ocean University (Shanghai, China) approved all the experimental protocols used in this study. Animal procedures abide by the Guidelines on Ethical Treatment of Experimental Animals established by the Ministry of Science and Technology, China.

AUTHOR CONTRIBUTIONS

JZ and WL designed the experiments. JZ and JL drafted the manuscript and carried out the experiments. JL, TX, and KY analyzed experimental data. WL and JZ provided scientific concepts and finalized the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.913233/full#supplementary-material

REFERENCES

Awoyemi, O. M., Kumar, N., Schmitt, C., Subbiah, S., and Crago, J. (2019). Behavioral, Molecular and Physiological Responses of Embryo-Larval Zebrafish Exposed to Types I and II Pyrethroids. *Chemosphere* 219, 526–537. doi:10.1016/j.chemosphere.2018.12.026

Bailey, H., and Secor, D. H. (2016). Coastal Evacuations by Fish during Extreme Weather Events. *Sci. Rep.* 6, 30280. doi:10.1038/srep30280

Blomqvist, S. R., Vidarsson, H., Fitzgerald, S., Johansson, B. R., Ollerstam, A., Brown, R., et al. (2004). Distal Renal Tubular Acidosis in Mice that Lack the Forkhead Transcription Factor Foxi1. *J. Clin. Invest.* 113 (11), 1560–1570. doi:10.1172/jci20665

Bolasina, S. N., Tagawa, M., and Yamashita, Y. (2007). Changes on Cortisol Level and Digestive Enzyme Activity in Juveniles of Japanese Flounder, *Paralichthys olivaceus*, Exposed to Different Salinity Regimes. *Aquaculture* 266 (1-4), 255–261. doi:10.1016/j.aquaculture.2007.01.046

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P. A. (1998). Prolactin (PRL) and its Receptor: Actions, Signal Transduction Pathways and Phenotypes Observed in PRL Receptor Knockout Mice. *Endocr. Rev.* 19 (3), 225–268. doi:10.1210/edrv.19.3.0334

Breves, J. P., McCormick, S. D., and Karlstrom, R. O. (2014). Prolactin and Teleost Ionocytes: New Insights into Cellular and Molecular Targets of Prolactin in Vertebrate Epithelia. *General Comp. Endocrinol.* 203, 21–28. doi:10.1016/j.ygcen.2013.12.014

Castanheira, M. F., Herrera, M., Costas, B., Conceição, L. E. C., and Martins, C. I. M. (2013). Can We Predict Personality in Fish? Searching for Consistency over Time and across Contexts. *PLoS One* 8 (4), e62037. doi:10.1371/journal.pone.0062037

Chang, W.-J., and Hwang, P.-P. (2011). Development of Zebrafish Epidermis. *Birth Defects Res. Part C Embryo Today Rev.* 93 (3), 205–214. doi:10.1002/bdrc.20215

Ching, B., Chen, X. L., Yong, J. H. A., Wilson, J. M., Hiong, K. C., Sim, E. W. L., et al. (2013). Increases in Apoptosis, Caspase Activity and Expression of P53 and Bax, and the Transition between Two Types of Mitochondrion-Rich Cells, in the Gills of the Climbing Perch, Anabas Testudineus, during a Progressive Acclimation from Freshwater to Seawater. *Front. Physiol.* 4, 135. doi:10.3389/fphys.2013.00135

Chou, M.-Y., Hsiao, C.-D., Chen, S.-C., Chen, I.-W., Liu, S.-T., and Hwang, P.-P. (2008). Effects of Hypothermia on Gene Expression in Zebrafish Gills: upregulation in Differentiation and Function of Ionocytes as Compensatory Responses. *J. Exp. Biol.* 211 (19), 3077–3084. doi:10.1242/jeb.019950

Cruz, S. A., Chao, P.-L., and Hwang, P.-P. (2013a). Cortisol Promotes Differentiation of Epidermal Ionocytes through Foxi3 Transcription Factors in Zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 164 (1), 249–257. doi:10.1016/j.cbpa.2012.09.011

Cruz, S. A., Lin, C.-H., Chao, P.-L., and Hwang, P.-P. (2013b). Glucocorticoid Receptor, but Not Mineralocorticoid Receptor, Mediates Cortisol Regulation of Epidermal Ionocyte Development and Ion Transport in Zebrafish (*Danio Rerio*). *PLOS ONE* 8 (10), e77997. doi:10.1371/journal.pone.0077997

Esaki, M., Hoshijima, K., Nakamura, N., Munakata, K., Tanaka, M., Ookata, K., et al. (2009). Mechanism of Development of Ionocytes Rich in Vacuolar-type H^+-ATPase in the Skin of Zebrafish Larvae. *Dev. Biol.* 329 (1), 116–129. doi:10.1016/j.ydbio.2009.02.026

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste. *Physiol. Rev.* 85 (1), 97–177. doi:10.1152/physrev.00050.2003

Evans, D. H. (2002). Cell Signaling and Ion Transport across the Fish Gill Epithelium. *J. Exp. Zool.* 293 (3), 336–347. doi:10.1002/jez.10128

Fatsini, E., Rey, S., Ibarra-Zatarain, Z., Boltaña, S., Mackenzie, S., and Duncan, N. J. (2020). Linking Stress Coping Styles with Brain mRNA Abundance of Selected

Transcripts for Senegalese Sole (*Solea Senegalensis*) Juveniles. *Physiol. Behav.* 213, 112724. doi:10.1016/j.physbeh.2019.112724

Guh, Y.-J., and Hwang, P.-P. (2017). Insights into Molecular and Cellular Mechanisms of Hormonal Actions on Fish Ion Regulation Derived from the Zebrafish Model. *General Comp. Endocrinol.* 251, 12–20. doi:10.1016/j.ygcen.2016.08.009

Guh, Y. J., Lin, C. H., and Hwang, P. P. (2015). Osmoregulation in Zebrafish: Ion Transport Mechanisms and Functional Regulation. *Excli. J.* 14, 627–659. doi:10.17179/excli2015-246

Higashimoto, Y., Nakao, N., Ohkubo, T., Tanaka, M., and Nakashima, K. (2001). Structure and Tissue Distribution of Prolactin Receptor mRNA in Japanese Flounder (*Paralichtys Olivaceus*): Conserved and Preferential Expression in Osmoregulatory Organs. *General Comp. Endocrinol.* 123 (2), 170–179. doi:10.1006/gcen.2001.7660

Hiroi, J., and McCormick, S. D. (2012). New Insights into Gill Ionocyte and Ion Transporter Function in Euryhaline and Diadromous Fish. *Respir. Physiol. Neurobiol.* 184 (3), 257–268. doi:10.1016/j.resp.2012.07.019

Horng, J.-L., Lin, L.-Y., and Hwang, P.-P. (2009). Functional Regulation of H^+-ATPase-rich Cells in Zebrafish Embryos Acclimated to an Acidic Environment. *Am. J. Physiol.-Cell Physiol.* 296 (4), C682–C692. doi:10.1152/ajpcell.00576.2008

Hwang, P.-P., and Chou, M.-Y. (2013). Zebrafish as an Animal Model to Study Ion Homeostasis. *Pflugers Arch. - Eur. J. Physiol.* 465 (9), 1233–1247. doi:10.1007/s00424-013-1269-1

Hwang, P.-P., and Lee, T.-H. (2007). New Insights into Fish Ion Regulation and Mitochondrion-Rich Cells. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 148 (3), 479–497. doi:10.1016/j.cbpa.2007.06.416

Ibarra-Zatarain, Z., Fatsini, E., Rey, S., Chereguini, O., Martin, I., Rasines, I., et al. (2016). Characterization of Stress Coping Style in Senegalese Sole (*Solea Senegalensis*) Juveniles and Breeders for Aquaculture. *R. Soc. open Sci.* 3 (11), 160495. doi:10.1098/rsos.160495

Katoh, F., and Kaneko, T. (2003). Short-term Transformation and Long-Term Replacement of Branchial Chloride Cells in Killifish Transferred from Seawater to Freshwater, Revealed by Morphofunctional Observations and a Newly Established "Time-Differential Double Fluorescent Staining" Technique. *J. Exp. Biol.* 206 (Pt 22), 4113–4123. doi:10.1242/jeb.00659

Kittilsen, S., Johansen, I. B., Braastad, B. O., and Øverli, Ø. (2012). Pigments, Parasites and Personalitiy: towards a Unifying Role for Steroid Hormones? *PLoS One* 7 (4), e34281. doi:10.1371/journal.pone.0034281

Koolhaas, J. M., Korte, S. M., De Boer, S. F., Van Der Vegt, B. J., Van Reenen, C. G., Hopster, H., et al. (1999). Coping Styles in Animals: Current Status in Behavior and Stress-Physiology. *Neurosci. Biobehav. Rev.* 23 (7), 925–935. doi:10.1016/s0149-7634(99)00026-3

Kumai, Y., Nesan, D., Vijayan, M. M., and Perry, S. F. (2012). Cortisol Regulates Na^+ Uptake in Zebrafish, *Danio rerio*, Larvae via the Glucocorticoid Receptor. *Mol. Cell. Endocrinol.* 364 (1-2), 113–125. doi:10.1016/j.mce.2012.08.017

LeBlanc, S., Höglund, E., Gilmour, K. M., and Currie, S. (2012). Hormonal Modulation of the Heat Shock Response: Insights from Fish with Divergent Cortisol Stress Responses. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* 302 (1), R184–R192. doi:10.1152/ajpregu.00196.2011

Lee, H., and Kimelman, D. (2002). A Dominant-Negative Form of P63 Is Required for Epidermal Proliferation in Zebrafish. *Dev. Cell* 2 (5), 607–616. doi:10.1016/S1534-5807(02)00166-1

Lee, D.-W., Choi, Y.-U., Park, H.-S., Park, Y.-S., and Choi, C. Y. (2022). Effect of Low pH and Salinity Conditions on the Antioxidant Response and Hepatocyte Damage in Juvenile Olive Flounder *Paralichthys olivaceus*. *Mar. Environ. Res.* 175, 105562. doi:10.1016/j.marenvres.2022.105562

Lewis, L., and Kwong, R. (2018). Zebrafish as a Model System for Investigating the Compensatory Regulation of Ionic Balance during Metabolic Acidosis. *Int. J. Mol. Sci.* 19 (4), 1087. doi:10.3390/ijms19041087

Li, W., Li, N., Liang, L., Yu, Q., Ren, P., Shi, H., et al. (2019). Regulation of P53 in the Red-Eared Slider (*Trachemys scripta Elegans*) in Response to Salinity Stress. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 221, 49–58. doi:10.1016/j.cbpc.2019.03.011

Lin, C.-H., Kuan, W.-C., Liao, B.-K., Deng, A.-N., Tseng, D.-Y., and Hwang, P.-P. (2016). Environmental and Cortisol-Mediated Control of Ca^{2+} Uptake in tilapia (*Oreochromis mossambicus*). *J. Comp. Physiol. B* 186 (3), 323–332. doi:10.1007/s00360-016-0963-7

Martins, C. I. M., Conceição, L. E. C., and Schrama, J. W. (2011). Consistency of Individual Variation in Feeding Behaviour and its Relationship with Performance Traits in Nile tilapia *Oreochromis niloticus*. *Appl. Animal Behav. Sci.* 133 (1-2), 109–116. doi:10.1016/j.applanim.2011.05.001

Maugars, G., Manirafasha, M.-C., Grousset, E., Boulo, V., and Lignot, J.-H. (2018). The Effects of Acute Transfer to Freshwater on Ion Transporters of the Pharyngeal Cavity in European Seabass (*Dicentrarchus labrax*). *Fish. Physiol. Biochem.* 44 (5), 1393–1408. doi:10.1007/s10695-018-0529-6

McCormick, S. D., and Bradshaw, D. (2006). Hormonal Control of Salt and Water Balance in Vertebrates. *General Comp. Endocrinol.* 147 (1), 3–8. doi:10.1016/j.ygcen.2005.12.009

Mechesso, A. F., Kim, Y. G., and Park, S. C. (2019). Effects of Butaphosphan and Cyanocobalamin Combination on Plasma Immune and Biochemical Parameters of Olive Flounder (*Paralichthys olivaceus*) Subjected to Crowding Stress. *Aquac. Res.* 50, 1611–1617. doi:10.1111/are.14038

Motohashi, E., Hasegawa, S., Mishiro, K., and Ando, H. (2009). Osmoregulatory Responses of Expression of Vasotocin, Isotocin, Prolactin and Growth Hormone Genes Following Hypoosmotic Challenge in a Stenohaline Marine Teleost, Tiger Puffer (*Takifugu rubripes*). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 154 (3), 353–359. doi:10.1016/j.cbpa.2009.07.003

Øverli, Ø., Sørensen, C., and Nilsson, G. E. (2006). Behavioral Indicators of Stress-Coping Style in Rainbow Trout: Do Males and Females React Differently to Novelty? *Physiol. Behav.* 87 (3), 506–512. doi:10.1016/j.physbeh.2005.11.012

Pelis, R. M., and McCormick, S. D. (2001). Effects of Growth Hormone and Cortisol on Na^+–K^+–$2Cl^-$ Cotransporter Localization and Abundance in the Gills of *Atlantic Salmon*. *General Comp. Endocrinol.* 124 (2), 134–143. doi:10.1006/gcen.2001.7703

Prunet, P., Sandra, O., Rouzic, P. L., Marchand, O., and Laudet, V. (2000). Molecular Characterization of the Prolactin Receptor in Two Fish Species, tilapia *Oreochromis niloticus* and Rainbow Trout, *Oncorhynchus mykiss*: A Comparative Approach. *Can. J. Physiol. Pharmacol.* 78 (12), 1086–1096. doi:10.1139/y00-093

Reis, M. I. R., do Vale, A., Pinto, C., Nascimento, D. S., Costa-Ramos, C., Silva, D. S. P., et al. (2007). First Molecular Cloning and Characterisation of Caspase-9 Gene in Fish and its Involvement in a Gram Negative Septicaemia. *Mol. Immunol.* 44 (7), 1754–1764. doi:10.1016/j.molimm.2006.07.293

Rupia, E. J., Binning, S. A., Roche, D. G., and Lu, W. (2016). Fight-flight or Freeze-Hide? Personality and Metabolic Phenotype Mediate Physiological Defence Responses in Flatfish. *J. Anim. Ecol.* 85 (4), 927–937. doi:10.1111/1365-2656.12524

Santos, C. R. A., Ingleton, P. M., Cavaco, J. E. B., Kelly, P. A., Edery, M., and Power, D. M. (2001). Cloning, Characterization, and Tissue Distribution of Prolactin Receptor in the Sea Bream (*Sparus Aurata*). *General Comp. Endocrinol.* 121 (1), 32–47. doi:10.1006/gcen.2000.7553

Seikai, T. (2002). Flounder Culture and its Challenges in Asia. *Rev. Fish. Sci.* 10, 421–432. doi:10.1080/20026491051721

Shu, Y., Lou, Q., Dai, Z., Dai, X., He, J., Hu, W., et al. (2016). The Basal Function of Teleost Prolactin as a Key Regulator on Ion Uptake Identified with Zebrafish Knockout Models. *Sci. Rep.* 6, 18597. doi:10.1038/srep18597

Silva, P. I. M., Martins, C. I. M., Engrola, S., Marino, G., Øverli, Ø., and Conceição, L. E. C. (2010). Individual Differences in Cortisol Levels and Behaviour of Senegalese Sole (*Solea Senegalensis*) Juveniles: Evidence for Coping Styles. *Appl. Animal Behav. Sci.* 124 (1-2), 75–81. doi:10.1016/j.applanim.2010.01.008

Sloman, K. A., Morgan, T. P., McDonald, D. G., and Wood, C. M. (2003). Socially-induced Changes in Sodium Regulation Affect the Uptake of Water-Borne Copper and Silver in the Rainbow Trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 135 (4), 393–403. doi:10.1016/s1532-0456(03)00139-x

Sloman, K. A., Scott, G. R., McDonald, D. G., and Wood, C. M. (2004). Diminished Social Status Affects Ionoregulation at the Gills and Kidney in Rainbow Trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* 61 (4), 618–626. doi:10.1139/f04-032

Takahashi, H., and Sakamoto, T. (2013). The Role of "mineralocorticoids" in Teleost Fish: Relative Importance of Glucocorticoid Signaling in the Osmoregulation and "central"actions of Mineralocorticoid Receptor. *General Comp. Endocrinol.* 181, 223–228. doi:10.1016/j.ygcen.2012.11.016

Takle, H., and Andersen, Ø. (2007). Caspases and Apoptosis in Fish. *J. Fish. Biol.* 71 (sc), 326–349. doi:10.1111/j.1095-8649.2007.01665.x

Tse, D. L. Y., Chow, B. K. C., Chan, C. B., Lee, L. T. O., and Cheng, C. H. K. (2000). Molecular Cloning and Expression Studies of a Prolactin Receptor in Goldfish (*Carassius auratus*). *Life Sci.* 66 (7), 593–605. doi:10.1016/S0024-3205(99)00632-3

Tseng, Y.-C., and Hwang, P.-P. (2008). Some Insights into Energy Metabolism for Osmoregulation in Fish. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 148 (4), 419–429. doi:10.1016/j.cbpc.2008.04.009

Williams, J., Hindell, J. S., Jenkins, G. P., Tracey, S., Hartmann, K., and Swearer, S. E. (2017). The Influence of Freshwater Flows on Two Estuarine Resident Fish Species Show Differential Sensitivity to the Impacts of Drought, Flood and Climate Change. *Environ. Biol. Fish.* 100 (9), 1121–1137. doi:10.1007/s10641-017-0632-z

Yuan, M., Jia, Q., Wang, T., Lu, Q., Tang, L., Wang, Y., et al. (2017). Dynamic Responses of Prolactin, Growth Hormone and Their Receptors to Hyposmotic Acclimation in the Olive Flounder *Paralichthys olivaceus*. *General Comp. Endocrinol.* 254, 8–13. doi:10.1016/j.ygcen.2017.09.005

Zeng, J., Herbert, N. A., and Lu, W. (2019). Differential Coping Strategies in Response to Salinity Challenge in Olive Flounder. *Front. Physiol.* 10, 1378. doi:10.3389/fphys.2019.01378

Zou, H., He, F., Lan, Z., Tang, L., and Lu, W. (2019). The Personality of Japanese Flounder (*Paralichthys olivaceus*) and Gene Expression Related with Osmoregulatory Capacity in the Gills. *Aquaculture* 500, 221–227. doi:10.1016/j.aquaculture.2018.10.013

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Fish Behavior as a Neural Proxy to Reveal Physiological States

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Behaviors are the integrative outcomes of the nervous system, which senses and responds to the internal physiological status and external stimuli. Teleosts are aquatic organisms which are more easily affected by the surrounding environment compared to terrestrial animals. To date, behavioral tests have been widely used to assess potential environmental risks using fish as model animals. In this review, we summarized recent studies regarding the effects of internal and external stimuli on fish behaviors. We concluded that behaviors reflect environmental and physiological changes, which have possible implications for environmental and physiological assessments.

Keywords: behaviors, physiology, environmental acclimation, neural circuits, toxicology

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INTRODUCTION

Maintaining stable internal conditions in the face of environmental fluctuations is important for animals to sustain life. Consequently, animals have evolved strategies to respond and acclimate to environmental changes. The large time scale of acclimation such as morphology, to be selected over generations to fit the animal into the specific niches. With the increase of acclimated flexibility, acclimation happens also in a relatively short time. Physiological and behavioral acclimation provides the mid-term and short-term acclimation, respectively, to confront the changing environments. Revealing the acclimation axis across physiology and behavior could not only delineate a more comprehensive picture of how a niche is determined for the animal but also raise the opportunity to pose the assumption in envisioning how the brain works to coordinate the body as a whole. However, compared to morphology, acclimated physiology and behavior are mostly hidden without a specific design of examination. In addition to the diversity in morphology, the physiological and behavioral diversities could show us the plasticity of brain circuit and abundance of designs for acclimating to the dynamic aquatic environments (Cooper et al., 2013; Salvanes et al., 2013; Sewall 2015; Tseng et al., 2020). The acclimation in a relatively short time scale could be seen as the balance of maneuver (external behavior) and talent (internal physiological acclimation) (Staddon, 2016).

Fish lives in aquatic habits and are more easily affected by the surrounding environments with diverse ion compositions and anthropogenic pollution compared to terrestrial animals (Hwang and Lee, 2007; Busch et al., 2016). Therefore, fish were chosen as animal models for environmental assessments (Scholz et al., 2008; Green and Planchart, 2018). In this review, we summarized the acclimation strategies of behavior and internal autonomous regulation under the several states of homeostasis in teleost. We discussed how the internal regulation co-opts the behavior in acclimation with a general view and extend it to the current understandings of the applications in environmental toxicology.

EFFECTS OF THE INTERNAL STATUS ON BEHAVIORS

Animals tend to maintain conditions that are best for their survival *via* physiological regulation, which is called "homeostasis". Behaviors are integrative outcomes and rapidly respond to

physiological changes for homeostasis. Alteration of physiological status constantly leads to behavioral response. Therefore, the process of physiological regulation can cause behavioral changes.

Effects of Physiological Conditions on Fish Behaviors

Body fluid regulation is essential for organism survival. Hyperosmolality of body fluids activates osmotic-sensing neurons in the vascular organ of the lamina terminalis (VOLT) and subfornical organ (SFO), leading to vasopressin release and a drinking response (Bazyan and Rogal, 2015; Abbott et al., 2016). Recent research indicated that the terminus of drinking behavior is not directly regulated by changes in blood osmolality (Kim et al., 2021). Water intake rapidly reduces the activity of glutamatergic neurons in the SFO to baseline activity and vasopressin levels in the plasma before plasma osmolality recovers, thus preventing hypo-osmolality (Mandelblat-Cerf et al., 2017). Unlike terrestrial animals, most fish can drink surrounding water by a swallowing reflex without inducing a feeling of thirst or water-seeking behaviors. Mudskippers, a kind of amphibian teleost, can use buccal water for gas exchange when on land. Buccal drying motivates them move to water and drink, as occurs with tetrapods, through regulating angiotensin II and vasopressin (Katayama et al., 2018). This suggests a highly conserved osmoregulatory mechanism between teleost fishes and mammals. This case shows nervous system integrate physiological conditions and mediate behavioral outcomes to maintain homeostasis, including in early vertebrates.

Nutrients is necessary for life; thus, animal may be more proactive for survival when limited or depleted nutrients. A shortage of food drives animals to exhibit risky foraging behaviors (Katz et al., 2013; Padilla et al., 2016; Balaban-Feld et al., 2019). Hungry goldfish (*Carassius auratus*) left a group of conspecifics and shelter for foraging opportunities in the presence of a predator (Balaban-Feld et al., 2019). Agouti-related peptide (AgRP) neurons in the ventromedial part of the arcuate nucleus in the hypothalamus respond to energy deficits, activates feeding behavior and regulates metabolism (Belgardt et al., 2009; Berrios et al., 2021). In addition, AgRP expressed neurons send the projection to the brain regions implicated in aggression, fear and stress responses, which might modulate risky food seeking behaviors (Padilla et al., 2016; Shainer et al., 2019). Orexin/hypocretin, a neuropeptide secreted in the hypothalamus, regulates appetite and modulates fighting behavior that increase the winning rate of social conflicts after food deprivation (Kotz et al., 2002; Nakajo et al., 2020). Starvation increases orexin/hypocretin-positive neuron activity in the hypothalamus, which mediates neural plasticity in the dorsal interpeduncular nucleus (dIPN) and potentiates the winner's pathway from the dorsal habenula to the dIPN (Nakajo et al., 2020). Taken together, animals take aggressive behavioral strategies to cope with starvation through the neuroendocrine system.

Behavior patterns during stress acclimation exhibited a property of energy reallocation to enhance behaviors that are more essential to survival. The Hypothalamic–pituitary–adrenal/interrenal (HPA/HPI) axis is main pathway to regulate physiological state and behaviors for coping with stress (Suarez-Bregua et al., 2018; Ganesh, 2021). Various stressor activated the HPA/HPI axis, which induced anxiety like behavior, reduced foraging and reproductive behaviors (Beitinger, 1990; Harris and Carr, 2016; Abreu et al., 2018; Filipsson et al., 2020). Corticotropin-releasing factor (CRF) and cortisol administration inhibited foraging behaviors, including food searching, prey capture, and food intake, in teleost fish (McCormick et al., 1998; Gregory and Wood, 1999; Bernier and Peter, 2001; Bernier et al., 2004). The HPA/HPI axis inhibited food intake may be due to slow energy support during digestion that was not efficient for emergent demands (Sapolsky et al., 2000). Elevated CRF and glucocorticoids inhibited food intake by stimulating leptin production and insulin releasing (Arase et al., 1988; Dallman et al., 1993; Michel and Cabanac, 1999). When animals are under internal unfavorable conditions, they activate and turn on the HPA/HPI axis and subsequently regulate the behavioral patterns to cope with an urgent stress.

Effects of Abnormal Physiological Conditions on Fish Behaviors

Behaviors are integrative outcomes of physiological regulation in responding to external or internal stimuli, and they can also reflect abnormal or inefficient physiological and neural regulation of the disease. Behavioral tests are established and used in clinical medicine for a full diagnosis (Hort et al., 2010; Gossink et al., 2016; Perez-Lloret et al., 2021). Zebrafish and medaka have been used as models to investigate the etiology and pathophysiology of Parkinson's disease (PD). Typically, there are two types of PD models: neurotoxin-induced and gene-based models to reduce dopaminergic neurons and dopamine release to mimic corresponding PD symptoms (Lam et al., 2005; McKinley et al., 2005; Matsui et al., 2012; Vaz et al., 2018; Najib et al., 2020; Razali et al., 2021). PD model fish also showed locomotion deficits, including a reduction in the swimming speed, an increase in the no-moving time, and impairment of touch sensations, which are similar to symptoms of PD patients (Lam et al., 2005; Milanese et al., 2012; Hughes et al., 2020; Robea et al., 2020; Wasel and Freeman, 2020). Thus, these behavioral characteristics may reflect motor function deficits of the central nervous system (CNS) and selective dopaminergic neuron losses.

The immune response may mediate behavioral responses (Dantzer, 2006). It was found that concentrations of cytokines of fish with high avoidance differed from those of low-avoidance fish in a novel-object test (Kirsten et al., 2018a). Bacterin-treated fish showed alterations of brain activities and lowered social preferences and exploratory behaviors toward novel objects (Kirsten et al., 2018b). Tilapia lake virus infection caused decreases in zebrafish locomotion and food intake, abnormal swimming patterns, and histopathological changes in the zebrafish brain (Mojzesz et al., 2021). This suggests that the immune system may mediate defensive responses at the behavioral level to minimize potential risks of infection and reallocate energy to healing. These cases mentioned previously reveal strong connections and high linkages between

physiological states and behavioral changes, and behavioral response could reflect the current physiological states.

EFFECTS OF ENVIRONMENTAL INFLUENCES ON BEHAVIOR

Compared to terrestrial animals, most of fish are ectotherms and live in water, so they may be more easily affected and more sensitive to environmental changes (Hansen et al., 2016; Olusanya and van Zyll de Jong, 2018). The physiological status of fish is directly and rapidly affected by environmental factors, including temperature, ions, gases, and chemicals (Lushchak and Bagnyukova, 2006; Valavanidis et al., 2006; Keen et al., 2017; Tong et al., 2020; Alfonso et al., 2021; Shih et al., 2021). Behavioral responses caused by changes in the physiological status have been widely published. Such studies suggested that environmental fluctuations may cause behavioral alterations through physiological regulation, and behavioral tests have high potential for assessing physiological states and environmental stimuli.

Effects of Global Climate Changes on Fish Behaviors

Behavioral changes often point to underlying physiological alterations that can be used to evaluate ecological risks. Global climate change has attracted much attention recently, and warming and acidification are two very important issues among the multitude of ecosystem-level stressors (Byrne, 2011; IPCC, 2014).

Warm temperatures cause guppy schools to swim closer together and faster under a predator threat (Weetman et al., 1998; Weetman et al., 1999). This behavior might be due to the need for increased feeding at elevated temperatures and may be associated with an increased risk assessment of predators during feeding. In rainbow trout, higher temperatures increased the time spent feeding in risky, open habitats and increased mortality (Biro et al., 2007). Juvenile European sea bass undertake riskier behaviors such as reducing their shoaling cohesiveness, swimming higher above the bottom, and reducing the distance between the shoal and a predator at higher temperatures (Malavasi et al., 2013). Warm temperatures also decrease oxygen solubility and cause a rapid depletion of oxygen levels of fish (Verberk et al., 2011). Oxygen is crucial for metabolism, and the nervous system is very vulnerable to hypoxia. Acute hypoxia caused over 50% of deaths in adult zebrafish in 10 min, and surviving fish showed significant stress responses in the brain (increased cell proliferation and astrocyte numbers) and lower exploratory behaviors at 6 h after recovery (Braga et al., 2013; Lee et al., 2018). A lower responsiveness and impairment of directionality to a startling mechanical stimulus in the escape performance of grey mullet (*Liza aurata*) and European sea-bass (*Dicentrarchus labrax*) were found when exposed to progressive hypoxia (Lefrancois et al., 2005; Lefrançois and Domenici, 2006). To prevent the hypoxia damage, behavioral response may be activated upon hypoxia stress. Chemosensory neuroepithelial cells (NECs) located in primary gill filaments (Dunel-Erb et al., 1982) sensed oxygen level (Jonz et al., 2004) and initiated hyperventilation (Perry et al., 2009). Lower partial pressure of oxygen in water inhibited K^+ permeability, elevated membrane potential, eventually activated voltage-gated Ca^{2+} channel and release neurotransmitter (possibly 5-HT or ACh) from NECs (Jonz et al., 2004; Shakarchi et al., 2013; Porteus et al., 2014; Jonz et al., 2015). Zebrafish larvae rapidly increased their body movements and beating of their pectoral fins to gain more oxygen within few second of a hypoxic stimulus (Erickstad et al., 2015). Low oxygen levels reduced the structure and size of schools to prevent an exacerbation of hypoxic conditions in herring (Domenici et al., 2002) and *C. auratus* (Israeli and Kimmel, 1996). Weakly electric fish (*Marcusenius victoriae* and *Petrocephalus degeni*) decreased the active acquisition of sensory information to save energy in a hypoxic condition (Ackerly et al., 2018; Clarke et al., 2020). Escape responses in fish are facilitated by anaerobically fueled muscles (Domenici and Blake, 1997; Marras et al., 2011; Marras et al., 2013); thus, the behavioral alteration is most probably due to a malfunctioning at the neurosensory level rather than to an impairment of muscle function (Domenici et al., 2007). Avoidance and energy-saving behavioral responses reflects current hypoxia challenges and can possibly be used to predict physiological condition.

Increased anthropogenic CO_2 emissions not only elevate the temperature but also acidify aquatic environments. Water acidification may alter different sensory modalities, especially to olfactory system, which directly exposed and analyzed chemical composition of surrounding environment (Ache and Young, 2005; Briffa et al., 2012). For teleost, the sense of smell plays important roles in feeding and reproduction, predator avoidance. Indeed, many studies reported that water acidification affect olfactory mediated behavior in marine fish (Porteus et al., 2018; Velez et al., 2019). Juvenile reef fish were usually repelled by predator odor and chemical alarm cues (chemical compounds released from the damaged epidermal tissue); however, coral reef damselfish reared under elevated CO_2 became attracted to predator odor (Dixson et al., 2010; Welch et al., 2014) and had reduced aversion to chemical alarm cues (Ferrari et al., 2011; Welch et al., 2014). Juvenile pink salmon reared in high-CO_2 waters exhibited reduced electrophysiological responses to amino acids that are important for identifying natal rivers when they return from the ocean to spawn (Ou et al., 2015). Ocean acidification induced slight changes in Cl^- and/or HCO^{3-} gradients across membranes of neurons altered $GABA_A$ receptor functions and cognition of fish (Evans et al., 2005; Nilsson et al., 2012). Elevated CO_2 decreased the intensity of anti-predator behaviors in juvenile damselfish in response to visual predation risks (Ferrari et al., 2012). Californian rockfish and barramundi showed increased anxiety and sheltering behaviors under high CO_2 levels (Hamilton et al., 2014; Rossi et al., 2015). These studies reveal the changes in olfactory and neural system functioning leads to inappropriate behavioral responses to environmental chemical stimuli.

Effects of Anthropogenic Pollution on Fish Behaviors

Freshwater ecosystems such as rivers, lakes, and wetlands are vulnerable to human pollution (Dudgeon, 2019; Reid et al., 2019).

Pesticides and herbicides used in agriculture can contaminate water through runoff from treated plants and soil (Aktar et al., 2009). Studies in India revealed that more than 90% of water and fish samples contained one and more often several pesticides (Kole et al., 2001). Studies of deltamethrin, an insecticide, found increased glutamate levels in the brains and led to hyperactive social behaviors in adult zebrafish (Lei et al., 2022). Fipronil causes neuronal hyperexcitation by inhibiting GABAergic neurotransmission and induces notochord degeneration and locomotor defects in zebrafish embryos and larvae (Tingle et al., 2003; Stehr et al., 2006). Moreover, a marked decrease of swimming endurance and anxiolytic effects were also found in fipronil-exposed adult zebrafish (Cuenca et al., 2022). The widely used herbicide, acetochlor, increased the time spent in the dark zone, suggesting promotion of anxiolytic behaviors (Huang et al., 2021). These behavioral changes caused by pesticide and herbicide exposure may lessen chances for survival of aquatic animals and threaten their populations as a whole.

Heavy metals from anthropic activities, such as the steel industry, mining activities, and smelters, are widely found in the environment and cause neurotoxicities (Squadrone et al., 2013; Zhong et al., 2018; Jiang et al., 2019). It was found that aluminum accumulates in the nervous system of fish and causes damage to nerve tissues, behavior, and cognition (Closset et al., 2021). Silver impairs social preferences, social recognition, learning, and memory in adult zebrafish (Fu et al., 2021). Cadmium induces CNS impairment and neuroinflammation and impairs social behaviors, escape behaviors, and predator responses in rainbow trout, sea bass, and zebrafish (Sloman et al., 2003; Blechinger et al., 2007; Faucher et al., 2008; Xu et al., 2022). Lead-exposed zebrafish exhibited decreased learning and altered color preferences (Bault et al., 2015; Xu et al., 2016). Mercury exposure causes Hg accumulation in the brain and an altered anxiety status, decreased foraging efficiency, and increased prey capture speed in seabream and fathead minnows (Grippo and Heath, 2003; Amlund et al., 2015; Pereira et al., 2016). Heavy metals caused imbalance between the reactive oxygen species (ROS) and the antioxidants, which increased oxidative stress and induced apoptosis (Karri et al., 2016). The damage on neurovascular system may lead to heavy metals pass through the blood–brain barrier and directly affected brain cells (Olung et al., 2021). In addition, heavy metals disturbed neurotransmission and caused cognition dysfunction in sublethal concentrations (Hayat et al., 2003; Toscano and Guilarte, 2005; Karri et al., 2016). Those studies demonstrated the neurotoxicity of heavy metals on behavioral level.

ENVIRONMENTAL RISK ASSESSMENTS WITH BEHAVIORAL MONITORING

Behaviors are a type of functional physiological organization that enables animals to react and acclimate to complex environmental (external) and physiological (internal) stimuli (Hellou, 2011; Morton et al., 2016). Behavioral response with sublethal pollutant concentrations were reported to be fast and sensitive biomarkers for water quality, ecotoxicological and chemical risk assessments (Hellou, 2011; Melvin and Wilson, 2013; Pyle and Ford, 2017; Legradi et al., 2018; Xia et al., 2018). Some behavioral outcomes have been measured in contaminant-exposed fish, including swimming speeds, startle responses, prey capture abilities, learning, and memory, which related to the probability of escaping a predator or capturing prey for feeding (Legradi et al., 2018; Bownik and Wlodkowic, 2021; Fitzgerald et al., 2021). Considering the increasing numbers of environmental contaminants and their unknown neurotoxic potentials, identifying and improving neurotoxicity testing strategies and methods are important for eco-neurotoxicity assessments in the future (Pyle and Ford, 2017; Legradi et al., 2018; Xia et al., 2018; Fitzgerald et al., 2021).

CONCLUDING REMARKS

In conclusion, behaviors are integrative outcomes of physiological responses in responding to internal or external stimuli and they also reflect abnormal physiological states (**Supplementary Table S1**). Thus, it is possible to implicate the alteration of behavioral patterns for environmental and physiological assessments.

AUTHOR CONTRIBUTIONS

M-YC: Conceptualization, Investigation, Writing—original draft, Supervision, Project administration, Funding acquisition. C-WF: Investigation, Writing—original draft. J-LH: Writing—original draft, Investigation, Supervision. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

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REFERENCES

Abbott, S. B. G., Machado, N. L. S., Geerling, J. C., and Saper, C. B. (2016). Reciprocal Control of Drinking Behavior by Median Preoptic Neurons in Mice. *J. Neurosci.* 36, 8228–8237. doi:10.1523/jneurosci.1244-16.2016

Abreu, M. S., Oliveira, T. A., Koakoski, G., Barreto, R. E., and Barcellos, L. J. G. (2018). Modulation of Cortisol Responses to an Acute Stressor in Zebrafish Visually Exposed to Heterospecific Fish during Development. *Zebrafish* 15, 228–233. doi:10.1089/zeb.2017.1509

Ache, B. W., and Young, J. M. (2005). Olfaction: Diverse Species, Conserved Principles. *Neuron* 48, 417–430. doi:10.1016/j.neuron.2005.10.022

Ackerly, K. L., Krahe, R., Sanford, C. P., and Chapman, L. J. (2018). Effects of Hypoxia on Swimming and Sensing in a Weakly Electric Fish. *J. Exp. Biol.* 221 (14), jeb172130. doi:10.1242/jeb172130

Aktar, M. W., Sengupta, D., and Chowdhury, A. (2009). Impact of Pesticides Use in Agriculture: Their Benefits and Hazards. *Interdiscip. Toxicol.* 2, 1–12. doi:10.2478/v10102-009-0001-7

Alfonso, S., Gesto, M., and Sadoul, B. (2021). Temperature Increase and its Effects on Fish Stress Physiology in the Context of Global Warming. *J. Fish. Biol.* 98, 1496–1508. doi:10.1111/jfb.14599

Amlund, H., Lundebye, A.-K., Boyle, D., and Ellingsen, S. (2015). Dietary Selenomethionine Influences the Accumulation and Depuration of Dietary Methylmercury in Zebrafish (*Danio rerio*). *Aquat. Toxicol.* 158, 211–217. doi:10.1016/j.aquatox.2014.11.010

Arase, K., York, D. A., Shimizu, H., Shargill, N., and Bray, G. A. (1988). Effects of Corticotropin-Releasing Factor on Food Intake and Brown Adipose Tissue Thermogenesis in Rats. *Am. J. Physiology-Endocrinology Metabolism* 255, E255–E259. doi:10.1152/ajpendo.1988.255.3.e255

Balaban-Feld, J., Mitchell, W. A., Kotler, B. P., Vijayan, S., Tov Elem, L. T., and Abramsky, Z. (2019). State-dependent Foraging Among Social Fish in a Risky Environment. *Oecologia* 190, 37–45. doi:10.1007/s00442-019-04395-z

Bault, Z. A., Peterson, S. M., and Freeman, J. L. (2015). Directional and Color Preference in Adult Zebrafish: Implications in Behavioral and Learning Assays in Neurotoxicology Studies. *J. Appl. Toxicol.* 35, 1502–1510. doi:10.1002/jat.3169

Bazyan, A. S., and Rogal, A. V. (2015). Neurochemical Mechanisms of a Need, Motivation and Goal-Directed Behavior. *Usp. Fiziol. Nauk.* 46, 3–21.

Beitinger, T. L. (1990). Behavioral Reactions for the Assessment of Stress in Fishes. *J. Gt. Lakes. Res.* 16, 495–528. doi:10.1016/s0380-1330(90)71443-8

Belgardt, B. F., Okamura, T., and Brüning, J. C. (2009). Hormone and Glucose Signalling in POMC and AgRP Neurons. *J. Physiol. (Lond)* 587, 5305–5314. doi:10.1113/jphysiol.2009.179192

Bernier, N. J., Bedard, N., and Peter, R. E. (2004). Effects of Cortisol on Food Intake, Growth, and Forebrain Neuropeptide Y and Corticotropin-Releasing Factor Gene Expression in Goldfish. *General Comp. Endocrinol.* 135, 230–240. doi:10.1016/j.ygcen.2003.09.016

Bernier, N. J., and Peter, R. E. (2001). The Hypothalamic-Pituitary-Interrenal axis and the Control of Food Intake in Teleost Fish. *Comp. Biochem. Physiology Part B Biochem. Mol. Biol.* 129, 639–644. doi:10.1016/s1096-4959(01)00360-8

Berrios, J., Li, C., Madara, J. C., Garfield, A. S., Steger, J. S., Krashes, M. J., et al. (2021). Food Cue Regulation of AGRP Hunger Neurons Guides Learning. *Nature* 595, 695–700. doi:10.1038/s41586-021-03729-3

Biro, P. A., Post, J. R., and Booth, D. J. (2007). Mechanisms for Climate-Induced Mortality of Fish Populations in Whole-Lake Experiments. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9715–9719. doi:10.1073/pnas.0701638104

Blechinger, S. R., Kusch, R. C., Haugo, K., Matz, C., Chivers, D. P., and Krone, P. H. (2007). Brief Embryonic Cadmium Exposure Induces a Stress Response and Cell Death in the Developing Olfactory System Followed by Long-Term Olfactory Deficits in Juvenile Zebrafish. *Toxicol. Appl. Pharmacol.* 224, 72–80. doi:10.1016/j.taap.2007.06.025

Bownik, A., and Wlodkowic, D. (2021). Applications of Advanced Neuro-Behavioral Analysis Strategies in Aquatic Ecotoxicology. *Sci. Total Environ.* 772, 145577. doi:10.1016/j.scitotenv.2021.145577

Braga, M. M., Rico, E. P., Córdova, S. D., Pinto, C. B., Blaser, R. E., Dias, R. D., et al. (2013). Evaluation of spontaneous recovery of behavioral and brain injury profiles in zebrafish after hypoxia. *Behav. Brain Res.* 253, 145–151.

Briffa, M., de la Haye, K., and Munday, P. L. (2012). High CO2 and Marine Animal Behaviour: Potential Mechanisms and Ecological Consequences. *Mar. Pollut. Bull.* 64, 1519–1528. doi:10.1016/j.marpolbul.2012.05.032

Busch, W., Schmidt, S., Kühne, R., Schulze, T., Krauss, M., and Altenburger, R. (2016). Micropollutants in European Rivers: A Mode of Action Survey to Support the Development of Effect-Based Tools for Water Monitoring. *Environ. Toxicol. Chem.* 35, 1887–1899. doi:10.1002/etc.3460

Byrne, M. (2011). in *Impact of Ocean Warming and Ocean Acidification on Marine Invertebrate Life History Stages: Vulnerabilities and Potential for Persistence in a Changing Ocean" in Oceanography and Marine Biology*. Editors R. N. Gibson, R. J. A. Atkinson, and J. D. M. Gordon (Boca Raton: CRC Press), 1–42.

Clarke, S. B., Chapman, L. J., and Krahe, R. (2020). The Effect of Normoxia Exposure on Hypoxia Tolerance and Sensory Sampling in a Swamp-Dwelling Mormyrid Fish. *Comp Biochem Physiol, Part A Mol Integr Physiol* 240, 110586.

Closset, M., Cailliau, K., Slaby, S., and Marin, M. (2021). Effects of Aluminium Contamination on the Nervous System of Freshwater Aquatic Vertebrates: A Review. *Int. J. Mol. Sci.* 23. doi:10.3390/ijms23010031

Cooper, C. A., Wilson, J. M., and Wright, P. A. (2013). Marine, Freshwater and Aerially Acclimated Mangrove rivulus (Kryptolebias Marmoratus) Use Different Strategies for Cutaneous Ammonia Excretion. *Am. J. Physiology-Regulatory, Integr. Comp. Physiology* 304, R599–R612. doi:10.1152/ajpregu.00228.2012

Cuenca, A. L. R., Simonato, J. D., and Meletti, P. C. (2022). Acute Exposure of Embryo, Larvae and Adults of *Danio rerio* to Fipronil Commercial Formulation Reveals Effects on Development and Motor Control. *Ecotoxicology* 31, 114–123. doi:10.1007/s10646-021-02497-0

Dallman, M. F., Strack, A. M., Akana, S. F., Bradbury, M. J., Hanson, E. S., Scribner, K. A., et al. (1993). Feast and Famine: Critical Role of Glucocorticoids with Insulin in Daily Energy Flow. *Front. Neuroendocrinol.* 14, 303–347. doi:10.1006/frne.1993.1010

Dantzer, R. (2006). Cytokine, Sickness Behavior, and Depression. *Neurol. Clin.* 24, 441–460. doi:10.1016/j.ncl.2006.03.003

Dixson, D. L., Munday, P. L., and Jones, G. P. (2010). Ocean Acidification Disrupts the Innate Ability of Fish to Detect Predator Olfactory Cues. *Ecol. Lett.* 13, 68–75. doi:10.1111/j.1461-0248.2009.01400.x

Domenici, P., and Blake, R. (1997). The Kinematics and Performance of Fish Fast-Start Swimming. *J. Exp. Biol.* 200, 1165–1178.

Domenici, P., Ferrari, R. S., Steffensen, J. F., and Batty, R. S. (2002). The Effect of Progressive Hypoxia on School Structure and Dynamics in Atlantic Herring Clupea Harengus. *Proc. Biol. Sci.* 269, 2103–2111.

Domenici, P., Lefrançois, C., and Shingles, A. (2007). Hypoxia and the Antipredator Behaviours of Fishes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 362, 2105–2121.

Dudgeon, D. (2019). Multiple Threats Imperil Freshwater Biodiversity in the Anthropocene. *Curr. Biol.* 29, R960–R967. doi:10.1016/j.cub.2019.08.002

Dunel-Erb, S., Bailly, Y., and Laurent, P. (1982). Neuroepithelial Cells in Fish Gill Primary Lamellae. *J. Appl. Physiology* 53, 1342–1353. doi:10.1152/jappl.1982.53.6.1342

Erickstad, M., Hale, L. A., Chalasani, S. H., and Groisman, A. (2015). A Microfluidic System for Studying the Behavior of Zebrafish Larvae Under Acute Hypoxia. *Lab. Chip.* 15, 857–866.

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste. *Physiol. Rev.* 85, 97–177. doi:10.1152/physrev.00050.2003

Faucher, K., Fichet, D., Miramand, P., and Lagardère, J.-P. (2008). Impact of Chronic Cadmium Exposure at Environmental Dose on Escape Behaviour in Sea Bass (*Dicentrarchus labrax* L.; Teleostei, Moronidae). *Environ. Pollut.* 151, 148–157. doi:10.1016/j.envpol.2007.02.017

Ferrari, M. C. O., Dixson, D. L., Munday, P. L., McCORMICK, M. I., Meekan, M. G., Sih, A., et al. (2011). Intrageneric Variation in Antipredator Responses of Coral Reef Fishes Affected by Ocean Acidification: Implications for Climate Change Projections on Marine Communities. *Glob. Chang. Biol.* 17, 2980–2986. doi:10.1111/j.1365-2486.2011.02439.x

Ferrari, M. C. O., McCormick, M. I., Munday, P. L., Meekan, M. G., Dixson, D. L., Lönnstedt, O., et al. (2012). Effects of Ocean Acidification on Visual Risk Assessment in Coral Reef Fishes. *Funct. Ecol.* 26, 553–558. doi:10.1111/j.1365-2435.2011.01951.x

Filipsson, K., Bergman, E., Greenberg, L., Österling, M., Watz, J., and Erlandsson, A. (2020). Temperature and Predator-Mediated Regulation of Plasma Cortisol and Brain Gene Expression in Juvenile Brown Trout (*Salmo trutta*). *Front. Zool.* 17, 25. doi:10.1186/s12983-020-00372-y

Fitzgerald, J. A., Könemann, S., Krümpelmann, L., Županič, A., and Berg, C. (2021). Approaches to Test the Neurotoxicity of Environmental Contaminants in the Zebrafish Model: from Behavior to Molecular Mechanisms. *Environ. Toxicol. Chem.* 40, 989–1006. doi:10.1002/etc.4951

Fu, C.-W., Horng, J.-L., Tong, S.-K., Cherng, B.-W., Liao, B.-K., Lin, L.-Y., et al. (2021). Exposure to Silver Impairs Learning and Social Behaviors in Adult Zebrafish. *J. Hazard. Mater.* 403, 124031. doi:10.1016/j.jhazmat.2020.124031

Ganesh, C. B. (2021). The Stress - Reproductive axis in Fish: The Involvement of Functional Neuroanatomical Systems in the Brain. *J. Chem. Neuroanat.* 112, 101904. doi:10.1016/j.jchemneu.2020.101904

Gossink, F. T., Dols, A., Kerssens, C. J., Krudop, W. A., Kerklaan, B. J., Scheltens, P., et al. (2016). Psychiatric Diagnoses Underlying the Phenocopy Syndrome of Behavioural Variant Frontotemporal Dementia. *J. Neurol. Neurosurg. Psychiatry* 87, 64–68. doi:10.1136/jnnp-2014-308284

Green, A. J., and Planchart, A. (2018). The Neurological Toxicity of Heavy Metals: A Fish Perspective. *Comp. Biochem. Physiology Part C Toxicol. Pharmacol.* 208, 12–19. doi:10.1016/j.cbpc.2017.11.008

Gregory, T. R., and Wood, C. M. (1999). The Effects of Chronic Plasma Cortisol Elevation on the Feeding Behaviour, Growth, Competitive Ability, and Swimming Performance of Juvenile Rainbow Trout. *Physiological Biochem. Zoology* 72, 286–295. doi:10.1086/316673

Grippo, M. A., and Heath, A. G. (2003). The Effect of Mercury on the Feeding Behavior of Fathead Minnows (*Pimephales promelas*). *Ecotoxicol. Environ. Saf.* 55, 187–198. doi:10.1016/s0147-6513(02)00071-4

Hamilton, T. J., Holcombe, A., and Tresguerres, M. (2014). CO 2 -induced Ocean Acidification Increases Anxiety in Rockfish via Alteration of GABA A Receptor Functioning. *Proc. R. Soc. B* 281, 20132509. doi:10.1098/rspb.2013.2509

Hansen, M. J., Morrell, L. J., and Ward, A. J. W. (2016). The Effect of Temporally Variable Environmental Stimuli and Group Size on Emergence Behavior. *Behav. Ecol.* 27, 939–945.

Harris, B. N., and Carr, J. A. (2016). The Role of the Hypothalamus-Pituitary-Adrenal/interrenal axis in Mediating Predator-Avoidance Trade-Offs. *General Comp. Endocrinol.* 230-231, 110–142. doi:10.1016/j.ygcen.2016.04.006

Hayat, S., Wigley, C. B., and Robbins, J. (2003). Intracellular Calcium Handling in Rat Olfactory Ensheathing Cells and its Role in Axonal Regeneration. *Mol. Cell. Neurosci.* 22, 259–270. doi:10.1016/s1044-7431(03)00051-4

Hellou, J. (2011). Behavioural Ecotoxicology, an "Early Warning" Signal to Assess Environmental Quality. *Environ. Sci. Pollut. Res.* 18, 1–11. doi:10.1007/s11356-010-0367-2

Hort, J., O'Brien, J. T., Gainotti, G., Pirttila, T., Popescu, B. O., Rektorova, I., et al.EFNS Scientist Panel on Dementia (2010). EFNS Guidelines for the Diagnosis and Management of Alzheimer's Disease. *Eur. J. Neurol.* 17, 1236–1248. doi:10.1111/j.1468-1331.2010.03040.x

Huang, T., Wang, S., Souders, C. L., Ivantsova, E., Wengrovitz, A., Ganter, J., et al. (2021). Exposure to Acetochlor Impairs Swim Bladder Formation, Induces Heat Shock Protein Expression, and Promotes Locomotor Activity in Zebrafish (*Danio rerio*) Larvae. *Ecotoxicol. Environ. Saf.* 228, 112978. doi:10.1016/j.ecoenv.2021.112978

Hughes, G. L., Lones, M. A., Bedder, M., Currie, P. D., Smith, S. L., and Pownall, M. E. (2020). Machine Learning Discriminates a Movement Disorder in a Zebrafish Model of Parkinson's Disease. *Dis. Model. Mech.* 13. doi:10.1242/dmm.045815

Hwang, P.-P., and Lee, T.-H. (2007). New Insights into Fish Ion Regulation and Mitochondrion-Rich Cells. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 148, 479–497. doi:10.1016/j.cbpa.2007.06.416

IPCCCore Writing Team (2014). "Climate Change 2014: Synthesis Report," in *Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Inter-govern-mental Panel on Climate Change* (Geneva, Switzerland: IPCC), 151.

Israeli, D., and Kimmel, E. (1996). Monitoring the Behavior of Hypoxia-Stressed *Carassius auratus* Using Computer Vision. *Aquac. Eng.* 15, 423–440. doi:10.1016/s0144-8609(96)01009-6

Jiang, D., Wang, Y., Zhou, S., Long, Z., Liao, Q., Yang, J., et al. (2019). Multivariate Analyses and Human Health Assessments of Heavy Metals for Surface Water Quality in the Xiangjiang River Basin, china. *Enviro Toxic Chem.* 38, 1645–1657. doi:10.1002/etc.4461

Jonz, M. G., Fearon, I. M., and Nurse, C. A. (2004). Neuroepithelial Oxygen Chemoreceptors of the Zebrafish Gill. *J. Physiol. (Lond)* 560, 737–752. doi:10.1113/jphysiol.2004.069294

Jonz, M. G., Zachar, P. C., Da Fonte, D. F., and Mierzwa, A. S. (2015). Peripheral Chemoreceptors in Fish: A Brief History and a Look Ahead. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 186, 27–38. doi:10.1016/j.cbpa.2014.09.002

Karri, V., Schuhmacher, M., and Kumar, V. (2016). Heavy Metals (Pb, Cd, as and MeHg) as Risk Factors for Cognitive Dysfunction: A General Review of Metal Mixture Mechanism in Brain. *Environ. Toxicol. Pharmacol.* 48, 203–213. doi:10.1016/j.etap.2016.09.016

Katayama, Y., Sakamoto, T., Saito, K., Tsuchimochi, H., Kaiya, H., Watanabe, T., et al. (2018). Drinking by Amphibious Fish: Convergent Evolution of Thirst Mechanisms during Vertebrate Terrestrialization. *Sci. Rep.* 8, 625. doi:10.1038/s41598-017-18611-4

Katz, M. W., Abramsky, Z., Kotler, B. P., Rosenzweig, M. L., Alteshtein, O., and Vasserman, G. (2013). Optimal Foraging of Little Egrets and Their Prey in a Foraging Game in a Patchy Environment. *Am. Nat.* 181, 381–395. doi:10.1086/669156

Keen, A. N., Klaiman, J. M., Shiels, H. A., and Gillis, T. E. (2017). Temperature-induced Cardiac Remodelling in Fish. *J. Exp. Biol.* 220, 147–160. doi:10.1242/jeb.128496

Kim, A., Madara, J. C., Wu, C., Andermann, M. L., and Lowell, B. B. (2021). Neural Basis for Regulation of Vasopressin Secretion by Anticipated Disturbances in Osmolality. *eLife* 10. doi:10.7554/elife.66609

Kirsten, K., Fior, D., Kreutz, L. C., and Barcellos, L. J. G. (2018a). First Description of Behavior and Immune System Relationship in Fish. *Sci. Rep.* 8, 846. doi:10.1038/s41598-018-19276-3

Kirsten, K., Soares, S. M., Koakoski, G., Carlos Kreutz, L., and Barcellos, L. J. G. (2018b). Characterization of Sickness Behavior in Zebrafish. *Brain, Behav. Immun.* 73, 596–602. doi:10.1016/j.bbi.2018.07.004

Kole, R. K., Banerjee, H., and Bhattacharyya, A. (2001). Monitoring of Market Fish Samples for Endosulfan and Hexachlorocyclohexane Residues in and Around Calcutta. *Bull. Environ. Contam. Toxicol.* 67, 554–559. doi:10.1007/s001280159

Kotz, C. M., Teske, J. A., Levine, J. A., and Wang, C. (2002). Feeding and Activity Induced by Orexin A in the Lateral Hypothalamus in Rats. *Regul. Pept.* 104, 27–32. doi:10.1016/s0167-0115(01)00346-9

Lam, C. S., Korzh, V., and Strahle, U. (2005). Zebrafish Embryos Are Susceptible to the Dopaminergic Neurotoxin MPTP. *Eur. J. Neurosci.* 21, 1758–1762. doi:10.1111/j.1460-9568.2005.03988.x

Lee, Y., Lee, S., Park, J. -W., Hwang, J. -S., Kim, S. -M., Lyoo, I. K., Lee, C. -J., and Han, I. -O. (2018). Hypoxia-Induced Neuroinflammation and Learning-Memory Impairments in Adult Zebrafish are Suppressed by Glucosamine. *Mol. Neurobiol.* 55, 8738–8753.

Lefrancois, C., Shingles, A., and Domenici, P. (2005). The Effect of Hypoxia on Locomotor Performance and Behaviour During Escape in Liza Aurata. *J. Fish Biol.* 67, 1711–1729.

Lefrancois, C., and Domenici, P. (2006). Locomotor Kinematics and Behaviour in the Escape Response of European Sea Bass, *Dicentrarchus labrax* L., Exposed to Hypoxia . *Mar. Biol.* 149, 969–977.

Legradi, J. B., Di Paolo, C., Kraak, M. H. S., van der Geest, H. G., Schymanski, E. L., Williams, A. J., et al.fnm (2018). An Ecotoxicological View on Neurotoxicity Assessment. *Environ. Sci. Eur.* 30, 46.doi:10.1186/s12302-018-0173-x

Lei, L., Zhu, B., Qiao, K., Zhou, Y., Chen, X., Men, J., et al. (2022). New Evidence for Neurobehavioral Toxicity of Deltamethrin at Environmentally Relevant Levels in Zebrafish. *Sci. Total Environ.* 822, 153623. doi:10.1016/j.scitotenv.2022.153623

Lushchak, V. I., and Bagnyukova, T. V. (2006). Effects of Different Environmental Oxygen Levels on Free Radical Processes in Fish. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* 144, 283–289. doi:10.1016/j.cbpb.2006.02.014

Malavasi, S., Cipolato, G., Cioni, C., Torricelli, P., Alleva, E., Manciocco, A., et al. (2013). Effects of Temperature on the Antipredator Behaviour and on the Cholinergic Expression in the European Sea Bass (*Dicentrarchus labrax* L.) Juveniles. *Ethology* 119, 592–604. doi:10.1111/eth.12100

Mandelblat-Cerf, Y., Kim, A., Burgess, C. R., Subramanian, S., Tannous, B. A., Lowell, B. B., et al. (2017). Bidirectional Anticipation of Future Osmotic Challenges by Vasopressin Neurons. *Neuron* 93, 57–65. doi:10.1016/j.neuron.2016.11.021

Marras, S., Killen, S. S., Claireaux, G., Domenici, P., and McKenzie, D. J. (2011). Behavioural and Kinematic Components of the Fast-Start Escape Response in Fish: Individual Variation and Temporal Repeatability. *J. Exp. Biol.* 214, 3102–3110.

Marras, S., Killen, S. S., Domenici, P., Claireaux, G., and McKenzie, D. J. (2013). Relationships Among Traits of Aerobic and Anaerobic Swimming Performance in Individual European Sea Bass *Dicentrarchus labrax*. *PLoS ONE* 8, e72815.

Matsui, H., Gavinio, R., and Takahashi, R. (2012). Medaka Fish Parkinson's Disease Model. *Exp. Neurobiol.* 21, 94–100. doi:10.5607/en.2012.21.3.94

McCormick, S. D., Shrimpton, J. M., Carey, J. B., O'Dea, M. F., Sloan, K. E., Moriyama, S., et al. (1998). Repeated Acute Stress Reduces Growth Rate of Atlantic Salmon Parr and Alters Plasma Levels of Growth Hormone, Insulin-like Growth Factor I and Cortisol. *Aquaculture* 168, 221–235. doi:10.1016/s0044-8486(98)00351-2

McKinley, E. T., Baranowski, T. C., Blavo, D. O., Cato, C., Doan, T. N., and Rubinstein, A. L. (2005). Neuroprotection of MPTP-Induced Toxicity in Zebrafish Dopaminergic Neurons. *Brain Res. Mol. Brain Res.* 141, 128–137. doi:10.1016/j.molbrainres.2005.08.014

Melvin, S. D., and Wilson, S. P. (2013). The Utility of Behavioral Studies for Aquatic Toxicology Testing: a Meta-Analysis. *Chemosphere* 93, 2217–2223. doi:10.1016/j.chemosphere.2013.07.036

Michel, C., and Cabanac, M. (1999). Effects of Dexamethasone on the Body Weight Set Point of Rats. *Physiol. Behav.* 68, 145–150. doi:10.1016/s0031-9384(99)00159-6

Milanese, C., Sager, J. J., Bai, Q., Farrell, T. C., Cannon, J. R., Greenamyre, J. T., et al. (2012). Hypokinesia and Reduced Dopamine Levels in Zebrafish Lacking β- and γ1-synucleins. *J. Biol. Chem.* 287, 2971–2983. doi:10.1074/jbc.m111.308312

Mojzesz, M., Widziolek, M., Adamek, M., Orzechowska, U., Podlasz, P., Prajsnar, T. K., et al. (2021). Tilapia Lake Virus-Induced Neuroinflammation in Zebrafish: Microglia Activation and Sickness Behavior. *Front. Immunol.* 12, 760882. doi:10.3389/fimmu.2021.760882

Morton, F. B., Brosnan, S. F., Prétôt, L., Buchanan-Smith, H. M., O'Sullivan, E., Stocker, M., et al. (2016). Using Photographs to Study Animal Social Cognition and Behaviour: Do Capuchins' Responses to Photos Reflect Reality? *Behav. Process.* 124, 38–46. doi:10.1016/j.beproc.2015.10.005

Najib, N. H. M., Nies, Y. H., Abd Halim, S. A. S., Yahaya, M. F., Das, S., Lim, W. L., et al. (2020). Modeling Parkinson's Disease in Zebrafish. *CNS Neurol. Disord. Drug Targets* 19, 386–399. doi:10.2174/1871527319666200708124117

Nakajo, H., Chou, M.-Y., Kinoshita, M., Appelbaum, L., Shimazaki, H., Tsuboi, T., et al. (2020). Hunger Potentiates the Habenular Winner Pathway for Social Conflict by Orexin-Promoted Biased Alternative Splicing of the AMPA Receptor Gene. *Cell. Rep.* 31, 107790. doi:10.1016/j.celrep.2020.107790

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sørensen, C., Watson, S.-A., et al. (2012). Near-future Carbon Dioxide Levels Alter Fish Behaviour by Interfering with Neurotransmitter Function. *Nat. Clim. Chang.* 2, 201–204. doi:10.1038/nclimate1352

Olung, N. F., Aluko, O. M., Jeje, S. O., Adeagbo, A. S., and Ijomone, O. M. (2021). Vascular Dysfunction in the Brain; Implications for Heavy Metal Exposures. *Curr. Hypertens. Rev.* 17, 5–13. doi:10.2174/1573402117666210225085528

Olusanya, H. O., and van Zyll de Jong, M. (2018). Assessing the Vulnerability of Freshwater Fishes to Climate Change in Newfoundland and Labrador. *PLoS ONE* 13, e0208182.

Ou, M., Hamilton, T. J., Eom, J., Lyall, E. M., Gallup, J., Jiang, A., et al. (2015). Responses of Pink Salmon to CO2-induced Aquatic Acidification. *Nat. Clim. Chang.* 5, 950–955. doi:10.1038/nclimate2694

Padilla, S. L., Qiu, J., Soden, M. E., Sanz, E., Nestor, C. C., Barker, F. D., et al. (2016). Agouti-related Peptide Neural Circuits Mediate Adaptive Behaviors in the Starved State. *Nat. Neurosci.* 19, 734–741. doi:10.1038/nn.4274

Pereira, P., Puga, S., Cardoso, V., Pinto-Ribeiro, F., Raimundo, J., Barata, M., et al. (2016). Inorganic Mercury Accumulation in Brain Following Waterborne Exposure Elicits a Deficit on the Number of Brain Cells and Impairs Swimming Behavior in Fish (White seabream-Diplodus sargus). *Aquat. Toxicol.* 170, 400–412. doi:10.1016/j.aquatox.2015.11.031

Perez-Lloret, S., van de Warrenburg, B., Rossi, M., Rodríguez-Blázquez, C., Zesiewicz, T., Saute, J. A. M., et al. (2021). Assessment of Ataxia Rating Scales and Cerebellar Functional Tests: Critique and Recommendations. *Mov. Disord.* 36, 283–297. doi:10.1002/mds.28313

Porteus, C. S., Abdallah, S. J., Pollack, J., Kumai, Y., Kwong, R. W. M., Yew, H. M., et al. (2014). The Role of Hydrogen Sulphide in the Control of Breathing in Hypoxic Zebrafish (*Danio rerio*). *J. Physiol. (Lond)* 592, 3075–3088. doi:10.1113/jphysiol.2014.271098

Porteus, C. S., Hubbard, P. C., Uren Webster, T. M., van Aerle, R., Canário, A. V. M., Santos, E. M., et al. (2018). Near-future CO2 Levels Impair the Olfactory System of a Marine Fish. *Nat. Clim. Chang.* 8, 737–743. doi:10.1038/s41558-018-0224-8

Pyle, G., and Ford, A. T. (2017). Behaviour Revised: Contaminant Effects on Aquatic Animal Behaviour. *Aquat. Toxicol.* 182, 226–228. doi:10.1016/j.aquatox.2016.11.008

Razali, K., Othman, N., Mohd Nasir, M. H., Doolaanea, A. A., Kumar, J., Ibrahim, W. N., et al. (2021). The Promise of the Zebrafish Model for Parkinson's Disease: Today's Science and Tomorrow's Treatment. *Front. Genet.* 12, 655550. doi:10.3389/fgene.2021.655550

Reid, A. J., Carlson, A. K., Creed, I. F., Eliason, E. J., Gell, P. A., Johnson, P. T. J., et al. (2019). Emerging Threats and Persistent Conservation Challenges for Freshwater Biodiversity. *Biol. Rev. Camb. Philos. Soc.* 94, 849–873. doi:10.1111/brv.12480

Robea, M.-A., Balmus, I.-M., Ciobica, A., Strungaru, S., Plavan, G., Gorgan, L. D., et al. (2020). Parkinson's Disease-Induced Zebrafish Models: Focussing on Oxidative Stress Implications and Sleep Processes. *Oxid. Med. Cell. Longev.* 2020, 1370837. doi:10.1155/2020/1370837

Rossi, T., Nagelkerken, I., Simpson, S. D., Pistevos, J. C. A., Watson, S.-A., Merillet, L., et al. (2015). Ocean Acidification Boosts Larval Fish Development but Reduces the Window of Opportunity for Successful Settlement. *Proc. Biol. Sci.* 282, 20151954. doi:10.1098/rspb.2015.1954

Salvanes, A. G. V., Moberg, O., Ebbesson, L. O. E., Nilsen, T. O., Jensen, K. H., and Braithwaite, V. A. (2013). Environmental Enrichment Promotes Neural Plasticity and Cognitive Ability in Fish. *Proc. Biol. Sci.* 280, 20131331. doi:10.1098/rspb.2013.1331

Sapolsky, R. M., Romero, L. M., and Munck, A. U. (2000). How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions. *Endocr. Rev.* 21, 55–89. doi:10.1210/edrv.21.1.0389

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., and Voelker, D. (2008). The Zebrafish Embryo Model in Environmental Risk Assessment-Aapplications beyond Acute Toxicity Testing. *Environ. Sci. Pollut. Res. Int.* 15, 394–404. doi:10.1007/s11356-008-0018-z

Sewall, K. B. (2015). Social Complexity as a Driver of Communication and Cognition. *Integr. Comp. Biol.* 55, 384–395. doi:10.1093/icb/icv064

Shainer, I., Michel, M., Marquart, G. D., Bhandiwad, A. A., Zmora, N., Ben-Moshe Livne, Z., et al. (2019). Agouti-Related Protein 2 Is a New Player in the Teleost Stress Response System. *Curr. Biol.* 29, 2009–2019. e7. doi:10.1016/j.cub.2019.05.021

Shakarchi, K., Zachar, P. C., and Jonz, M. G. (2013). Serotonergic and Cholinergic Elements of the Hypoxic Ventilatory Response in Developing Zebrafish. *J. Exp. Biol.* 216, 869–880. doi:10.1242/jeb.079657

Shih, S.-W., Yan, J.-J., Wang, Y.-H., Tsou, Y.-L., Chiu, L., Tseng, Y.-C., et al. (2021). Estrogen-related Receptor γ2 Controls NaCl Uptake to Maintain Ionic Homeostasis. *J. Endocrinol.* 251, 149–159. doi:10.1530/joe-21-0112

Sloman, K. A., Scott, G. R., Diao, Z., Rouleau, C., Wood, C. M., and McDonald, D. G. (2003). Cadmium Affects the Social Behaviour of Rainbow Trout, *Oncorhynchus mykiss*. *Aquat. Toxicol.* 65, 171–185. doi:10.1016/s0166-445x(03)00122-x

Squadrone, S., Prearo, M., Brizio, P., Gavinelli, S., Pellegrino, M., Scanzio, T., et al. (2013). Heavy Metals Distribution in Muscle, Liver, Kidney and Gill of European Catfish (Silurus Glanis) from Italian Rivers. *Chemosphere* 90, 358–365. doi:10.1016/j.chemosphere.2012.07.028

Staddon, J. E. R. (2016). *Adaptive Behavior and Learning*. Cambridge: Cambridge University Press.

Stehr, C. M., Linbo, T. L., Incardona, J. P., and Scholz, N. L. (2006). The Developmental Neurotoxicity of Fipronil: Notochord Degeneration and Locomotor Defects in Zebrafish Embryos and Larvae. *Toxicol. Sci.* 92, 270–278. doi:10.1093/toxsci/kfj185

Suarez-Bregua, P., Guerreiro, P. M., and Rotllant, J. (2018). Stress, Glucocorticoids and Bone: A Review from Mammals and Fish. *Front. Endocrinol. (Lausanne)* 9, 526. doi:10.3389/fendo.2018.00526

Tingle, C. C. D., Rother, J. A., Dewhurst, C. F., Lauer, S., and King, W. J. (2003). "Fipronil: Environmental Fate, Ecotoxicology, and Human Health Concerns," in *Reviews of Environmental Contamination and Toxicology. 1–66*. Editor G. W. Ware (New York, NYNew York: Springer). doi:10.1007/978-1-4899-7283-5_1

Tong, S.-K., Lee, H.-L., Lee, Y.-C., Wu, L.-C., Tsou, Y.-L., Lu, S.-W., et al. (2020). Arginine Vasopressin Modulates Ion and Acid/Base Balance by Regulating Cell Numbers of Sodium Chloride Cotransporter and H+-ATPase Rich Ionocytes. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21113957

Toscano, C. D., and Guilarte, T. R. (2005). Lead Neurotoxicity: from Exposure to Molecular Effects. *Brain Res. Brain Res. Rev.* 49, 529–554. doi:10.1016/j.brainresrev.2005.02.004

Tseng, Y.-C., Yan, J.-J., Furukawa, F., and Hwang, P.-P. (2020). Did Acidic Stress Resistance in Vertebrates Evolve as Na+/H+ Exchanger-Mediated Ammonia Excretion in Fish? *Bioessays* 42, e1900161. doi:10.1002/bies.201900161

Valavanidis, A., Vlahogianni, T., Dassenakis, M., and Scoullos, M. (2006). Molecular Biomarkers of Oxidative Stress in Aquatic Organisms in Relation to Toxic Environmental Pollutants. *Ecotoxicol. Environ. Saf.* 64, 178–189. doi:10.1016/j.ecoenv.2005.03.013

Vaz, R. L., Outeiro, T. F., and Ferreira, J. J. (2018). Zebrafish as an Animal Model for Drug Discovery in Parkinson's Disease and Other Movement Disorders: A Systematic Review. *Front. Neurol.* 9, 347. doi:10.3389/fneur.2018.00347

Velez, Z., Roggatz, C. C., Benoit, D. M., Hardege, J. D., and Hubbard, P. C. (2019). Short- and Medium-Term Exposure to Ocean Acidification Reduces Olfactory Sensitivity in Gilthead Seabream. *Front. Physiol.* 10, 731. doi:10.3389/fphys.2019.00731

Verberk, W. C. E. P., Bilton, D. T., Calosi, P., and Spicer, J. I. (2011). Oxygen Supply in Aquatic Ectotherms: Partial Pressure and Solubility Together Explain Biodiversity and Size Patterns. *Ecology* 92, 1565–1572. doi:10.1890/10-2369.1

Wasel, O., and Freeman, J. L. (2020). Chemical and Genetic Zebrafish Models to Define Mechanisms of and Treatments for Dopaminergic Neurodegeneration. *Int. J. Mol. Sci.* 21. doi:10.3390/ijms21175981

Weetman, D., Atkinson, D., and Chubb, J. C. (1998). Effects of Temperature on Anti-predator Behaviour in the Guppy, *Poecilia reticulata*. *Anim. Behav.* 55, 1361–1372. doi:10.1006/anbe.1997.0666

Weetman, D., Atkinson, D., and Chubb, J. C. (1999). Water Temperature Influences the Shoaling Decisions of Guppies, *Poecilia reticulata*, under Predation Threat. *Anim. Behav.* 58, 735–741. doi:10.1006/anbe.1999.1191

Welch, M. J., Watson, S.-A., Welsh, J. Q., McCormick, M. I., and Munday, P. L. (2014). Effects of Elevated CO2 on Fish Behaviour Undiminished by Transgenerational Acclimation. *Nat. Clim. Chang.* 4, 1086–1089. doi:10.1038/nclimate2400

Xia, C., Fu, L., Liu, Z., Liu, H., Chen, L., and Liu, Y. (2018). Aquatic Toxic Analysis by Monitoring Fish Behavior Using Computer Vision: A Recent Progress. *J. Toxicol.* 2018, 2591924. doi:10.1155/2018/2591924

Xu, X., Weber, D., Burge, R., and VanAmberg, K. (2016). Neurobehavioral Impairments Produced by Developmental Lead Exposure Persisted for Generations in Zebrafish (*Danio rerio*). *Neurotoxicology* 52, 176–185. doi:10.1016/j.neuro.2015.12.009

Xu, Y., Zhao, H., Wang, Z., Gao, H., Liu, J., Li, K., et al. (2022). Developmental Exposure to Environmental Levels of Cadmium Induces Neurotoxicity and Activates Microglia in Zebrafish Larvae: From the Perspectives of Neurobehavior and Neuroimaging. *Chemosphere* 291, 132802. doi:10.1016/j.chemosphere.2021.132802

Zhong, W., Zhang, Y., Wu, Z., Yang, R., Chen, X., Yang, J., et al. (2018). Health Risk Assessment of Heavy Metals in Freshwater Fish in the Central and Eastern North China. *EcotoxicolEnviron. Saf.* 157, 343–349. doi:10.1016/j.ecoenv.2018.03.048

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Neurochemical Signalling Associated With Gill Oxygen Sensing and Ventilation: A Receptor Focused Mini-Review

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Despite the large body of work describing vertebrate ventilatory responses to hypoxia, remarkably little is known about the receptors and afferent pathways mediating these responses in fishes. In this review, we aim to summarize all receptor types to date implicated in the neurotransmission or neuromodulation associated with O_2 sensing in the gills of fish. This includes serotonergic, cholinergic, purinergic, and dopaminergic receptor subtypes. Recent transcriptomic analysis of the gills of zebrafish using single-cell RNA sequencing has begun to elucidate specific receptor targets in the gill; however, the absence of receptor characterization at the cellular level in the gill remains a major limitation in understanding the neurochemical control of hypoxia signalling.

Keywords: neuroepithelial cell, chemoreception, serotonergic receptors, hypoxia, cholinergic receptors, zebrafish

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INTRODUCTION

Adequate oxygen delivery to tissue is important for maintaining cellular processes and homeostasis. Thus, an animal's ability to detect low oxygen, and respond appropriately, is crucial for survival. Vertebrate respiratory chemoreceptors detect such chemical changes in the environment, or arterial blood supply, and upon stimulation initiate corrective autonomic reflexes, such as the hyperventilatory response (HVR) to hypoxia (Perry et al., 2009; Nurse, 2010; Jonz, 2018). The most well described vertebrate chemoreceptors are the type 1 (or glomus) cells of the mammalian carotid body. Hypoxic activation of these cells involves the modulation of K^+ conductance, membrane depolarization, and resulting Ca^{2+}-dependent neurotransmitter release to act on sensory terminals of the carotid sinus nerve (Lopez-Barneo et al., 1988; Gonzalez et al., 1994; Nurse, 2010; Kumar and Prabhakar, 2012).

The neurochemistry involved in transmitting the hypoxic signal in the carotid body is well characterized. Adenosine triphosphate (ATP) is released by type 1 cells to act on afferent terminal purinergic P2X3 receptors, and is one of the main excitatory neurotransmitters involved in hypoxic signalling (Zhang et al., 2000; Nurse, 2005). Adenosine, produced by the breakdown of extracellular ATP, acts on adenosine A2a receptors to further enhance the hypoxic response by type 1 cells (Nurse, 2014). Acetylcholine (ACh), co-released with ATP, is also largely responsible for excitatory signalling in the carotid body *via* post-synaptic nicotinic receptors (Zhang et al., 2000). Dopamine, acting on D_2 receptors, plays a modulatory role in mediating the response to hypoxia in the carotid body (Benot and López-Barneo, 1990; Iturriaga and Alcayaga, 2004). Additionally, embedded within the respiratory epithelium of neonatal mammals lie clusters of chemosensory neuroepithelial bodies (NEBs), of which serotonin (5-

TABLE 1 | Summary of receptor types reported to effect changes in ventilation amplitude and/or frequency and their location within the gill, if available. The first column indicates receptor type, as characterized by pharmacological studies, gene expression analysis or immunohistochemistry.

Receptor type	Effect on ventilation[a]	Location within gill	References
Serotonergic receptors (5-HT)			
α-methyl-5-HT (5-HT_2 receptor agonist)	↑		McDonald et al. (2010)
Ketanserin (5-HT_2-R antagonist)	↓		McDonald et al. (2010), Shakarchi et al. (2013)
1-phenylbiguanide (5-HT_3 receptor agonist)	↑		Janvier et al. (1996)
Metoclopramide (5-HT_3 receptor antagonist)	↓		Janvier et al. (1996)
MDL72222 (5-HT_3 receptor antagonist)	↓		Jonz et al. (2015)
CR759836.1 (gene encoding $5HT_{3A}$-like receptors)		Neurons	Pan et al. (2022)
htr1ab (gene encoding 5-HT_{1A})		NECs	Pan et al. (2022)
Cholinergic receptors (ACh)			
Nicotine (nicotinic ACh receptor agonist)	↑		Burleson and Milsom (1995a)
Hexamethonium (nicotinic ACh receptor antagonist)	↓		Shakarchi et al. (2013)
Muscarine (muscarinic ACh receptor agonist)	↑		Burleson and Milsom (1995a)
Atropine (muscarinic ACh receptor antagonist)	No effect		Burleson and Milsom (1995a), McKenzie et al. (1995), Burleson and Smatresk (1990)
	↓		Rahbar et al. (2016)
chrna7 (gene encoding nicotinic ACh receptor subunit α7)	-	NECs	Lauriano et al. (2021)
chrna2b (gene encoding nicotinic ACh receptor subunit α2b)	-	NECs/neurons	Pan et al. (2022)
chrna6 (gene encoding nicotinic ACh receptor subunit α6)	-	NECs	Pan et al. (2022)
chrnb3a (gene encoding nicotinic ACh receptor subunit β3a)	-	NECs	Pan et al. (2022)
chrnb4 (gene encoding nicotinic ACh receptor subunit β4)	-	Neurons	Pan et al. (2022)
Purinergic receptors			
ATPγS (broad spectrum purinergic receptor agonist)	↑	-	Coe et al. (2017)
PPADS (purinergic P2X2/3 receptor antagonist)	↓	-	Coe et al. (2017)
P2X3 immunolabelling	-	NECs/neurons	Jonz and Nurse (2003), Rahbar et al. (2016)
Aminophylline (adenosine A1/2 receptor antagonist)	↓	-	Stecyk and Farrel (2006), Stensløkken et al. (2004)
SCH58261 (adenosine A2a receptor antagonist)	↓	-	Coe et al. (2017)
Dopamine receptors			
DA	↓	-	Shackarchi et al. (2013)
drd4a (gene encoding dopamine D_2-like family D_4 receptor)	-	NECs	Pan et al. (2022)
drd3 (gene encoding dopamine D_2-like family D_3 receptor)	-	Neurons	Pan et al. (2022)

[a]*Effect on ventilation measured as amplitude or frequency.*

hydroxytryptamine, 5-HT) is the major neurotransmitter released during hypoxia (Fu et al., 2002).

In contrast, remarkably little is known about the receptors and afferent pathways mediating HVR in fishes. In this review, we aim to summarize the available evidence that has linked specific receptor types in the gills to neurochemical signalling associated with O_2 sensing in fish.

FISH OXYGEN CHEMORECEPTORS

Neuroepithelial cells (NECs) were first identified in the gill by Dunel-Erb et al. (1982), who noted a similar morphology to mammalian pulmonary chemoreceptors. NECs were characterized by the presence of dense-cored vesicles and contained the monoamine, serotonin (5-hydroxytrymptamine, 5-HT; Dunel Erb et al., 1982). Due to the homology between the carotid body and the first gill arch in fish, NECs are believed to be homologues of type 1 cells (Milsom and Burleson, 2007). More recent evidence has suggested they may have a closer evolutionary link to pulmonary NEBs (Hockman et al., 2017).

The first direct evidence for the involvement of gill NECs in O_2 chemoreception came from studies using zebrafish (*Danio rerio*; Jonz et al., 2004). Using whole-cell patch-clamp recordings of NECs isolated from the gills, it was shown that NECs responded to a decreased PO_2 by inhibition of background K^+ channels and membrane depolarization. Subsequent studies showed similar O_2 sensitivity of gill NECs in channel catfish (*Ictalurus punctatus*; Burleson et al., 2006), and isolated NECs from adult goldfish (*Carassius auratus*) responded to hypoxia by Ca^{2+}-dependent vesicular recycling (Zachar et al., 2017), which is consistent with neurotransmitter release and activation of sensory nerve fibres to initiate the hypoxic response. Currently there is no direct evidence of the neurotransmitters released during hypoxia and the receptors they act on remain uncharacterized.

RECEPTOR CONTROL OF OXYGEN SENSING AND VENTILATION

Despite the lack of receptor characterization in the gill, there are numerous studies identifying neurotransmitters and neuroendocrine factors that may be associated with O_2 sensing in the gills of fish (recently reviewed by Porteus et al., 2012; Pan and Perry, 2020). The following sections will summarize the major candidate receptor subtypes suggested to play a role in

facilitating and mediating the ventilatory responses to hypoxia. These studies are summarized in **Table 1**.

Serotonergic Receptors

Serotonergic receptors are divided into seven distinct classes, 5-HT_{1-7}, largely based on their structural and operational characteristics within the serotonergic system (Peroutka and Howell, 1994; Hoyer et al., 2002). 5-HT receptors belong to the G-protein-coupled receptor (GPCR) superfamily, with the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel (Hoyer et al., 2002). Of the seven distinct classes, there are also a number of different 5-HT_{1-7} receptor subtypes, each having a distinct pattern of distribution and function in the nervous system, giving rise to many different signalling capabilities (reviewed by Barnes and Sharp, 1999). Some post-synaptic 5-HT receptor subtypes are known to cause neuronal depolarization (5-HT_{2A}, 5-HT_{2C}, 5-HT_3 and 5-HT_4 receptors) leading to excitatory signalling, or neuronal hyperpolarization (5-HT_{1A} receptor) inhibiting signalling (Barnes and Sharp, 1999).

As NECs characteristically contain 5-HT, it has been long hypothesized that 5-HT is involved in the neurotransmission of the hypoxic signal. Early evidence of this came from isolated gills of rainbow trout (*Oncorhynchus mykiss*), where 5-HT elicited a modest transient burst of chemoreceptor activity (Burleson and Milsom, 1995a). Additionally, rainbow trout intra-arterial injections of 5-HT also caused an elevation in gill ventilation and heart rate (Burleson and Milsom, 1995b).

Pharmacological studies targeting specific 5-HT receptor subtypes have revealed a potential excitatory role of 5-HT_2 and 5-HT_3 receptors. In the European eel (*Anguilla anguilla*), intravenous administration of 5-HT elicited a large increase in ventilatory frequency and amplitude (Janvier et al., 1996). Janvier et al. also showed the 5-HT-induced hyperventilatory response could be mimicked by the 5-HT_3 receptor agonist, 1-phenylbiguanide, and blocked by the 5-HT_3 receptor antagonist, metoclopramide. In the Gulf toadfish (*Opsanus beta*), injection with the 5-HT_2 receptor agonist, α-methyl-5-HT, increased ventilation amplitude and this response was attenuated by the 5-HT_2 receptor antagonist, ketanserin (McDonald et al., 2010). Similarly, 5-HT mimicked hypoxia by increasing ventilation frequency of 7 days post-fertilization (d.p.f) zebrafish larvae, and subsequent addition of ketanserin reversibly reduced ventilation frequency (Shakarchi et al., 2013). In zebrafish larvae, 5-HT_3 receptor blockade with tropanyl 3,5-dichlorobenzoate (MDL 72222) also reduced the hyperventilatory response to acute hypoxia (Jonz et al., 2015). These results suggest an excitatory role for 5-HT_2 and 5-HT_3 receptors; however, it is unknown if these effects were limited to the gills, as whole animal experiments do not exclude potential targets in the central nervous system (CNS). Additionally, in the gills of the Antarctic fish (*Pagothenia borchgrevinki*), branchial vasoconstriction was mediated by 5-HT_2 receptors (Sundin et al., 1998); and previous studies demonstrated the innervation of the filament arteries by serotonergic neurons in trout and zebrafish (Bailly et al., 1989; Jonz and Nurse, 2003). Given the effects of hypoxia on blood flow in the gills (Booth, 1979), an additional role of 5-HT_2 receptors in controlling vascular responses to hypoxia is conceivable.

Recent single-cell transcriptomic analysis of chemosensory NECs and other cell types of the gills of adult zebrafish has begun to provide compelling evidence for potential actions of these receptor types by localizing them to, and within, the gill. The gene encoding 5-HT_{3A}-like receptors shows highest expression in gill neurons (Pan et al., 2022). As NECs are innervated by at least one population of intrabranchial neurons, as well as extrabranchial nerve fibres (Jonz et al., 2004), there is a plausible mechanism for chemotransduction *via* 5-HT_{3A} receptors in the gill. This would be similar to the carotid body, where post-synaptic excitation during hypoxia is mediated primarily by ionotropic 5-HT_3 receptors and, to a lesser extent, 5-HT_2 (Zhong et al., 1999; Nurse, 2005). Further work localizing 5-HT_{3A} receptors to the specific neuronal population innervating NECs would be needed to confirm this.

Interestingly, the gene encoding the inhibitory 5-HT_{1A} receptor, *htr1ab*, was found to be most abundant in NECs above any other cell type in the gill (Pan et al., 2022). This may suggest a specific O_2-sensing role for these receptors, possibly through a negative feedback or autoreceptor mechanism, which has not yet been explored pharmacologically.

Cholinergic Receptors

Cholinergic receptors are subdivided into nicotinic and muscarinic types. Nicotinic receptors (nAChRs) are ligand-gated ion channels formed by the assembly of five transmembrane subunits and mediate fast neurotransmission in the central and peripheral nervous systems (Ho et al., 2020). Many different nAChR subtypes exist, each consisting of a specific combination of subunits (α1-10, β1-4, γ, δ, and ε; Kalamida et al., 2007). Muscarinic receptors (mAChRs) are G-protein coupled receptors, of which five main subtypes exist (M1-5), and have actions in the central and peripheral nervous systems (Caulfield, 1993).

A role for ACh in gill neurotransmission is not as clear as in the mammalian carotid body. Non-serotonergic, NEC-like cells containing the vesicular ACh transporter, VAChT, have been identified in zebrafish (Shakarchi et al., 2013; Zachar et al., 2017) and mangrove rivulus (*Kryptolebias marmoratus*; Regan et al., 2011), though it is currently unclear if these cells function as O_2 chemoreceptors. Ventilatory responses to ACh and nicotine have been reported in several species (Burleson and Milsom, 1995b; Shakarchi et al., 2013). The effects of muscarine, however, appear to be more uncertain. In rainbow trout, muscarine elicited moderate afferent nerve activity in gill arch preparations and increased ventilation frequency in whole animal experiments. The muscarinic antagonist, atropine, prevented stimulation by hypoxia; however, it had no effect on ventilation (Burleson and Milsom, 1995a; 1995b). Similarly, atropine had no effect on ventilatory responses to hypoxia in the Adriatic sturgeon (*Acipenser naccarii;* McKenzie et al., 1995) or the channel catfish (Burleson and Smatresk, 1990). In zebrafish, atropine abolished the ventilatory response to hypoxia, but only at very high concentrations (Rahbar et al., 2016). Administration of atropine also abolished the hypercarbia-induced ventilatory

responses observed in Pacific spiny dogfish (*Squalus acanthias*; McKendry et al., 2001).

Additionally, there appears to be a time-dependent emergence of cholinergic control of ventilation during development. Exogenous application of ACh did not affect ventilation frequency in early stage zebrafish larvae (7–10 d.p.f); however, in late stage larvae (14–21 d.p.f) ACh had a stimulatory effect on ventilation frequency. The nicotinic ACh receptor antagonist, hexamethonium, did not inhibit hypoxia-induced hyperventilation at 10 d.p.f. but did after 12 d.p.f. (Shakarchi et al., 2013).

In the Asian catfish (*Heteropneustes fossilis*) the nicotinic receptor α7 subunit is expressed in the NECs and mucous cells in the gill and respiratory air sac (Lauriano et al., 2021). In addition, semi-quantitative PCR detected low expression of the AChR γ-like subunit in the gills of adult transparent *Pristella maxillaris* (Ma et al., 2021). RNA sequencing of the adult zebrafish gills has further narrowed down potential ACh receptor subunit targets within the gill. The nicotinic receptor α2b subunit gene, *chrna2b*, was highly expressed in NECs and neurons; whereas the α6 subunit gene (*chrna6*) and β3a subunit gene (*chrnb3a*) were expressed primarily in NECs, and the β4 subunit gene (*chrnb4*) was mainly present in neurons (Pan et al., 2022). The location of these subunits supports a model in which VAChT-positive cells release ACh during hypoxic stimulation, leading to excitatory post-synaptic or paracrine effects on ACh receptors of neurons or NECs (Pan et al., 2022). Like NECs, VAChT-positive cells of the zebrafish gills are closely apposed to nerve fibres (Zachar et al., 2017).

Purinergic Receptors

There are two families of purinergic receptors—P1 (adenosine) receptors, including four subtypes (A1, A2a, A2b and A3), and P2 (ATP) receptors, which are further subdivided into ionotropic P2X (P2X1-7) and metabotropic P2Y (P2Y1-12) receptors (reviewed by Burnstock, 2018). ATP is a major excitatory neurotransmitter in the mammalian carotid body (Zhang et al., 2000; Nurse, 2005), and adenosine, produced by the extracellular breakdown of ATP, enhances the excitatory response (Nurse, 2014). Such a role in the gills of fish has been less explored.

The broad-spectrum purinergic agonist, ATPγS, elicited a hyperventilatory response in zebrafish larvae. Further, the purinergic receptor antagonist, PPADS, which targets purinergic P2X2 and P2X3 receptors, inhibited the hyperventilatory response to hypoxia (Coe et al., 2017). Immunohistochemical staining of P2X3 receptors showed co-localization with NECs and 5-HT-positive neurons (Rahbar et al., 2016). P2X3 receptors were also found in 5-HT-positve cells in the tips of zebrafish lamellae (Jonz and Nurse, 2003).

In the common carp (*Cyprinus carpio*), purinergic blockade with aminophylline, an A1 and A2 receptor antagonist, reversed the increases in respiration rate that occurred with the onset of hypoxia (Stecyk and Farrell, 2006). Adenosine injection initiated a biphasic response in ventilation frequency (a decrease followed by an increase) in the epaulette shark (*Hemiscyllium ocellatum*), which was also blocked by aminophylline (Stensløkken et al., 2004). Moreover, in zebrafish, the A2a receptor antagonist, SCH58261, inhibited the ventilatory response to hypoxia (Coe et al., 2017).

The above studies provide evidence for an excitatory role of P2X2/3 and A1/2 receptors in control of the hyperventilatory response to hypoxia in fish. Further, these results suggest a similar mechanism to the carotid body, where pre- and post-synaptic A2a receptors are believed to enhance the response to hypoxia (Nurse, 2014).

Dopaminergic Receptors

Dopamine receptors are G-protein coupled and include the D_1-like receptor subtypes (D_1 and D_5) which activate adenylyl cyclase, and the D_2-like subfamily (D_2, D_3, and D_4) which inhibit adenylyl cyclase and activate K^+ channels (reviewed by Martel and Gatti McArthur, 2020). Dopamine is an important inhibitory neuromodulator in carotid body hypoxia signalling (Nurse, 2010). Dopamine released by type 1 cells has an autocrine-paracrine action on dopaminergic D_2 receptors located on type 1 cells to inhibit Ca^{2+} channels, leading to negative feedback regulation of further neurotransmitter release during hypoxia (Benot and López-Barneo, 1990).

Early evidence for a role of dopamine in the gills was shown in isolated gills of rainbow trout, where dopamine caused a small and brief burst in chemoreceptor activity followed by a mild inhibition of receptor discharge (Burleson and Milsom, 1995a). In zebrafish larvae, exogenous application of dopamine has been shown to decrease ventilation frequency as early as 7 d.p.f. (Shakarchi et al., 2013). RNA sequencing of the adult zebrafish gill detected dopamine receptors in the D_2-like family, including *drd4a* in NECs and *drd3* highly expressed in neurons (Pan et al., 2022). This recent work using zebrafish suggests an inhibitory dopaminergic mechanism in the gill, possibly via D_2-like receptors, and is an area for potential future developments. Pharmacologically targeting the specific dopamine receptor subtypes controlling the ventilatory responses to hypoxia may be an interesting extension of this work.

CONCLUSION

Since the initial discovery of gill NECs, much work has been done to identify the neurotransmitters and their respective receptor types involved in mediating the physiological responses to hypoxia. Recent RNA sequencing has begun to further localize some of these receptor subtypes to NECs and neurons within the gill; however, future work is needed to continue to localize specific receptor subtypes within the gill and provide physiological evidence of their involvement in O_2 sensing.

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REFERENCES

Bailly, Y., Dunel-Erb, S., Geffard, M., and Laurent, P. (1989). The Vascular and Epithelial Serotonergic Innervation of the Actinopterygian Gill Filament with Special Reference to the Trout, Salmo Gairdneri. *Cell Tissue Res.* 258, 349–363. doi:10.1007/BF00239455

Barnes, N. M., and Sharp, T. (1999). A Review of Central 5-HT Receptors and Their Function. *Neuropharmacology* 38, 1083–1152. doi:10.1016/S0028-3908(99)00010-6

Benot, A. R., and Lopez-Barneo, J. (1990). Feedback Inhibition of Ca2+ Currents by Dopamine in Glomus Cells of the Carotid Body. *Eur. J. Neurosci.* 2, 809–812. doi:10.1111/j.1460-9568.1990.tb00473.x

Booth, J. H. (1979). The Effects of Oxygen Supply, Epinephrine, and Acetylcholine on the Distribution of Blood Flow in Trout Gills. *J. Exp. Biol.* 83, 31–39. doi:10.1242/jeb.83.1.31

Burleson, M. L., and Milsom, W. K. (1995b). Cardio-ventilatory Control in Rainbow Trout: II. Reflex Effects of Exogenous Neurochemicals. *Respir. Physiol.* 101, 289–299. doi:10.1016/0034-5687(95)00029-D

Burleson, M. L., and Milsom, W. K. (1995a). Cardio-ventilatory Control in Rainbow Trout: I. Pharmacology of Branchial, Oxygen-Sensitive Chemoreceptors. *Respir. Physiol.* 100, 231–238. doi:10.1016/0034-5687(95)91595-X

Burleson, M. L., Mercer, S. E., and Wilk-Blaszczak, M. A. (2006). Isolation and Characterization of Putative O2 Chemoreceptor Cells from the Gills of Channel Catfish (*Ictalurus punctatus*). *Brain Res.* 1092, 100–107. doi:10.1016/J.BRAINRES.2006.03.085

Burleson, M. L., and Smatresk, N. J. (1990). Evidence for Two Oxygen-Sensitive Chemoreceptor Loci in Channel Catfish,*Ictalurus punctatus*. *Physiol. Zool.* 63, 208–221. doi:10.1086/physzool.63.1.30158162

Burnstock, G. (2018). Purine and Purinergic Receptors. *Brain Neurosci. Adv.* 2, 2398212818817494. doi:10.1177/2398212818817494

Caulfield, M. P. (1993). Muscarinic Receptors-Characterization, Coupling and Function. *Pharmacol. Ther.* 58, 319–379. doi:10.1016/0163-7258(93)90027-B

Coe, A. J., Picard, A. J., and Jonz, M. G. (2017). Purinergic and Adenosine Receptors Contribute to Hypoxic Hyperventilation in Zebrafish (*Danio rerio*). *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 214, 50–57. doi:10.1016/j.cbpa.2017.09.013

Dunel-Erb, S., Bailly, Y., and Laurent, P. (1982). Neuroepithelial Cells in Fish Gill Primary Lamellae. *J. Appl. Physiology* 53, 1342–1353. doi:10.1152/jappl.1982.53.6.1342

Fu, X. W., Nurse, C. A., Wong, V., and Cutz, E. (2002). Hypoxia-induced Secretion of Serotonin from Intact Pulmonary Neuroepithelial Bodies in Neonatal Rabbit. *J. Physiology* 539, 503–510. doi:10.1113/jphysiol.2001.013071

Gonzalez, C., Almaraz, L., Obeso, A., and Rigual, R. (1994). Carotid Body Chemoreceptors: from Natural Stimuli to Sensory Discharges. *Physiol. Rev.* 74, 829–898. doi:10.1152/physrev.1994.74.4.829

Ho, T. N. T., Abraham, N., and Lewis, R. J. (2020). Structure-Function of Neuronal Nicotinic Acetylcholine Receptor Inhibitors Derived from Natural Toxins. *Front. Neurosci.* 14. 609005. Available at: https://www.frontiersin.org/article/10.3389/fnins.2020.609005.doi:10.3389/fnins.2020.609005

Hockman, D., Burns, A. J., Schlosser, G., Gates, K. P., Jevans, B., Mongera, A., et al. (2017). Evolution of the Hypoxia-Sensitive Cells Involved in Amniote Respiratory Reflexes. *Elife* 6, 1–28. doi:10.7554/eLife.21231

Hoyer, D., Hannon, J. P., and Martin, G. R. (2002). Molecular, Pharmacological and Functional Diversity of 5-HT Receptors. *Pharmacol. Biochem. Behav.* 71, 533–554. doi:10.1016/S0091-3057(01)00746-8

Iturriaga, R., and Alcayaga, J. (2004). Neurotransmission in the Carotid Body: Transmitters and Modulators between Glomus Cells and Petrosal Ganglion Nerve Terminals. *Brain Res. Rev.* 47, 46–53. doi:10.1016/j.brainresrev.2004.05.007

Janvier, J.-J., Peyraud-Waïtzenegger, M., and Soulier, P. (1996). Mediation of Serotonin-Induced Hyperventilation via 5-HT3-Receptor in European Eel *Anguilla anguilla*. *J. Comp. Physiol. B* 165, 640–646. doi:10.1007/BF00301132

Jonz, M. G., Fearon, I. M., and Nurse, C. A. (2004). Neuroepithelial Oxygen Chemoreceptors of the Zebrafish Gill. *J. Physiol.* 560, 737–752. doi:10.1113/jphysiol.2004.069294

Jonz, M. G. (2018). Insights into the Evolution of Polymodal Chemoreceptors. *Acta Histochem.* 120, 623–629. doi:10.1016/j.acthis.2018.08.008

Jonz, M. G., and Nurse, C. A. (2003). Neuroepithelial Cells and Associated Innervation of the Zebrafish Gill: A Confocal Immunofluorescence Study. *J. Comp. Neurol.* 461, 1–17. doi:10.1002/cne.10680

Jonz, M. G., Zachar, P. C., Da Fonte, D. F., and Mierzwa, A. S. (2015). Peripheral Chemoreceptors in Fish: A Brief History and a Look Ahead. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 186, 27–38. doi:10.1016/j.cbpa.2014.09.002

Kalamida, D., Poulas, K., Avramopoulou, V., Fostieri, E., Lagoumintzis, G., Lazaridis, K., et al. (2007). Muscle and Neuronal Nicotinic Acetylcholine Receptors. *FEBS J.* 274, 3799–3845. doi:10.1111/j.1742-4658.2007.05935.x

Kumar, P., and Prabhakar, N. R. (2012). Peripheral Chemoreceptors: Function and Plasticity of the Carotid Body. *Compr. Physiol.* 2, 141–219. doi:10.1002/cphy.c100069

Lauriano, E. R., Capillo, G., Icardo, J. M., Fernandes, J. M. O., Kiron, V., Kuciel, M., et al. (2021). Neuroepithelial Cells (NECs) and Mucous Cells Express a Variety of Neurotransmitters and Neurotransmitter Receptors in the Gill and Respiratory Air-Sac of the Catfish Heteropneustes Fossilis (Siluriformes, Heteropneustidae): a Possible Role in Local Immune Defence. *Zoology* 148, 125958. doi:10.1016/J.ZOOL.2021.125958

López-Barneo, J., López-López, J. R., Ureña, J., and Gonzalez, C. (1988). Chemotransduction in the Carotid Body: K + Current Modulated by P O 2 in Type I Chemoreceptor Cells. *Science* 241 (4865), 580–582. doi:10.1126/science.2456613

Ma, K., Chen, Y., Zhou, L., Liu, Z., and Liu, Z. (2021). Cloning and Characterization of Nicotinic Acetylcholine Receptor γ-like Gene in Adult Transparent Pristella Maxillaris. *Gene* 769, 145193. doi:10.1016/J.GENE.2020.145193

Martel, J. C., and Gatti McArthur, S. (2020). Dopamine Receptor Subtypes, Physiology and Pharmacology: New Ligands and Concepts in Schizophrenia. *Front. Pharmacol.* 11, 1003. Available at:. doi:10.3389/fphar.2020.01003https://www.frontiersin.org/article/10.3389/fphar.2020.01003

McDonald, M. D., Gilmour, K. M., Walsh, P. J., and Perry, S. F. (2010). Cardiovascular and Respiratory Reflexes of the Gulf Toadfish (*Opsanus beta*) during Acute Hypoxia. *Respir. Physiology Neurobiol.* 170, 59–66. doi:10.1016/j.resp.2009.12.012

McKendry, J. E., Milsom, W. K., and Perry, S. F. (2001). Branchial CO(2) Receptors and Cardiorespiratory Adjustments during Hypercarbia in Pacific Spiny Dogfish (*Squalus acanthias*). *J. Exp. Biol.* 204, 1519–1527. doi:10.1242/jeb.204.8.1519

McKenzie, D. J., Taylor, E. W., Bronzi, P., and Bolis, C. L. (1995). Aspects of Cardioventilatory Control in the Adriatic Sturgeon (*Acipenser naccarii*). *Respir. Physiol.* 100, 45–53. doi:10.1016/0034-5687(94)00121-F

Milsom, W. K., and Burleson, M. L. (2007). Peripheral Arterial Chemoreceptors and the Evolution of the Carotid Body. *Respir. Physiology Neurobiol.* 157, 4–11. doi:10.1016/J.RESP.2007.02.007

Nurse, C. A. (2005). Neurotransmission and Neuromodulation in the Chemosensory Carotid Body. *Aut. Neurosci.* 120, 1–9. doi:10.1016/J.AUTNEU.2005.04.008

Nurse, C. A. (2010). Neurotransmitter and Neuromodulatory Mechanisms at Peripheral Arterial Chemoreceptors. *Exp. Physiol.* 95, 657–667. doi:10.1113/expphysiol.2009.049312

Nurse, C. A. (2014). Synaptic and Paracrine Mechanisms at Carotid Body Arterial Chemoreceptors. *J. Physiol.* 592, 3419–3426. doi:10.1113/jphysiol.2013.269829

Pan, W., Godoy, R. S., Cook, D. P., Scott, A. L., Nurse, C. A., and Jonz, M. G. (2022). Single-cell Transcriptomic Analysis of Neuroepithelial Cells and Other Cell Types of the Gills of Zebrafish (*Danio rerio*) Exposed to Hypoxia. *Sci. Rep.* 12(1):10144. doi:10.1038/s41598-022-13693-1Accepted 26 May

Pan, Y. K., and Perry, S. F. (2020). Neuroendocrine Control of Breathing in Fish. *Mol. Cell. Endocrinol.* 509, 110800. doi:10.1016/j.mce.2020.110800

Peroutka, S. J., and Howell, T. A. (1994). The Molecular Evolution of G Protein-Coupled Receptors: Focus on 5-hydroxytryptamine Receptors. *Neuropharmacology* 33, 319–324. doi:10.1016/0028-3908(94)90060-4

Perry, S. F., Jonz, M. G., and Gilmour, K. M. (2009). "Chapter 5 Oxygen Sensing and the Hypoxic Ventilatory Response," *Fish Physiology*. 27, 193–253. doi:10.1016/S1546-5098(08)00005-8

Porteus, C. S., Brink, D. L., and Milsom, W. K. (2012). Neurotransmitter Profiles in Fish Gills: Putative Gill Oxygen Chemoreceptors. *Respir. Physiology Neurobiol.* 184, 316–325. doi:10.1016/J.RESP.2012.06.019

Rahbar, S., Pan, W., and Jonz, M. G. (2016). Purinergic and Cholinergic Drugs Mediate Hyperventilation in Zebrafish: Evidence from a Novel Chemical Screen. *PLoS One* 11, e0154261. doi:10.1371/journal.pone.0154261

Regan, K. S., Jonz, M. G., and Wright, P. A. (2011). Neuroepithelial Cells and the Hypoxia Emersion Response in the Amphibious fishKryptolebias Marmoratus. *J. Exp. Biol.* 214, 2560–2568. doi:10.1242/jeb.056333

Shakarchi, K., Zachar, P. C., and Jonz, M. G. (2013). Serotonergic and Cholinergic Elements of the Hypoxic Ventilatory Response in Developing Zebrafish. *J. Exp. Biol.* 216, 869–880. doi:10.1242/jeb.079657

Stecyk, J. A. W., and Farrell, A. P. (2006). Regulation of the Cardiorespiratory System of Common Carp (*Cyprinus carpio*) during Severe Hypoxia at Three Seasonal Acclimation Temperatures. *Physiological Biochem. Zoology* 79, 614–627. doi:10.1086/501064

Stensløkken, K.-O., Sundin, L., Renshaw, G. M. C., and Nilsson, G. E. (2004). Adenosinergic and Cholinergic Control Mechanisms during Hypoxia in the Epaulette Shark (*Hemiscyllium ocellatum*), with Emphasis on Branchial Circulation. *J. Exp. Biol.* 207, 4451–4461. doi:10.1242/jeb.01291

Sundin, L., Davison, W., Forster, M., and Axelsson, M. (1998). A Role of 5-HT2 Receptors in the Gill Vasculature of the Antarctic Fish Pagothenia Borchgrevinki. *J. Exp. Biol.* 201, 2129–2138. doi:10.1242/jeb.201.14.2129

Zachar, P. C., Pan, W., and Jonz, M. G. (2017). Characterization of Ion Channels and O2 Sensitivity in Gill Neuroepithelial Cells of the Anoxia-Tolerant Goldfish (*Carassius auratus*). *J. Neurophysiology* 118, 3014–3023. doi:10.1152/jn.00237.2017

Zhang, M., Zhong, H., Vollmer, C., and Nurse, C. A. (2000). Co-release of ATP and ACh Mediates Hypoxic Signalling at Rat Carotid Body Chemoreceptors. *J. Physiology* 525, 143–158. doi:10.1111/j.1469-7793.2000.t01-1-00143.x

Zhong, H., Zhang, M., and Nurse, C. A. (1999). Electrophysiological Characterization of 5-HT Receptors on Rat Petrosal Neurons in Dissociated Cell Culture. *Brain Res.* 816, 544–553. doi:10.1016/S0006-8993(98)01232-3

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Molecular and morphological investigations on the renal mechanisms enabling euryhalinity of red stingray *Hemitrygon akajei*

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Most cartilaginous fishes live in seawater (SW), but a few exceptional elasmobranchs (sharks and rays) are euryhaline and can acclimate to freshwater (FW) environments. The plasma of elasmobranchs is high in NaCl and urea concentrations, which constrains osmotic water loss. However, these euryhaline elasmobranchs maintain high levels of plasma NaCl and urea even when acclimating to low salinity, resulting in a strong osmotic gradient from external environment to body fluid. The kidney consequently produces a large volume of dilute urine to cope with the water influx. In the present study, we investigated the molecular mechanisms of dilute urine production in the kidney of Japanese red stingray, *Hemitrygon akajei*, transferred from SW to low-salinity environments. We showed that red stingray maintained high plasma NaCl and urea levels by reabsorbing more osmolytes in the kidney when transferred to low salinity. RNA-seq and qPCR analyses were conducted to identify genes involved in NaCl and urea reabsorption under the low-salinity conditions, and the upregulated gene expressions of Na^+-K^+-Cl^- cotransporter 2 (*nkcc2*) and Na^+/K^+-ATPase (*nka*) were found in the FW-acclimated individuals. These upregulations occurred in the early distal tubule (EDT) in the bundle zone of the kidney, which coils around the proximal and collecting tubules to form the highly convoluted structure of batoid nephron. Considering the previously proposed model for urea reabsorption, the upregulation of *nkcc2* and *nka* not only causes the reabsorption of NaCl in the EDT, but potentially also supports enhanced urea reabsorption and eventually the production of dilute urine in FW-acclimated individuals. We propose advantageous characteristics of the batoid-type nephron that facilitate acclimation to a wide range of salinities, which might have allowed the batoids to expand their habitats.

KEYWORDS

cartilaginous fish, euryhalinity, osmoregulation, urine, nephron, membrane transporter, FW adaptation, Batoidea

Introduction

The class Chondrichthyes, commonly known as cartilaginous fishes, currently contains over 1,250 living species (Ebert and Dando, 2020), and more than 90% of these are obligate marine species (Ballantyne and Robinson, 2010). Their osmoregulatory strategy is characterized by the presence of high concentrations of NaCl and urea in the body fluid to cope with the high external osmolality in marine environment (ureosmotic strategy) (Payan et al., 1973; Pang et al., 1977). The high levels of urea (300–450 mM) are essential for maintaining their body fluid iso- or slightly hyperosmotic to the surrounding seawater (SW) (Ballantyne and Fraser, 2012), thereby avoiding dehydration (Greenwell et al., 2003; Anderson et al., 2007). Meanwhile, a limited number of elasmobranch (the sharks, rays, and skates) species can tolerate low-salinity water and are known as euryhaline elasmobranchs. Indeed, euryhaline elasmobranchs, such as bull shark *Carcharhinus leucas* (Thorson, 1971), Atlantic stingray *Hypanus sabinus* (Piermarini and Evans, 1998), and largetooth sawfish *Pristis microdon* (Smith, 1931), have been reported to inhabit freshwater (FW) environments during parts of their life cycles. The most distinctive physiological feature of euryhaline elasmobranchs is that they retain relatively high internal NaCl and urea levels (osmolality >600 mOsm) in FW environment (Hazon et al., 2003), which is approximately twice the plasma osmolalities of FW teleosts (Ballantyne and Fraser, 2012). FW-acclimated euryhaline elasmobranchs thus face a steep inwardly directed osmotic gradient between internal and external environments.

Several previous studies suggest that the gill, rectal gland, and kidney contribute to maintaining the high internal NaCl level in the low-salinity environment. In the case of gills, elasmobranchs have two types of branchial ionocytes and the number of these cells increased in FW-acclimated Atlantic stingray (Piermarini and Evans, 2001; Choe et al., 2005) and 30% SW-acclimated Japanese banded houndshark *Triakis scyllium* (Takabe et al., 2016). Furthermore, branchial mRNA levels of Na^+/K^+-ATPase (*nka*) and Na^+/H^+ exchanger type-3 (*nhe3*) were significantly increased following the acclimation to low-salinity environment in bull shark (Reilly et al., 2011), Atlantic stingray (Choe et al., 2005), and houndshark (Takabe et al., 2016). These findings suggest the potential contribution of branchial ionocytes in euryhaline elasmobranchs to NaCl uptake in low-salinity environments. Rectal gland is another osmoregulatory organ dedicated to the excretion of excess NaCl in marine environment. The size and NKA activity of rectal gland decreased following the FW-acclimation in bull shark (Oguri, 1964; Pillans et al., 2005) and Atlantic stingray (Piermarini and Evans, 2000), implying that the excretion of NaCl from the rectal gland is suppressed in low-salinity environments to maintain the high internal NaCl level. Meanwhile, kidney is the only organ able to excrete excess water from the body with concomitant retention of ions and urea (Olson, 1999). It is well known that the kidney of teleost fishes produces large amounts of dilute urine to overcome overhydration in the hypo-osmotic environments (Edwards and Marshall, 2012). In euryhaline Atlantic stingrays, both the glomerular filtration rate (GFR) and urine flow rate (UFR) were markedly increased after transfer from ambient SW to 50%-diluted SW, suggesting that the kidney of stingrays vigorously excretes excess water gained from the diluted environment as in the case of FW teleost fishes (Janech et al., 2006). The concomitant increases in reabsorption of NaCl and urea to produce dilute urine in the hypo-osmotic environment were also demonstrated in the Atlantic stingray (Janech et al., 2006) and bull shark (Imaseki et al., 2019). In bull shark, Na^+-Cl^- cotransporter (NCC) was suggested to be a key renal transporter facilitating the reabsorption of both NaCl and urea in the FW-acclimated bull shark (Imaseki et al., 2019). However, whether the molecular mechanism to produce dilute urine is shared between bull shark and other euryhaline elasmobranch species remains unknown.

Recently, we reported that the kidney of Japanese red stingray *Hemitrygon akajei* has a higher capacity to reabsorb NaCl and urea than that of cloudy catshark *Scyliorhinus torazame* in SW. The difference in osmolyte compositions of the plasma and urine between stingray and catshark was supported by previous data obtained in little skate *Leucoraja erinacea* (Stolte et al., 1977), whitespotted bamboo shark *Chiloscyllium plagiosum* (Wong and Chan, 1977), spiny dogfish *Squalus acanthias* (Thurau et al., 1969), and bull shark (Imaseki et al., 2019), suggesting that the kidneys of batoids (rays and skates) have higher capacity to reabsorb NaCl and urea than those of selachians (sharks) (Aburatani et al., 2020). The enhanced renal reabsorption is likely related to the anatomical characteristics of the batoid nephrons, where the early distal tubule (EDT) is highly convoluted and coiled around the proximal and collecting tubules (Aburatani et al., 2020). The higher capacity to reabsorb NaCl and urea is also advantageous for acclimation to low-salinity environments. In fact, several phylogenetically diverse species of batoids including largetooth sawfish, Atlantic stingray, and giant freshwater stingray *Urogymnus polylepis* are euryhaline (Last et al., 2016; Grant et al., 2019), whereas only few species of the family Carcharhinidae are known to be euryhaline among selachians (Ballantyne and Fraser, 2012). These facts indicate that euryhaline batoids have distinct characteristics from bull shark in renal mechanisms for adaptation to low-salinity environment.

Here, we performed transfer experiments of red stingrays from SW to low-salinity environments and conducted a comprehensive search for differentially expressed genes (DEGs) by RNA sequencing (RNA-seq). The mechanism to produce dilute urine was examined by integrating transcriptomic, physiological, and histochemical approaches. This new knowledge of renal mechanisms in euryhaline batoids will further improve our understanding of how elasmobranchs acquired euryhalinity during their evolutionary history.

Materials and methods

Animals

Male and female red stingrays, *Hemitrygon akajei* (Muller & Henle, 1841), were caught in a bay at Ushimado and transported to the Atmosphere and Ocean Research Institute (AORI), The University of Tokyo. One or two stingrays were kept in a 500-L experimental tank filled with recirculating natural SW (35–36‰) at 20°C under a constant photoperiod (12 L: 12 D) without feeding. Stingrays of both sexes that were caught in January, May, and November 2018 (average disc width = 33.6 ± 2.6 cm, average body weight = 1.5 ± 0.4 kg; N = 13) were used for a transfer experiment from SW to FW (Experiment 1). For urine collection (Experiment 2), only female stingrays caught in November and December 2021 and February 2022 (average disc width = 49.7 ± 3.3 cm, average body weight = 4.6 ± 0.6 kg; N = 11) were used to avoid possible contamination of seminal fluid into the urine sample in males (Kempton, 1953). All procedures for animal experiments were approved by the Animal Ethics Committee of Atmosphere and Ocean Research Institute of The University of Tokyo (P19-2). The present study was carried out in compliance with the ARRIVE guidelines.

Transfer experiment 1: Tissue sampling for the analysis of gene expressions

Before the transfer experiment, stingrays were accommodated in the experimental tanks for at least 3 days in order to acclimate to the new environments. During the first 3 days of experiment, the salinity of the experimental tank water was reduced by 10–20% per day by replacement with FW to achieve a salinity of approximately 50% SW on day 3. The replaced FW was dechlorinated at the same temperature as the experimental tanks. The salinity was held for 4 days (day 4–7) to allow the stingrays acclimating to a brackish environment. Then, from day 8–11, the salinity of the tank water was further lowered 10–15% per day and reached nearly FW condition (osmolality, less than 25 mOsm/kg; Cl^-: less than 13 mM) on day 11. Our preliminary experiment suggested that the two-step dilution protocol is suitable for successful acclimation of stingrays to FW environment without mortality. In this transfer regime, the stingrays were exposed to hypo-osmotic conditions for at least 7 days (after day 3). On day 11, more than 6 h after reaching a FW-acclimated condition, the stingrays were euthanized with 0.02% (w/v) ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich, MO, United States) buffered with an equal amount of sodium bicarbonate (Sigma-Aldrich). Blood samples were obtained from tail vasculature using a syringe and 22G needle and were centrifuged at 10,000 × g for 10 min at 4°C to obtain the plasma. Plasma samples were stored at −30°C or −80°C until use. The whole kidney was dissected out and separated into the left and right halves. One half was immediately frozen with liquid nitrogen and stored at -80°C until RNA extraction, while the other half was fixed in modified Bouin's solution (saturated picric acid: formalin = 3: 1) for 2 days at 4°C and then preserved in 70% ethanol at 4°C until use. The SW control individuals were kept in SW without dilution for 14 days in the experimental tank.

Cations (sodium, calcium, and magnesium) concentrations of holding water and plasma were measured with an atomic absorption spectrophotometer (Z5300, Hitachi, Tokyo, Japan). Chloride ion concentration was measured by a digital chloridometer (C-50AP, Jokoh, Japan). Osmolality was measured by a vapor pressure osmometer (5,600, Wescor, UT, United States). Urea concentration was measured by a colorimetric method according to Rahmatullah and Boyde (1980).

Transfer experiment 2: Urine collection from SW control and 5% SW-acclimated stingrays

To collect urine from conscious individuals, the following surgical procedure was conducted during the transfer experiment. To lower the osmotic stress that may add to the surgical stress, the final dilution of environmental water was stopped at 5% SW instead of FW. After the initial 3 days of the acclimation period, stingrays were transferred to a low-salinity environment as described in experiment 1, except that replacement of FW was stopped at 5% SW (osmolality, 53.4 ± 3.7 mOsm/kg; Cl^-, 26.0 ± 2.1 mM). After reaching to 5% SW, stingrays were anesthetized and blood samples were obtained from tail vasculature using a syringe and 22G needle with 0.2% (w/v) potassium EDTA as an anticoagulant. Blood samples were centrifuged at 10,000 × g for 10 min at 4°C to obtain the plasma. A polyethylene cannula (SP-45 or SP-55, Natsume Seisakusyo, Tokyo, Japan) was inserted into the protruded opening of urinary tract and was tied to the surrounding tissue by surgical suture. The other side of cannula was connected and secured to a 2-ml sampling tube where excreted urine was collected into (see also Aburatani et al., 2020). Stingrays were recovered from anesthesia

by irrigating the gills with aerated holding water and then returned to a 100L-container tank (700 cm × 500 cm × 410 cm) for urine collection. Excreted urine was collected from conscious animals up to 48 h after the blood sampling. Plasma and urine samples were stored at −30°C until use. Ions, urea, and osmolality of the holding water, plasma, and urine were measured as described above. To estimate the reabsorption rates of osmolytes, we calculated solute reabsorption rates of major osmolytes by borrowing the reported values for glomerular filtration rate (GFR) and urine flow rate (UFR) in Atlantic stingray (Janech et al., 2006). The average values of the Atlantic stingray reared in harbor water were adopted for the calculation in the SW control individuals, while those in 50% diluted harbor water were adopted for the calculation in the 5% SW-acclimated red stingray. The solute reabsorption rates were calculated using (P_{osm} × GFR)–(U_{osm} × UFR) where P_{osm} and U_{osm} are concentrations of the osmolytes in plasma and urine, respectively.

RNA-seq analysis

Total RNA was extracted from the frozen kidney with ISOGEN (Nippon Gene, Toyama, Japan) and treated with DNase I to digest genomic DNA. Purification was conducted using the Zymo RNA Clean & Concentrator Column (Zymo Research, CA, United States). The quantity and quality of the RNA were examined with the Qubit RNA HS Assay Kit on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, MA, United States) and the RNA 6000 Nano Kit on a 2,100 Bioanalyzer (Agilent Technologies, CA, United States). Sequencing libraries were prepared using 1 μg of the RNA with the TruSeq Stranded mRNA Sample Prep Kit (Illumina, CA, United States) and the TruSeq single-index adaptor (Illumina). The libraries were sequenced with HiSeq1500 (Illumina), and single reads of 80 bases were obtained. The adaptor sequences and low-quality reads were trimmed with Trim Galore! v0.4.0 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), and sequence quality was checked by fastq quality filter v0.11.3 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The trimmed reads were then assembled *de novo* using Trinity v2.3.2 (Grabherr et al., 2011) to obtain transcript contigs. The contigs were annotated using ncbi-blast-2.6.0+ (McGinnis and Madden, 2004). The trimmed reads were mapped to the assembled contigs using bowtie2 v2.3.0 (Langmead and Salzberg, 2012), and the expressions were quantified with eXpress v1.5.1 (Cappé and Moulines, 2009). The differential expression analysis was then conducted by edgeR v3.0.0 (Robinson et al., 2010; McCarthy et al., 2012). The raw sequenced reads were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers DRX363233, DRX363234, DRX363235, DRX363236, DRX363237, and DRX363238.

To investigate candidate gene contributing to FW acclimation, contigs annotated as genes known for ions and urea transport were shortlisted. Amino acid sequences of human *Homo sapiens* and western clawed frog *Xenopus tropicalis* were used as a query for local TBLASTN search, and the sequences of top-hit contigs were sent to NCBI BLASTX to obtain an annotation. When different contigs were annotated as the same protein or when the contig was annotated as a "hypothetical protein," their orthologies were checked by inferring gene trees based on deduced amino acid sequences of the contigs using a web tool; ORTHOSCOPE (Inoue and Satoh, 2019; http://yurai.aori.u-tokyo.ac.jp/orthoscope/Vertebrata.html)

cDNA cloning and RNA probe synthesis

Total RNA was extracted from the frozen kidney as described above. Complementary DNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) from 2 μg of total RNA pretreated with TURBO DNase-free kit (Thermo Fisher Scientific). Primer sets were designed to amplify cDNAs encoding NCC (858 bp; Genbank accession No. LC706818), elongation factor 1α1 subunit (EF1α1, 847 bp; Genbank accession No. LC706817), and β-actin (ACTB, 850 bp; Genbank accession No. LC715734) based on the contig sequence data from the transcriptome database. PCR was performed using KAPA Taq Extra (Kapa Biosystems, MA, United States) with the kidney cDNA as templates. The amplified products were ligated into pGEM-T Easy Vector (Promega, WI, United States) and transformed into *Escherichia coli* XL1-Blue cells. Plasmids were prepared from positive bacterial culture using FastGene Plasmid Mini Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. The cloned cDNAs were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and an automated DNA sequencer (ABI PRISM 3100, Applied Biosystems, CA, United States). Primer sets used in this study are shown in Supplementary Table S1.

To synthesize a digoxigenin (DIG)-labeled RNA probe, the insert region of the sequenced plasmid was amplified with Primestar GXL (Takara Bio, Shiga, Japan) using the vector-specific M13 Forward and Reverse primers and subsequently purified with Wizard SV Gel and PCR Clean-up System (Promega). The purified DNA contains T7 and SP6 promotor sequences flanking the insert. Antisense and sense RNA probes were then synthesized from the purified DNA fragments using DIG RNA Labeling Kit (Roche Applied Science, Mannheim, Germany) with either T7 or SP6 RNA polymerase according to the manufacturer's protocols.

Quantitative real-time PCR

The gene expression levels were measured by quantitative real-time PCR (qPCR) using 7900HT Fast Real Time PCR System (Applied Biosystems) with a KAPA SYBR FAST qPCR Kit (Kapa Biosystems). The plasmids containing target sequences were serially diluted as standard templates for quantification in qPCR assay. The copy numbers were calculated using Sequence Detection System software v2.4 (Applied Biosystems). To explore the suitable gene for internal control in qPCR assay, the mRNA expression levels of β-actin (*actb*) and elongation factor 1α1 (*ef1α1*) were compared (Supplementary Figure S1). Since *ef1α1* mRNA expression was more stable between SW control and FW-acclimated individuals than that of *actb*, we adopted *ef1α1* as the reference gene for normalization. Primer sets for qPCR assay were designed using PrimerQuest (https://www.idtdna.com/Primerquest/Home/Index). Six individuals (three males and three females) were used for both the SW control and FW transfer.

In situ hybridization

The fixed kidney was dehydrated with a series of ethanol, cleared in methyl benzoate, and embedded in Paraplast (McCormick Scientific, IL, United States). Serial sections were made at 7 μm thickness. Eight sections (four from SW control and four from FW-acclimated stingrays) were mounted onto a single MAS-GP-coated slide (Matsunami Glass, Osaka, Japan) for comparison of the signal intensities between SW and FW individuals with the same tissue section processing. Deparaffinized sections were treated with 2.5 μg/ml proteinase K (Sigma-Aldrich) and then hybridized with DIG-labeled RNA probes in hybridization buffer (50% formamide, 5 × SSC, 40 μg/ml bovine calf thymus DNA) at 58°C for 2 days. After hybridization, sections were serially washed in 2 × SSC for 30 min at room temperature, 2 × SSC for 1 h at 65°C, and 0.1 × SSC for 1 h at 65°C. The hybridized RNA probes were detected using Anti-Digoxigenin-AP, Fab fragment (1:5,000, Roche Applied Science). Hybridization signals were visualized with 4-nitro blue tetrazolium chloride and X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate. Sections were counterstained with Kernechtrot Stain Solution (MUTO PURE CHEMICALS, Tokyo, Japan) and mounted using Permount (Fisher Chemical, NJ, United States). For image analysis described below, stained sections were mounted using CC/Mount (Diagnostic BioSystems, CA, United States) without counterstaining. Micrographs were obtained using a digital camera (DXM1200; Nikon, Tokyo, Japan).

Image analysis

Image analysis was performed to examine the signal intensity among nephron segments. The micrographs were captured using identical conditions and analyzed with ImageJ package Fiji (Schindelin et al., 2012). Basal and apical membranes were designated using the polygon selection tool. Areas and total signal intensities were measured between the basal and apical membranes to calculate the mean tubular intensity/area. Three randomly selected images were examined for each individual to consider variations among tubules.

Statistical analysis

Values were expressed as means ± s. e.m. After the assumption of normality with Shapiro–Wilk test, data were compared using two-tailed Student's or Welch's *t*-test according to whether the covariance were found or not. When the values were not normally distributed, the Mann–Whitney *U*-test was used instead. $p < 0.05$ was considered as statistically significant. Holm method was applied to the calculated *p* values for multiple comparisons. Statistical analysis was performed using Kyplot 6.0 software (Kyenslab, Tokyo, Japan).

Results

Changes in plasma and urine compositions following the acclimation to low-salinity environments

Tables 1, 2 show the osmolality and ion concentrations of plasma and urine in experiments 1 and 2, respectively. In SW control individuals, the plasma was composed of high levels of NaCl and urea, which contribute to an osmolality that was nearly identical to the holding SW (1,012.8 ± 5.4 mOsm/kg in experiment 1 and 1,054.5 ± 5.4 mOsm/kg in experiment 2). All plasma parameters, except for Ca^{2+} and Mg^{2+} in experiment 2, were significantly decreased after acclimation to the low-salinity environments. However, considerable concentrations of Na^+, Cl^-, and urea were maintained in the plasma even in low-salinity environments, resulting in a steep osmotic gradient between the plasma and surrounding environment (for plasma, 605.7 ± 8.0 mOsm/kg in experiment 1 and 623.5 ± 20.7 mOsm/kg in experiment 2; for environmental water, 17.0 ± 4.0 mOsm/kg in experiment 1 and 53.4 ± 3.7 in experiment 2, respectively).

In SW control red stingray, the urine osmolality and Na^+ level were nearly identical to those of plasma, while urine Cl^- and urea levels were significantly lower than those in plasma. In contrast, Ca^{2+} tended to be higher in urine than plasma ($p = 0.069$ after correction by Holm method) and Mg^{2+} was significantly higher in urine than in plasma (approximately 5.3 and 294.0 times concentrated in urine for Ca^{2+} and Mg^{2+}, respectively). Following the acclimation to 5% SW, urine levels of osmolality, Mg^{2+}, and Cl^- were significantly decreased (Table 2). Urine levels of Na^+ were 10 times lower in individuals acclimated to 5% SW than SW control individuals, but no significant difference was observed due to the high variation in SW control

TABLE 1 Plasma compositions of red stingray in Experiment 1

	N	Osmolality (mOsm/kg)	Na^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)	Cl^- (mM)	Urea (mM)
Plasma							
SW	6	1,042.2 ± 6.7	329.8 ± 5.7	3.5 ± 0.3	1.1 ± 0.1	278.3 ± 4.5	281.7 ± 7.7
FW	6	605.7 ± 8.0**	216.4 ± 4.1***	2.4 ± 0.3*	0.7 ± 0.1*	175.4 ± 3.0***	142.3 ± 2.2**
Environmental water							
SW	3	1,012.8 ± 5.4	545.0 ± 5.6	6.8 ± 2.4	56.5 ± 0.3	572.7 ± 1.5	<0.0
FW	3	17.0 ± 4.0	7.2 ± 2.5	0.6 ± 0.0	0.8 ± 0.2	11.2 ± 0.9	<0.0

Values are presented as means ± s.e.m. Statistically significant differences between SW and FW individuals are shown with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

TABLE 2 Compositions of plasma and urine collected from SW- and 5% SW-acclimated red stingray in Experiment 2

	N	Osmolality (mOsm/kg)	Na^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)	Cl^- (mM)	Urea (mM)
Plasma							
SW	5	1,045.9 ± 5.0	363.3 ± 10.7	4.1 ± 0.7	0.9 ± 0.2	300.8 ± 10.8	324.1 ± 12.8
5% SW	6	623.5 ± 20.7***	210.3 ± 6.9***	2.4 ± 0.2	0.5 ± 0.1	173.1 ± 6.4***	194.0 ± 7.8***
Urine							
SW	5	1,040.0 ± 18.6	344.7 ± 96.4	21.7 ± 7.1	264.6 ± 55.8†	81.9 ± 18.9†††	84.0 ± 32.5†
5% SW	6	172.8 ± 13.4***†††	32.3 ± 4.3†††	1.1 ± 0.2††	0.7 ± 0.2*	20.2 ± 4.8*†††	91.9 ± 9.0†††
Environmental water							
SW	5	1,054.5 ± 5.4	552.1 ± 4.0	8.7 ± 2.0	52.9 ± 0.4	551.3 ± 2.4	<1.0
5% SW	6	53.4 ± 3.7	22.6 ± 2.0	1.4 ± 0.2	2.6 ± 0.2	26.0 ± 2.1	<1.0

Values are presented as means ± s.e.m. Comparisons within the same parameters were corrected by Holm method. Statistically significant differences between SW, and 5% SW, individuals, and between plasma and urine are shown with asterisks and daggers, respectively (*, †$p < 0.05$, ††$p < 0.01$, ***, †††$p < 0.001$).

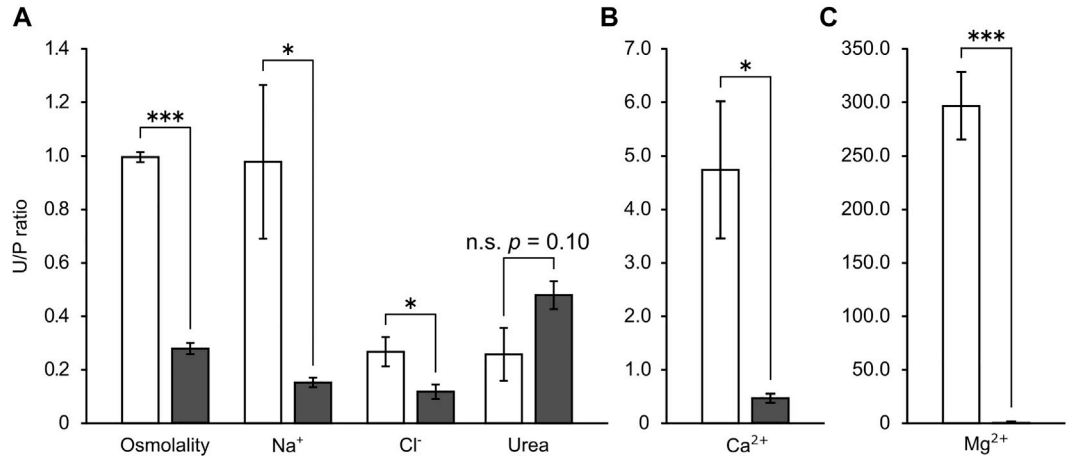

FIGURE 1
The urine/plasma (U/P) ratios of major osmolytes **(A)** and divalent ions **(B,C)** in the stingray. Open and filled bar represent values from SW- and 5% SW-acclimated stingrays, respectively. Asterisks indicate statistically significant differences between SW- and 5% SW-acclimated stingrays (*$p < 0.05$, ***$p < 0.001$).

TABLE 3 Estimated reabsorption rate of major osmolytes in the red stingrays.

		Reabsorption rate (μmol/hour/kg)		
	N	Na^+	Cl^-	Urea
SW	5	1,070.1 ± 122.1	1,069.3 ± 30.0	1,156.0 ± 52.0
5% SW	6	2,347.0 ± 70.3***	1982.8 ± 79.0***	1,662.1 ± 133.2**

Values are presented as means ± s. e.m. Asterisks indicate statistically significant differences between SW- and 5% SW-acclimated stingrays (**$p < 0.01$, ***$p < 0.001$). For the calculation, reported values of GFR [SW; 3.8 (ml/hour/kg), 5% SW; 12.4 (ml//hour/kg)] and UFR [SW; 0.9 (ml/hour/kg), 5% SW; 8.1 (ml//hour/kg)] were adopted from Atlantic stingray acclimated to natural and 50% diluted harbor water, respectively (Janech et al., 2006).

individuals. Overall, the decreases in osmolality and ion concentrations were greater in urine than in plasma. In consequence, the urine/plasma (U/P) ratios of osmolality and ions were significantly lower in 5% SW-acclimated individuals than in SW control individuals (SW versus 5% SW groups; osmolality, 0.99 ± 0.02 vs. 0.28 ± 0.02; Na^+, 0.98 ± 0.29 vs. 0.15 ± 0.02; Cl^-, 0.27 ± 0.05 vs. 0.12 ± 0.03; Ca^{2+}, 4.75 ± 1.28 vs. 0.46 ± 0.08; Mg^{2+}, 297.6 ± 31.54 vs. 1.67 ± 0.56) (Figure 1). However, urea concentration in urine was similar between SW control and 5% SW-acclimated individuals, and thus, the U/P ratio of urea slightly but insignificantly increased in 5% SW-acclimated individuals (SW versus 5% SW groups; 0.26 ± 0.10 vs. 0.48 ± 0.05) (Figure 1A).

Currently, values of GFR and UFR of conscious red stingrays in SW and FW environments are not available. Therefore, with the GFRs and UFRs reported in euryhaline Atlantic stingray (Janech et al., 2006), we estimated the reabsorption rate of Na^+, Cl^-, and urea (Table 3). In Janech et al. (2006), GFR and UFR values were determined in SW- and 50% SW-acclimated stingrays. Although the salinity of diluted media was greatly different between the previous study (50% SW) and the current investigation (5% SW), we considered that borrowing the GFR and UFR values from the study of Atlantic stingrays is insightful on estimating the changes in the urine production capacity under different salinities as the GFR and UFR were significantly increased following the transfer of stingrays from SW to 50% SW (Janech et al., 2006). In the SW control group of the red stingrays, urea is reabsorbed to a slightly greater extent than NaCl, whereas the reabsorption rate of Na^+ and Cl^- was greater than that of urea in the FW-acclimated stingrays. Compared to the SW control, the reabsorption rates of Na^+, Cl^-, and urea were significantly increased in the 5% SW-acclimated stingrays by 2.2-, 1.9-, and 1.4-fold, respectively (Table 3).

Expression profiles of transporters involved in NaCl and urea reabsorption

To search for the candidate genes that are responsible for the enhanced reabsorption of NaCl and urea in low-salinity environments, the transcriptomes of the kidney were examined in SW control and FW-acclimated individuals. The analysis of the sample correlation matrix on the overall expression profiles showed a clear clustering between SW control and FW-acclimated stingrays (Supplementary Figure S2). By using the selection criteria of DEGs described in our previous study (Imaseki et al., 2019), we identified 131 annotated genes upregulated in FW after the application of dual cut-off [false discovery rate (FDR) < 0.05 and log2 fold change (logFC) > 1.5 of count per million (CPM) value] (Supplementary Table S2). Among them, we focused on solute carrier (*slc*) family genes as they play pivotal roles in osmolyte transport in osmoregulatory epithelia (Hediger et al., 2004). The following eight *slc* genes were found to be upregulated: facilitated glucose transporter member 1 (*slc2a1*, *glut1*, 5.3-fold), sodium-coupled monocarboxylate transporter 1 (*slc5a8*, *smct1*, 5.7-fold), sodium-sulfate cotransporter (*slc13a1*, *nas1*, 12.8-fold), monocarboxylate transporter 4 (*slc16a3*, *mot4*, 5.9-fold), sulfate transporter (*slc26a2*, *dtdst*, 7.8-fold), ammonium transporter Rh type B (*slc42a2*, *rhbg*, 3.3-fold), large neutral amino acids transporter small subunit 4 (*slc43a2*, *lat4*, 2.9-fold), and sodium-dependent lysophosphatidylcholine symporter 1-B (*slc59a1*, *nls1b*, 7.7-fold) (Supplementary Table S2). However, no candidate genes encoding known transporters or channels for Cl^- or urea transport were found among the DEGs. To further explore the transcriptome, we subsequently extracted expression data of the following *slc* family genes known to be involved in NaCl and urea transport: *slc5* (sodium glucose cotransporter), *slc9* (sodium/proton exchanger), *slc12* (electroneutral cation-coupled chloride cotransporter), and *slc14* (urea transporter) (Table 4). In addition, expression data of chloride channel (*clc*), epithelial sodium channel (*enac*), *nka*, and FXYD domain-containing ion transport regulator [*fxyd*; often regarded as the third NKA subunit (Pirkmajer and Chibalin, 2019)] were also extracted (Table 4). Among the *slc* genes involved in NaCl transport, *nkcc2* (*slc12a1*) has the highest Transcripts Per Kilobase Million (TPM) values, and the values in FW-acclimated stingray were 1.9-fold higher than those of SW control individuals. The TPM values of NKA alpha subunit 1 (*nkaα1*) and *fxyd3* were comparable to those of *nkcc2*, and 2.5-fold increase in the TPM values of *nkaα1* was observed in FW-acclimated individuals. The TPM values of *nkaβ1* subunit, *fxyd2*, and *fxyd3* were increased 3.7-, 8.1-, and 1.3-fold in FW-acclimated stingrays, respectively. The expression of the *ncc* (*slc12a3*) was decreased in the FW-acclimated stingrays, and the TPM values are much lower than those of *nkcc2*.

To obtain the molecular information on membrane transporters for divalent ions, we also extracted the expression data of the following gene families: *slc8* (sodium/calcium exchanger), *slc13* (sodium sulfate cotransporter), *slc26* (sulfate/anion exchanger), *slc41* (sodium/magnesium exchanger), Cyclin M divalent cation transport mediator (*cnnm*), Transient receptor potential cation channel subfamily M (*trpm*), Transient receptor

TABLE 4 Expression of genes putatively linked to NaCl and urea reabsorption in the transcriptomic analysis.

Transcript contig ID	Annotation	Gene symbol	TPM value					
			SW1	SW2	SW3	FW1	FW2	FW3
DN56997_c6_g2	Solute carrier family 5 (sodium/glucose cotransporter), member 1 [*Leucoraja erinacea*]	*slc5a1*, *sglt1*	3.1	4.1	1.3	5.6	4.7	24.8
DN48755_c5_g1	Sodium/glucose cotransporter 2 isoform X2 [*Chiloscyllium plagiosum*]	*slc5a2*, *sglt2*	23.8	49.7	50.0	73.7	97.3	93.2
DN57379_c1_g2	Sodium/myo-inositol cotransporter-like isoform X1 [*Amblyraja radiata*]	*slc5a3*, *smit1*	71.4	53.2	56.0	61.4	69.8	31.7
DN56128_c7_g1	High-affinity choline transporter 1-like [*Amblyraja radiata*]	*slc5a7*, *cht*	0.9	1.0	4.6	1.5	1.9	4.4
DN47922_c8_g2	Sodium-coupled monocarboxylate transporter 1 isoform X1 [*Amblyraja radiata*]	*slc5a8*, *smct1*	7.0	5.2	4.2	18.9	25.4	48.8
DN51220_c0_g1	Sodium/glucose cotransporter 4 [*Amblyraja radiata*]	*slc5a9*, *sglt4*	25.0	31.0	39.8	19.4	15.5	12.2
DN55339_c6_g1	Sodium/glucose cotransporter 5 isoform X1 [*Carcharodon carcharias*]	*slc5a10*, *sglt5*	2.2	1.4	1.3	1.7	2.6	2.0
DN57283_c3_g2	Sodium/myo-inositol cotransporter 2-like isoform X1 [*Amblyraja radiata*]	*slc5a11*, *smit2*	139.2	194.1	77.4	201.1	180.4	102.6
DN57206_c4_g2	Sodium-coupled monocarboxylate transporter 2 isoform X2 [*Chiloscyllium plagiosum*]	*slc5a12*, *smct2*	18.1	8.2	36.6	6.3	24.3	20.4
DN51815_c0_g1	Sodium/hydrogen exchanger 1 [*Amblyraja radiata*]	*slc9a1*, *nhe1*	2.0	2.0	2.1	3.5	3.1	2.9
DN46781_c0_g1	Sodium/hydrogen exchanger 2-like [*Amblyraja radiata*]	*slc9a2*, *nhe2*	2.9	3.8	5.3	2.1	2.7	3.9
DN52182_c12_g3	Na^+/H^+ exchanger type 3 [*Hypanus sabinus*]	*slc9a3*, *nhe3*	9.4	8.9	19.4	4.3	4.9	5.9
DN54402_c2_g1	Sodium/hydrogen exchanger 7 isoform X1 [*Carcharodon carcharias*]	*slc9a7*, *nhe7*	1.8	0.9	0.8	0.8	0.6	0.3
DN47838_c4_g1	Sodium/hydrogen exchanger 8 isoform X1 [*Amblyraja radiata*]	*slc9a8*, *nhe8*	9.7	10.0	9.3	5.3	6.3	6.6
DN46008_c0_g1	Sodium/hydrogen exchanger 9-like isoform X1 [*Carcharodon carcharias*]	*slc9a9*, *nhe9*	2.6	3.1	4.6	2.1	1.6	4.0
DN56595_c4_g1	**Solute carrier family 12 member 1***	***slc12a1***, ***nkcc2***	367.1	297.5	496.9	777.2	720.7	667.9
DN48109_c9_g1	Na^+:K^+:2Cl- cotransporter 1 [*Himantura signifer*]	*slc12a2*, *nkcc1*	6.1	7.6	5.5	4.5	5.3	6.6
DN50058_c2_g1	Solute carrier family 12 member 3 [*Amblyraja radiata*]	*slc12a3*, *ncc*	20.2	13.8	31.5	4.3	10.7	1.4
DN53154_c3_g2	PREDICTED: solute carrier family 12 member 6 isoform X1 [*Latimeria chalumnae*]	*slc12a6*, *kcc3*	8.3	7.1	7.1	7.3	6.4	6.7
DN53606_c0_g1	Solute carrier family 12 member 7 isoform X2 [*Amblyraja radiata*]	*slc12a7*, *kcc4*	4.7	8.6	8.2	8.5	8.4	6.0
DN51351_c0_g1	Solute carrier family 12 member 8 isoform X1 [*Carcharodon carcharias*]	*slc12a8*, *ccc9*	2.5	6.3	5.3	3.2	3.8	3.4
DN53601_c4_g5	Solute carrier family 12 member 9-like isoform X1 [*Amblyraja radiata*]	*slc12a9*, *ccc6*	1.4	0.9	0.8	0.8	0.7	0.9
DN52195_c5_g1	**Urea transporter***	***slc14a1***, ***ut***	16.1	19.8	24.0	21.1	23.7	31.6
DN54090_c2_g1	H (+)/Cl (-) exchange transporter 3 isoform X4 [*Chiloscyllium plagiosum*]	*clc-3*	11.9	10.8	10.6	8.6	10.5	8.4
DN48716_c0_g1	H (+)/Cl (-) exchange transporter 5 isoform X1 [*Scyliorhinus canicula*]	*clc-5*	6.3	5.7	6.3	6.0	5.4	7.5
DN52817_c0_g2	Chloride transport protein 6 [*Amblyraja radiata*]	*clc-6*	6.5	8.0	5.8	4.6	4.5	4.8
DN46657_c0_g1	H (+)/Cl (-) exchange transporter 7 isoform X1 [*Amblyraja radiata*]	*clc-7*	9.7	9.6	8.2	12.8	8.6	6.2
DN57075_c9_g2	Chloride channel protein ClC-Kb-like [*Carcharodon carcharias*]	*clc-kb***	7.6	9.3	11.4	8.9	6.1	7.1
DN56714_c7_g2	Chloride channel K isoform X1 [*Carcharodon carcharias*]	*clc-kb***	39.1	53.3	54.0	62.4	80.4	97.1
DN51921_c1_g2	Amiloride-sensitive sodium channel subunit alpha-like [*Scyliorhinus canicula*]	*enacα*	12.1	16.5	19.6	17.2	22.7	30.3
DN54588_c5_g2	Amiloride-sensitive sodium channel subunit beta [*Amblyraja radiata*]	*enacβ*	6.4	7.3	2.4	9.5	8.0	6.5
DN54241_c13_g1	**Na^+/K^+ transporting subunit alpha 1***	***nkaα1***	306.9	345.1	383.2	815.8	847.1	1,009.2
DN45503_c0_g1	Na^+/K^+-ATPase alpha-subunit 2 [*Himantura signifer*]	*nkaα2*	0.8	1.2	1.3	0.4	0.6	0.9
DN53748_c3_g2	RecName: Full = Sodium/potassium-transporting ATPase subunit beta-1; AltName: Full = Sodium/potassium-dependent ATPase subunit beta-1 [*Tetronarce californica*]	*nkaβ1*	133.3	134.9	144.9	417.7	486.2	604.8
DN52334_c7_g1	Hypothetical protein [*Chiloscyllium punctatum*]	*nkaβ3***	4.4	6.4	7.2	3.9	2.2	7.5
DN51046_c4_g1	Protein ATP1B4 [*Scyliorhinus canicula*]	*nkaβ4*	9.8	9.2	5.5	5.6	6.2	5.9

(Continued on following page)

TABLE 4 (*Continued*) Expression of genes putatively linked to NaCl and urea reabsorption in the transcriptomic analysis.

Transcript contig ID	Annotation	Gene symbol	TPM value					
			SW1	SW2	SW3	FW1	FW2	FW3
DN46337_c6_g3	FXYD domain-containing ion transport regulator 6-like [*Rhincodon typus*]	*fxyd2***	8.6	19.2	40.5	108.3	63.3	387.0
DN58223_c7_g1	FXYD domain-containing ion transport regulator 3-like [*Chiloscyllium plagiosum*]	*fxyd3*	365.1	321.1	439.3	496.4	466.4	548.3
DN55291_c9_g1	FXYD domain-containing ion transport regulator 6 [*Carcharodon carcharias*]	*fxyd6***	0.1	0.2	0.1	1.2	1.3	2.8

Expression levels were shown in Transcripts Per Kilobase Million (TPM). *Previously cloned sequence (Aburatani et al., 2020). **Orthology was checked by ORTHOSCOPE (Inoue and Satoh, 2019).

potential cation channel subfamily V (*trpv*), and ATPase plasma membrane calcium transporting (*pmca*) (Table 5). Among the genes with the TPM values higher than 20, we observed that the expression levels of *slc13a3*, *slc13a4*, *slc26a6*, and *slc41a1* were decreased, whereas those of *slc26a1* and *trpm6* were increased.

Changes in gene expression of *nkcc2*, *nkaα1*, *ut*, and *ncc*

Using qPCR, we subsequently investigated the expression levels of *nkcc2*, *nkaα1*, urea transporter (*ut*), and *ncc* that have been reported to be important for NaCl and urea reabsorption of the kidney in cartilaginous fishes (Hyodo et al., 2014; Imaseki et al., 2019; Aburatani et al., 2020). The expression levels of *nkcc2* and *nkaα1* mRNAs in the kidney of FW-acclimated stingray were significantly higher than those of SW control individuals (1.7- and 2.6-fold, respectively; Figures 2A, B). However, no significant difference was observed in the expression of *ut* mRNA between SW control and FW-acclimated stingrays (Figure 2C). The expression of *ncc* mRNA was significantly lower in the FW-acclimated stingrays than in the SW control individuals (Figure 2D). These expression patterns examined by qPCR were consistent with those of RNA-seq analyses. No difference was observed in gene expression levels between sexes both in SW control (*nkcc2*, $p = 0.16$; *nkaα1*, $p = 0.71$; *ut*, $p = 0.41$; *ncc*, $p = 0.69$) and FW-acclimated stingrays (*nkcc2*, $p = 0.92$; *nkaα1*, $p = 0.20$; *ut*, $p = 0.50$; *ncc*, $p = 0.54$).

Distribution and expression levels of *nkcc2*, *nkaα1*, *ut*, and *ncc* mRNAs in the stingray nephron

Intense *nkcc2* mRNA signals were observed in the largest columnar cells of early distal tubule (EDT) in the bundle zone (open arrowhead labeled "e" in Figures 3A,B), and weak signals for *nkcc2* mRNA were continued to the ascending late distal tubules (LDT) in the sinus zone (open arrows in Figures 3C, D), which are relatively smaller diameter than the proximal tubules II (PII) (filled arrowhead in Figures 3C, D). The signal intensity of *nkcc2* mRNA in the EDT of FW-acclimated individuals was considerably stronger than that of SW control individuals, while no obvious difference was found in the LDT between SW control and FW-acclimated individuals. *nkaα1* mRNA signals were found in a wide range of renal tubules, and the strongest signals were observed in the EDT in the bundle zone (Figures 3E, F). In the bundle zone, the signal intensities of *nkaα1* mRNA were higher in the proximal tubule I (PI) (open arrowheads labeled "p" in Figure 3F), collecting tubule (tubules "c" in Figures 3E, F), and EDT (tubules "e" in Figures 3E, F) of FW-acclimated individuals than those of SW control. The increases in signal intensity of *nkaα1* mRNA were the most prominent in the EDT of the bundle zone, and this coincided well with the increased *nkcc2* mRNA signals in the FW-acclimated individuals. In the sinus zone, *nkaα1* mRNA signals were increased in the ascending and descending LDT (open and filled arrows, respectively) and PII segment (filled arrowhead) in the FW-acclimated individuals (Figures 3G, H). Of note, we controlled the time for chromogenic reaction of *nkcc2* and *nkaα1* mRNA signals in order to compare signal intensities between SW and FW individuals. If the reaction time was extended, *nkcc2* and *nkaα1* mRNA signals were noticeable in the EDT even in SW control individuals, but differences in signal intensity were diminished between SW control and FW-acclimated individuals.

The *ut* mRNA signals were found in the collecting tubule (CT) and preceding transitional region between LDT and CT in the vicinity of renal corpuscles both in SW control and FW-acclimated individuals. The collecting tubules were characterized by the cuboidal epithelial cells located in the center of the tubular bundle and the signal intensity of *ut* mRNA was stronger in FW-acclimated individuals (open arrowhead labeled "c" in Figures 3I, J). In contrast, *ut* mRNA signals in the transitional region between LDT and CT in sinus zone were decreased in FW-acclimated individuals (filled arrow in Figures 3K, L). In SW control stingray, *ncc* mRNA signal was detected in the *ut* mRNA-positive tubule of the sinus zone (filled arrow in Figures 3K, O), but not in the collecting tubule of the bundle zone (open

TABLE 5 Expression of genes putatively linked to divalent ion reabsorption in the transcriptomic analysis.

Transcript contig ID	Annotation	Gene symbol	TPM value					
			SW1	SW2	SW3	FW1	FW2	FW3
DN41214_c0_g1	Sodium/calcium exchanger 1-like isoform X4 [*Carcharodon carcharias*]	*slc8a1, ncx1*	1.2	0.2	0	0	0.8	0.5
DN131733_c0_g1	Sodium/calcium exchanger 3-like isoform X1 [*Carcharodon carcharias*]	*slc8a3, ncx3*	0.3	0.4	0	0.1	0.6	0.2
DN57715_c13_g2	Solute carrier family 13 member 1 [*Gopherus evgoodei*]	*slc13a1, nas1*	0.4	0.5	0.3	6.1	4.5	3.3
DN55252_c11_g1	Solute carrier family 13 member 2 [*Amblyraja radiata*]	*slc13a2, nadc1*	5.8	9.9	3.1	10.7	10.3	17.2
DN57644_c12_g1	Solute carrier family 13 member 3 [*Amblyraja radiata*]	*slc13a3, nadc3*	412.1	304.4	309.2	136.2	98.1	51.7
DN47621_c0_g1	Solute carrier family 13 member 4 [*Chiloscyllium plagiosum*]	*slc13a4, sut1*	98.7	83.7	208.6	14.1	12.0	8.0
DN55553_c8_g1	Sulfate transporter-like [*Amblyraja radiata*]	*slc26a1, sat-1***	32.4	6.6	12.3	183.3	124.7	89.5
DN39385_c0_g1	Sulfate transporter [*Amblyraja radiata*]	*slc26a2, dtdst***	0.2	0.2	0.4	0.1	0.2	0.1
DN55312_c0_g2	Prestin isoform X1 [*Amblyraja radiata*]	*slc26a5, pres*	6.2	5.6	3.6	8.4	10.3	5.8
DN51950_c3_g1	Solute carrier family 26 member 6 [*Chiloscyllium plagiosum*]	*slc26a6*	76.6	117.8	111.5	7.6	7.5	2.4
DN53138_c4_g1	Solute carrier family 26 member 9 [*Amblyraja radiata*]	*slc26a9*	5.2	5.0	4.4	2.7	1.9	0.7
DN46515_c10_g1	Solute carrier family 26 member 10 [*Carcharodon carcharias*]	*slc26a10*	0.8	0.5	0.6	0.2	0.1	0.1
DN56056_c5_g2	Sodium-independent sulfate anion transporter [*Rhincodon typus*]	*slc26a11*	5.9	9.3	5.3	7.6	8.6	6.3
DN53519_c12_g1	Solute carrier family 41 member 1 [*Amblyraja radiata*]	*slc41a1***	131.0	88.8	70.4	31.5	27.9	14.4
DN49105_c7_g2	Solute carrier family 41 member 1 isoform X1 [*Carcharodon carcharias*]	*slc41a3***	1.0	1.4	0.6	0.3	0.5	1.6
DN46730_c7_g1	Metal transporter CNNM1 [*Amblyraja radiata*]	*cnnm1*	4.3	5.1	6.0	2.1	5.2	3.9
DN46730_c8_g2	Metal transporter CNNM2 isoform X1 [*Amblyraja radiata*]	*cnnm2*	7.3	5.8	8.5	7.0	6.3	5.7
DN46612_c11_g1	Metal transporter CNNM3-like isoform X1 [*Amblyraja radiata*]	*cnnm3*	1.0	1.3	1.1	1.4	1.1	1.8
DN53777_c5_g1	Transient receptor potential cation channel subfamily M member 6 [*Amblyraja radiata*]	*trpm6*	8.1	11.0	7.7	21.4	20.7	11.7
DN52096_c10_g1	Transient receptor potential cation channel subfamily M member 7 isoform X3 [*Amblyraja radiata*]	*trpm7*	13.5	18.0	12.1	9.6	12.3	7.7
DN47960_c6_g1	Transient receptor potential cation channel subfamily V member 4 [*Carcharodon carcharias*]	*trpv4*	0.6	0.5	0.3	0.4	0.3	0.6
DN133231_c0_g1	Transient receptor potential cation channel subfamily V member 5-like isoform X2 [*Amblyraja radiata*]	*trpv5*	0	0	0	0	0	0.2
DN47186_c0_g1	Transient receptor potential cation channel subfamily V member 6 [*Scyliorhinus canicula*]	*trpv6*	0.4	0.2	1.0	3.3	4.9	5.4
DN46788_c0_g3	Plasma membrane calcium-transporting ATPase 1 isoform X2 [*Amblyraja radiata*]	*pmca1*	4.6	4.5	6.3	3.8	4.4	5.0
DN52137_c2_g2	Plasma membrane calcium-transporting ATPase 2 isoform X3 [*Chiloscyllium plagiosum*]	*pmca2*	10.4	3.9	13.8	2.3	2.1	0.8

Expression levels were shown in TPM. **Orthology was checked by ORTHOSCOPE (Inoue and Satoh, 2019).

arrowhead labeled "c" in Figures 3I, M). However, *ncc* mRNA signals were almost undetectable in FW-acclimated individuals (Figures 3N, P).

Because the results of *in situ* hybridization indicate segment-specific upregulation of *nkcc2*, we analyzed the signal intensity of *nkcc2* mRNA expressions in each segment, namely, EDT and LDT (Figure 4). As with the observations of *in situ* hybridization images, the signal intensity of *nkcc2* mRNA was significantly increased in the EDT inside the tubular bundles. This was not observed in the LDT of the sinus zone.

Discussion

In the present study, we found that the red stingray is similar to other euryhaline elasmobranchs as the red stingray maintained high levels of plasma NaCl and urea that make up an osmolality over 600 mOsm in low-salinity environments. Euryhaline elasmobranchs produce dilute urine to cope with a massive water influx caused by the steep osmotic gradient between body fluid and low-salinity environments. Consistent with the previous findings in sawfish (Smith, 1931), Atlantic stingray (Janech and Piermarini, 2002),

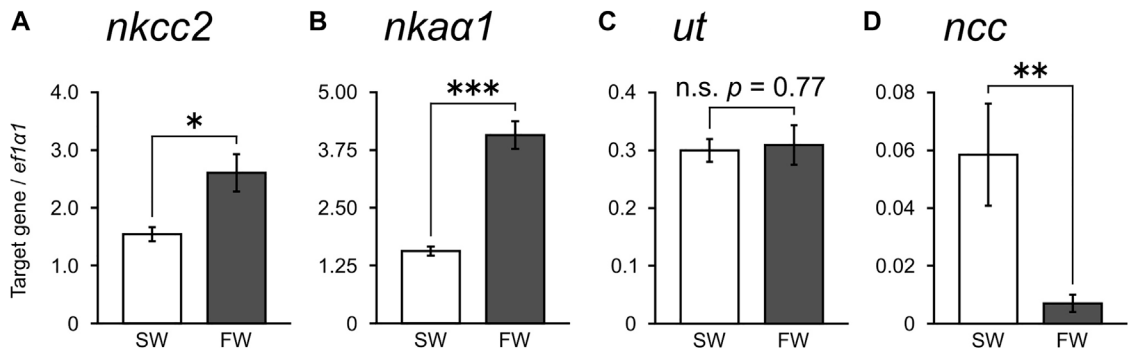

FIGURE 2
Expression of transporter mRNAs in the kidney measured by real-time qPCR. mRNAs for *nkcc2* **(A)**, *nkaα1* **(B)**, *ut* **(C)**, and *ncc* **(D)** were quantified and normalized against those of elongation factor 1α1 (*ef1α1*). *N* = 6 in each group. Asterisks indicate statistically significant differences between SW control and FW-acclimated stingrays (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

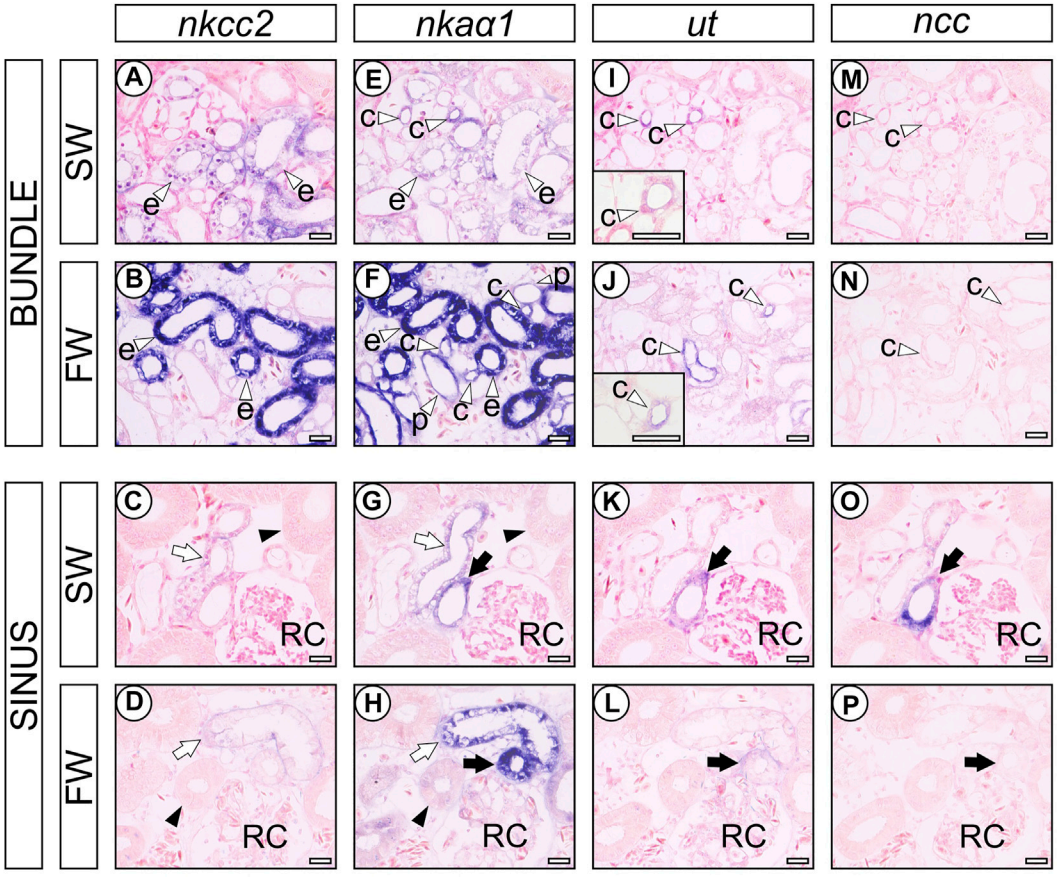

FIGURE 3
In situ hybridization analysis of transporter genes in the nephrons of SW control and FW-acclimated stingrays. *nkcc2* **(A–D)**, *nkaα1* **(E–H)**, *ut* **(I-L)**, and *ncc* **(M-P)** in SW control **(A,C,E,G,I,K,M,O)** and FW-acclimated **(B,D,F,H,J,L,N,P)** stingray. Open arrowheads indicate the PI (labeled with "p"), EDT (labeled with "e"), and CT (labeled with "c") in the bundle zone **(A,B,E,F,I,J,M,N)**. Filled arrowheads indicate the PII **(C,D,G,H)**. Open and filled arrows indicate the ascending and descending LDT, respectively **(C,D,G,H,K,L,O,P)**. RC, renal corpuscle. Bars, 50 μm. Note that 1) the *nkcc2* mRNA was intensely expressed in the EDT of FW-acclimated stingrays, but no difference was observed in LDT between SW control and FW-acclimated stingrays, and 2) concomitantly, prominent *nkaα1* mRNA signals were observed in the EDT of FW-acclimated stingrays.

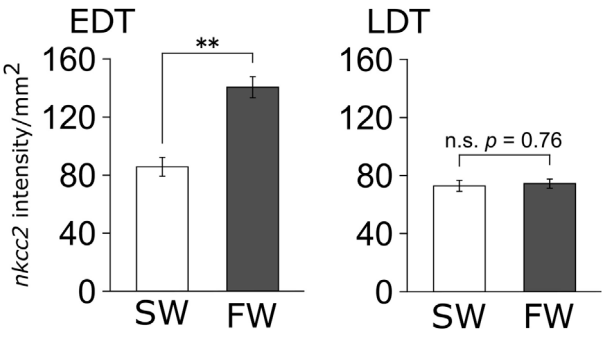

FIGURE 4
Signal intensity of *nkcc2* mRNA expressions in EDT and LDT. The signals of *nkcc2* mRNA expression in the EDT (left panel) and LDT (right panel) were quantified with ImageJ. Open and filled columns represent SW control and FW-acclimated individuals, respectively. $N = 4$ in each group. Statistically significant difference between SW and FW is shown with asterisks (**$p < 0.01$).

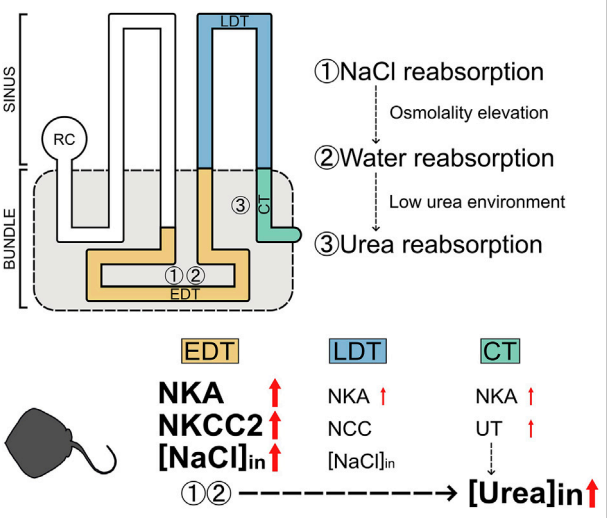

FIGURE 5
Schematic summary for the enhancement of NaCl and urea reabsorption in the kidneys of red stingray following acclimation to low-salinity environment. $[NaCl]_{in}$ and $[Urea]_{in}$ indicate reabsorptions of NaCl and urea, respectively.

and bull shark (Imaseki et al., 2019), red stingray excreted dilute urine following the acclimation to diluted environments. Our results further implied that the red stingray seems to possess a higher capacity to produce dilute urine than bull shark, for which physiological and molecular investigation of kidney function was conducted following the acclimation to FW in a similar manner to the present study (Imaseki et al., 2019). When we compared the U/P ratio of each parameter, the U/P ratios of osmolality, Na^+, and Cl^- were significantly decreased following the acclimation to 5% SW in red stingray. In FW-acclimated bull sharks, the Na^+ and Cl^- concentrations in the urine were 5- and 7-fold higher than those of 5% SW-acclimated red stingrays (Imaseki et al., 2019), leading to our hypothesis that the mechanisms of diluted urine production are different between bull shark and red stingray.

In the present study, GFR and UFR values were not available in conscious red stingrays acclimated to SW and low-salinity environments. Therefore, we borrowed the GFR and UFR values reported in the Atlantic stingray reared in ambient SW and 50% SW to estimate differences in the solute reabsorption rate between the SW control and 5% SW-acclimated red stingrays. The estimated values in Table 3 indicate that the transfer of red stingray from SW to 5% SW caused significant (1.4- to 2.2-fold) increases in reabsorption of NaCl and urea from the glomerular filtrate, suggesting that the red stingrays enhanced reabsorption of both NaCl and urea in low-salinity environments. However, it should be noted that our reported solute reabsorption rates in 5% SW-acclimated stingray may be underestimated. Since red stingrays in FW or 5% SW have larger osmotic difference between body fluid and holding water than the case of Atlantic stingray, the red stingrays must have experienced a greater influx of water. Therefore, it is reasonable to assume that the red stingray in FW or 5% SW should have a higher GFR and UFR.

Anatomical and molecular characteristics of the red stingray nephron to produce dilute urine

RNA-seq and subsequent qPCR analyses revealed that *nkcc2* (*slc12a1*) is the most abundantly expressed gene among candidate solute carrier (*slc*) family proteins involved in the NaCl reabsorption. The expression of *nkcc2* mRNA was significantly increased following the acclimation to FW environments. NKCC2 is an apically localized membrane protein contributing to active reabsorption of NaCl in the distal segment of FW teleost nephron (Takvam et al., 2021) and the thick ascending limb of the loop of Henle in the mammalian nephron (Bazúa-Valenti et al., 2016). In these diluting segments, NKCC2 reabsorbs NaCl from the glomerular filtrate in coordination with the basolateral NKA. Our data also indicated higher mRNA levels of *nkaα1*, *nkaβ1*, *fxyd2*, and *fxyd3* in FW-acclimated individuals, implying that the apical NKCC2 and basolateral NKA system was enhanced for producing dilute urine in the FW.

We previously found that the red stingray possesses a highly convoluted and elongated EDT segment, where NKCC2 and NKA are coexpressed for reabsorbing NaCl from the glomerular

filtrate (Aburatani et al., 2020). In the present study, *in situ* hybridization data demonstrated that *nkaα1* and *nkcc2* mRNA levels were upregulated in the EDT of the stingray nephron following the acclimation to FW. Although *nkcc2* mRNA was also found in LDT, the upregulation was only observed in the EDT. On the other hand, in bull shark, no increase was observed in the expression levels of *nkcc2* mRNA in the EDT following the transfer from SW to FW, implying that the difference in the expression levels of *nkcc2* mRNA causes the higher urinary concentrations of Na^+ and Cl^- in the FW-acclimated bull sharks than the 5% SW-acclimated red stingrays. Low levels of Cl^- in the urine ware also reported in sawfish *Pristis microdon* and Atlantic stingray *Hypanus sabinus* captured in FW environments (Smith, 1931; Janech and Piermarini, 2002). Although molecular and anatomical investigations are needed in sawfish and Atlantic stingray, we hypothesize that the remarkable ability of batoid nephrons to produce dilute urine is attributable to the enhanced expression of *nkcc2* and *nka* mRNAs in the well-developed EDT.

In addition to NaCl, the estimated reabsorption rate for urea was also increased in the stingray acclimated to low-salinity environments. The mechanism for the higher urea reabsorption in low-salinity environments is probably explainable with the urea reabsorption model proposed in cartilaginous fish (Figure 5; Hyodo et al., 2014). This model is composed of three steps: 1) active reabsorption of NaCl, 2) passive reabsorption of water, and 3) facilitative urea reabsorption in the bundle zone. The first step is active transport of NaCl from primary urine into the interstitial space in the microenvironment wrapped by the impermeable peritubular sheath (Lacy and Reale, 1986). NKCC2 and NKA expressed in the EDT are responsible for this step, resulting in elevation of interstitial osmolality inside the peritubular sheath. The elevated interstitial osmolality leads to water reabsorption via the aquaporin-expressing segment such as the EDT (Cutler et al., 2022), which creates a low-urea interstitial fluid inside the peritubular sheath. After these two steps, urea is left in processed filtrate and being concentrated. In the final step, urea is reabsorbed through the facilitative UT expressed in the collecting tubule (Hyodo et al., 2004; Kakumura et al., 2015; Aburatani et al., 2020) using the concentration gradient of urea as a driving force. Therefore, it is highly probable that the enhanced NaCl reabsorption in the EDT (the first step) consequently caused a greater amount of urea reabsorption in the kidney of FW (or 5% SW)-acclimated stingray (Figure 5).

In bull shark, NCC appeared to be a key molecule contributing to the successful FW acclimation (Imaseki et al., 2019). Approximately 10-fold increase was detected in the expression of *ncc* mRNA, and the *ncc* mRNA signals were remarkably abundant in the LDT (the fourth loop), which concomitantly expressed high level of *nka* mRNA. The NCC and NKA system in the FW bull shark is thought to increase the NaCl reabsorption in the sinus zone. In the stingray, on the other hand, *ncc* expression is limited to the transitional portion between LDT and CT as with houndshark (Imaseki et al., 2019). Furthermore, the expression levels of *ncc* were decreased following the FW acclimation. These results suggest that the mechanisms to produce dilute urine are different between red stingray and bull shark. Since the LDT is located outside of the peritubular sheath, the enhanced NaCl reabsorption in the LDT cannot affect the interstitial osmolality in the microenvironment wrapped by the peritubular sheath, which could be the reason why the estimated amount of reabsorbed urea from the primary urine is lower in FW-acclimated bull shark (Imaseki et al., 2019) than 5% SW-acclimated red stingray.

In FW-acclimated red stingrays, upregulation of *nkaα1* mRNA signals was observed not only in the EDT, but also in most segments in the bundle zone. Relatively high *nkaα1* expression was found in the PI and CT in the bundle zone of the kidney from FW-acclimated red stingray, suggesting that enhancement of active solute transports is not limited to the EDT for elevating the interstitial osmolality. Although currently an apically located transporter/channel, which functions in coordination with NKA, remains to be clarified in the PI and CT of FW-acclimated red stingray, reabsorption of any solutes could result in the further elevation of interstitial osmolality.

It has been well-documented that excretion of divalent ions is a crucial renal function of SW fishes, including cartilaginous fishes (Beyenbach, 2000; McDonald, 2007), as SW contains higher divalent ions than the body fluid. Indeed, the U/P ratio of divalent cations (in particular Mg^{2+}) was higher than that of monovalent cations in the SW control red stingray. Following the transfer to 5% SW, the U/P ratios of Ca^{2+} and Mg^{2+} decreased by 90.3 and 99.4%, respectively, indicating that excretory functions of divalent ions were strongly suppressed as the divalent ion influxes stopped in low-salinity environments. The genes encoding *slc13a3*, *13a4*, *26a6*, and *41a1* were abundantly expressed in the kidney of SW control stingrays, while FW transfer decreased the expression of those genes, suggesting that they may contribute to the excretion of divalent ions into the urine. In holocephalan elephant fish (*Callorhinchus milii*), the PII within the sinus zone expressed SLC26A1 and SLC26A6 at basolateral and apical membranes, respectively, and they were related to sulfate secretion (Hasegawa et al., 2016), supporting the putative role of SLC26A6 in divalent ion excretion in the red stingray nephron. In red stingray, however, the expression of *slc26a1* was upregulated in FW-acclimated individuals. Further studies are needed to investigate the mechanisms regulating divalent ion homeostasis in SW and low-salinity environments in batoids.

Perspectives: Evolution of euryhalinity in elasmobranchs

The subclass Elasmobranchii is composed of Selachii and Batoidea. Several species in Batoidea are known to be euryhaline, while only bull shark and their close relatives such as *Glyphis* are known euryhaline selachians. In addition, Grant et al. (2019) recently categorized 19 batoids as estuarine generalists, which suggests that they may have certain abilities to survive in wide ranges of salinity. The

categorized batoid species are phylogenetically diverse, including Dasyatidae, Pristidae, Rajidae, Rhinidae, and Rhinopteridae, while no selachians satisfied their criteria. These facts suggest that batoids have some intrinsic quality to accommodate low-salinity environments. Our previous investigation showed that in the batoid-type nephron, the EDT is more convoluted than that in selachians (Aburatani et al., 2020). Furthermore, we demonstrated that the red stingray has remarkable capacity to produce dilute urine, which is partly related to the enhanced expression of *nkcc2* and *nka* mRNAs in the highly convoluted EDT. These characteristics of the stingray nephron indicate that the batoid-type nephron could be advantageous for expanding their habitat to a wide range of salinity environments. Our data showed that red stingray and bull shark use different renal mechanisms for the acclimation to low-salinity environments, supporting the notion that batoids and selachians have acquired euryhalinity through the separate evolutionary trajectories (Ballantyne and Fraser, 2012). Further comparative studies on renal morphology and physiological responses to different environmental conditions in other euryhaline elasmobranchs are necessary to deepen our understanding of the osmoregulatory strategies in cartilaginous fishes.

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Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of Atmosphere and Ocean Research Institute of The University of Tokyo (P19-2).

Author contributions

NA, WT, and SH conceptualized and designed the study. NA, WT, MK-SW, CT, MK, and SK performed the experiments and collected data. NA, WT, KS, WG, and TS kept the animals and contribute to sample collection. NA wrote the first draft of the manuscript, and WT, MK-SW, and SH largely contributed to the revision process. All the authors contributed substantial input to the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.953665/full#supplementary-material

References

Aburatani, N., Takagi, W., Wong, M. K. S., Kadota, M., Kuraku, S., Tokunaga, K., et al. (2020). Facilitated NaCl uptake in the highly developed bundle of the nephron in Japanese red stingray *hemitrygon akajei* revealed by comparative anatomy and molecular mapping. *Zool. Sci.* 37 (5), 458–466. doi:10.2108/zs200038

Anderson, W. G., Taylor, J. R., Good, J. P., Hazon, N., and Grosell, M. (2007). Body fluid volume regulation in elasmobranch fish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 148 (1), 3–13. doi:10.1016/j.cbpa.2006.07.018

Ballantyne, J. S., and Fraser, D. I. (2012). Euryhaline elasmobranchs. *Fish. Physiol.* 32, 125–198. doi:10.1016/B978-0-12-396951-4.00004-9

Ballantyne, J. S., and Robinson, J. W. (2010). Freshwater elasmobranchs: a review of their physiology and biochemistry. *J. Comp. Physiol. B* 180 (4), 475–493. doi:10.1007/s00360-010-0447-0

Bazúa-Valenti, S., Castañeda-Bueno, M., and Gamba, G. (2016). Physiological role of SLC12 family members in the kidney. *Am. J. Physiol. Ren. Physiol.* 311 (1), F131–F144. doi:10.1152/ajprenal.00071.2016

Beyenbach, K. W. (2000). Renal handling of magnesium in fish: from whole animal to brush border membrane vesicles. *Front. Biosci.* 5 (712-719), D712–D719. doi:10.2741/beyenbach

Cappé, O., and Moulines, E. (2009). On-line expectation–maximization algorithm for latent data models. *J. Roy. Stat. Soc. B* 71 (3), 593–613. doi:10.1111/j.1467-9868.2009.00698.x

Choe, K. P., Kato, A., Hirose, S., Plata, C., Sindic, A., Romero, M. F., et al. (2005). NHE3 in an ancestral vertebrate: primary sequence, distribution, localization, and function in gills. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289 (5), R1520–R1534. doi:10.1152/ajpregu.00048.2005

Cutler, C. P., Kurt, K., Campbell, K. E., and Ojo, T. (2022). Aquaporin (AQP) channels in the spiny dogfish, *Squalus acanthias* II: localization of AQP3, AQP4 and AQP15 in the kidney. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 258, 110701. doi:10.1016/j.cbpb.2021.110701

Ebert, D. A., and Dando, M. (2020). *Field guide to sharks, rays & chimaeras of Europe and the Mediterranean*. Princeton University Press. doi:10.1515/9780691211824

Edwards, S. L., and Marshall, W. S. (2012). Principles and patterns of osmoregulation and euryhalinity in fishes. *Fish. Physiol.* 32, 1–44. doi:10.1016/B978-0-12-396951-4.00001-3

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* 29 (7), 644–652. doi:10.1038/nbt.1883

Grant, M. I., Kyne, P. M., Simpfendorfer, C. A., White, W. T., and Chin, A. (2019). Categorising use patterns of non-marine environments by elasmobranchs and a review of their extinction risk. *Rev. Fish. Biol. Fish.* 29 (3), 689–710. doi:10.1007/s11160-019-09576-w

Greenwell, M. G., Sherrill, J., and Clayton, L. A. (2003). Osmoregulation in fish: mechanisms and clinical implications. *Vet. Clin. North Am. Exot. Anim. Pract.* 6 (1), 169–189. doi:10.1016/S1094-9194(02)00021-X

Hasegawa, K., Kato, A., Watanabe, T., Takagi, W., Romero, M. F., Bell, J. D., et al. (2016). Sulfate transporters involved in sulfate secretion in the kidney are localized in the renal proximal tubule II of the elephant fish (*Callorhinchus milii*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 311 (1), R66–R78. doi:10.1152/ajpregu.00477.2015

Hazon, N., Wells, A., Pillans, R. D., Good, J. P., Anderson, W. G., Franklin, C. E., et al. (2003). Urea based osmoregulation and endocrine control in elasmobranch fish with special reference to euryhalinity. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 136 (4), 685–700. doi:10.1016/S1096-4959(03)00280-X

Hediger, M. A., Romero, M. F., Peng, J. B., Rolfs, A., Takanaga, H., Bruford, E. A., et al. (2004). The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. *Pflugers Arch.* 447 (5), 465–468. doi:10.1007/s00424-003-1192-y

Hyodo, S., Kakumura, K., Takagi, W., Hasegawa, K., and Yamaguchi, Y. (2014). Morphological and functional characteristics of the kidney of cartilaginous fishes: with special reference to urea reabsorption. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307 (12), R1381–R1395. doi:10.1152/ajpregu.00033.2014

Hyodo, S., Katoh, F., Kaneko, T., and Takei, Y. (2004). A facilitative urea transporter is localized in the renal collecting tubule of the dogfish *Triakis scyllia*. *J. Exp. Biol.* 207 (2), 347–356. doi:10.1242/jeb.00773

Imaseki, I., Wakabayashi, M., Hara, Y., Watanabe, T., Takabe, S., Kakumura, K., et al. (2019). Comprehensive analysis of genes contributing to euryhalinity in the bull shark, *Carcharhinus leucas*; Na^+-Cl^- co-transporter is one of the key renal factors upregulated in acclimation to low-salinity environment. *J. Exp. Biol.* 222 (12), jeb201780. doi:10.1242/jeb.201780

Inoue, J., and Satoh, N. (2019). Orthoscope: an automatic web tool for phylogenetically inferring bilaterian orthogroups with user-selected taxa. *Mol. Biol. Evol.* 36 (3), 621–631. doi:10.1093/molbev/msy226

Janech, M. G., Fitzgibbon, W. R., Ploth, D. W., Lacy, E. R., and Miller, D. H. (2006). Effect of low environmental salinity on plasma composition and renal function of the atlantic stingray, a euryhaline elasmobranch. *Am. J. Physiol. Ren. Physiol.* 291 (4), F770–F780. doi:10.1152/ajprenal.00026.2006

Janech, M. G., and Piermarini, P. M. (2002). Renal water and solute excretion in the Atlantic stingray in fresh water. *J. Fish. Biol.* 61 (4), 1053–1057. doi:10.1111/j.1095-8649.2002.tb01863.x

Kakumura, K., Takabe, S., Takagi, W., Hasegawa, K., Konno, N., Bell, J. D., et al. (2015). Morphological and molecular investigations of the holocephalan elephant fish nephron: the existence of a countercurrent-like configuration and two separate diluting segments in the distal tubule. *Cell Tissue Res.* 362 (3), 677–688. doi:10.1007/s00441-015-2234-4

Kempton, R. T. (1953). Studies on the elasmobranch kidney. II. Reabsorption of urea by the smooth dogfish. *Biol. Bull.* 104 (1), 45–56. doi:10.2307/1538690

Lacy, E. R., and Reale, E. (1986). The elasmobranch kidney. III Fine structure of the peritubular sheath. *Anat. Embryol.* 173 (3), 299–305. doi:10.1007/BF00318913

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with bowtie 2. *Nat. Methods* 9 (4), 357–359. doi:10.1038/nmeth.1923

Last P., Naylor G., Séret B., White W., de Carvalho M., and Stehmann M. (Editors) (2016). *Rays of the world* (Clayton South: CSIRO publishing). doi:10.1071/9780643109148

McCarthy, D. J., Chen, Y., and Smyth, G. K. (2012). Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* 40 (10), 4288–4297. doi:10.1093/nar/gks042

McDonald, M. D. (2007). "The renal contribution to salt and water balance," in *Fish osmoregulation* (Boca Raton: CRC Press), 309–332. doi:10.1201/9780429063909

McGinnis, S., and Madden, T. L. (2004). Blast: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res.* 32 (Suppl. l_2), W20–W25. doi:10.1093/nar/gkh435

Oguri, M. (1964). Rectal glands of marine and fresh-water sharks: comparative histology. *Science* 144 (3622), 1151–1152. doi:10.1126/science.144.3622.1151

Olson, K. R. (1999). "Rectal gland and volume homeostasis," in *Sharks, skates, and rays: The biology of elasmobranch fishes* (Baltimore: John Hopkins University Press), 329

Pang, P. K., Griffith, R. W., and Atz, J. W. (1977). Osmoregulation in elasmobranchs. *Am. Zool.* 17 (2), 365–377. doi:10.1093/icb/17.2.365

Payan, P., Goldstein, L., and Forster, R. P. (1973). Gills and kidneys in ureosmotic regulation in euryhaline skates. *Am. J. Physiol.* 224 (2), 367–372. doi:10.1152/ajplegacy.1973.224.2.367

Piermarini, P. M., and Evans, D. H. (2000). Effects of environmental salinity on Na (+)/K (+)-ATPase in the gills and rectal gland of a euryhaline elasmobranch (*Dasyatis sabina*). *J. Exp. Biol.* 203 (19), 2957–2966. doi:10.1242/jeb.203.19.2957

Piermarini, P. M., and Evans, D. H. (2001). Immunochemical analysis of the vacuolar proton-ATPase B-subunit in the gills of a euryhaline stingray (*Dasyatis sabina*): effects of salinity and relation to Na+/K+-ATPase. *J. Exp. Biol.* 204 (19), 3251–3259. doi:10.1242/jeb.204.19.3251

Piermarini, P. M., and Evans, D. H. (1998). Osmoregulation of the atlantic stingray (*Dasyatis sabina*) from the freshwater lake jesup of the st. Johns river, Florida. *Physiol. Zool.* 71 (5), 553–560. doi:10.1086/515973

Pillans, R. D., Good, J. P., Anderson, W. G., Hazon, N., and Franklin, C. E. (2005). Freshwater to seawater acclimation of juvenile bull sharks (*Carcharhinus leucas*): Plasma osmolytes and Na+/K+-ATPase activity in gill, rectal gland, kidney and intestine. *J. Comp. Physiol. B* 175 (1), 37–44. doi:10.1007/s00360-004-0460-2

Pirkmajer, S., and Chibalin, A. V. (2019). Hormonal regulation of Na+-K+-ATPase from the evolutionary perspective. *Curr. Top. Membr.* 83, 315–351. doi:10.1016/bs.ctm.2019.01.009

Rahmatullah, M., and Boyde, T. R. C. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chim. Acta.* 107 (1-2), 3–9. doi:10.1016/0009-8981(80)90407-6

Reilly, B. D., Cramp, R. L., Wilson, J. M., Campbell, H. A., and Franklin, C. E. (2011). Branchial osmoregulation in the euryhaline bull shark, *Carcharhinus leucas*: a molecular analysis of ion transporters. *J. Exp. Biol.* 214 (17), 2883–2895. doi:10.1242/jeb.058156

Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26 (1), 139–140. doi:10.1093/bioinformatics/btp616

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: An open-source platform for biological-image analysis. *Nat. Methods* 9 (7), 676–682. doi:10.1038/nmeth.2019

Smith, H. W. (1931). The absorption and excretion of water and salts by the elasmobranch fishes: I. fresh water elasmobranchs. *Am. J. Physiology-Legacy Content* 98 (2), 279–295. doi:10.1152/ajplegacy.1931.98.2.279

Stolte, H., Galaske, R. G., Eisenbach, G. M., Lechene, C., Schmidt-Nielsen, B., Boylan, J. W., et al. (1977). Renal tubule ion transport and collecting duct function in the elasmobranch little skate, *Raja erinacea*. *J. Exp. Zool.* 199 (3), 403–410. doi:10.1002/jez.1401990314

Takabe, S., Inokuchi, M., Yamaguchi, Y., and Hyodo, S. (2016). Distribution and dynamics of branchial ionocytes in houndshark reared in full-strength and diluted seawater environments. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 198, 22–32. doi:10.1016/j.cbpa.2016.03.019

Takvam, M., Wood, C. M., Kryvi, H., and Nilsen, T. O. (2021). Ion transporters and osmoregulation in the kidney of teleost fishes as a function of salinity. *Front. Physiol.* 12, 664588. doi:10.3389/fphys.2021.664588

Thorson, T. B. (1971). Movement of bull sharks, carcharhinus leucas, between caribbean sea and lake nicaragua demonstrated by tagging. *Copeia* 1971 (2), 336. doi:10.2307/1442846

Thurau, K., Antkowiak, D., and Boylan, J. W. (1969). Demonstration of a renal osmoregulatory mechanism in the spiny dogfish, *Squalus acanthias*. *Bull. Mr. Desert Isl. Biol. Lab.* 9, 63–64.

Wong, T. M., and Chan, D. K. O. (1977). Physiological adjustments to dilution of the external medium in the lip-shark *Hemiscyllium plagiosum* (Bennett). II. branchial, renal and rectal gland function. *J. Exp. Zool.* 200 (1), 85–95. doi:10.1002/jez.1402000111

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Inhibition of gastric acid secretion with omeprazole affects fish specific dynamic action and growth rate: Implications for the development of phenotypic stomach loss

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An acid-secreting stomach provides many selective advantages to fish and other vertebrates; however, phenotypic stomach loss has occurred independently multiple times and is linked to loss of expression of both the gastric proton pump and the protease pepsin. Reasons underpinning stomach loss remain uncertain. Understanding the importance of gastric acid-secretion to the metabolic costs of digestion and growth will provide information about the metabolic expense of acid-production and performance. In this study, omeprazole, a well characterized gastric proton pump inhibitor, was used to simulate the agastric phenotype by significantly inhibiting gastric acidification in Nile tilapia. The effects on post-prandial metabolic rate and growth were assessed using intermittent flow respirometry and growth trials, respectively. Omeprazole reduced the duration (34.4%) and magnitude (34.5%) of the specific dynamic action and specific growth rate (21.3%) suggesting a decrease in digestion and assimilation of the meal. Gastric pH was measured in control and omeprazole treated fish to confirm that gastric acid secretion was inhibited for up to 12 h post-treatment ($p < 0.05$). Gastric evacuation measurements confirm a more rapid emptying of the stomach in omeprazole treated fish. These findings reinforce the importance of stomach acidification in digestion and growth and present a novel way of determining costs of gastric digestion.

KEYWORDS

***Oreochromis niloticus*, specific growth rate, metabolic rate, H^+/K^+-ATPase, stomach**

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Introduction

The stomach is a highly conserved vertebrate organ that provides a number of key advantages in the digestion of nutrients (Wilson and Castro 2010). Emerging in an elasmobranch ancestor approximately 350 million years ago (Koelz 1992), the stomach is defined by the production of hydrochloric acid (HCl) and pepsinogen (Smit 1968) by specialized oxynticopeptic cells. Pepsinogen, the inactive zymogen, is cleaved by HCl to produce activated pepsin, a proteolytic digestive enzyme (Sanny et al., 1975; Kageyama 2002). The stomach plays a key role in the breakdown and digestion of many food components including protein (Barrington 1942; Bakke et al., 2010), animal exoskeletons, plant cell walls (Lobel 1981), and solubilization of elements including phosphorus (Sugiura 2006) and calcium (Koelz 1992). Protein denaturation relies heavily on low pH in the stomach and phosphorus solubilization at low pH makes different supplemental forms more bio-available (Cho and Bureau 2001).

Despite the clear advantages of acid-peptic digestion, paradoxically many teleost species are agastric due to secondary loss of the acid-peptic digestive phenotype (Wilson and Castro 2010). Notably, these loss events result in not only phenotypic loss of the stomach, but also the loss of key stomach genes from the genome indicating a permanence of this loss (Castro et al., 2014). The key genes in this loss are the *atp4a* and *atp4b* that encode the respective α and β subunits of the gastric proton pump (H^+/K^+-ATPase), as well as various pepsin encoding genes (Castro et al., 2012, 2014). The gastric proton pump that produces the characteristic HCl in the stomach lumen is energetically expensive, as it relies on ATP-hydrolysis for ion movement, and there are costs associated with the maintenance of protective mucus and the neutralizing of the acid in the small intestine by bicarbonate. In vertebrate species that maintain gastric acid production, the energetic costs of gastric acid secretion are outweighed by the increased efficiency of nutrient utilization, particularly protein via pepsin digestion, and thus enhanced growth rates. It is hypothesized that the costs of gastric acidification might outweigh the benefits under some circumstances, ultimately resulting in the loss of these genes over time.

To better understand the role of the stomach in digestion, metabolic costs of acid-peptic digestion need to be determined. The increase in metabolic rate observed after the ingestion of a meal has been documented in many animals (Secor 2009). Termed the specific dynamic action (SDA) or the thermic effect of feeding, this increase in energy expenditure is thought to be the "cost of digestion" from mastication and secretion to digestion and assimilation (Secor 2009; Chabot et al., 2016). SDA represents the metabolic rate associated with feeding, which exceeds the standard metabolic rate (SMR), a basic maintenance requirement measured as the minimum rate of oxygen consumption of non-feeding, unstressed animals at rest, below which physiological function is impaired (Priede 1985). SDA has been previously used in fin fish aquaculture to identify optimal meal sizes, meal composition, ideal water temperature, and water pH that minimizes the SDA response, allowing for increased energy to be allocated to growth (e.g., Jobling 1981; Chakraborty et al., 1995; Tirsgaard et al., 2015). Despite these studies focussing on optimizing feeding responses, there have been limited efforts to determine the metabolic cost of gastric digestion, with one earlier study in Burmese python (Secor 2003), which estimated the stomach contributed 55% of SDA for a meal equivalent to 25% of body mass (BM) although this high value is disputed (see Wang and Rindom 2021). However, in order to isolate the direct metabolic costs of stomach acidification, the proton pump inhibitor omeprazole, can be used to inhibit this acid production (Andrade et al., 2004; Wood et al., 2009; Schubert 2017). Omeprazole provides a tool for unique comparisons of growth rates and SDA patterns in animals by inhibiting stomach acidification to create a pharmacological knock-down of gastric-acid secretion thereby mimicking the agastric condition.

Omeprazole, a benzimidazole derivative, acts to prevent gastric acid secretion by binding cysteine residues on the luminal side of the gastric proton pump H^+/K^+-ATPase, irreversibly blocking pump function (Lindberg et al., 1987). Omeprazole is a highly specific drug that works only in the acidic environment of the canaliculus of the acid-secreting oxynticopeptic or parietal cells making it an excellent candidate for analyzing energy usage during acid-digestion (Lindberg et al., 1986; Morii et al., 1989). Omeprazole is widely used in human medicine to reduce stomach acid production (McTavish et al., 1991) and has been used in spiny dogfish (25 mg kg^{-1} BM; *Squalus acanthias*, Wood et al., 2009) and common boa (22 mg kg^{-1} BM; *Boa constrictor*, Andrade et al., 2004) to study the post-feeding alkaline tide. The former study confirmed the acid-reducing effect of omeprazole in fish and highlighted future applicability of the drug. Omeprazole has also been used to study extragastric (gill and kidney) H^+/K^+-ATPase in Nile tilapia (*Oreochromis niloticus*) (Barnawi et al., 2020). In humans, omeprazole has been shown to delay overall assimilation of protein and cause potential protein malabsorption (Evenepoel et al., 1998). However, from a nutritional point of view in humans its impacts are likely negligible (Evenepoel et al., 1998) although not without risks in vulnerable groups (Heidelbaugh 2013).

In the present study, omeprazole was used to reveal the contribution of gastric acidification to patterns of the SDA and growth in the Nile tilapia (*O. niloticus*), a fish with a highly acidic stomach (pH 2.0; Moriarty 1973) and rapid growth rate with significant commercial importance in aquaculture (Maclean et al., 2002). A multi-experimental approach was used to confirm inhibition of gastric acid secretion and examine the

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TABLE 1 Guaranteed analysis of the commercial balanced diet used. Bluewater Feed Company Trout 2M 50-19.

Crude protein	Min	50.0%
Crude Fat	Min	19.0%
Crude Fiber	Max	2.3%
Sodium	Actual	0.4%
Calcium	Actual	1.5%
Phosphorus	Actual	1.2%
Vitamin A	Min	4,000 IU/kg
Vitamin D	Min	2,500 IU/kg
Vitamin E	Min	131 IU/kg

effects of this treatment on growth and digestion. Four experiments are outlined: 1) The effects of omeprazole on gastric pH were measured to confirm inhibition of gastric acid secretion. 2) Next, the SDA was measured to examine the overall metabolic costs of digestion with inhibited gastric acid secretion. To better understand the effects of omeprazole on SDA and long-term effects of the treatment 3) growth rate was assessed over an eight-week feeding trial and 4) gastric evacuation rate determined. With respect to SDA, we predicted a decreased contribution of the H^+/K^+-ATPase to SDA peak $\dot{M}O_2$ and increase in time to peak of the post-prandial metabolic response because acidification occurs earlier in the digestive process. As inhibition of gastric acidification by omeprazole should reduce activation of pepsin and thus protein digestion, we also predicted reduced protein breakdown which should translate into a prolonged SDA duration and a decrease in growth rate. With the delay in acidification, we predicted a decrease in gastric evacuation rates with omeprazole treatment since acidification would take longer.

Materials and methods

Animals

Juvenile male Nile tilapia (*O. niloticus*) were obtained from Sandplains Aquaculture (Mossley, ON) and acclimated in 500 L recirculation tanks with mechanical and biological filtration, aeration, and 20% daily water changes in the Wilfrid Laurier University animal care facility. Water was prepared by mixing two parts reverse osmosis (RO) water with one-part city of Waterloo dechlorinated tap water to reduce water conductivity and hardness. The tilapia water had a final conductivity of approximately 300 μS cm^{-1}, pH 7.7, Na^+ 2.86 mM Cl^- 0.85 mM, Ca^{2+} hardness 300 mg L^{-1} $CaCO_3$ and alkalinity of 80 mg L^{-1} $CaCO_3$. Temperature was maintained at 26°C using a thermostat (InkBird Tech, Shenzhen, PRC) and immersion heater (300W Eheim Gmbh, Deizisau, Germany) in each tank. The light regime was 12 h light: 12 h dark. All experiments were conducted under the Canadian Council for Animal Care guidelines using protocols approved by the Laurier Animal Care Committee (AUP R14002 and R18003).

Preparation of control and omeprazole dosed diets

In all trials, the control and omeprazole treated diets were prepared using the same procedure. Omeprazole and control diets were prepared using a commercial balanced diet (Bluewater Feed Company, Desboro, ON, Canada) formulated with the guaranteed analysis presented in Table 1. Omeprazole was added to the treated diet at a dose of 25 mg kg^{-1} BM day^{-1} (Wood et al., 2009) based on either a 1% or 2% BM daily ration by initially dissolving it in 95% ethyl alcohol (EtOH) and spraying it onto the feed. The feed was then air dried to allow for EtOH evaporation and was stored at 4°C in sufficient amounts to feed fish for 1 week. EtOH was added to the control diet and was air-dried to control for any effects of residual EtOH on gastric acidification, energy expenditure and/or growth.

Experimental procedures to measure gut pH

Understanding the extent to which omeprazole affects gastric pH was critical for this study. The pH trial was conducted using two groups of 30 Nile tilapia (30.4 ± 5.9 g) randomly assigned to one of the treatment groups. Feed (sham control and omeprazole) was given at a set time each day and daily ration was recorded. After 2 weeks on the treatment rations, fish were removed from the tanks at 3, 6-, 12-, 24- and 48-h post-feeding for determination of gastric and anterior intestinal pH (n = 6). Serial dissection was chosen for this experiment as the 'T' shape and relatively small size of the stomach (Morrison and Wright 1999; 1.4% (v:w) BM for a mean fish mass of 143 g, n = 34, unpublished data) makes *in vivo* pH measurement potentially harmful and stressful to the fish.

At each time point, the tilapia were euthanized with an overdose of MS222 (1:5000 w:v; Syndel, Nanaimo BC Canada) buffered with $NaHCO_3$ followed by spinal transection, and fish mass and standard length were recorded. Glass combination pH electrodes (Biotrode, Hamilton) and Radiometer PHM85 pH meters were used to measure pH. The pH was measured first in the full excised stomach including chyme, the emptied stomach, and finally in the separated stomach contents. The anterior intestine was also removed and ligated 3 cm from the pyloric sphincter to form a gut sac. The pH of the chyme in this sac was recorded. The pH electrodes were rinsed with deionized water after each

measurement and calibration was checked twice daily using precision standards (Hanna Instruments, Woonsocket RI). Negligible electrode drift was observed. The pH of the feed was determined in a slurry created by mixing 1 g of feed pulverized with a mortar and pestle in 10 ml of Milli Q water at ambient temperature (24°C).

The buffer capacity of the feed was also determined by manually titrating this 1 g pellet mixture using 1 N HCl and the same pH system described above with a Radiometer TTA80 titration assembly. The resulting titration curve (Supplementary Figure S1) was used to calculate the amount of acid required to acidify 1 g of pellets from its original pH based on the full stomach pH measurement over the 0–3 h and 0–6 h time intervals. Two linear regressions were fitted to the data covering the pH ranges of 6.2–5.0 [y = −0.0120 x + 6.238. r^2 0.97] and pH 5.0–2.0 [y = −0.00251x+5.346 r^2 0.99]. Gastric acid secretion is expressed as μEq H^+ g^{-1} feed h^{-1}.

Respirometry

Oxygen consumption rates ($\dot{M}_{O2}$; μmol O_2 min^{-1} kg^{-1}) in Nile tilapia were determined using automated intermittent flow respirometry (Steffensen 1989). Respirometry was performed using a set of four 1-L respirometry chambers, each with a galvanic oxygen electrode connected to a 4-channel oxygen analyzer (AMP-DAQ4) and flush and recirculation pumps controlled through a DAQ-M unit using AutoResp™ software (version 2.2.0; Loligo® Systems Viborg Denmark). The setup corresponded to previous studies measuring $\dot{M}_{O2}$ in fish (Tirsgaard et al., 2015; Rosewarne et al., 2016). The galvanic oxygen electrodes were calibrated using a 1g L^{-1} solution of sodium sulfite in water (0%) and in air (100% air saturation). $\dot{M}O_2$ was measured every 10.5 min cycling between 4 min flush, 0.5 min wait, and 6 min measurement periods. Specifically, $\dot{M}_{O2}$ was calculated using the rate of the declining oxygen content during the measurement period (Svendsen et al., 2015). The associated R^2 values above 0.95 were used for $\dot{M}_{O2}$ measurements. To avoid physiological effects of hypoxia, water oxygen levels were maintained above 80% air saturation (O_{2sat}) during the measurement period, similar to previous respirometry studies (Tirsgaard et al., 2015; Baktoft et al., 2016). The oxygen consumption rates were used as a proxy for aerobic metabolic rate and thus energy use (Nelson 2016; Schwartzbach et al., 2020). The respirometry systems were submerged in a 450 L trough tank of circulating, filtered and aerated water kept at a consistent 26°C using a temperature regulator (TMP-REG, Loligo®Systems). The trough tank was partially covered to minimize visual disturbances of the fish, although dim light corresponding to the diel cycle remained present in the tank.

Experimental procedures to measure specific dynamic action

Respirometry trials utilizing randomized paired control and omeprazole feedings were performed to determine acute effects of omeprazole on the metabolic rate of Nile tilapia. Eight Nile tilapia (19.5 ± 4.29 g) were used for the paired control and omeprazole feedings. The experiment started by weighing and placing the Nile tilapia into the individual respirometry chambers. The following 48 h were used for fasting and to collect $\dot{M}O_2$ data for estimates of SMR. The fish were then voluntarily fed meals of a commercial balanced diet (Bluewater Feed Company) equaling 2% BM via a feeding port. In a randomized fashion, half of the meals were dosed with omeprazole (TCI, Portland OR United States). Fish were fed voluntarily inside the respirometry chambers *via* a feeding port, eliminating the need for sham feedings for stress control (Chabot et al., 2016). Meals were consumed completely within 30 min. The metabolic rates of the fish were recorded for 96 h post-feeding, allowing the oxygen consumption rate to peak after feeding and then return to the SMR following the post-prandial response. Each fish was given both an omeprazole-dosed and a control sham treated meal over the course of the experiment to allow for pairwise comparison between the treatments in each individual fish.

SMR and SDA variables were calculated using the R package "FishMO2", which uses non-parametric quantile regression to correct for circadian activity patterns when calculating SDA variables (Chabot et al., 2016). Tau (t) was set at 0.2 to calculate SMR and a lambda (λ) value of 24 h was used as the activity cycle variable as the standard protocol suggests. The R package was used to estimate SMR, peak time post feeding, postprandial peak, net postprandial peak, SDA duration and SDA magnitude of the individual fish. The variables were then compared between acute omeprazole and control feedings.

Growth trial

To further understand the long-term effects on stomach acidification, growth rates of fish on control and omeprazole treatments were determined. To track individual growth rates all fish were anaesthetized with MS222 (1:10,000) and implanted with 8 mm PIT (passive integrated transponder) tags (Biomark, Boise, ID, United States) into the peritoneal cavity through an incision made with a #11 scalpel blade. Fish were allowed 1 week to recover. The growth trial was conducted using two groups of 23 Nile tilapia (n = 23) (33.0 ± 10.4 g) fed either a control feed, the commercial balanced diet (Bluewater Feed Company), or fed the control diet supplemented with omeprazole (TCI). All fish were kept at a constant water temperature of 26°C using a

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thermostat (InkBird Tech) and immersion heaters (300W Eheim Gmbh). The fish in both groups were fed manually at a rate of 2% BM per day separated over two meals. No differences were seen in feeding behaviors between treatments, and the entire ration was consumed at each feeding. The feeding trial was conducted for 8 weeks, with biometric parameters (standard length and mass) taken every 2 weeks, which allowed for adjustment of the ration size (2%) for the growing fish.

Over the 8 weeks, the BM data was compiled, and specific growth rate (SGR) was calculated as

$$SGR = \frac{\left(\ln M_f - \ln M_i\right)}{T}$$

where M_f is the final mass (g), M_i is the initial mass, and T is the time (days) between measurements. Fulton's condition factor (K) (Fulton, 1904) was calculated as

$$K = \frac{100,000^{*}M}{L^3}$$

where M is the wet mass (g) and L is the corresponding standard length (mm) of the fish.

Growth rates and condition factor were calculated over the entire eight-week period to determine if growth rates were consistent over the feeding study. These measures were used to compare the increments of mass gain between different treatment groups based on growth per day (SGR). At the end of the trial, the tilapia were euthanized with an overdose of buffered MS222 (1:5000 w:v) followed by spinal transection, and fish mass, and standard length was recorded.

Experimental procedures to measure gastric evacuation

A gastric evacuation trial was conducted using two groups of 60 Nile tilapia (46.2 ± 13.7 g) randomly assigned to one of the treatment groups (control or omeprazole). The daily feeding rate throughout the trial was 1% BM day^{-1}. This meal size was the equivalent to the meal size given in the growth trial that was provided twice daily (2% × 1% BM). Serial dissection at five selected timepoints 1, 3-, 6-, 12-, and 24-h post-feeding (n = 10) was used to assess gastric contents gravimetrically. At each time point, tilapia from each group were euthanized with an overdose of buffered MS222 (1: 5000 w:v) followed by spinal transection, and fish mass and standard length were recorded. The stomach of the fish was then excised, and all stomach contents were collected into pre-weighed tubes for mass determination. Stomach contents were then weighed, and frozen on dry ice for later dry matter analysis. Dry matter of the stomach contents was determined by drying the sample at 105°C until constant mass was reached (AOAC, 2000).

Statistical analysis

Data is presented as means ± standard deviation (SD). Respirometry data (SMR, peak time post feeding, peak value, net peak, SDA duration and SDA magnitude) were analyzed using paired Student's t-tests, while growth (SGR), chyme pH, and biometric (K) data were analyzed using a two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls (SNK) post hoc test. SGR and chyme pH data were transformed to satisfy conditions for a parametric test. Gastric evacuation and stomach (full, empty) and intestine pH data were either not normally distributed or lacked homogeneity of variance even after transformation and were therefore analyzed using two separate Kruskal Wallis one-way ANOVAs on ranks for control and omeprazole groups over time. When appropriate, either a Student's t-test or Wilcoxon rank sum exact test was used to compare treatment differences at each time point. Results were considered significant at $p \leq 0.05$. Sigmaplot 11.0 software was used for statistical analyses (Systat Software Inc., Palo Alto, CA).

Results

The pellets fed to the fish had a starting pH of 6.261 ± 0.016 (n = 3). In control fish, at the first sampling point 3 h post feeding a 1% BM meal, the pH of the stomach was around pH 4 as measured in the stomach full or emptied, and the separated chyme (Figures 1A–C, respectively). From 6 h onward, the full stomach pH significantly declined further to less than pH 2. This was reflected in the stomach chyme readings but not the emptied stomach pH. In the omeprazole treatment group, stomach pH was significantly higher than the control fish from 3 to 12 h post feeding in the pH five to six range. By 24 h stomach pH was significantly lower and not different from the control fish. The patterns of full stomach pH changes in control and omeprazole groups reflected patterns seen in chyme pH rather than the emptied stomach. The calculated gastric acid secretion rates over the 0–3 h and 0–6 h post prandial periods were depressed by over 90% in the omeprazole treated fish compared to the sham controls (Figure 1E).

The pH in the anterior intestinal was lower 3–6 h post feeding compared to later time points in control fish, which reflects the input of the acidic chyme from the stomach and peaked at a higher pH of 7.073 at 24 h (Figure 1D). Only during the first 12 h post feeding was the intestinal pH in omeprazole treated fish higher than in controls. Note that the 6 h time point was marginally non-significant (p = 0.052). There were also no significant changes in intestinal pH in omeprazole treated fish over time.

During the respirometry trial, tilapia voluntarily consumed the full meal provided in the respirometry chambers within 30 min. The SMR values measured in the 48 h period prior to feeding were not significantly different between the two treatment groups establishing

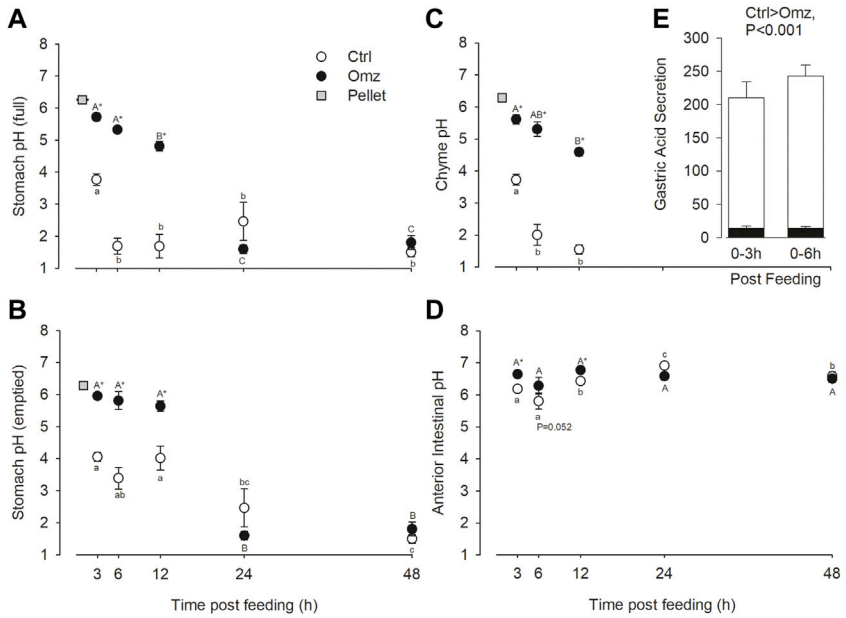

FIGURE 1
Nile tilapia (*Oreochromis niloticus*) gut pH measurements and gastric acid secretion rates. PIT tagged Nile tilapia were fed a 1% BM ration of pellets that were either sham treated (Control; white fill) or dosed with omeprazole at 25 mg kg^{-1} BM (Omeprazole; black fill) for 30 days. Tilapia were sampled at 3-h, 6-h, 12-h, 24-h and 48-h post-feed. The pH of the full stomach **(A)**, the emptied stomach (mucosa only) **(B)**, the isolated stomach chyme **(C)**, and the anterior intestine with contents **(D)** were measured. Note, stomach chyme was not present past 12 h post-feed, so no data is present at 24 and 48 h **(E)** Gastric acid secretion rates (μEq H^+ g^{-1} feed h^{-1}) were estimated over the 0–3 h and 0–6 h post prandial periods from pH change in **(A)** and the pellet titration curve (Supplementary Figure S1). Data were analyzed by two-way ANOVA with SNK post hoc test; n = 6. Time points with unshared letters are significantly different from each other. Asterisks signify significant differences between the two treatment groups.

a comparable baseline (Figure 2; Table 2). The SDA peak or net peak and time to peak values were not significantly different between the two treatment groups. Net peak data is not shown. However, the SDA duration in tilapia fed the omeprazole dosed meals was 34.4% shorter and the overall SDA magnitude or scope was 34.5% lower compared to the paired control meal.

The specific growth rates were calculated from the eight-week growth trial (Figure 3). Fish fed equally well on both diets, and rations were completely consumed during each feeding. Over the eight-week period the omeprazole group exhibited a significant reduction in SGR by 21.3% (Ctrl 2.12 ± 0.45; Omeprazole 1.67 ± 0.32 $p < 0.001$). There were also some time dependent changes in SGR over the eight-week period with a significant decrease in SGR over time with no interaction of time and treatment. At the end of the trial, the omeprazole associated decrease in growth rates resulted in a lower total biomass gain of 502.65 g (n = 23) or an average of 20 g less mass added per individual, in comparison to the control group. There was no significant difference in condition factor during the feeding trial (Ctrl 2.39 ± 0.53; Omeprazole 2.35 ± 0.52, $p = 0.819$).

The gastric evacuation data is presented in Figure 4. In the control fish there was a decrease in stomach content wet mass after 1 h post feeding which was relatively stable until 12 h but by 24 h the stomach was essentially empty. In the case of the omeprazole treated fish, the amount of stomach contents did not change over the first 6 h post feeding but had decreased significantly by 12 h and was essentially absent by 24 h. At the 12 h time point there was significantly less chyme in the stomach of the omeprazole treated fish compared to the control ($p = 0.013$), indicating a more rapid gastric evacuation.

Discussion

The gastric proton pump inhibitor omeprazole predictably inhibited post-prandial gastric acidification in juvenile Nile tilapia. This greater than 90% inhibition of acidification corresponded with a decrease in both SDA duration and magnitude. It followed that there was a more rapid gastric evacuation or emptying following a meal. Although there was a decrease in the magnitude of the SDA, this was not reflected in a digestive energy savings that could then be allocated to increased growth but rather a reduction in the SGR by more than 20% was observed in the omeprazole treated fish. Collectively, these data suggest that simulation of an agastric phenotype in fish by pharmacological "knock down" of the gastric proton pump with omeprazole may be constrained by poor digestion, which highlights the high degree of conservation of the gastric phenotype in vertebrates. This could imply that the emergence of the agastric

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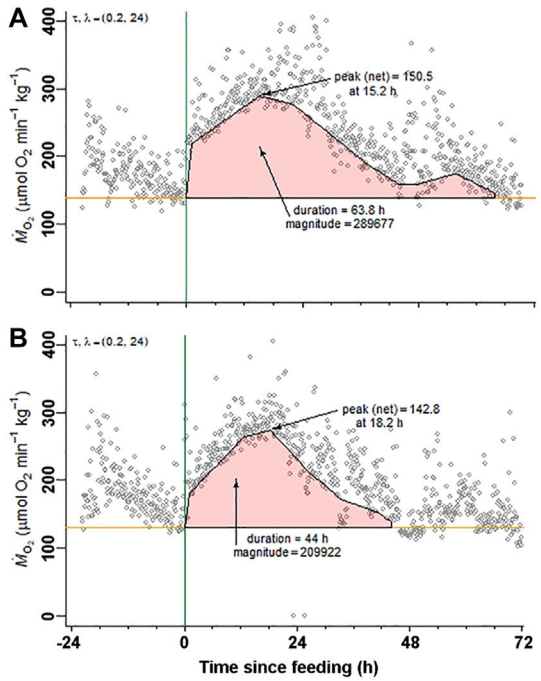

FIGURE 2
Nile tilapia (*Oreochromis niloticus*) Specific Dynamic Action measurements. A representative set of metabolic rate measurements (M_{O2}; µmol O_2 min^{-1} kg^{-1}) over time from a Nile tilapia (19.5 g) following a 2% body mass (BM) voluntarily fed meal of **(A)** control pellets and **(B)** 25 mg kg^{-1} BM omeprazole dosed pellets. Standard metabolic rate (SMR) and specific dynamic action (SDA) parameters (time to peak, peak, net peak, duration, and magnitude) were calculated according to Chabot et al. (2016) using the R package "FishMO2".

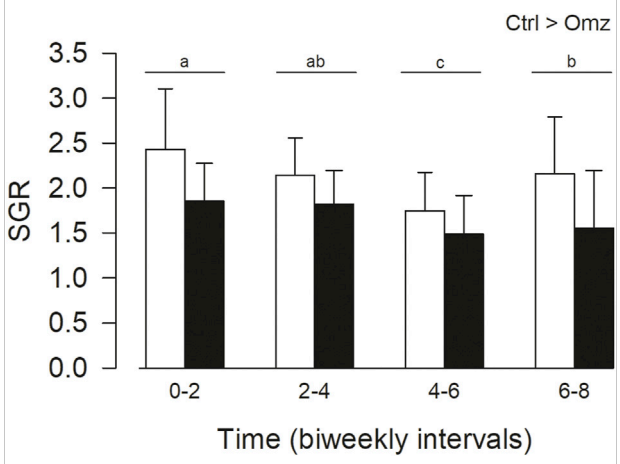

FIGURE 3
Nile tilapia (*Oreochromis niloticus*) growth trial. PIT tagged Nile tilapia were fed daily a 2% BM ration of pellets that were either sham treated (Control; Ctrl, white bars) or dosed with omeprazole at 25 mg kg^{-1} BM (Omeprazole; solid bars) over an eight-week period and weighed every 2 weeks. Specific growth rates (SGR) were calculated over bi-weekly intervals. Data was analyzed by two-way ANOVA with SNK post hoc test; n = 23. There were significant treatment (Ctrl>Omz) and time effects but without interactions. The time intervals with unshared letters are significantly different from each other.

phenotype in fishes is correlated with the availability of easily digestible diets, but this hypothesis requires future testing.

The inhibition of gastric acidification with omeprazole was consistent with earlier studies (*Squalus acanthias* Wood et al., 2009; *Boa constrictor*, Andrade et al., 2004). The patterns of pH changes indicated that the pH of the full stomach was largely reflective of the chyme pH rather than the gastric mucosa (emptied stomach). We also estimated gastric acid secretion from the pH changes of the stomach contents and estimated buffer capacity of 1 g of pellets which we predicted would be relatively stable over the 6 h post prandial period based on our gastric evacuation data set. In control fish, 210–240 µEq H^+ were secreted to acidify 1 g of pellets. In contrast omeprazole treated fish only secreted 14 µEq H^+, representing a >90% decrease in acid secretion. The degree of inhibition is in keeping with human studies, where omeprazole doses of 20–40 mg resulted in 80%–100% reductions in stimulated acid secretion (Olbe et al., 1989). It followed that the chyme in the anterior intestine was significantly less acidic in omeprazole treated fish. This reduction in the acidity of the intestinal chyme might be

TABLE 2 Standard metabolic rate (SMR) and Specific Dynamic Action (SDA) parameters measured in Nile tilapia (*Oreochromis niloticus*) voluntarily fed a meal of 2% BM of either sham-treated pellets (Control) or pellets treated with omeprazole at a final dose of 25 mg kg^{-1} BM. The variables were compared using paired t-tests n = 8.

Specific dynamic action variables

Treatment	SMR ($\dot{M}O_2$)	Peak time (h)	Peak ($\dot{M}O_2$)	Duration (h)	SDA (x10^3) (mg O_2 kg^{-1})
Control	129.2 ± 5.2	12.1 ± 2.1	248.2 ± 9.0	63.8 ± 3.8	246 ± 28
Omeprazole	135.9 ± 9.6	15.2 ± 1.3	248.3 ± 11.4	41.8 ± 2.6	161 ± 25
p	0.556	0.192	0.992	0.002	0.015

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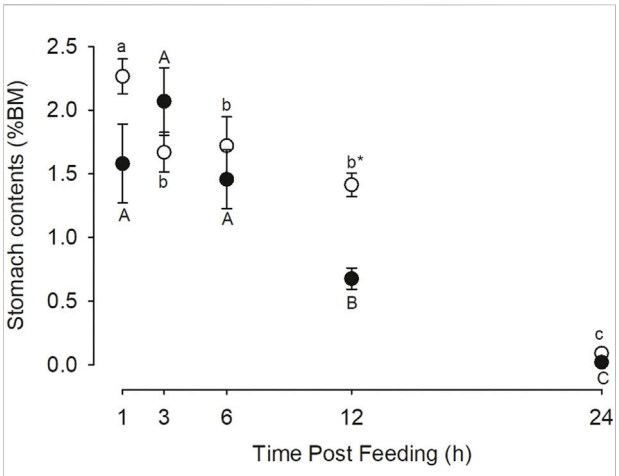

FIGURE 4
Nile tilapia (*Oreochromis niloticus*) stomach evacuation. PIT tagged Nile tilapia were fed a 1% BM ration of pellets that were either sham treated (Control; white circles) or dosed with omeprazole at 25 mg kg^{-1} BM (Omeprazole; black circles) for 30 days. Serial dissections at five selected timepoints 1, 3-, 6-, 12-, and 24-h post-feeding (n = 10) was used to assess gastric contents gravimetrically. These values represent the wet mass of stomach contents as a percentage of animal body mass. Significant differences between treatments were only found at the 12-h post-feeding timepoint ($p < 0.05$). Time points with unshared letters are significantly different from each other.

an explanation for the increased gastric evacuation rate observed. It is known that gastric evacuation or emptying is under neuro-endocrine control, and that enteroendocrine cells in the anterior intestine can delay gastric emptying by secreting "brake" hormones such as cholecystokinin (CCK) in response to acidic chyme entering the intestine (Olsson et al., 1999; Goyal et al., 2019). However, future work should confirm if CCK release by enteroendocrine cells is decreased in omeprazole treated fish to validate this hypothesis.

The SDA represents the total energy expended on the ingestion, digestion, absorption, and assimilation of the meal (reviewed by McCue 2006; Secor 2009; Wang and Rindom 2021). The present study revealed a decrease in both SDA duration and magnitude with omeprazole treatment, suggesting that the meals were digested and assimilated in a shorter period and were evacuated from the alimentary canal more rapidly. This observed decrease in total duration was unexpected, as we predicted that inhibition of gastric acidification would retard digestion and thus prolong the duration of digestion and assimilation. This contrasts with mammalian studies, in which omeprazole did delay gastric emptying following a solid meal (Benini et al., 1996; Sanaka et al., 2010). However, this clearly did not happen in tilapia and was confirmed by the shorter stomach residency time in the omeprazole-treated fish. The amount of chyme in the stomach was not different between treatments at the earlier time points (1–6-h) but by 12 h post-feeding the omeprazole treated group only had half as much chyme left compared to the control. No detectable difference was seen at 24-h post-feeding as the stomach in both treatment groups was almost completely emptied. In fish exposed to low temperature, hypoxia or hypercapnia, an increase in SDA duration has been observed (Jordan and Steffensen 2007; Tirsgaard et al., 2015a, b). However, these variables were controlled in the present study and cannot account for the observed changes.

There was no significant difference in the time to peak or peak of the SDA suggesting there was no detectable direct impact of H^+/K^+-ATPase inhibition directly on $\dot{M}O_2$. However, it cannot be rule out that there could be other oxygen consuming processes masking these changes such as intestinal processes acting sooner because of the earlier gastric evacuation. The significant decrease in SDA magnitude indicates that there should be either additional energy available for growth since it is not being partitioned toward digestion, and/or there is simply less digestion occurring overall. Significantly the growth trial data revealed a decrease in the SGR of omeprazole-treated fish. The reduced SDA duration and magnitude can be explained by a decrease in nutrient absorption and/or assimilation resulting in reduced growth when compared to the control group. Previous work in channel catfish (Brown and Cameron 1991a, b) and Burmese pythons (McCue et al., 2005) have shown that the SDA can be mimicked by an infusion of amino acids and that this increase can be significantly blocked by the protein synthesis inhibitor cycloheximide. These results indicate the major contribution of protein assimilation to SDA. Future digestibility trials will address whether protein assimilation has indeed been adversely impacted by omeprazole. The use of cycloheximide in Nile tilapia can also be used to confirm the SDA response.

Dietary acidification is an approach that has been used to help understand the cost of gastric acid secretion in barramundi *Lates calcarifer* (Goodrich et al., 2022). The SDA response of the animals after being fed an acidified diet (from pH 5.82 to 4.09) showed a significant reduction in the overall magnitude of the SDA response by ~45% in comparison to the control group fed a 3.5% ration (Goodrich et al., 2022), which is similar to the observed decreases in the current study (34.5%). Along with the alterations to SDA response, a significant reduction in the alkaline tide response was observed, with blood pH and bicarbonate not showing significant increases post feeding seen in control fish. In dogfish fed a 2% ration, Wood et al. (2009) found a reduction in the alkaline tide using omeprazole. Citing a previous study in rainbow trout where dietary acidification resulted in decreased mRNA expression of *atp4a* (Sugiura 2006), Goodrich et al. (2022)

suggested that the reduction in energy expenditure may be in part due to a similar phenomenon occurring in the fish during their trial. Future studies on SDA response with omeprazole treatment in teleost fishes should be done observing the alkaline tide for more clear comparisons of the results with Goodrich et al. (2022).

In the study of Andrade et al. (2004) in the snake *B. constrictor*, although omeprazole inhibited the alkaline tide caused by gastric HCO_3^- secretion into the blood, it had no significant effect on the SDA unlike in the present study. In addition to taxonomic (fish versus reptile) and trophic (frequent small meals versus infrequent large meals) differences, the discrepancy with the present study can be at least partially explain by differences in experimental design. In the present study on tilapia, a repeated measures design allowed for individuals to serve as their own controls and thus provided greater statistical power to detect differences against a background of inter-individual variation. Goodrich et al. (2022) took the same repeated measures approach in their barramundi SDA study with similar successful results in detecting treatment differences. Notably, we were unable to detect differences if we used an unpaired *t*-test (unpublished observations). Also, the *B. constrictor* study made use of two groups of animals, that unfortunately had differing SMRs that could potentially complicate the interpretation of results as well (Andrade et al., 2004). However, more recent data in *B. constrictor* indicates that the contribution of the gastric proton pump to SDA is negligible since buffering the meal with either carbonate or bone meal, which increase acid secretion, did not alter SDA (Henriksen et al., 2015; Nørgaard et al., 2016) and that pyloric ligation abolished SDA (Enok et al., 2013). The latter study on pyloric ligation indicates that post-gastric process are the main contributors to SDA consistent with the findings with cycloheximide discussed earlier (Brown and Cameron 1991a, b; McCue et al., 2005).

In the present study, we found the voluntary feeding within the respirometry chamber was important to the success of the SDA measurements. Repeated force feeding by intubation was problematic resulting in regurgitation, and markedly elevated $\dot{M}O_2$ (MR and JMW, unpublished observations; Chabot et al., 2016). In addition, the required dilution of the ground moist pellets for liquid delivery *via* the feeding cannula necessitated delivery of a smaller meal size (<2% BM) since tilapia have a small stomach (Morrison and Wright 1999). We were also able to feed repeated meals allowing for repeated measures statistical analysis.

The growth rates we report are within the range of values reported in other studies with *O. niloticus* (e.g., Makori et al., 2017). The reduced growth in the omeprazole-treated fish could be explained by decreased protein digestion and absorption from the feed, as acid denaturation is the first step in protein digestion in gastric fish species (Bakke et al., 2010). While gastric pH plays a small role in denaturing structural bonds in proteins, the importance lies in the activation of pepsin for protein digestion (Sanny et al., 1975; Kageyama 2002). Gastric acid digestion increases the amount of soluble polypeptide that get broken down into di- and oligo-peptides, increasing the speed of intestinal absorption of dietary amino acids (Grabner and Hofer 1989; Hamdan et al., 2009). Pepsin cleaves off di- and oligo-peptides at preferential bonds between hydrophobic and aromatic amino acids including phenylalanine, tyrosine, and tryptophan (Kageyama 2002). As pepsin is one of the three main proteases in the digestive tract, along with intestinal trypsin and chymotrypsin, this decrease in digestive function of the omeprazole treated fish may prevent proper breakdown of protein, leading to reduced absorption (Kageyama 2002; Yúfera and Darías, 2007). With the omeprazole treatment and resultant suppression of stomach acidification, protein digestion occurring in the tilapia gut may have been reduced as less pepsinogen was activated, which may have resulted in reduced time in the intestine for absorption and decreased overall digestive transit times. However, it has yet to be determined if the latter has been affected by omeprazole treatment and future work is required. Omeprazole treatment did not appear to influence feeding behavior or appetite, as Nile tilapia are voracious eaters and readily took up the pellets during feeding. This behavior did not change during the course of the trial with complete meal consumption observed.

A key requirement for growth in fish species is adequate digestible phosphorus from the diet. The reduction in growth observed in the present study could in part be caused by a decrease in phosphorus uptake. Phosphorus is a very important nutrient for fish, as it is directly involved in all energy related processes involving ATP and is an essential component of cell membranes and nucleic acids in addition to its obvious role in bone growth and remodeling (National Research Council, 2011; Hossain and Yoshimatsu, 2014). As phosphorus is scarce in fresh water, the diet is the most important source, and digestible forms are essential to maintain health of the fish. The main form of dietary phosphorus in fish feeds is hydroxyapatite or bone phosphate in fish meal, which requires strong acidity in order to be adequately solubilized for intestinal absorption (Cho and Bureau, 2001; Sugiura, 2006; Hua and Bureau, 2010). This observation coupled with the inhibition of acid production in the omeprazole treated fish in the current study emphasizes the importance of determining the digestibility of this key nutrient moving forward.

In humans, omeprazole is widely used in the chronic treatment of gastric ulcers and gastroesophageal reflux disease

(GERD) (Schubert 2017). Although clinical studies have shown that omeprazole can delay overall protein assimilation and potentially cause protein malabsorption (Evenepoel et al., 1998), which would be consistent with the present study in tilapia, there are generally negligible impacts from a nutritional perspective (Evenepoel et al., 1998; Gibbons and Gold 2003). This discrepancy can be explained by the use of restricted ration (2% BM day^{-1}) in the present study, which is generally not a factor in the western diet. Thus, in humans, malabsorption would be masked by a dietary excess in nutrients.

Conclusions and future perspectives

The use of omeprazole was effective in altering digestion in the treated group in both acute feeding post-prandial metabolic rate (i.e., SDA variables) and over the course of the long-term growth and gastric evacuation trials. The results indicate that omeprazole impaired the production of gastric acid (achlorhydria) and potentially the activation of pepsin in the stomach of these fish, significantly compromising gastric function to the point where they could be considered "functionally agastric". The omeprazole treatment offers a novel way to explore the metabolic expense of the acidic environment of the stomach. This study paves the way for future studies to address the nature of the effects of gastric acid inhibition on growth, specifically examining protein digestibility and assimilation and the microbiome. Since the stomach also acts as a barrier for pathogen entry into the intestine, future work can also address this role in enteric disease susceptibility in fishes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Wilfrid Laurier University Animal Care Committee.

Author contributions

The study was designed by KM, MR, and JMW. Data was collected by KM, MR, and EP and analyzed by KM and JMW. The draft manuscript was prepared by KM and revised by JMW and JCS.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.966447/full#supplementary-material

References

Andrade, D. V., De Toledo, L. P., Abe, A. S., and Wang, T. (2004). Ventilatory compensation of the alkaline tide during digestion in the snake *Boa constrictor*. *J. Exp. Biol.* 207, 1379–1385. doi:10.1242/jeb.00896

AOAC (Association of Analytical Chemists) (2000). *Official methods of analysis of the association of official analytical chemists*. 14th ed. Rockville, MD, USA: Association of Analytical Chemists.

Bakke, A. M., Glover, C., and Krogdahl, Å. (2010). "Feeding, digestion, and absorption of nutrients," in *Fish physiology: The multifunctional gut of fish*. Editors M. Grosell, A. Farrell, and C. Brauner (Academic Press), 57–110.

Baktoft, H., Jacobsen, L., Skov, C., Koed, A., Jepsen, N., Berg, S., et al. (2016). Phenotypic variation in metabolism and morphology correlating with animal swimming activity in the wild: Relevance for the OCLTT (oxygen- and capacity-limitation of thermal tolerance), allocation and performance models. *Conserv. Physiol.* 4, cov055–14. doi:10.1093/conphys/cov055

Barnawi, E. A., Doherty, J. E., Ferreira, P. G., and Wilson, J. M. (2020). Extra-gastric expression of the proton pump H^+/K^+-ATPase in the gills and kidney of the teleost *Oreochromis niloticus*. *J. Exp. Biol.* 223, jeb214890. doi:10.1242/jeb.214890

Barrington, E. J. W. (1942). Gastric digestion in the lower vertebrates. *Biol. Rev.* 17, 1–27. doi:10.1111/j.1469-185x.1942.tb00429.x

Benini, L., Castellani, G., Bardelli, E., Sembenini, C., Brentegani, M. T., Caliari, S., et al. (1996). Omeprazole causes delay in gastric emptying of digestible meals. *Dig. Dis. Sci.* 41, 469–474. doi:10.1007/BF02282320

Brown, C. R., and Cameron, J. N. (1991a). The induction of specific dynamic action in channel catfish by infusion of essential amino acids. *Physiol. Zool.* 64, 276–297. doi:10.1086/physzool.64.1.30158524

Brown, C. R., and Cameron, J. N. (1991b). The relationship between specific dynamic action (SDA) and protein synthesis rates in the channel catfish. *Physiol. Zool.* 64, 298–309. doi:10.1086/physzool.64.1.30158525

Castro, L. F. C., Goncalves, O., Mazan, S., Tay, B.-H., Venkatesh, B., and Wilson, J. M. (2014). Recurrent gene loss correlates with the evolution of stomach phenotypes in gnathostome history. *Roy. Soc. B.* 281, 20132669–9. doi:10.1098/rspb.2013.2669

Castro, L. F. C., Lopes-Marques, M., Gonçalves, O., and Wilson, J. M. (2012). The evolution of pepsinogen C genes in vertebrates: Duplication, loss and functional diversification. *PloS one* 7, e32852. doi:10.1371/journal.pone.0032852

Chabot, D., Koenker, R., and Farrell, A. P. (2016). The measurement of specific dynamic action in fishes. *J. Fish. Biol.* 88, 152–172. doi:10.1111/jfb.12836

Chakraborty, S. C., Ross, L. G., and Ross, B. (1995). Energy budget and metabolism in common carp, *Cyprinus carpio* L., fed on different dietary protein levels and at different ration levels. *Aquac. Nutr.* 1, 179–187. doi:10.1111/j.1365-2095.1995.tb00042.x

Cho, C. Y., and Bureau, D. P. (2001). A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. *Aquac. Res.* 32, 349–360. doi:10.1046/j.1355-557x.2001.00027.x

Cho, C. Y. (1992). Feeding systems for rainbow trout and other salmonids with reference to current estimates of energy and protein requirements. *Aquaculture* 100, 107–123. doi:10.1016/0044-8486(92)90353-m

Enok, S., Simonsen, L. S., and Wang, T. (2013). The contribution of gastric digestion and ingestion of amino acids on the postprandial rise in oxygen consumption, heart rate and growth of visceral organs in pythons. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 165A, 46–53. doi:10.1016/j.cbpa.2013.01.022

Evenepoel, P., Claus, D., Geypens, B., Maes, B., Hiele, M., Rutgeerts, P., et al. (1998). Evidence for impaired assimilation and increased colonic fermentation of protein, related to gastric acid suppression therapy. *Aliment. Pharmacol. Ther.* 12, 1011–1019. doi:10.1046/j.1365-2036.1998.00377.x

Fulton, T. W. (1904). The rate of growth of fishes. *22nd Annu. Rep. Fish. Board Scotl.*, 141–241.

Gibbons, T. E., and Gold, B. D. (2003). The use of proton pump inhibitors in children: A comprehensive review. *Paediatr. Drugs* 5, 25–40. doi:10.2165/00128072-200305010-00003

Goodrich, H. R., Wilson, R. W., Smullen, R., Barnes, A. C., and Franklin, C. E. (2022). Acidified fish feeds reduce the energetic and physiological costs of digestion in juvenile barramundi (*Lates calcarifer*). *Aquaculture* 546, 737400–737414. doi:10.1016/j.aquaculture.2021.737400

Goyal, R. K., Guo, Y., and Mashimo, H. (2019). Advances in the physiology of gastric emptying. *Neurogastroenterol. Motil.* 31, e13546. doi:10.1111/nmo.13546

Grabner, M., and Hofer, R. (1989). Stomach digestion and its effect upon protein hydrolysis in the intestine of rainbow trout (*Salmo gairdneri Richardson*). *Comp. Biochem. Physiol. Part A Physiol.* 92, 81–83. doi:10.1016/0300-9629(89)90745-7

Hamdan, M., Moyano, F. J., and Schuhardt, D. (2009). Optimization of a gastrointestinal model applicable to the evaluation of bioaccessibility in fish feeds. *J. Sci. Food Agric.* 89, 1195–1201. doi:10.1002/jsfa.3574

Heidelbaugh, J. J. (2013). Proton pump inhibitors and risk of vitamin and mineral deficiency: Evidence and clinical implications. *Ther. Adv. Drug Saf.* 4, 125–133. doi:10.1177/2042098613482484

Henriksen, P. S., Enok, S., Overgaard, J., and Wang, T. (2015). Food composition influences metabolism, heart rate and organ growth during digestion in Python regius. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 183, 36–44. doi:10.1016/j.cbpa.2014.12.031

Hopkins, K. D. (1992). Reporting fish growth: A review of the basics. *J. World Aquac. Soc.* 23, 173–179. doi:10.1111/j.1749-7345.1992.tb00766.x

Hossain, M. A., and Yoshimatsu, T. (2014). Dietary calcium requirement in fishes. *Aquac. Nutr.* 20 (1), 1–11. doi:10.1111/anu.12135

Hua, K., and Bureau, D. P. (2010). Quantification of differences in digestibility of phosphorus among cyprinids, cichlids, and salmonids through a mathematical modelling approach. *Aquaculture* 308 (3–4), 152–158. doi:10.1016/j.aquaculture.2010.07.040

Jobling, M. (1981). The influences of feeding on the metabolic rate of fishes: A short review. *J. Fish. Biol.* 18, 385–400. doi:10.1111/j.1095-8649.1981.tb03780.x

Jordan, A. D., and Steffensen, J. F. (2007). Effects of ration size and hypoxia on specific dynamic action in the cod. *Physiol. Biochem. Zool.* 80, 178–185. doi:10.1086/510565

Kageyama, T. (2002). Pepsinogens, progastricsins, and prochymosins: Structure, function, evolution, and development. *Cell. Mol. Life Sci.* 59, 288–306. doi:10.1007/s00018-002-8423-9

Koelz, H. R. (1992). Gastric acid in vertebrates. *Scand. J. Gastroenterol. Suppl.* 27, 2–6. doi:10.3109/00365529209095998

Lindberg, P., Brändström, A., and Wallmark, B. (1987). Structure-activity relationships of omeprazole analogues and their mechanism of action. *Trends Pharmacol. Sci.* 8, 399–402. doi:10.1016/0165-6147(87)90107-6

Lindberg, P., Nordberg, P., Alminger, T., Brandstrom, A., and Wallmark, B. (1986). The mechanism of action of the gastric acid secretion inhibitor omeprazole. *J. Med. Chem.* 29, 1327–1329. doi:10.1021/jm00158a001

Lobel, P. S. (1981). Trophic biology of herbivorous reef fishes: Alimentary pH and digestive capabilities. *J. Fish. Biol.* 19, 365–397. doi:10.1111/j.1095-8649.1981.tb05842.x

Makori, A. J., Abuom, P. O., Kapiyo, R., Anyona, D. N., and Dida, G. O. (2017). Effects of water physico-chemical parameters on tilapia (*Oreochromis niloticus*) growth in earthen ponds in Teso North Sub-County, Busia County. *Fish. Aquat. Sci.* 20, 30. doi:10.1186/s41240-017-0075-7

McCue, M. D., Bennett, A. F., and Hicks, J. W. (2005). The effect of meal composition on specific dynamic action in Burmese pythons (*Python molurus*). *Physiol. Biochem. Zool.* 78, 182–192. doi:10.1086/427049

McCue, M. D. (2006). Specific dynamic action: A century of investigation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 144, 381–394. doi:10.1016/j.cbpa.2006.03.011

McTavish, D., Buckley, M. M.-T., and Heel, R. C. (1991). Omeprazole. An updated review of its pharmacology and therapeutic use in acid-related disorders. *Omeprazole. Drugs* 42, 138–170. doi:10.2165/00003495-199142010-00008

Morii, M., Takata, H., and Takeguchi, N. (1989). Acid activation of omeprazole in isolated gastric vesicles, oxyntic cells, and gastric glands. *Gastroenterology* 96, 1453–1461. doi:10.1016/0016-5085(89)90512-x

Morrison, C. M., and Wright, J. R., Jr (1999). A study of the histology of the digestive tract of the Nile tilapia. *J. Fish. Biol.* 54, 597–606. doi:10.1111/j.1095-8649.1999.tb00638.x

National Research Council (2011). *Nutrient requirements of fish and shrimp*. Washington, DC: National Academies Press. doi:10.17226/13039

Nelson, J. A. (2016). Oxygen consumption rate v. rate of energy utilization of fishes: A comparison and brief history of the two measurements. *J. Fish. Biol.* 88, 10–25. doi:10.1111/jfb.12824

Nørgaard, S., Andreassen, K., Malte, C. L., Enok, S., and Wang, T. (2016). Low cost of gastric acid secretion during digestion in ball pythons. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 194, 62–66. doi:10.1016/j.cbpa.2016.01.003

Olbe, L., Cederberg, C., Lind, T., and Olausson, M. (1989). Effect of omeprazole on gastric acid secretion and plasma gastrin in man. *Scand. J. Gastroenterol. Suppl.* 24, 27–32. doi:10.3109/00365528909091240

Olsson, C., Aldman, G., Larsson, A., and Holmgren, S. (1999). Cholecystokinin affects gastric emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* 202, 161–170. doi:10.1242/jeb.202.2.161

Priede, I. G. (1985). "Metabolic scope in fishes," in *Fish energetics: New perspectives*. Editors P. Tytler and P. Calow (London: Croom Helm), 33–64.

Rosewarne, P. J., Wilson, J. M., and Svendsen, J. C. (2016). Measuring maximum and standard metabolic rates using intermittent flow respirometry: A student laboratory investigation of aerobic metabolic scope and environmental hypoxia in aquatic breathers. *J. Fish. Biol.* 88, 265–283. doi:10.1111/jfb.12795

Sanaka, M., Yamamoto, T., and Kuyama, Y. (2010). Effects of proton pump inhibitors on gastric emptying: A systematic review. *Dig. Dis. Sci.* 55, 2431–2440. doi:10.1007/s10620-009-1076-x

Sanny, C. G., Hartsuck, J. A., and Tang, J. (1975). Conversion of pepsinogen to pepsin. Further evidence for intramolecular and pepsin-catalyzed activation. *J. Biol. Chem.* 250, 2635–2639. doi:10.1016/s0021-9258(19)41649-9

Schubert, M. L. (2017). Physiologic, pathophysiologic, and pharmacologic regulation of gastric acid secretion. *Curr. Opin. Gastroenterol.* 33, 430–438. doi:10.1097/MOG.0000000000000392

Schwartzbach, A., Behrens, J., and Svendsen, J. (2020). Atlantic cod *Gadus morhua* save energy on stone reefs: Implications for the attraction versus production debate in relation to reefs. *Mar. Ecol. Prog. Ser.* 635, 81–87. doi:10.3354/meps13192

Secor, S. M. (2003). Gastric function and its contribution to the postprandial metabolic response of the Burmese python *Python molurus*. *J. Exp. Biol.* 206, 1621–1630. doi:10.1242/jeb.00300

Secor, S. M. (2009). Specific dynamic action: A review of the postprandial metabolic response. *J. Comp. Physiol. B* 179, 1–56. doi:10.1007/s00360-008-0283-7

Smit, H. (1968). Gastric secretion in the lower vertebrates and birds. In *Handbook of Physiology section 6 alimentary canal vol. V bile, digestion, ruminal physiology* (C. F. Code, ed.), pp. 2791–2805.

Steffensen, J. F. (1989). Some errors in respirometry of aquatic breathers: How to avoid and correct for them. *Fish. Physiol. Biochem.* 59, 49–59. doi:10.1007/BF02995809

Sugiura, S. H., Roy, P. K., and Ferraris, R. P. (2006). Dietary acidification enhances phosphorus digestibility but decreases H^+, K^+-ATPase expression in rainbow trout. *J. Exp. Biol.* 209, 3719–3728. doi:10.1242/jeb.02436

Svendsen, J. C., Tirsgaard, B., Cordero, G. A., and Steffensen, J. F. (2015). Intraspecific variation in aerobic and anaerobic locomotion: Gilthead sea bream (*Sparus aurata*) and Trinidadian guppy (*Poecilia reticulata*) do not exhibit a trade-off between maximum sustained swimming speed and minimum cost of transport. *Front. Physiol.* 6, 43–12. doi:10.3389/fphys.2015.00043

Taylor, J. R., and Grosell, M. (2009). The intestinal response to feeding in seawater gulf toadfish, *Opsanus beta*, includes elevated base secretion and increased epithelial oxygen consumption. *J. Exp. Biol.* 212, 3873–3881. doi:10.1242/jeb.034579

Tirsgaard, B., Moran, D., and Steffensen, J. F. (2015). Prolonged SDA and reduced digestive efficiency under elevated CO_2 may explain reduced growth in Atlantic cod (*Gadus morhua*). *Aquat. Toxicol.* 158, 171–180. doi:10.1016/j.aquatox.2014.11.009

Tirsgaard, B., Svendsen, J. C., and Steffensen, J. Fl. (2015). Effects of temperature on specific dynamic action in the Atlantic cod *Gadus morhua*. *Fish. Physiol. Biochem.* 41, 41–50. doi:10.1007/s10695-014-0004-y

Wang, T., and Rindom, E. (2021). The physiological response to digestion in snakes: A feast for the integrative physiologist. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 254, 110891. doi:10.1016/j.cbpa.2020.110891

Wilson, J. M., and Castro, L. F. C. (2010). Morphological diversity of the gastrointestinal tract in fishes. *Fish. Physiol.* 30, 1–55.

Wood, C. M., Schultz, A. G., Munger, R. S., and Walsh, P. J. (2009). Using omeprazole to link the components of the post-prandial alkaline tide in the spiny dogfish, *Squalus acanthias*. *J. Exp. Biol.* 212, 684–692. doi:10.1242/jeb.026450

Yúfera, M., and Darías, M. J. (2007). Changes in the gastrointestinal pH from larvae to adult in Senegal sole (*Solea senegalensis*). *Aquaculture* 267, 94–99. doi:10.1016/j.aquaculture.2007.02.009

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Metabolic trade-offs associated with homeostatic adjustments in pelagic and benthic cephalopods: Comparative evaluations of NH_4^+/H^+ transport machinery in gills

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Cephalopods are ancient mollusks that can be found in many different ecological niches in the ocean ranging from the intertidal zone to the deep-sea abyss. In order to adapt to a lifestyle in various habitats, cephalopods have evolved a variety of locomotory modes to accommodate their respective habitats. Most cephalopods have relatively high metabolic rates due to their less efficient swimming mode by jet propulsion. This lifestyle is characterized by a high level of energy expenditure, fueled exclusively by protein diets that are rapidly digested and may produce metabolic nitrogenous waste NH_3/NH_4^+ accumulation and acid-base disturbances. This study observed that the NH_4^+ transport rate in pelagic bigfin reef squid *(Sepioteuthis lessoniana)* is two times faster than benthic common octopus (*Octopus vulgaris*). Inhibition of Na^+/H^+ exchangers (NHEs) showed significant disruption of NH_4^+ and H^+ excretory processes in gills of octopus but not in squid. However, inhibition of vacuolar-type H^+-ATPase (VHA) significantly disrupts NH_4^+ and H^+ transport rates in gills of both animals. Accordingly, for NH_4^+ and H^+ homeostasis, benthic octopus with lower aerobic respiration rates utilize both active and Na^+-driven secondary transport machinery. In order to avoid NH_4^+ accumulated in the blood, pelagic squids with higher aerobic respiration rates prefer active NH_4^+ and H^+ transport mechanisms that consume ATP intensively.

KEYWORDS

cephalopods, ecological niches, acid-base regulations, ammonium excretion, perfusion

Introduction

The marine ecosystem was dominated by cephalopods during the Ordovician period, primarily by primitive nautiloidea (Kroeger and Yun-Bai, 2009; Histon, 2012). In place of nautiloidea, modern coleoid cephalopods, such as octopuses and squids, still serve as apex predators at the top of the food chain (Villanueva et al., 2017). They have evolved advanced sensory and locomotor abilities, which are regarded as physiological traits in terms of convergent evolution for competition with fish in the marine environment (Hu et al., 2014; Hu et al., 2015; Chung et al., 2020). Due to their well-developed nervous system and specialized locomotion patterns (crawling and jet propulsion), coleoid cephalopods have the advantage over most marine invertebrates or even teleosts (Xavier et al., 2015). Today, over 800 species of coleoid cephalopods have evolved a variety of lifestyles in order to survive in the different ecological niches of the ocean system (Wood et al., 2000; Jereb and Roper, 2010). Octopod and loliginid cephalopods have been observed in distinct ecological niches across all families. Octopodidae animals are mainly benthic and cave-dwelling cephalopods that crawl and rest in a restful posture, thus avoiding predation risk or waiting for prey (Scheel and Bisson, 2012). Additionally, loliginids are pelagic cephalopods with locomotive flexibility. The jet-propulsion swimming pattern enables them to maintain substantial advantages that confer maneuverability for a variety of athletic activities, such as chasing prey, projecting ink for escape, and irrigation of egg capsules (Wells, 1990). Furthermore, most loliginid cephalopods possess fin-like extensions on their mantle. Therefore, in addition to jet propulsion, fin locomotion allows loliginid cephalopods to move in a manner similar to that of fish. However, fin-derived locomotion creates more drag while shifting distantly. Therefore, pelagic loliginid cephalopods evolved relatively small lobe fins for hovering and mainly used jet-propulsion when making long migrations (O'Dor, 2002; Mather et al., 2010).

There is also the fact that cephalopods have comparative high metabolic rates compared to other marine animals, which is a result of their athletic locomotion (Potts, 1965). As an example, cephalopods swim by jet propulsion, which is an inefficient and energy-consuming mode of locomotion (Seibel and Drazen, 2007). It should also be noted that the energy sources of cephalopods are primarily derived from protein metabolism rather than carbohydrate or lipid metabolism (Boucher-Rodoni and Mangold, 1989; Lee, 1995). Protein metabolism generates nitrogenous compounds such as ammonia (NH_3)/ammonium (NH_4^+), urea, uric acid, and so on (Wright, 1995). Therefore, the metabolically-derived nitrogenous wastes could accumulate in blood to the lethal level, causing further metabolic dysregulation and extracellular acid-base disturbances (Boucher-Rodoni and Mangold, 1995; Lee, 1995; Wells and Clarke, 1996). And the conversion of ammonium into free amino acids by specific metabolic pathways is thought to be an important means of maintaining ammonium homeostasis. A close relationship exists between the synthesis of glutamate (Glu) and glutamine (Gln) and the production and utilization of ammonium (Huang et al., 2020). As a consequence, most modern coleoid cephalopods would have a mechanism for excreting or utilizing nitrogenous waste, and they should also have remarkable features for regulating metabolic ammonium and acidosis. According to earlier studies, branchial hearts, renal appendages, and gills would be involved in the excretion of ammonium for the maintenance of homeostasis in cephalopods (Potts, 1965). However, there is still uncertainty about the homeostatic trade-offs between adaptive energy metabolism and movement by fins and jet propulsion, or even crawling locomotion, in coleoid cephalopods, which may reflect species distribution from natural selection.

Acid-base and ammonium regulation are essential features of aquatic animals and contribute to a range of intracellular pH values (Hwang and Perry, 2010; Hwang and Lin, 2013; Hu et al., 2015). It relates to a wide range of physiological processes, including signaling transduction, metabolic adjustments, and animal activities (Tseng and Hwang, 2008; Chang and Hwang, 2011; Tovey and Brauner, 2018). Early studies in teleosts indicated that the gill epithelium is the primary tissue responsible for gas exchange, ion and acid-base regulation, and nitrogenous product excretion (Evans et al., 2005; Hwang and Lin, 2013). In a perturbed aquatic system, those transepithelial ion regulatory proteins play the most important role in maintaining ion and pH homeostasis. For example, the vacuole H^+-ATPase (VHA), Na^+/H^+ exchanger (NHE), rhesus protein (RhP), Na^+/K^+ pump (NKA) and other ion regulatory proteins have been demonstrated to be involved in blood pH stabilization and ammonium excretion in teleosts (Liu et al., 2013; Tseng et al., 2013; Liu et al., 2016). In eukaryotic organisms, the VHA is an essential component of the acid-base regulation, as it drives proton flux on the epithelial membrane coupled to ATP hydrolysis (Nishi and Forgac, 2002). And the NHE is a membrane protein that maintains the acid-base and NH_4^+ balance in teleosts by facilitating transepithelial Na^+ uptake and H^+ and/or NH_4^+ secretion (Tseng et al., 2020). Using the advanced homeostasis model in teleosts, (Hwang et al., 2011) further delineated the functions of specialized epithelial ionocytes that maintain acid-base and ammonium balance by active or passive transport. Fish-derived homeostasis mechanisms have also been identified in other aquatic invertebrates, such as cephalopods and crustaceans (Wright, 1995; Wright and Wood, 2009; Hu et al., 2014; Hu et al., 2015). Recently, a study of gills, liver and blood of cuttlefish (*Sepia pharaonis*) demonstrated that this sepiid cephalopod family could convert ammonia to urea *via* uricolysis of the ornithine-urea cycle (OUC) and glutamine formation pathway

for ammonia detoxification under high ambient ammonia nitrogen stress. (Peng et al., 2017). Moreover, convergent evolution between fish and cephalopods has led to the development of a well-developed branchial acid-base regulatory system coordinated by specific regulatory proteins in the gill tissue of modern coleoid cephalopods, such as VHA, NHE, RhP and other ion regulatory proteins, which is the primary site for ammonium regulation (Hu et al., 2014; Hu et al., 2015; Hu et al., 2017).

Due to the fact that cephalopods require sufficient energy to achieve their respective locomotion types, the gill tissue is thought to be the most sensitive organ for NH_4^+ and H^+ regulation (Hu et al., 2014). It is still unclear how they may differ in relation to different behavioral paradigms. The present study examined the metabolic features and responses of gill epithelial NH_4^+ and H^+ transport in pelagic and benthic cephalopods. The gills are relatively complex organs compared to other excretory organs since they contain ctenidial arteries and ctenidial veins associated with branchial and systemic hearts, respectively. Based on our previous study on common octopus (Hu et al., 2017), a gill perfusion approach with specific inhibitors against regulatory proteins was applied to determine the functions of responsive proteins in the acid-base regulation of the coleoid squid, *S. lessoniana* and octopus, *O. vulgaris*. As a way to better understand the NH_4^+ and acid-base regulation mechanisms in cephalopod gills, the perfusion saline was prepared according to the osmolarity, ion content, and pH value (~pH 7.6) of blood from octopus and squid. This study hypothesizes that the distinct ammonium and acid-base homeostasis abilities of pelagic squid and benthic octopus may be related to their different locomotion patterns and lifestyles, which may explain why these two cephalopod species remain as the apex marine predators in the open marine system.

Materials and methods

Experimental animals

The adult bigfin reef squid (*Sepioteuthis lessoniana*; mantle lengths ranging from 200 to 250 mm) and common octopus (*Octopus vulgaris*; mantle lengths ranging from 150 to 180 mm) were collected from Bisha and Heping Island harbors in Keelung, Taiwan. The bigfin reef squid and common octopus were raised at the Marine Research Station, ICOB, Academia Sinica, and were kept respectively in aerated circulatory fiber reinforced plastics (FRP) buckets (total volume approximately 1000 L seawater, pH 8.0 to 8.1, sand filters, constant 12/12h light-dark cycle) and in flow-through systems (total volume approximately 37000 L, pH 8.0. to 8.1). The animals were fed twice daily with a diet consisting of white leg shrimp (*Litopenaeus vannamei*) and Japanese horse mackerel (*Trachurus japonicus*). Animals weighing 500 to 800 grams were kept for at least two months before they were sacrificed for experiments. The experimental protocols were approved by the Biosafety Committee and Institutional Animal Care and Use Committee of Academia Sinica (approval no. BSF17-12-1158).

Oxygen consumption estimations

The resting metabolic rates (RMRs) of octopus (Octopodidae) and squid (Loliginidae) cephalopods were determined by observing the oxygen consumption rate when the animals were incubated in a closed swim tunnel (an intermittent-flow respirometry system) with continuous water flow (as shown in Supplementary Figure S1). During the experiment, the oxygen consumption of animals was measured until the air saturation level dropped to 70%, a criteria level applicable to embryonic squid and adult fish (Kuan et al., 2022; Wang et al., 2022). In the beginning, the animals were gently transferred to the swimming tunnel and were temporarily adapted for ten minutes. The estimations would begin after habituation, and the intact system would be maintained at 26°C. The oxygen probes were calibrated according to the manufacturer's instructions (available on the PreSens website https://www.presens.de/products/detail/oxy-4-mini) and then placed in the swimming tunnel and connected to a mini channel fiber-optic oxygen transmitter (PreSens, Regensburg, Germany). Additionally, the bacteria control would be tested under the same conditions as the background for 30 minutes to ensure that the respiration of the bacteria could be ignored.

Examination of amino acids content in blood collected from the vena cava

After dissecting the funnel and mantle from the ventral side, blood samples were taken at approximately 500 µL from different blood transit stations (anterior vena cava, ctenidial artery or ctenidial vein) of the circulatory system (Figure 1A) by using syringes. In this study, glutamate and glutamine levels in blood were determined from samples collected from the anterior vena cava in order to determine the levels of these substances before entering the systemic heart of cephalopods. In order to extract the amino acids in the blood of anterior vena cava, ethanol was used, along with norvaline as an internal standard, and the sample was centrifuged at 4300 g for 10 minutes. The 2 mL fixed quantity supernatant was transferred to a new tube and dried in a vacuum concentrator. The dried samples were reconstituted in 100 mL of 8 mM HCl, filtered with a 0.2 micron syringe filter, and then analyzed using the AccQ tag Ultra Reagent Kit (Water, Milford, MA, USA). Derivatized samples were injected into a high-performance liquid chromatography (HPLC) system (ACQUITY UPLC H-Class System, Waters) equipped with a TUV detector. The amino

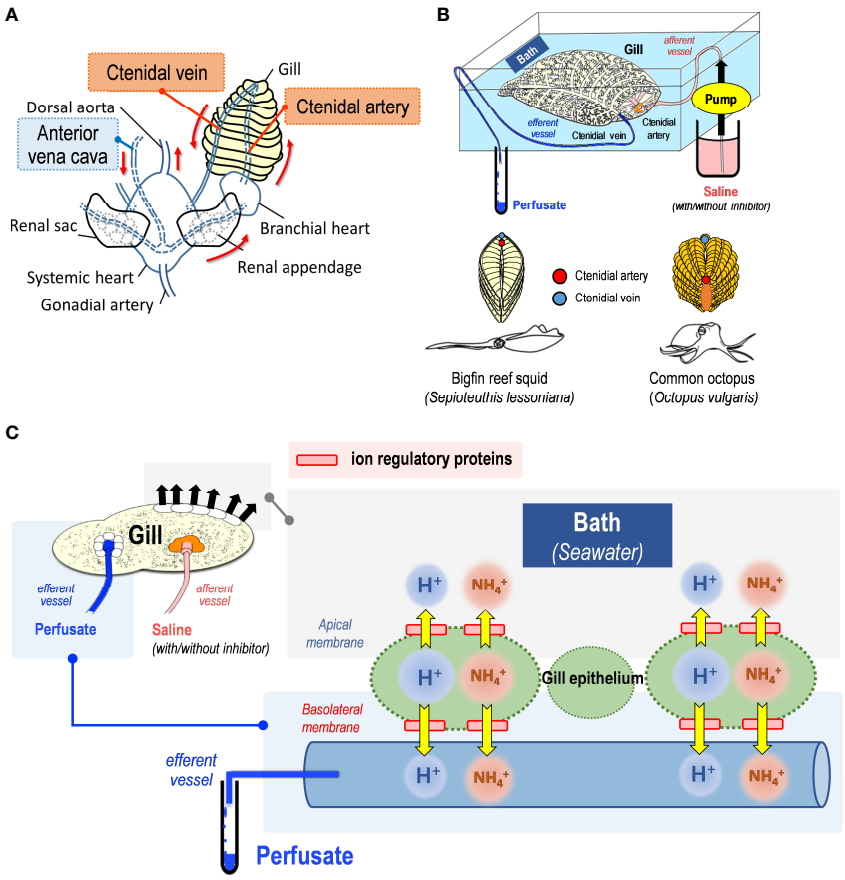

FIGURE 1
Representative images of blood circulation in cephalopod gills and *ex-vivo* approach. **(A)** The closed circulation system of cephalopods. The arrows indicate the direction of blood flow in the vessels. Vessels (anterior vena cava, ctenidial artery and ctenidial vein) where blood samples were taken were highlighted in background colors. **(B)** The schematic model of the *ex-vivo* perfusion experiment in the gills. During the experiments, the gills were incubated in a filtering seawater bath. The perfusate was collected from the efferent side of the tubing. **(C)** The sample collected from perfusate represents the fluid transport from the basolateral side of the gill epithelium to the circulating blood *via* the ctenidial vein.

acids were identified and measured by comparing with the retention times and peak areas of the standards (WAT088122, Waters).

Evaluation of NH_4^+ concentrations in blood of gill artery and vein

For the measurement of NH_4^+ concentrations, 25 µL of blood was mixed with 100 µL of reagent containing orthophthaldialdehyde, sodium sulphite, and sodium borate. After incubation in the dark for two hours at room temperature, the fluorescence was measured at excitation and emission wavelengths of 350 and 420 nm using a microplate reader (Moleculer Device, Spectra Max, M5) (Holmes et al., 1999; Hu et al., 2014).

Preparation of perfusion salines

The perfusion salines have similar ingredients and osmolarity to real cephalopod blood (Hu et al., 2017) depending on the native ion concentration and pH value (pH 7.6) in the *in vivo* blood test for different cephalopods. The blood sample extraction procedure begins with centrifugation of the samples for one minute at 12000 rpm. The supernatant was then diluted with double-deionized water (1:10000). Afterwards, major cation contents (Na^+, K^+, Ca^{2+}, Mg^{2+}) were determined using an atomic absorption spectrophotometer (Hitachi Z-8000, Tokyo, Japan). For the measurement of anion ion Cl^-, a double-beam spectrophotometer was used (NanoDrop 2000/2000c UV-Vis Spectrophotometer, Thermo Scientific). For the generation of the standard curve, the standard solutions of major ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-; Merck Darmstadt, Germany) were used.

Corning 965 carbon dioxide analyzer (Olympic Analytical Service, England) was used to estimate HCO_3^- concentration from total dissolved carbon measurements. The perfusion salines used in the cephalopod perfusion experiments are different between bigfin reef squid and common octopus, as shown in Supplementary Table S1.

Gill perfusion experiments and pharmacological evaluations

Gills were carefully removed from the mantle cavity and connective tissues for the perfusion experiments (Figure 1A). The gills were carefully cut at the 1st order ctenidial arteries and veins that transport blood between the gills and branchial heart. After the gills had been completely dissected from the mantle cavity, *ex-vivo* gill perfusion experiments could be performed. The ctenidial arteries and veins were connected with one end of special-treated polyethylene tubes (0.86 mm inner diameter*1.52 mm outer diameter, PE-100) and tied tightly by woven strings. Another end of the polyethylene tubes was attached to PVC tubes connected to thicker silicon tubes (2 mm in diameter). Silicon tubes fitted with a peristaltic pump (Multi-channel peristaltic system, MINIPULS® Evolution) was used to pump the perfusion saline into the gill at a rate of 12 mL per hour by setting the pump to 10 revolutions per minute (rpm). Perfusion experiments were conducted by incubating the gills in an aerated container filled with 50 mL of filtering seawater (Figure 1B).

For the purpose of ensuring that the regulatory proteins are inhibited by the specific inhibitors (ethylisopropyl amiloride (EIPA) for NHE; bafilomycin A1 (BafA1) for VHA), the sample collection should begin after the saline has been in the gill for at least 30 minutes. We prepared perfusion saline by adding different dosages of EIPA that could compete for Na^+ binding sites to determine whether NHEs are responsible for regulating cephalopod gill function (Schaffhauser et al., 2016) and could be acted on squid as well (Hu et al., 2011). The role of VHA in the regulation of homeostasis in the gills of squid and octopus was examined by preparing perfusion saline containing different dosages of BafA1, which is noncovalently binding with VHA to prohibit H^+ conduction. As part of the perfusion experiments, the pH value of saline would be immediately measured by a pH meter and adjusted with 0.1 M hydrochloric acid and $NaOH_{(aq)}$. After the perfusion experiments had been completed, the perfusate and bath were collected and immediately measured for pH and NH_4^+ concentration. The sample collected from the perfusate represents the fluid that would be transported back to the body *via* the ctenidial vein. This represents the functions of the basolateral membrane side to the perfusate blood (Figure 1C). Alternatively, the sample collected from the bath represents the fluid that would be expelled or exchanged by the apical side of gills with the surrounding environment (seawater, bath) (Figure 1C).

Statistical analysis

GraphPad Prism 7.00 (GraphPad, San Diego, CA, USA) was used for statistical analyses. Data are presented as mean ± S.D. Student's t-test was used to test for significance between squid and octopus in terms of oxygen consumption rates, glutamate/glutamine contents, ammonium transport rates, and ammonium concentration between the ctenidial artery and ctenidial vein. The effect of different dosages of EIPA and BafA1 on NH_4^+ and H^+ transport rates in gills was determined using one-way ANOVA followed by Tukey's pairwise comparison. Differences were considered to be significant at $p< 0.05$.

Results

The resting metabolic appearances of octopus and squid

The resting metabolic rates (RMRs) were determined by observing the oxygen consumption rates of experimental animals. The oxygen consumption rate of octopus (33.69 ± 6.51 μmol O_2/h g) is significantly lower ($p<0.001$) than that of squid (183.23 ± 95.67 μmol O_2/h g) (Figure 2A). During the experimental process, both species remain calm and do not exhibit any active behavior with distance movement.

Glutamate and glutamine contents in blood collected from anterior vena cava

The glutamine concentrations in blood collected from the anterior vena cava of octopus (7.42 ± 1.27 μmol/g) and squid (20.19 ± 4.54 μmol/g) were both relatively higher than glutamate concentrations (3.23 ± 0.83 μmol/g in octopus; 1.67 ± 0.77 μmol/g in squid) (Figure 2B). Notably, the glutamate content in blood collected from the anterior vena cava of octopus is 1.9-fold higher than that of squid (Figure 2B). By contrast, the glutamine content in the anterior vena cava blood of octopus is 2.7-fold lower than that of squid (Figure 2B).

NH_4^+ concentrations in blood of octopus and squid gills

Blood was collected from the ctenidial arteries and veins of octopus and squid, respectively, in order to better understand the NH_4^+ concentrations in the gills. The NH_4^+ concentration in

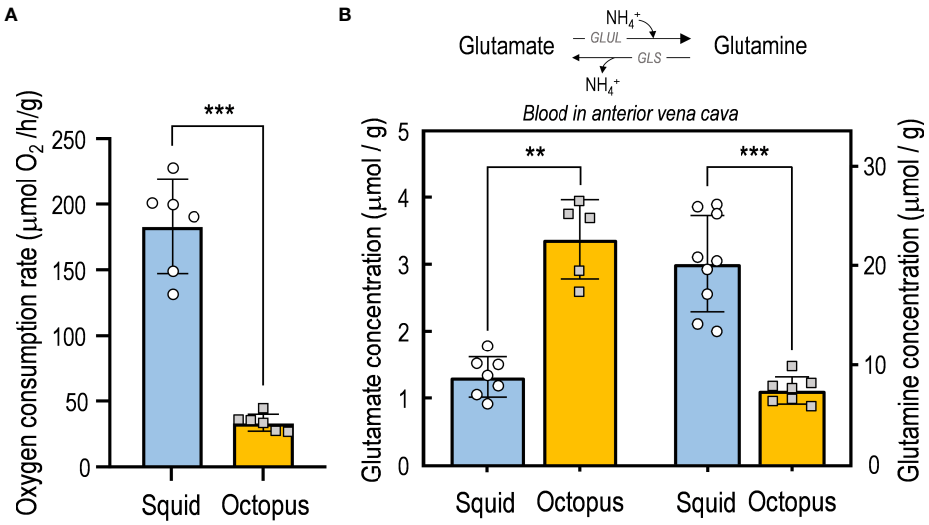

FIGURE 2
Metabolic features in squid and octopus. **(A)** The resting metabolic rates (RMRs) of adult bigfin reef squid (*S. lessoniana*) and common octopus (*O. vulgaris*) were evaluated by measuring their oxygen consumption rates under the animals were incubated in a closed swim tunnel with continuous water flow. **(B)** The glutamate and glutamine contents in blood were collected from the anterior vena cava. The estimated values are presented as mean ± SD (n = 5~9). Asterisks indicate significance levels of $p < 0.01$ (**) and $p < 0.001$ (***).

ctenidial veins (~250 μM) of octopus was lower than that in ctenidial arteries (~300 μM) (Figure 3A). Moreover, in squid, the NH_4^+ concentration in ctenidial veins (~20 μM) was found to be also lower than that in ctenidial arteries (~25 μM) (Figure 3A). Furthermore, the NH_4^+concentration in the blood of octopus gills (250~300 μM) is approximately ten times higher than that of squid (20~25 μM).

NH_4^+ transport patterns of gills by the perfusion approach

As shown in Figure 3B, the NH_4^+ transport rate in the epithelium from the basolateral site to the perfusate blood of octopus was 0.1105 ± 0.0023 μmol NH_4^+ h^{-1} g_{FM}^{-1}, and 0.2795 ± 0.1249 μmol NH_4^+ h^{-1} g_{FM}^{-1} in *S. lessoniana*. Squid gills showed a higher NH_4^+ transport rate into perfusate blood than octopus gills.

Pharmacological studies of sodium-hydrogen exchanger (NHE) in *NH_4^+* and H^+ transport on cephalopod gills

In squid gills, the average values of NH_4^+ transport rates from the basolateral side to the perfusate blood were 0.251~0.301 μmol NH_4^+ h^{-1} g_{FM}^{-1} under 2.5, 5 and 10 μM EIPA treatments, which were not significantly different from the control group (Figure 4A; Supplementary Table S2). Besides, the average values of NH_4^+ transport rates from the apical side to the bath seawater under 2.5, 5 and 10 μM EIPA treatments were 0.081~0.173 μmol NH_4^+ h^{-1} g_{FM}^{-1} (Figure 4A; Supplementary Table S2), which were also not significantly different from the control value (0.141 ± 0.066 μmol NH_4^+ h^{-1} g_{FM}^{-1}). Additionally, the average values of H^+ transport rates in squid gills were maintained within the range of 0.001~0.008 μmol H^+ h^{-1} g_{FM}^{-1} and 0.006~0.015 μmol H^+ h^{-1} g_{FM}^{-1} from the basolateral side to the perfusate and from the apical side to the bath, respectively, under EIPA treatments (Figure 4B; Supplementary Table S2).

Moreover, EIPA was also perfused to examine the effects of NHE inhibition on the NH_4^+ and H^+ transport in octopus gills. The NH_4^+ transport rates from the basolateral side to the perfusate blood decreased by approximately 30%, 45% and 84% under 2.5, 5 and 10 μM EIPA treatments, respectively, which showed a significantly decreased tendency (p=0.0018) compared with the control group (Figure 4A; Supplementary Table S2). Moreover, the NH_4^+ transport rates from the apical side to the bath were 0.062~0.070 μmol NH_4^+ h^{-1} g_{FM}^{-1} under different dosages of EIPA treatments, which were not significantly different from the control ones (0.054 ± 0.006 μmol NH_4^+ h^{-1} g_{FM}^{-1}) (Figure 4A; Supplementary Table S2). For the estimation of H^+ transport rates, compared to the control group, a decrease of approximately 39%, 30%, and 91% from the basolateral side to the perfusate blood was observed under 5, 7.5 and 10 μM EIPA perfused octopus gills, respectively. It was also observed that the H^+ transport from the apical side to the bath was reduced by approximately 32%

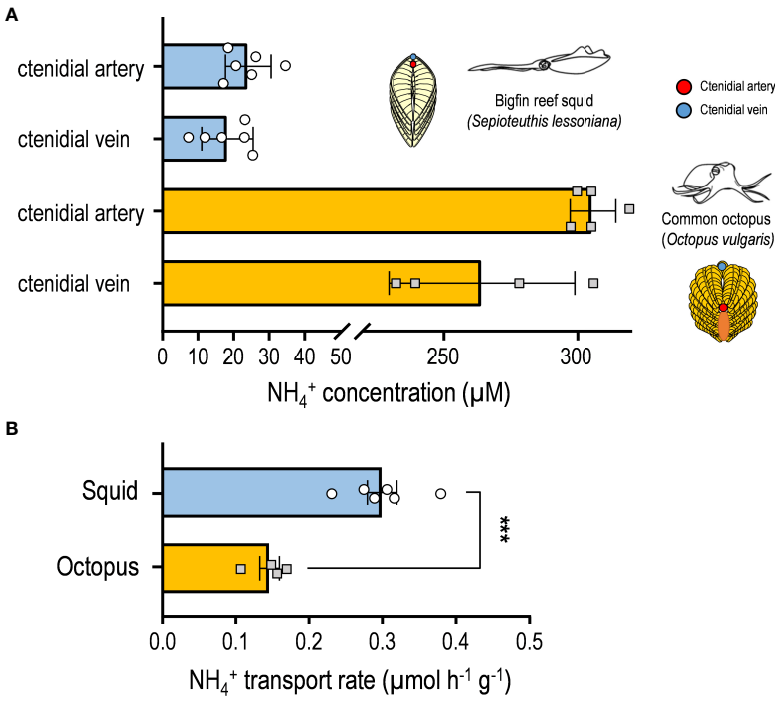

FIGURE 3
NH_4^+ concentration and transport rates in cephalopod gills. **(A)** The NH_4^+ concentration in blood collected from the ctenidial artery and ctenidial vein of squid and octopus. **(B)** The NH_4^+ transport rates were determined using the fluid collected from the perfusate in perfused gills. The estimated values are presented as mean ± SD (n = 4~6). Asterisks indicate significance levels of $p < 0.001$ (***).

~95% under different dosages of EIPA treatment (Figure 4B; Supplementary Table S2).

Pharmacological studies of vacuolar H^+ ATPase (VHA) in NH_4^+ and H^+ transport on cephalopod gills

In squid gills, under 2.5 and 5 μM of BafA1 treatment, the NH_4^+ transport rate from the basolateral side to the perfusate was significantly increased by about 93% and 91%, respectively, compared with the control counterpart. However, the dosage of 7.5 μM of BafA1 treatment would not affect the NH_4^+ transport rate (Figure 5A; Supplementary Table S3). While estimating the NH_4^+ transport rate from the apical side to the bath seawater, the 2.5 μM of BafA1 treatment would result in an increased level (0.268 ± 0.072 μmol NH_4^+ h^{-1} g_{FM}^{-1}) of about 2.88-fold higher than the control counterpart (0.093 ± 0.034 μmol NH_4^+ h^{-1} g_{FM}^{-1}). Other dosages (5 and 7.5 μM) of BafA1 treatments would not affect the NH_4^+ transport from the apical side to the bath (Figure 5A). While we further examined the H^+ transport rates from the basolateral side to the perfusate blood, the 2.5 and 5 μM of BafA1 treatment would apparently result in about 9.0- and 4.9-fold decrease compared to the control group, respectively (Figure 5A). And the dosage of 7.5 μM of BafA1 treatment would not affect the H^+ transport from the basolateral side to the perfusate. Besides, the average values of H^+ transport rates from the apical side to the bath seawater were 0.005~0.009 μmol H^+ h^{-1} g_{FM}^{-1} under 2.5, 5 and 7.5 μM BafA1 treatments, which were not significantly different from the control group (Figure 5A; Supplementary Table S3).

Moreover, in octopus gills, the NH_4^+ transport rate from the basolateral side to the perfusate would decrease to 0.064 ± 0.042 μmol NH_4^+ h^{-1} g_{FM}^{-1}(42% decrement) and 0.061 ± 0.010 μmol NH_4^+ h^{-1} g_{FM}^{-1} (44% decrement) under 2.5 and 5 μM of BafA1 treatment, respectively. The 7.5 μM of BafA1 treatment would reversely increase (0.343 ± 0.081 μmol NH_4^+ h^{-1} g_{FM}^{-1}) the NH_4^+ transport from the basolateral side to the perfusate blood (Figure 5A). And the BafA1 treatments would not apparently affect the NH_4^+ transport from the apical side to the bath seawater (p=0.2870) (Supplementary Table S3). As for the BafA1 treatment effects on the H^+ transport rates in octopus gills, the BafA1 treatment would result in a significantly decreased fashion in H^+ transport rates (p=0.0102) from the basolateral side to the perfusate blood (Figure 5B; Supplementary Table S3). Moreover, the H^+ transport rates from the apical side to the bath seawater were also significantly affected by the BafA1 treatment (p=0024) (Figure 5B; Supplementary Table S3).

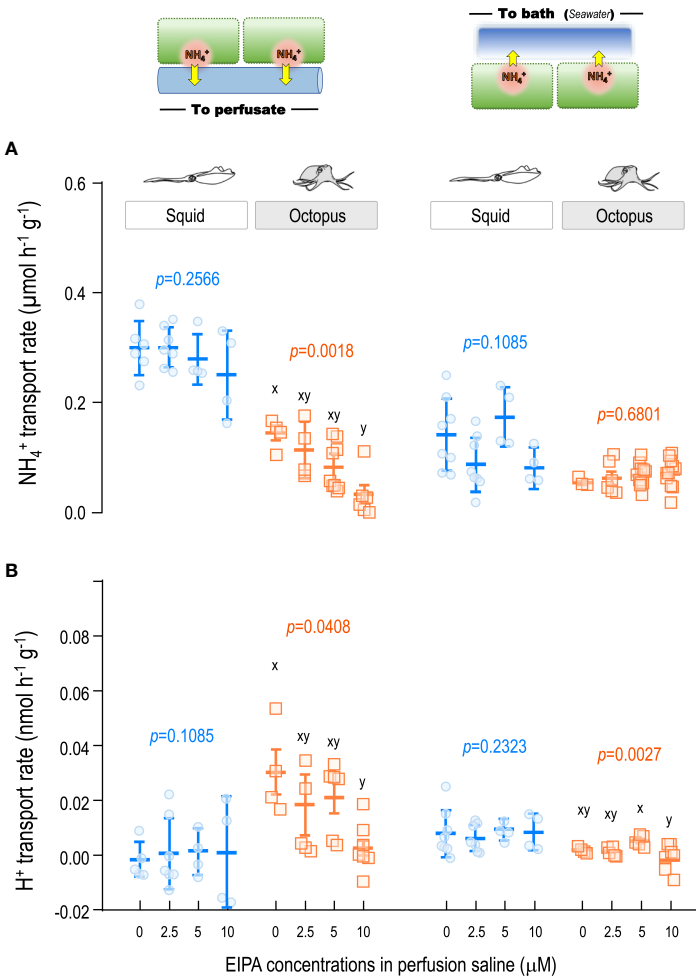

FIGURE 4
Effects of NHE inhibitor EIPA on the NH_4^+ and H^+ transport rates in gill epithelium of squid and octopus by *ex-vivo* perfusion approach. Different dosages of the NHE inhibitor EIPA were tested for their effects on the NH_4^+ **(A)** and H^+ **(B)** transport rates in squid and octopus gills by *ex-vivo* perfusion. Different letters indicate significant differences among perfused inhibitor dosages ($p < 0.05$; One-way ANOVA, Tukey's pairwise comparisons).

Discussion

The distinct strategies in cephalopods for NH_4^+ regulation and homeostasis

The modern coleoid cephalopods are widely distributed in the marine ecosystem, from the abyssal regions to the intertidal zones. By virtue of their particular locomotion patterns and intelligence, they behave with greater dominance than most marine animals of similar size (Villanueva et al., 2017). Octopods animals can swim by contracting their arms with their velar skins and thus expelling water or crawling as their primary modes of movement. In addition, the modern loliginid squids use their muscular mantle to pressurize the mantle cavity water ejecting through the siphons; therefore, they are able to move relatively rapidly with a jet propulsion pattern and discharge ink as a decoy or smoke screen (Bone et al., 1981). Several studies have proposed that cephalopods' jet propulsion or crawling motion patterns create high energy demands as a compensatory measure for their inferior competitive ability with other marine vertebrates (O'Dor et al., 1995; Webber et al., 2000). As a result, these special locomotion types may act as a double-edged sword, causing huge nitrogenous wastes or productions from protein catabolism, which may adversely affect body growth, nervous system abnormalities, or other possible intrinsic activities imbalances. Consequently, they must adopt different behaviors and adapt their homeostasis regulation strategies to discard or exploit nitrogenous wastes

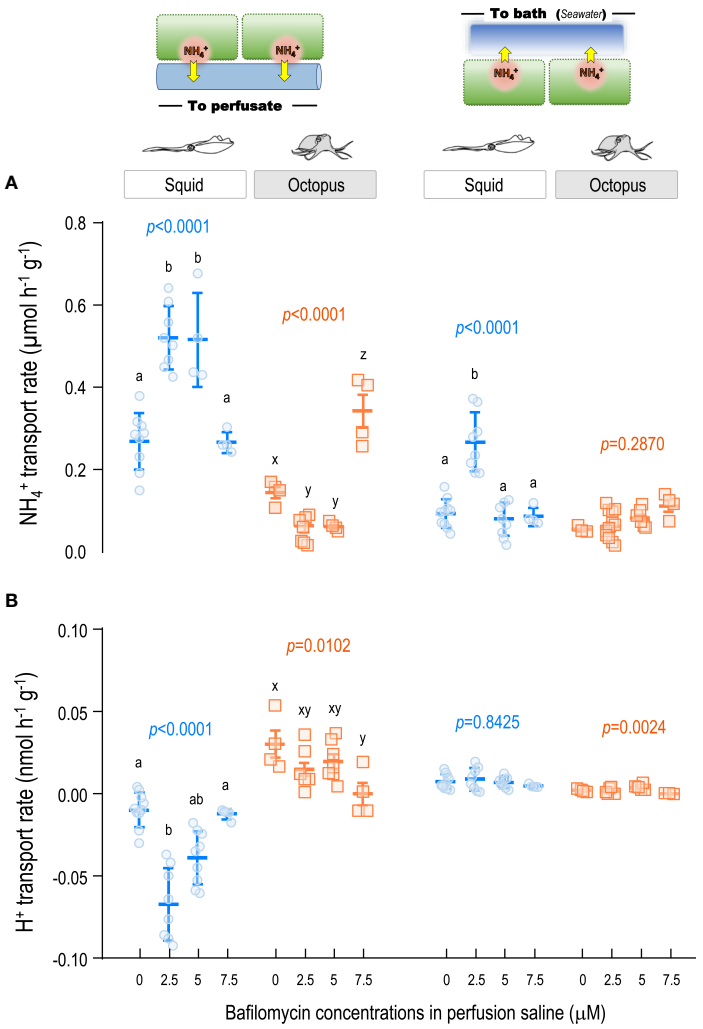

FIGURE 5
Effects of VHA inhibitor bafilomycin on the $NH_4^+$and H^+ transport rates in gill epithelium of squid and octopus by *ex-vivo* perfusion approach. Different dosages of the VHA inhibitor bafilomycin were tested for their effects on the NH_4^+ **(A)** and H^+ **(B)** transport rates in squid and octopus gills by *ex-vivo* perfusion. Different letters indicate significant differences among perfused inhibitor dosages ($p < 0.05$; One-way ANOVA, Tukey's pairwise comparisons).

in comparison with other marine animals, even between the Octopod and loliginid cephalopods.

As cephalopod species behave differently in their locomotion patterns, their natural habitats vary accordingly (O'Dor et al., 2002). The resting metabolism of Octopod and loliginid cephalopods may reflect differences in their respective life histories (Potts, 1965). In this study, the resting metabolic rate of octopus *O. vulgaris* was found to be significantly lower than that of squid *S. lessoniana* (Figure 2A). This feature suggested that their different locomotion strategies, such as crawling or jet-motion, may correlate with their different metabolic strategies. Despite the fact that jet propulsion is not an efficient locomotion pattern that requires too much energy from aerobic respiration, this pattern is capable of rapid acceleration and locomotion (O'Dor and Webber, 1991; Gemmell et al., 2021). Octopuses, on the other hand, are considered to be jet-propulsion abandonment members (Wells, 1990; Huffard, 2006). This species is an ambush predator with excellent camouflage and maneuverability, allowing it to stay in mud bottoms and caves for a long time (Kayes, 1973; Caldwell et al., 2015). In this respect, Octopods may be exposed to hypoxic and high ammonium conditions (Wells and Wells, 1983; Okutani, 1990; Seibel and Childress, 2000) and should therefore demonstrate extraordinary capacities for nitrogenous detoxification. In this study, the gills of Octopoda *O. vulgaris* was found to accumulate higher levels of NH_4^+ in blood compared to that of the pelagic

loliginid bigfin reef squid, *S. lessoniana* (Figure 4A). As a result of the "ammonium-keeping" ability of octopus blood, a chemical gradient against ambient high ammonium levels may be generated. A higher intact aerobic respiration rate in pelagic squid, and a higher NH_4^+ transport rate to the perfusate, may result in a lower NH_4^+ accumulation in the gills. Apart from that, NH_4^+ is an intermediate metabolite that can be converted into urea or amino acids for maintaining intact homeostasis or buoyancy capacity (Seibel et al., 2004; Peng et al., 2017). Consequently, further studies are needed to explore those nitrogen pathways in terms of ammonium regulation in cephalopods with different lifestyles.

Among these metabolic processes, ammonia-related amino acid synthesis is one of the most important biochemical processes for maintaining organismic NH_4^+ homeostasis (Levitt and Levitt, 2018; Voss et al., 2021). Under the Glu-Gln cycle, glutamate (Glu) is catabolized to glutamine (Gln), which coordinates with NH_4^+. Compared to benthic octopus, pelagic squid blood contains more glutamine, a potential NH_4^+ carrier, while octopus retains more glutamate for the potential binding plasticity toward NH_4^+. In terms of the metabolic trade-off concept, benthic octopus with lower aerobic respiration rates would prefer to utilize glutamate as an alternative metabolic substrate for energy supply. In contrast, pelagic squids would eliminate NH_4^+ accumulation by binding NH_4^+ to glutamine and increasing NH_4^+ transport through their gills. The metabolic trade-off adjustment may help prevent nitrogenous wastes from accumulating and may also benefit the Glu/Gln balance. Additionally, those bicarbonate-buffering molecules, such as carbonic acid (H_2CO_3), bicarbonate ion (HCO_3^-) and carbon dioxide (CO_2), are essential for the acid-base homeostatic mechanism in both aquatic vertebrates and invertebrates (Melzner et al., 2020; Tresguerres et al., 2020). However, ammonia could also serve as the non-bicarbonate buffer molecule binding to H^+, which may play a role in the acid-base balance (Hu et al., 2014; Tseng et al., 2020). Cephalopods are capable of producing and accumulating large quantities of nitrogenous NH_4^+ , in comparison with most marine fishes of similar body weight (Packard, 1972; Lee, 1995). Consequently, cephalopods must be equipped with efficient machinery for acid-base regulation and detoxification of nitrogenous products in comparison to their athletic competitor teleosts in similar ocean habitats. In this way, cephalopod mollusks may be able to alleviate abiotic perturbations resulting from high metabolic-derived cellular acidification and nitrogenous waste accumulation. It is unclear whether there is a relationship between NH_4^+ contents/transport significance and different locomotion types in cephalopods. The estimations of Glu and Gln levels in the blood of squids and octopuses may therefore reflect their lifestyles and locomotion patterns. Additional evidence is needed to prove this notion.

Moreover, we should also be aware that vertical migration in the ocean system is a major metabolic expenditure for many pelagic organisms, and a special mechanism for neutral buoyancy in cephalopods has been proposed (Seibel et al., 2004). In pelagic deep-sea crustacean species, the use of NH_4^+ has been reported to be effective in maintaining buoyancy (Sanders and Childress, 1988). Cranchiidae would also store ammoniacal fluid in their unique coelomic cavity, and NH_4^+ is subsequently accumulated in vacuoles in body tissues such as the mantle and arms (Voight et al., 1995; Seibel et al., 2004). This means nitrogenous NH_4^+ is not a metabolic waste product for cephalopods but a useful molecule that participates in amino acid metabolism, acid-base regulation, and even buoyancy locomotion.

NH_4^+ and H^+ transport machinery in the branchial epithelium of squid and octopus

In most aquatic animals, ammonia is a toxic chemical that has detrimental effects on the nervous system, metabolic disorders, and other severe pathological conditions (Wood et al., 1995; Weiner and Verlander, 2016). For marine animals with an active lifestyle, gills serve as an efficient organ to deal with this biological stressor. Based on earlier findings in cephalopods, the renal appendages and gills are the most important organs for regulating NH_4^+ levels. In addition to secreting ammonia, the renal appendages are also believed to produce urine by combining urea and water to regulate osmotic pressure (Potts, 1965). However, renal appendages are able to excrete less than 1% of ammonium. Therefore, it was concluded that the gill was the primary organ for excreting ammonium in cephalopods (Potts, 1965).

The excretion of ammonia can occur either in the form of non-ionized NH_3 or ammonium (NH_4^+) by direct diffusion through paracellular pathways or through Rhesus (Rh) glycoproteins that work with apical NHEs (Randall and Ip, 2006; Wright and Wood, 2009; Liu et al., 2013; Hu et al., 2017; Clifford et al., 2022), and the acid-trapping mechanism is hypothesized to be important. Under conditions of high environmental ammonia, the consumption of metabolic acid in pacific hagfish (*Eptatretus stoutii*) was significantly increased, accompanied by the conversion of NH_3 to NH_4^+ (Clifford et al., 2015). The mRNA and protein levels of renal Rhcg1 were stimulated in common carp (*Cyprinus carpio*) under acidic conditions, along with upregulation of several ion transporters, including the NHE, VHA, and NKA (Wright et al., 2014). Besides, the basolateral sodium-potassium ATPase (NKA) actively creates the concentration gradient of Na^+ (energy coming from ATP consumption), and the driving force to process the NHEs works by Na^+ gradient. Therefore, the secondary active transport manner by NHE homologs is essential for acid-base regulation; moreover, coordination of NH_4^+ excretion by combining H^+ with NH_3. According to a previous study in the crustacean *Carcinus maenas*, H^+ would

trap NH_3 within intracellular vesicles to form NH_4^+, thereby allowing H^+ secretion through this potential pathway for ammonia excretion (Weihrauch et al., 2002). As for cephalopods, recent molecular insights into the regulation of NH_4^+ and H^+ have also been well demonstrated (Hu et al., 2014; Hu et al., 2015; Hu et al., 2017). Coleoid cephalopods possess sophisticated vertebrate-like NH_4^+/H^+ regulatory mechanisms and rely extensively upon branchial epithelia for homeostasis control. In the semitubular environment of cephalopod gills, an acid-trapping mechanism for NH_4^+ has also been proposed based on morphological, functional, and molecular studies (Hu and Tseng, 2017). Thus, homeostasis in aquatic organisms is not only controlled by direct diffusion or by transepithelial transporters but also by the H^+ secretion machinery.

As part of this study, EIPA and BafA1 were applied to pharmacological perfusion experiments on squid and octopus gills. Inhibition of NHEs by EIPA appeared to affect NH_4^+ and H^+ transport in octopus gills. However, inhibition of NHE by EIPA appears to have no significant effect on either NH_4^+ or H^+ transport in the squid epithelium from basolateral or apical sites. In this regard, compared to the pelagic species, the crawling cephalopod octopus would rely on the secondary active transport route (NHE) *via* the electrochemical Na^+ gradient to maintain NH_4^+ and H^+ transport. In case of elevated ambient NH_4^+ and H^+ concentrations, octopuses with less aerobic respiration capacity have an energy-saving strategy to maintain homeostasis and hunt prey with less energy consumption. It should be noted that, while this pharmacological trial utilized the NHE inhibitor EIPA in octopus gills, we found that the NH_4^+ transport towards the apical site was not significantly affected as was shown toward the basolateral site and the effective feature in H^+ transport towards the apical site. Accordingly, it appears that another apical NH_4^+ transporter, such as the RhP homolog, that can facilitate NH_4^+ transport in the gill epithelium of octopus (Hu et al., 2017). Moreover, it is also inferred that the level of NH_4^+ excreted by the octopus gills is primarily determined by the transport of NH_4^+ from the branchial cells into the blood. It is possible that this basolateral machinery may be operated by other NH_4^+ transport molecules, such as Rhp1b, which has been suggested to be present in the branchiae of marine polychaetes (*Eurythoe complanata*) (Weihrauch and Allen, 2018). The "ammonium-keeping" significance of chemical gradients against environmental nitrogenous toxicity is the reason for a higher NH_4^+ concentration in the ctenidial vein of octopus than in squid. It would be physiologically meritorious to discover what caused the difference in NH_4^+ transport rates between octopus and squid. A possible explanation for this significance may be the presence of different NH_4^+ transport molecules with different isoforms (Weihrauch and Allen, 2018). NH_4^+ transport molecules have different functional characteristics, enabling organisms to adapt to a variety of habitats with different mobility styles, as well as have significantly greater physiological plasticity due to the well-regulated NH_4^+ content, essential for maintaining intact homeostasis and metabolic balance in various environmental conditions.

Apart from H^+ transport by NHE, the primary active transport by VHA with ATP-dependent operation appears to be irreplaceable. Observing the effects of the VHA inhibitor bafilomycin (BafA1) on NH_4^+ and H^+ transport in the gills of squid and octopus, the influence patterns were quite apparent. The BafA1 inhibits the H^+ transport rate toward the blood in squid gills, but the NH_4^+ transport rates in gill epithelium are reversely stimulated, suggesting that other alternative molecules, such as the RhP homologs, may contribute to the efficient NH_4^+ transport capacity of squid to maintain acid-base equilibrium (Hu et al., 2014). Meanwhile, the fact that BafA1 would inhibit both NH_4^+ and H^+ transport in octopus gills indicates that VHA is also essential for homeostasis in crawling cephalopods. Additionally, octopus VHA may be involved in the post-translational control of vesicular NH_4^+ excretion *via* a membrane trafficking pathway that represents an alternative method of excreting vesicular NH_4^+ (Hu et al., 2017). As for the NH_4^+ transport towards the apical site, it was not significantly affected. This may also be due to the fact that the NH_4^+ excretion in the octopus gills is predominantly transported from the branchial cells into the blood, and/or the apical NHE may also be involved in the excretion of NH_4^+. More importantly, the VHA in the gills of octopus showed distinct distribution within the endothelial cells of blood vessels (Hu et al., 2017). Accordingly, our preliminary data indicate that high concentrations (10 μM in saline) of perfused BafA1 affect the morphology of the gills of octopuses during the perfusion procedure. Therefore, the maximum inhibitory concentration of 7.5 μM BafA1 was used in both species during the VHA-inhibition studies.

It is true that the epithelial basolateral NKA actively creates the concentration gradient of Na^+ (energy derived from ATP consumption); however, the SW habitat provides a natural Na^+-rich driving force for the NHEs to work. Natural Na^+ gradients in SW drive the secondary active transport mechanism through the NHE homologs, which is crucial for the regulation of NH_4^+ and H^+. As a result of the energy constraint concerns, the implementation of NHE traits, as well as the energy-consuming VHA in the gills for body fluid homeostasis, offers a potential benefit for reducing energy consumption. It was evident from this feature that the octopus would take advantage of both secondary and primary active transport mechanisms to maintain NH_4^+ and H^+ homeostasis. As energy is a major constraint on physiological processes, trade-offs between energy allocations

to different physiological processes under the concern of energy efficiency could be considered to be a cellular adjustment. In order to achieve regular homeostasis, the benthic crawling octopuses may act as "misers," which means that they deliberate with the amount of energy consumed (Semmens et al., 2004; Onthank and Cowles, 2011). As an alternative, the acid-base and ammonium regulation in pelagic squid could be largely controlled by primary active transport in an energy-intensive manner since they possess higher aerobic respiration rate and more efficient NH_4^+ regulation machinery than benthic crawling octopus. Due to their similar athletic hunting strategies and jet propulsion locomotion pattern, the pelagic squid may behave as a "spendthrift" for energy consumption that is not concerned with the food source (Shulman et al., 2002; Gao et al., 2022). This characteristic implies that pelagic squids are able to generate enough energy for intact physiological adjustments, such as body growth and nitrogenous waste elimination.

Conclusion

Cephalopods are ancient mollusks that can be found in a wide variety of ecological niches in the ocean, ranging from the intertidal zone to the deep ocean. To adapt to their lifestyles in various types of habitats, modern cephalopods have evolved a variety of locomotory modes. In general, cephalopods have relatively high metabolic rates due to their less efficient swimming mode by jet propulsion. This energy-intensive lifestyle is primarily fueled by the rapid digestion of protein diets, which may lead to metabolic ammonia/ammonium accumulation and acid-base disturbances. The inhibition of secondary active transport machinery significantly impaired NH_4^+ and H^+ excretory processes in the gills of benthic octopus, but not in pelagic squid. However, the inhibition of primary active transport machinery significantly impairs the excretion of NH_4^+ and H^+ in both benthic and pelagic coleoid cephalopods. Pelagic squids prefer active transport for NH_4^+/H^+ excretion in an energy-dependent manner, whereas benthic octopuses use both secondary and primary active transport for homeostasis. Consequently, the pelagic squid, in contrast to the benthic octopus, may have developed NH_4^+/H^+-transport mechanisms in an energy-intensive manner to avoid toxic nitrogenous effects in the blood.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Academia Sinica Institutional Animal Care and Utilization Committee (approval no. BSF17-12-1158).

Author contributions

M-WL, P-LK, and P-HS conceived the study, designed, and carried out the metabolic and perfusion analysis. M-WL and Y-CT wrote the first draft of the paper, and all co-authors commented on and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.971764/full#supplementary-material

References

Bone, Q., Pulsford, A., and Chubb, A. D. (1981). Squid mantle muscle. *J. Mar. Biol. Assoc. U. K.* 61, 327–342. doi: 10.1017/S0025315400046981

Boucher-Rodoni, R., and Mangold, K. (1989). Respiration and nitrogen excretion by the squid *Loligo forbesi*. *Mar. Biol.* 103, 333–338. doi: 10.1007/BF00397267

Boucher-Rodoni, R., and Mangold, K. (1995). Ammonia production in cephalopods, physiological and evolutionary aspects. *Mar. Freshw. Behav. Physiol.* 25, 53–60. doi: 10.1080/10236249409378907

Caldwell, R. L., Ross, R., Rodaniche, A., and Huffard, C. L. (2015). Behavior and body patterns of the larger pacific striped octopus. *PloS One* 10, e0134152. doi: 10.1371/journal.pone.0134152

Chang, W. J., and Hwang, P. P. (2011). Development of zebrafish epidermis. *Birth Defects Res. C Embryo Today* 93, 205–214. doi: 10.1002/bdrc.20215

Chung, W.-S., Kurniawan, N. D., and Marshall, N. J. (2020). Toward an MRI-based mesoscale connectome of the squid brain. *Iscience* 23, 100816. doi: 10.1016/j.isci.2019.100816

Clifford, A. M., Goss, G. G., and Wilkie, M. P. (2015). Adaptations of a deep sea scavenger: High ammonia tolerance and activeNH_4^+excretion by the pacific hagfish (*Eptatretus stoutii*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 182, 64–74. doi: 10.1016/j.cbpa.2014.12.010

Clifford, A. M., Wilkie, M. P., Edwards, S. L., Tresguerres, M., and Goss, G. G. (2022). Dining on the dead in the deep: Active NH_4^+Excretion *via* Na^+/H^+ NH_4^+ exchange in the highly ammonia tolerant pacific hagfish, *Eptatretus stoutii*. *Acta Physiol.* 00, e13845. doi: 10.1111/apha.13845

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97–177. doi: 10.1152/physrev.00050.2003

Gao, X., Gong, Y., Chen, X., and Li, Y. (2022). Dietary shifts and niche partitioning throughout ontogeny reduce intraspecific competition in a pelagic generalist predator. *Mar. Ecol. Prog. Ser.* 692, 81–97. doi: 10.3354/meps14079

Gemmell, B. J., Dabiri, J. O., Colin, S. P., Costello, J. H., Townsend, J. P., and Sutherland, K. R. (2021). Cool your jets: Biological jet propulsion in marine invertebrates. *J. Exp. Biol.* 224, jeb222083. doi: 10.1242/jeb.222083

Histon, K. (2012). Paleoenvironmental and temporal significance of variably colored Paleozoic orthoconic nautiloid cephalopod accumulations. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* 367, 193–208. doi: 10.1016/j.palaeo.2012.07.008

Holmes, R. M., Aminot, A., Kérouel, R., Hooker, B. A., and Peterson, B. J. (1999). A simple and precise method for measuring ammonium in marine and freshwater ecosystems. *Can. J. Fish Aquat. Scci.* 56, 1801–1808. doi: 10.1139/f99-128

Huang, P. C., Liu, T. Y., Hu, M. Y., Casties, I., and Tseng, Y. C. (2020). Energy and nitrogenous waste from glutamate/glutamine catabolism facilitates acute osmotic adjustment in non-neuroectodermal branchial cells. *Sci. Rep.* 10, 1–17. doi: 10.1038/s41598-020-65913-1

Huffard, C. (2006). Locomotion by *Abdopus aculeatus* (Cephalopoda: Octopodidae): Walking the line between primary and secondary defenses. *J. Exp. Biol.* 209, 3697–3707. doi: 10.1242/jeb.02435

Hu, M. Y., Guh, Y.-J., Stumpp, M., Lee, J.-R., Chen, R.-D., Sung, P.-H., et al. (2014). Branchial NH_4^+-dependent acid–base transport mechanisms and energy metabolism of squid (*Sepioteuthis lessoniana*) affected by seawater acidification. *Front. Zool.* 11, 1–17. doi: 10.1186/s12983-014-0055-z

Hu, M. Y., Hwang, P.-P., and Tseng, Y.-C. (2015). Recent advances in understanding trans-epithelial acid-base regulation and excretion mechanisms in cephalopods. *Tissue Barriers* 3, e1064196. doi: 10.1080/21688370.2015.1064196

Hu, M. Y., Sung, P.-H., Guh, Y.-J., Lee, J.-R., Hwang, P.-P., Weihrauch, D., et al. (2017). Perfused gills reveal fundamental principles of pH regulation and ammonia homeostasis in the cephalopod *Octopus vulgaris*. *Front. Physiol.* 8, 162. doi: 10.3389/fphys.2017.00162

Hu, M., and Tseng, Y.-C. (2017). "Acid–base regulation and ammonia excretion in cephalopods: An ontogenetic overview,", in *Acid-base balance and nitrogen excretion in invertebrates* (Cham: Springer), 275–298. doi: 10.1007/978-3-319-39617-0_11

Hu, M. Y., Tseng, Y. C., Lin, L. Y., Chen, P. Y., Charmantier-Daures, M., Hwang, P. P., et al. (2011). New insights into ion regulation of cephalopod molluscs: A role of epidermal ionocytes in acid-base regulation during embryogenesis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R1700–R1709. doi: 10.1152/ajpregu.00107.2011

Hwang, P. P., Lee, T. H., and Lin, L. Y. (2011). Ion regulation in fish gills: Recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R28–R47. doi: 10.1152/ajpregu.00047.2011

Hwang, P.-P., and Lin, L.-Y. (2013). "Gill ionic transport, acid-base regulation, and nitrogen excretion". *The Physiology of Fishes 4th Edn,* eds D. H. Evans and J. B Claiborne (Boca Raton, FL: CRC Press) 4, 205–233.

Hwang, P. P., and Perry, S. F. (2010) (San Diego), 311–344.

Jereb, P., and Roper, C. F. (2010). "Cephalopods of the world-an annotated and illustrated catalogue of cephalopod species known to date," in *Myopsid and oegopsid squids*, Vol 2. (Rome: Fao).

Kayes, R. (1973). The daily activity pattern of *Octopus vulgar* is in a natural habitat. *Mar. Freshw. Behav. Physiol.* 2, 337–343. doi: 10.1080/10236247309386935

Kroeger, B., and Yun-Bai, Z. (2009). Pulsed cephalopod diversification during the Ordovician. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* 273, 174–183. doi: 10.1016/j.palaeo.2008.12.015

Kuan, P.-L., You, J.-Y., Wu, G.-C., and Tseng, Y.-C. (2022). Temperature increases induce metabolic adjustments in the early developmental stages of bigfin reef squid (*Sepioteuthis lessoniana*). *Sci. Total Environ.* 844, 156962. doi: 10.1016/j.scitotenv.2022.156962

Lee, P. (1995). Nutrition of cephalopods: Fueling the system. *Mar. Freshw. Behav. Physiol.* 25, 35–51. doi: 10.1080/10236249409378906

Levitt, D. G., and Levitt, M. D. (2018). A model of blood-ammonia homeostasis based on a quantitative analysis of nitrogen metabolism in the multiple organs involved in the production, catabolism, and excretion of ammonia in humans. *Clin. Exp. Gastroenterol.* 11, 193–215. doi: 10.2147/CEG.S160921

Liu, S. T., Horng, J. L., Chen, P. Y., Hwang, P. P., and Lin, L. Y. (2016). Salt secretion is linked to acid-base regulation of ionocytes in seawater-acclimated medaka: New insights into the salt-secreting mechanism. *Sci. Rep.* 6, 31433. doi: 10.1038/srep31433

Liu, S. T., Tsung, L., Horng, J. L., and Lin, L. Y. (2013). Proton-facilitated ammonia excretion by ionocytes of medaka (*Oryzias latipes*) acclimated to seawater. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 305, R242–R251. doi: 10.1152/ajpregu.00047.2013

Mather, J. A., Griebel, U., and Byrne, R. A. (2010). Squid dances: An ethogram of postures and actions of sepioteuthis sepioidea squid with a muscular hydrostatic system. *Mar. Freshw. Behav. Physiol.* 43, 45–61. doi: 10.1080/10236241003660771

Melzner, F., Mark, F. C., Seibel, B. A., and Tomanek, L. (2020). Ocean acidification and coastal marine invertebrates: Tracking CO_2 effects from seawater to the cell. *Annu. Rev. Mar. Sci.* 12, 499–523. doi: 10.1146/annurev-marine-010419-010658

Nishi, T., and Forgac, M. (2002). The vacuolar (H^+)-ATPases–nature's most versatile proton pumps. *Nat. Rev. Mol. Cell Biol.* 3, 94–103. doi: 10.1038/nrm729

O'Dor, R. (2002). Telemetered cephalopod energetics: Swimming, soaring, and blimping. *Integr. Comp. Biol.* 42, 1065–1070. doi: 10.1093/icb/42.5.1065

O'Dor, R., Adamo, S., Aitken, J., Andrade, Y., Finn, J., Hanlon, R., et al. (2002). Currents as environmental constraints on the behavior, energetics and distribution of squid and cuttlefish. *Bull. Mar. Sci.* 71, 601–617.

O'Dor, R., Hoar, J., Webber, D., Carey, F., Tanaka, S., Martins, H., et al. (1995). Squid *(Loligo forbesi*) performance and metabolic rates in nature. *Mar. Freshw. Behav. Physiol.* 25, 163–177. doi: 10.1080/10236249409378915

O'Dor, R., and Webber, D. (1991). Invertebrate athletes: Trade-offs between transport efficiency and power density in cephalopod evolution. *J. Exp. Biol.* 160, 93–112. doi: 10.1242/jeb.160.1.93

Okutani, T. (1990). Squids, cuttlefish and octopuses. *Mar. Freshw. Behav. Physiol.* 18, 1–17. doi: 10.1080/10236249009378778

Onthank, K. L., and Cowles, D. L. (2011). Prey selection in *Octopus rubescens*: Possible roles of energy budgeting and prey nutritional composition. *Mar. Biol.* 158, 2795–2804. doi: 10.1007/s00227-011-1778-4

Packard, A. (1972). Cephalopods and fish: The limits of convergence. *Biol. Rev.* 47, 241–307. doi: 10.1111/j.1469-185X.1972.tb00975.x

Peng, R. B., Le, K. X., Wang, P. S., Wang, Y., Han, Q. X., and Jiang, X. M. (2017). Detoxification pathways in response to environmental ammonia exposure of the cuttlefish, *Sepia pharaonis*: Glutamine and urea formation. *J. World Aquac Soc.* 48, 342–352. doi: 10.1111/jwas.12341

Potts, W. T. W. (1965). Ammonia excretion in *Octopus dolfeini*. *Comp. Biochem. Physiol.* 14, 339–354. doi: 10.1016/0010-406X(65)90209-4

Randall, D. J., and Ip, Y. K. (2006). Ammonia as a respiratory gas in water and air-breathing fishes. *Respir. Physiol. Neurobiol.* 154, 216–225. doi: 10.1016/j.resp.2006.04.003

Sanders, N., and Childress, J. (1988). Ion replacement as a buoyancy mechanism in a pelagic deep-sea crustacean. *J. Exp. Biol.* 138, 333–343. doi: 10.1242/jeb.138.1.333

Schaffhauser, D., Fine, M., Tabata, M., Goda, T., and Miyahara, Y. (2016). Measurement of rapid amiloride-dependent pH changes at the cell surface using a proton-sensitive field-effect transistor. *Biosensors* 6, 11. doi: 10.3390/bios6020011

Scheel, D., and Bisson, L. (2012). Movement patterns of giant pacific octopuses, *Enteroctopus dofleini* (Wülker 1910). *J. Exp. Mar. Biol. Ecol.* 416, 21–31. doi: 10.1016/j.jembe.2012.02.004

Seibel, B. A., and Childress, J. J. (2000). Metabolism of benthic octopods (Cephalopoda) as a function of habitat depth and oxygen concentration. *Deep Sea Res. Part I Oceanogr. Res. Pap* 47, 1247–1260. doi: 10.1016/S0967-0637(99)00103-X

Seibel, B. A., and Drazen, J. C. (2007). The rate of metabolism in marine animals: Environmental constraints, ecological demands and energetic opportunities. *Philos. Trans. R Soc. Lond B Biol. Sci.* 362, 2061–2078. doi: 10.1098/rstb.2007.2101

Seibel, B. A., Goffredi, S. K., Thuesen, E. V., Childress, J. J., and Robison, B. H. (2004). Ammonium content and buoyancy in midwater cephalopods. *J. Exp. Mar. Biol. Ecol.* 313, 375–387. doi: 10.1016/j.jembe.2004.08.015

Semmens, J., Pecl, G., Villanueva, R., Jouffre, D., Sobrino, I., Wood, J., et al. (2004). Understanding octopus growth: Patterns, variability and physiology. *Mar. Freshw. Res.* 55, 367–377. doi: 10.1071/MF03155

Shulman, G., Chesalin, M., Abolmasova, G., Yuneva, T., and Kideys, A. (2002). Metabolic strategy in pelagic squid of genus *Sthenoteuthis* (Ommastrephidae) as the basis of high abundance and productivity: An overview of the soviet investigations. *Bull. Mar. Sci.* 71, 815–836.

Tovey, K. J., and Brauner, C. J. (2018). Effects of water ionic composition on acid–base regulation in rainbow trout, during hypercarbia at rest and during sustained exercise. *J. Comp. Physiol. B* 188, 295–304. doi: 10.1007/s00360-017-1129-y

Tresguerres, M., Clifford, A. M., Harter, T. S., Roa, J. N., Thies, A. B., Yee, D. P., et al. (2020). Evolutionary links between intra-and extracellular acid–base regulation in fish and other aquatic animals. *J. Exp. Zool. A Ecol. Integr. Physiol.* 333, 449–465. doi: 10.1002/jez.2367

Tseng, Y.-C., Hu, M. Y., Stumpp, M., Lin, L.-Y., Melzner, F., and Hwang, P.-P. (2013). CO_2-driven seawater acidification differentially affects development and molecular plasticity along life history of fish (*Oryzias latipes*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 165, 119–130. doi: 10.1016/j.cbpa.2013.02.005

Tseng, Y. C., and Hwang, P. P. (2008). Some insights into energy metabolism for osmoregulation in fish. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 148, 419–429. doi: 10.1016/j.cbpc.2008.04.009

Tseng, Y. C., Yan, J. J., Furukawa, F., and Hwang, P. P. (2020). Did acidic stress resistance in vertebrates evolve as Na^+/H^+ exchanger-mediated ammonia excretion in fish? *BioEssays* 42, e1900161. doi: 10.1002/bies.201900161

Villanueva, R., Perricone, V., and Fiorito, G. (2017). Cephalopods as predators: A short journey among behavioral flexibilities, adaptions, and feeding habits. *Front. Physiol.* 8, 598. doi: 10.3389/fphys.2017.00598

Voight, J. R., Pörtner, H. O., and O'dor, R. K. (1995). A review of ammonia-mediated buoyancy in squids (cephalopoda: Teuthoidea). *Mar. Freshw. Behav. Physiol.* 25, 193–203. doi: 10.1080/10236249409378917

Voss, C. M., Arildsen, L., Nissen, J. D., Waagepetersen, H. S., Schousboe, A., Maechler, P., et al. (2021). Glutamate dehydrogenase is important for ammonia fixation and amino acid homeostasis in brain during hyperammonemia. *Front. Neurosci.* 15, 646291. doi: 10.3389/fnins.2021.646291

Wang, M.-C., Hsu, M.-T., Lin, C.-C., Hsu, S.-C., Chen, R.-D., Lee, J.-R., et al. (2022). Adaptive metabolic responses in a thermostabilized environment: Transgenerational trade-off implications from tropical tilapia. *Sci. Total Environ.* 806, 150672. doi: 10.1016/j.scitotenv.2021.150672

Webber, D. M., Aitken, J. P., and O'dor, R. K. (2000). Costs of locomotion and vertic dynamics of cephalopods and fish. *Physiol. Biochem. Zool.* 73, 651–662. doi: 10.1086/318100

Weihrauch, D., and Allen, G. J. (2018). Ammonia excretion in aquatic invertebrates: New insights and questions. *J. Exp. Biol.* 221, jeb169219. doi: 10.1242/jeb.169219

Weihrauch, D., Ziegler, A., Siebers, D., and Towle, D. W. (2002). Active ammonia excretion across the gills of the green shore crab *Carcinus maenas*: Participation of Na^+/K^+-ATPase, V-type h^+-ATPase and functional microtubules. *J. Exp. Biol.* 205, 2765–2775. doi: 10.1242/jeb.205.18.2765

Weiner, I. D., and Verlander, J. W. (2016). Recent advances in renal ammonia metabolism and transport. *Curr. Opin. Nephrol. Hypertens.* 25, 436. doi: 10.1097/MNH.0000000000000255

Wells, M. (1990). Oxygen extraction and jet propulsion in cephalopods. *Can. J. Zool.* 68, 815–824. doi: 10.1139/z90-117

Wells, M. J., and Clarke, A. (1996). Energetics: The costs of living and reproducing for an individual cephalopod. *Philos. Trans. R Soc. Lond B Biol. Sci.* 351, 1083–1104. doi: 10.1098/rstb.1996.0095

Wells, M., and Wells, J. (1983). The circulatory response to acute hypoxia in octopus. *J. Exp. Biol.* 104, 59–71. doi: 10.1242/jeb.104.1.59

Wood, J. W., Day, C. L., Lee, P., and O'dor, R. K. (2000). CephBase: Testing ideas for cephalopod and other species-level databases. *Oceanography* 13, 14–20. doi: 10.5670/oceanog.2000.03

Wood, C. M., Pärt, P., and Wright, P. A. (1995). Ammonia and urea metabolism in relation to gill function and acid-base balance in a marine elasmobranch, the spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.* 198, 1545–1558. doi: 10.1242/jeb.198.7.1545

Wright, P. A. (1995). Nitrogen excretion: Three end products, many physiological roles. *J. Exp. Biol.* 198, 273–281. doi: 10.1242/jeb.198.2.273

Wright, P. A., and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: Role of rhesus (Rh) glycoproteins. *J. Exp. Biol.* 212, 2303–2312. doi: 10.1242/jeb.023085

Wright, P. A., Wood, C. M., and Wilson, J. M. (2014). Rh Versus pH: The role of rhesus glycoproteins in renal ammonia excretion during metabolic acidosis in a freshwater teleost fish. *J. Exp. Biol.* 217, 2855–2865. doi: 10.1242/jeb.098640

Xavier, J. C., Allcock, A. L., Cherel, Y., Lipinski, M. R., Pierce, G. J., Rodhouse, P. G., et al. (2015). Future challenges in cephalopod research. *J. Mar. Biol. Assoc. U. K.* 95, 999–1015. doi: 10.1017/S0025315414000782

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Vitamin D regulates transepithelial acid secretion in zebrafish (*Danio rerio*) larvae

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Maintenance of an acid-base balance is essential for normal physiological processes in vertebrates. Freshwater fishes live in an aquatic environment with variable pH, and their buffering capacity for acid-base balance in body fluids is weak. Thus, after acid exposure, fishes secrete excess acid to prevent internal acidosis. Acid-secreting ionocytes present in the adult gills and embryonic skin are primarily responsible for acid secretion, and H^+-ATPase and Na^+/H^+ exchanger 3 (NHE3) are the two main transporters responsible for apical acid secretion. Vitamin D is a well-known hormone involved in the maintenance of Ca^{2+} homeostasis and is suggested to be involved in acid-base regulation by modulating the activity and/or mRNA expression of NHE3 in mammalian models. It remains unclear whether vitamin D is involved in acid secretion in fishes. The aim of the present study was to use zebrafish as a model to determine whether vitamin D and its receptors influence acid secretion. Our results indicated that the levels of 1α, 25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), the bioactive vitamin D, were significantly increased in 3 days post-fertilization zebrafish larvae after exposure to acidic freshwater (AFW, pH 4.0). Exogenous 1α,25$(OH)_2D_3$ (20 μg/L) incubation substantially enhanced the mRNA expression of acid-secreting transporters and acid secretion at the skin of the entire body and each H^+-ATPase-rich cell (HRC), a type of acid-secreting ionocyte. Furthermore, the expression of vitamin D receptors (VDRs) was identified in HRCs of zebrafish. When both VDRa and VDRb were knocked down, acid secretion and the mRNA expression of acid-secreting transporters were significantly decreased. Moreover, double knockdown of VDRa/b prevented the increase in acid secretion induced by AFW and 1α,25$(OH)_2D_3$ treatment. This study is the first to indicate that vitamin D is involved in acid secretion in fish.

KEYWORDS

zebrafish, vitamin D, acid secretion, H⁺-ATPase, ionocyte

Introduction

Fish living in freshwater environments frequently experience aquatic acidification, which is caused by natural and anthropogenic ambient factors. Maintenance of an internal pH homeostasis is vital for normal physiological processes (Karet, 2002). The acid-base buffering capacity of fish body fluids is low; therefore, fish easily have internal acidosis under ambient acidity and need to excrete excess acid (Evans et al., 2005; Perry and Gilmour, 2006; Hwang et al., 2011). While the gills are responsible for approximately 90% of the acid-base movement in adult fish, this function is mainly performed by the yolk skin during early embryonic stages (Evans et al., 2005; Hwang and Perry, 2010). Acid secretion at the cellular level in fish gills/skin is primarily accomplished by the excretion of protons across the apical membrane of ionocytes (Evans et al., 2005; Hwang et al., 2011). A specific subtype of ionocytes for acid secretion has been identified in the gills and embryonic skin of teleosts (Hwang et al., 2011). In acid-secreting ionocytes, apical H^+-ATPase (HA) and Na^+/H^+ exchanger 3 (NHE3) are the two pathways for transepithelial proton secretion (Hwang et al., 2011; Guh et al., 2015). In addition, basolateral anion exchanger 1 (AE1) transports HCO_3^- from ionocytes to body fluid that is also involved in proton secretion in teleosts (Lee et al., 2011; Hsu et al., 2014).

Previous studies have combined electrophysiological, gene knockdown, and pharmacological experiments to determine HA and NHE3b mediated proton secretion at the apical membrane of H^+-ATPase-rich cells (HRCs), the acid-secreting ionocytes, in zebrafish (Shih et al., 2008; Shih et al., 2012). In zebrafish, HA and NHE3b in HRCs are responsible for approximately 70% and 30% of the apical proton secretion, respectively (Shih et al., 2008; Shih et al., 2012). However, NHE3 is the major route for apical proton secretion in acid-secreting ionocytes of medaka (Lin C. C. et al., 2012; Lin et al., 2021). The function of other acid secretion-related transporters in ionocytes is homologous in fishes (Hsu et al., 2014; Liu et al., 2016; Yan and Hwang, 2019). Hormonal control plays an essential role in the maintenance of the acid-base balance in fishes (Guh et al., 2015; Yan and Hwang, 2019). Some hormones such as cortisol and endothelin-1 are induced after acid exposure in fishes (Guh et al., 2014; Lin et al., 2015; Lin et al., 2021). Overexpression and pharmacological approaches suggest that increased hormone levels can regulate the capacity for acid secretion by stimulating the mRNA expression and activity of acid-secreting transporters and/or ionocyte differentiation (Cruz et al., 2013; Guh et al., 2014; Lin et al., 2015; Lin et al., 2021).

Acid exposure simultaneously causes a decrease in Ca^{2+} absorption and increase in Ca^{2+} loss rate in zebrafish (Kwong and Perry, 2014). Moreover, acid exposure increases the gene expression and secretion of cortisol, a hypercalcemic hormone, but decreases the gene expression of stanniocalcin-1, a hypocalcemic hormone, in zebrafish (Kumai et al., 2012; Lin et al., 2015; Chou et al., 2015). Decreased whole-body Ca^{2+} levels in zebrafish after acid exposure may be compensated by an increase in the mRNA expression level of epithelial Ca^{2+} channels and the density of *ecac*-expressing ionocytes (Kwong and Perry, 2014). Thus, the different changes in cortisol and stanniocalcin-1 levels after acid exposure may reflect a compensatory mechanism for Ca^{2+} homeostasis. However, incubation and overexpression experiments have indicated that cortisol and stanniocalcin-1 are not only involved in the regulation of *ecac* transcripts but also *ha* and *nhe3b* transcripts in zebrafish (Lin et al., 2015; Chou et al., 2015). Furthermore, cortisol can affect the acid secretion capacity at each HRC in zebrafish (Lin et al., 2015). In contrast, knockdown of hypercalcemic parathyroid hormone 1 (PTH1) decreases HRC differentiation in zebrafish (Kwong and Perry, 2015). Calciotropic hormones may have multiple effects on ion regulation in fishes.

Vitamin D is a well-known hormone for Ca^{2+} homeostasis in mammals and has also been reported to elevate the body-fluid Ca^{2+} levels in carp, cod, and zebrafish (Swarup et al., 1991; Sundell et al., 1993; Lin C. H. et al., 2012). The vitamin D precursor is initially synthesized in the skin. Through a series of reactions, it is converted to 1α, 25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), the active form of vitamin D, by renal 1α-OHase (*CYP27B1*) (Holick, 2007). Previous studies have indicated that renal tissues and the liver are vital for 1α,25$(OH)_2D_3$ production (Hayes et al., 1986; du Bois et al., 1988; Takeuchi et al., 1991), and *cyp27b1* has also been identified in teleosts (Cheng et al., 2006; Lin C. H. et al., 2012). Vitamin D regulates target gene expression by binding to vitamin D receptors (VDRs) (Holick, 2007). There are two forms of VDR in teleosts (Taylor et al., 2001; Howarth et al., 2008). VDRa and VDRb are two paralogous VDRs in zebrafish. For Ca^{2+} uptake, vitamin D bound to VDRa has been proposed to regulate the gene expression of epithelial Ca^{2+} channels in zebrafish (Lin C. H. et al., 2012). However, in mammalian cell lines, teleost VDRs have been found to induce transcription of the expression construct with 1α,25$(OH)_2D_3$ (Reschly et al., 2007; Howarth et al., 2008; Krasowski et al., 2011). Although the two isoforms of medaka VDRs show different responses to 1α,25$(OH)_2D_3$, they still induce the transcripts of VDR responsive element-containing expression constructs in cell lines (Howarth et al., 2008). In mammals, metabolic acids are mainly excreted by the proximal tubules of the kidneys. NHE3 plays an essential role in

Abbreviations: 1α,25$(OH)_2D_3$, 1α, 25-dihydroxyvitamin D_3; AE1b, anion exchanger 1b; AFW, acidic freshwater; ANOVA, analysis of variance; dpf, days post-fertilization; FW, freshwater; HA, H^+-ATPase; HRCs, H^+-ATPase-rich cells; NHE3, Na^+/H^+ exchanger 3; PBS, phosphate-buffered saline; PTH1, parathyroid hormone 1; SEM, standard error of the mean; SIET, Scanning ion-selective electrode technique; VDR, vitamin D receptor.

apical proton secretion in the proximal tubules, and its activity provides the necessary driving force for bicarbonate reabsorption (Wagner et al., 2004; Bobulescu and Moe, 2006). Mice with targeted NHE3 disruption exhibit metabolic acidosis and decreased renal absorption of Na^+ and Ca^{2+} (Bobulescu and Moe, 2006; Pan et al., 2012). In addition, increased serum 1α,25 $(OH)_2D_3$ levels have been found in NHE3 knockout mice (Pan et al., 2012). Treatment with 1α,25 $(OH)_2D_3$ stimulates apically located sodium-hydrogen exchange (NHE) activity in renal epithelial cell lines of mammals (Binswanger et al., 1993). Vitamin D has been suggested to be involved in the regulation of the acid-base homeostasis by enhancing urinary acid excretion in mammals (Stim et al., 1994). In contrast, 1α,25 $(OH)_2D_3$ treatment inhibits the NHE activity in a Caco-2 colonic carcinoma cell line (Wali et al., 1992) and the rat ileum by downregulating the mRNA levels of NHE3 (Gill et al., 2002). The divergent responses of NHE to 1α,25$(OH)_2D_3$ in tissues and cell lines suggest the involvement of different cell-specific mechanism(s) in the regulation of NHE (Gill et al., 2002). To date, the major understanding of vitamin D in the ion regulation of fishes has focused on Ca^{2+} absorption (Lin and Hwang, 2016). Our understanding of whether acid exposure affects vitamin D synthesis in fishes is limited. Moreover, the effects of vitamin D on acid-secreting transporters and acid secretion in fishes remain unknown.

The zebrafish is an emerging model frequently used to explore the endocrine control on ion regulation in fishes because of its advantages in molecular, cellular, bioinformatic, and *in vivo* electrophysiological approaches (Guh et al., 2015; Yan and Hwang, 2019). In addition, acid-secreting transporters have been well identified in HRCs, and zebrafish can survive in acidic freshwater (AFW) with a pH as low as 4 (Guh et al., 2015). Thus, the present study used zebrafish as a model to investigate the following questions: (1) Does AFW (pH 4.0) change the mRNA expression of *cyp27b1* that encodes an enzyme for 1α,25 $(OH)_2D_3$ synthesis and 1α,25$(OH)_2D_3$ levels in zebrafish larvae? (2) Is vitamin D involved in regulating the mRNA expression of HA, NHE3b, and AE1b, a set of acid secretion-related transporters are expressed in HRC, and acid secretion in zebrafish larvae? (3) Does VDRa and/or VDRb mediate the effects of vitamin D on the regulation of acid secretion? Elucidating these questions would enhance our knowledge regarding the role of vitamin D in the acid-base regulation in fishes.

Materials and methods

Experimental animals

Zebrafish (*Danio rerio*) were kept in local tap water at 28.5°C under a 14:10 h light:dark photoperiod at the Department of Marine Biotechnology, National Kaohsiung University of Science and Technology, Kaohsiung, Taiwan. The experimental protocols were approved by the Institutional Animal Care and Utilization Committee of the university (approval no. 0109-AAAP-006).

Acclimation experiments

Freshwater (FW, pH 7.0) and acidic freshwater (AFW, pH 4.0) prepared from local tap water were used to determine the effects of an acidic medium. In accordance with previous studies (Lin et al., 2015; Lin et al., 2021), the acidic medium was prepared by adding H_2SO_4 to the FW; the concentrations of other ions in the AFW were same as those in the FW (see Supplemental Table S1). Fertilized zebrafish eggs were transferred to either FW or AFW and then incubated until sampling at 3 days post-fertilization (dpf). The media were changed twice daily. The pH values of all experimental media were verified using a pH meter (FP20; OHAUS, Parsippany, NJ, USA), and the ion concentrations were determined using an atomic absorption spectrometer (U-2000; Hitachi, Tokyo, Japan).

Vitamin D measurement

Following the method previously described by Lin C. H. et al., 2012, the amount of whole-body 1α,25$(OH)_2D_3$, the bioactive form of vitamin D, in zebrafish larvae was measured using an ELISA kit (E0467GE; EIAlab, Wuhan, China). Three dpf zebrafish larvae with FW and AFW treatments were anesthetized with 0.03% MS-222 and then washed several times with 1X phosphate-buffered saline (PBS). Twenty larvae were pooled in one vial as a single sample. The zebrafish larvae in vials were homogenized in 500 μL of 1X PBS. Next, diethyl ether (Merck, Darmstadt, Germany) was added to the homogenates and the mixtures were vortexed for 1 min. Then, the mixtures were centrifuged at 2000 × *g* at 4°C for 10 min. The diethyl ether phase was collected and dried under a nitrogen flux at room temperature. Finally, the dried samples were resuspended in the standard/sample solution of the ELISA kit, and 1α,25$(OH)_2D_3$ was quantified according to the manufacturer's instructions.

Vitamin D incubation experiment

Following the method previously described by Lin et al., 2012, 0 (control) and 20 μg/L of 1α,25$(OH)_2D_3$ (CAS No. 32222-06-3, Sigma, USA) were used for an incubation experiment. Zebrafish eggs were incubated in 1α,25$(OH)_2D_3$ media immediately after fertilization and were sampled at 3 dpf for subsequent analysis. The incubation media were changed with new 1α,25$(OH)_2D_3$ solutions daily to maintain constant

levels of $1\alpha,25(OH)_2D_3$. During incubation, no significant mortality or abnormal behavior was observed.

RNA extraction

After anesthetization with 0.03% MS-222, 20 zebrafish larvae were collected and homogenized in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then mixed with 0.2 mL chloroform and thoroughly shaken. After centrifugation at 4°C and 12,000 × *g* for 30 min, the supernatants were collected. The samples were then mixed with an equal volume of isopropanol. Pellets were precipitated by centrifugation at 4°C and 12,000 × *g* for 30 min, washed with 70% alcohol, and stored at -20°C until use.

Reverse-transcription polymerase chain reaction

Complementary DNA (cDNA) was prepared according to the manufacturer's protocol of SuperScript Reverse Transcriptase (Invitrogen). Briefly, 5 μg of total RNA was reverse-transcribed in a final volume of 20 μL containing 0.5 mM dNTPs, 2.5 μM oligo$(dT)_{20}$, 250 ng random primers, 5 mM dithiothreitol, 40 units RNase inhibitor, and 200 units reverse transcriptase (SuperScript Reverse Transcriptase, Invitrogen) for 1 h at 50°C, followed by a 70°C incubation for 15 min.

Quantitative real-time PCR

qPCR was performed using a LightCycler real-time PCR system (Roche, Penzberg, Germany) in a 10 μL mixture containing 5 μL 2X SYBR Green I Master (Roche), 300 nM of the primer pairs, and 20–30 ng cDNA. The standard curve for each gene was checked in a linear range with β-actin as an internal control. The present study followed previous studies (Chang et al., 2009; Lin C. H. et al., 2012; Lin et al., 2015) for the qPCR primer design (Supplemental Table S2). To show each gene expression at the same scale, the expression value was multiplied by different fold, 1000x for *cyp27b1*, 100x for *ha*, 5000x for *nhe3b*, 2000x for *ae1b* and 1000x for *gcm2*, finally.

In situ hybridization

Zebrafish *vdra* or *vdrb* fragments were obtained by PCR using a PCR primer design. The PCR fragment was inserted into pGEM-T Easy Vector (Promega, Madison, WI, USA). The inserted fragments were amplified with T7 and SP6 primers by PCR, and the products were used as templates for *in vitro* transcription with T7 and SP6 RNA polymerase (Roche) in the presence of digoxigenin-11-uridine triphosphate (Roche) to synthesize the probes. Three dpf zebrafish larvae were anesthetized on ice and fixed with 4% paraformaldehyde in a PBS (1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na_2HPO_4, and 0.002 mM KH_2PO_4; pH 7.4) solution at 4 °C overnight. Subsequently, we performed *in situ* hybridization as previously described (Lin C. H. et al., 2012).

Immunocytochemistry

After *in situ* hybridization, the samples were immediately subjected to immunocytochemistry. The zebrafish larvae were washed with PBS and then incubated with 3% bovine serum albumin for 2 h to block non-specific binding. The samples were then incubated overnight at 4°C with a polyclonal antibody against the α subunit of zebrafish H^+-ATPase (dilution at 1:100; synthetic peptide: AEMPADSGYPAYLGARLA). After washing with PBS for 30 min, the samples were further incubated with Alexa Fluor 488 goat anti-rabbit IgG antibodies (Molecular Probes; diluted at 1:200 with PBS) for 2 h at room temperature. Images were acquired using an Axioplan 2 microscope (Zeiss, Oberkochen, Germany).

Morpholino oligonucleotide knockdown

As previously described (Lin C. H. et al., 2012), zebrafish *vdra* morpholino oligonucleotide (MO) (5'- AACGGCACTAT TTTCCGTAAGCATC-3'), *vdrb* MO (5'- AACGTTCCGGT CGAACTCATCTGGC-3'), and a standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') were used for the MO knockdown experiment. A 4 ng/embryo (MO) dosage was used for the injection. The MOs (4 ng/embryo) were injected into the embryos at the 1–2 cell stage using an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). At this dose, the MO knockdown did not cause embryos either significant abnormal development or mortality. The MO-injected larvae at 3 dpf were sampled for subsequent analyses.

Scanning ion-selective electrode technique, skin surface H^+ gradients, and H^+ flux in ionocytes of zebrafish larvae

Scanning ion-selective electrode technique (SIET), recording of the H^+ gradients at the skin surface, and H^+ flux at the surface

of ionocytes in zebrafish larvae were performed as previously described (Lin et al., 2006). Briefly, SIET was performed at room temperature (26–28°C) in a small plastic recording chamber filled with 1 mL of a "recording medium" that contained 300 μM 3-(N-morpholino)propanesulfonic acid buffer (Sigma) and 0.1 mg/L ethyl 3-aminobenzoate (Tricaine, pH 7.0) (Sigma), as described previously (Lin et al., 2006). To record the H^+ gradients at the skin surface of a larva, a microelectrode was moved to a target position 10–20 μm from the skin. After recording the target point, the microelectrode was moved to record the background. In the present study, $\Delta[H^+]$ represents the difference between the measured H^+ gradients of the skin surface and background.

To record the H^+ flux at the surface of the ionocytes, the microelectrode was moved to a position 2 μm above the cell surface. At every position, the voltage difference in microvolts was measured by orthogonally probing the surface at 10-μm intervals. The recording was performed in 10 replicates, and the median of the repeats was used to calculate the ion flux of the cell. To calculate the ionic flux, the voltage differences were first converted into a concentration gradient ΔC ($\mu mol \cdot L^{-1} \cdot cm^{-3}$). ΔC was subsequently converted into ionic flux using Fick's law of diffusion in the following equation: $J = D(\Delta C)/\Delta X$, where J ($pmol \cdot cm^{-2} \cdot s^{-1}$) is the net flux of the ion, D is the diffusion coefficient of the ion (2.09×10^{-5} cm^2/s for H^+), and ΔX (cm) is the distance between the two points.

Statistical analysis

The values were expressed as mean ± standard error of the mean (SEM). The results were compared using one-way analysis of variance (ANOVA) with Tukey's pairwise test and Student's *t*-test. The significance level was set at $p < 0.05$.

Results

Effects of AFW on the *cyp27b1* transcripts and whole-body 1α,25(OH)$_2$D$_3$ levels in 3 dpf zebrafish larvae

To determine the effects of AFW (pH 4.0) treatment on the mRNA expression of *cyp27b1*, the key enzyme in the synthesis of 1α,25(OH)$_2$D$_3$, and whole-body 1α,25(OH)$_2$D$_3$ levels in zebrafish larvae, the zebrafish fertilized eggs were exposed to FW (pH 7.0) or AFW for 3 days. Zebrafish larvae exposed to AFW exhibited significantly elevated expression of *cyp27b1* and 1α,25(OH)$_2$D$_3$ level compared with zebrafish larvae exposed to FW (Figures 1A, B).

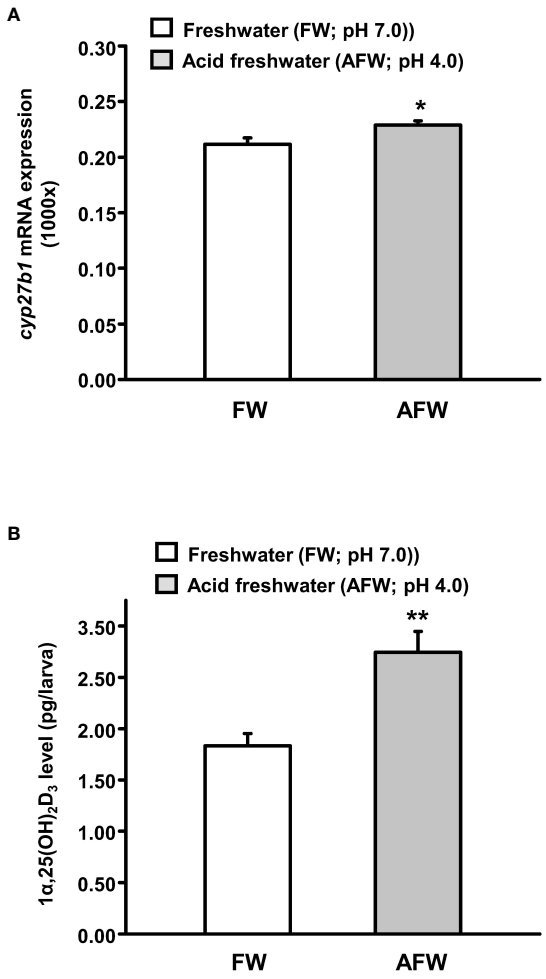

FIGURE 1
Effects of freshwater (FW, pH 7.0) and acidic freshwater (AFW, pH 4.0) on **(A)** the mRNA expression of *cyp27b1* and **(B)** whole body 1α, 25-dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$) level in 3 days post-fertilization (dpf) zebrafish larvae. The mRNA expression was analyzed by qPCR, and the values were normalized to β-actin. Values were expressed as the mean ± SEM (n = 6). Student's t-test, * $p < 0.05$; ** $p < 0.01$.

Effects of exogenous 1α,25(OH)$_2$D$_3$ treatment on the mRNA expression of genes related to acid secretion and proton secretion in 3 dpf zebrafish larvae

The mRNA expression of *cyp27b1* and whole-body 1α,25(OH)$_2$D$_3$ levels were significantly upregulated in zebrafish larvae in AFW (Figure 1). Therefore, we further investigated whether exogenous 1α,25(OH)$_2$D$_3$ (20 μg/L) treatment causes changes in the mRNA expression of transporters related to acid secretion

and the proton secretion. Compared with the control, exogenous 1α,25(OH)$_2$D$_3$ treatment predominantly increased the mRNA expression of the acid-secreting transporters, HA, NHE3b, and anion exchanger 1b (AE1b), and proton secretion on the skin surface of 3 dpf zebrafish larvae (Figures 2A, B).

Colocalization of VDR mRNA with acid-secreting ionocyte in zebrafish larvae

To further confirm the role of vitamin D in acid secretion, we examined whether the mRNA expression of *vdra* and/or *vdrb* is expressed in HRCs, the acid-secreting ionocytes, by double *in situ* hybridization and immunocytochemistry against VDR mRNA and H$^+$-ATPase, respectively, in 3 dpf zebrafish larvae. As shown in Figures 3A, B, partial *vdra* signals were localized in the HRCs but all *vdrb* signals were identified in the HRCs (Figures 3C, D).

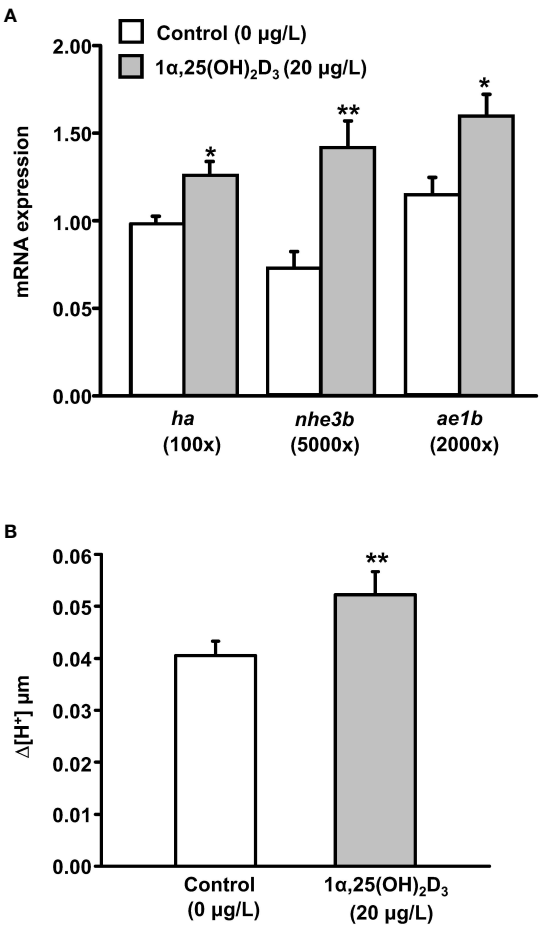

FIGURE 2
Effects of exogenous 1α,25(OH)$_2$D$_3$ treatment on **(A)** the mRNA expression of transporter genes and **(B)** proton secretion in the skin surface of 3 dpf zebrafish larvae. The mRNA expressions were analyzed by qPCR, and the values were normalized to β-actin. Scanning ion-selective electrode technique (SIET) was used to measure the H$^+$ activity on the skin. Values were expressed as the mean ± SEM (n = 6–12). Student's t-test, * p < 0.05; ** p < 0.01.

Effects of *vdra* and/or *vdrb* knockdown on the mRNA expression of genes related to acid secretion and proton secretion in 3 dpf zebrafish larvae

Exogenous 1α,25(OH)$_2$D$_3$ treatment significantly increased the mRNA expression of transporters related to acid secretion and the proton secretion on the skin surface of zebrafish larvae (Figure 2). Moreover, *vdra* and *vdrb* expressions were identified in HRCs (Figure 3). Thus, we further explored the effects of VDRa and/or VDRb on acid secretion in zebrafish larvae. Fertilized zebrafish eggs were injected with *vdra* or/and *vdrb* MO, and transporter expression and proton secretion were analyzed in 3 dpf larvae. The mRNA expression of *ha*, *nhe3b*, and *ae1b* and proton secretion from the skin surface did not change in *vdra* or *vdrb* morphants (Figure 4A). In contrast, double *vdra* and *vdrb* (*vdra*/*b*) knockdown caused a significant decrease in mRNA expression of acid-secreting transporters as well as the proton secretion from the skin surface (Figures 4A, B).

Effect of exogenous 1α,25(OH)$_2$D$_3$ treatment on *gcm2* expression in 3 dpf zebrafish larvae with *vdra*/*b* knockdown

To determine whether vitamin D can potentially promote the differentiation of acid-secreting ionocytes, we examined the effect of exogenous 1α,25(OH)$_2$D$_3$ (20 μg/L) treatment on the expression of *gcm2*, a key transcription factor controlling the differentiation of HRCs (Chang et al., 2009). Exogenous 1α,25(OH)$_2$D$_3$ treatment significantly stimulated *gcm2* transcripts, while the stimulation was prevented in 3 dpf zebrafish larvae with *vdra*/*b* knockdown (Figure 5).

Effects of exogenous 1α,25(OH)$_2$D$_3$ or AFW treatment on proton secretion of acid-secreting ionocytes in 3 dpf zebrafish larvae with *vdra*/*b* knockdown

Double *vdra*/*b* knockdown impaired proton secretion from the skin surface in zebrafish larvae (Figure 4B). To establish whether *vdra*/*b* knockdown mediated the effect of vitamin D and AFW on proton secretion of HRCs, *vdra*/*b* MO were co-injected into fertilized zebrafish eggs. Knockdown of *vdra*/*b* prevented the increase in proton secretion in HRCs induced by exogenous

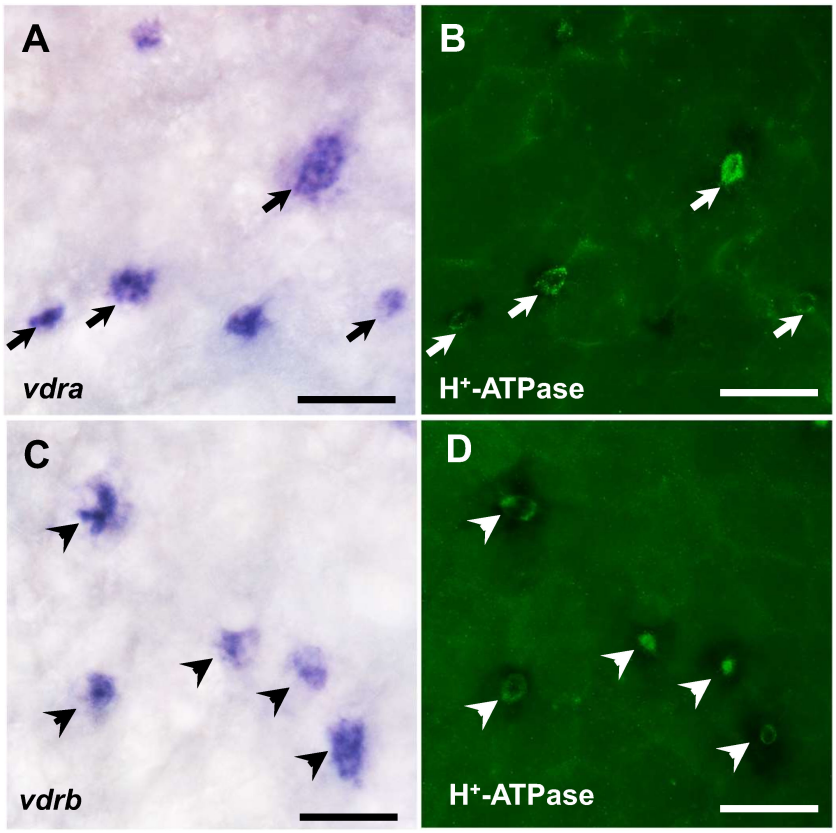

FIGURE 3
In situ hybridization staining of *vdra* and *vdrb* **(A, C)** and immunocytochemical staining of H^+-ATPase **(B, D)** in 3 dpf zebrafish larvae. Co-localization of *vdra* mRNA and H^+-ATPase protein signals in the same cell is indicated by arrows. Scale bar: 20 μm.

$1\alpha,25(OH)_2D_3$ (20 μg/L) treatment in 3 dpf zebrafish larvae (Figure 6A). In addition, the increased proton secretion in HRCs induced by AFW treatment was also blocked in *vdra*/*b* zebrafish morphants (Figure 6B).

Discussion

In the present study, the level of $1\alpha,25(OH)_2D_3$, the bioactive vitamin D, was increased in 3 dpf zebrafish larvae after exposure to AFW (pH 4.0). AFW exposure also elevated the mRNA expression of *cyp27b1*, which encodes the enzyme for $1\alpha,25(OH)_2D_3$ synthesis. These findings suggest that AFW treatment stimulates *cyp27b1* transcription, thereby enhancing the synthesis of $1\alpha,25(OH)_2D_3$ in zebrafish. In zebrafish larvae, acid secretion is stimulated after 3 and 4 days of AFW treatment (Horng et al., 2009; Lin et al., 2015). In addition, AFW treatment significantly stimulates HRC differentiation and the mRNA expression of acid-secreting transporters in HRCs of zebrafish larvae (Horng et al., 2009; Chang et al., 2009; Lin et al., 2015). The increased HRC differentiation and mRNA expression of acid-secreting transporters have been suggested to contribute to increased acid secretion in zebrafish (Lin et al., 2015). In the present study, $1\alpha,25(OH)_2D_3$ treatment substantially stimulated the gene expression of HA, NHE3b, and AE1b, the transporters related to acid secretion in HRCs. Furthermore, it significantly enhanced the capacity of acid secretion at the skin surface and each HRC. Therefore, we conclude that increased $1\alpha,25(OH)_2D_3$ level contributes to the enhanced acid secretion ability of zebrafish after exposure to AFW. This is the first study to demonstrate that $1\alpha,25(OH)_2D_3$ treatment increases acid secretion and the mRNA expression of acid-secreting transporters in fish.

$1\alpha,25(OH)_2D_3$ treatment elevated the acid secretion capacity at each HRC. HA and NHE3b are the major routes for apical acid secretion and responsible for approximately 70% and 30% apical acid secretion in HRCs of zebrafish, respectively (Shih et al., 2008; Shih et al., 2012). Previous results have revealed that $1\alpha,25(OH)_2D_3$ treatment significantly increased the mRNA expression of both *ha* and *nhe3b*, which was approximately 0.25-fold for *ha* expression and 1-fold for *nhe3b* expression. $1\alpha,25(OH)_2D_3$ treatment had a greater effect on *nhe3b*

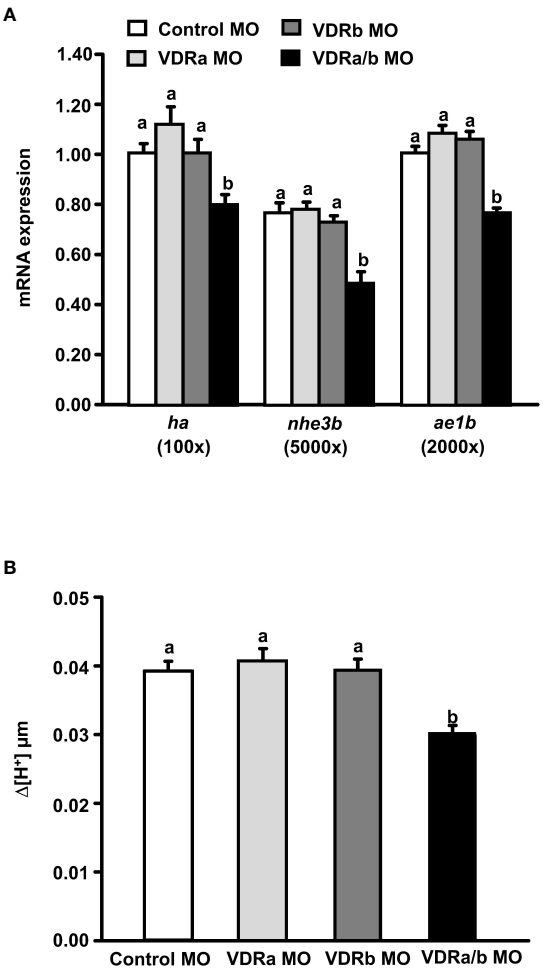

FIGURE 4
Effects of vitamin D receptor (VDR) morpholino oligonucleotides (MOs) on **(A)** the mRNA expression of transporters and **(B)** proton secretion in the skin surface of 3 dpf zebrafish larvae. The mRNA expressions were analyzed by qPCR, and the values were normalized to β-actin. SIET was used to measure the H^+ activity on the skin. Different letters indicate a significant difference ($p < 0.05$), as determined using one-way ANOVA followed by Tukey's multiple-comparison test. Values were expressed as the mean ± SEM (n = 6–12).

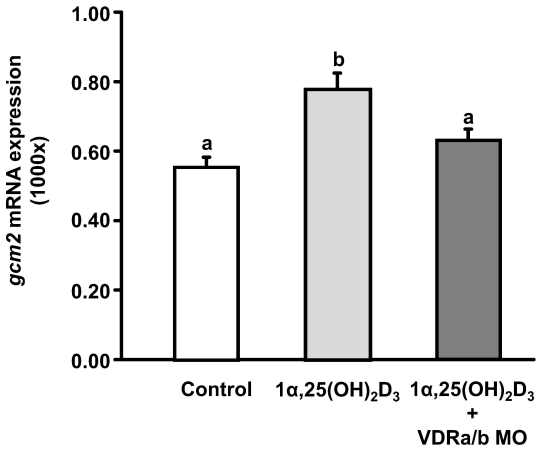

FIGURE 5
The effect of VDR MOs on *gcm2* expression in 3 dpf zebrafish larvae treated with $1\alpha,25(OH)_2D_3$. The mRNA expression was analyzed by qPCR, and the values were normalized to β-actin. Different letters indicate a significant difference ($p < 0.05$), as determined using one-way ANOVA followed by Tukey's multiple-comparison test. Values were expressed as the mean ± SEM (n = 6).

expression. However, Lin et al. (2015) reported that the mRNA expression level of *ha* was far greater than *nhe3b*—approximately 50-fold—in 3 dpf zebrafish larvae, and the present study also found a similar result. Thus, the increased *ha* expression by $1\alpha,25(OH)_2D_3$ treatment predominantly contributed to the increased acid secretion in zebrafish larvae. The mRNA expression of *ae1b* also increased in zebrafish larvae treated with $1\alpha,25(OH)_2D_3$. *ae1b* is expressed in the basolateral membrane of acid-secreting ionocytes in zebrafish and medaka (Lee et al., 2011; Hsu et al., 2014). Knockdown of *ae1b* decrease acid secretion in zebrafish, and *ae1b* is suggested to extrude cytosolic HCO_3^- out of cells to fulfill the function of epithelial acid secretion (Lee et al., 2011). Therefore, $1\alpha,25(OH)_2D_3$ treatment may increase the capacity for acid secretion at each HRC by enhancing the expression of apical and basolateral acid-secreting transporters in zebrafish larvae.

Acid secretion at skin surface of the entire body and at each HRC of zebrafish was elevated by $1\alpha,25(OH)_2D_3$ treatment. Moreover, $1\alpha,25(OH)_2D_3$ treatment induced the mRNA expression of acid-secreting transporters. Therefore, vitamin D may exert a genomic effect on mRNA expression and subsequently increase the capacity for acid secretion at each HRC. This is done *via* VDR, a ligand-activated transcription factor (Omdahl et al., 2002). Unlike mammals, there are two paralogous VDR forms in fishes because of genome duplication (Taylor et al., 2001; Howarth et al., 2008). By immunohistochemistry with a mammalian VDR antibody, VDR signals were detected in most tissues including the gills in zebrafish (Craig et al., 2008). However, this approach could not identify the respective expressions of paralogous VDRs. To reinforce our hypothesis, we performed double *in situ* hybridization and immunocytochemistry to determine the mRNA expression of VDRa and/or VDRb in HRCs. The mRNA expression signals of both VDRa and VDRb were identified in HRCs. However, when we knocked down either VDRa or VDRb, the mRNA expression of acid-secreting transporters and acid secretion were not affected. In contrast, double knockdown of VDRa and VDRb significantly decreased the mRNA expression and acid secretion. Knockdown of VDRa induced VDRb expression and vice versa (Supplemental Figure S1). The single knockdown of a VDR may be compensated by the expression of the other VDR form and the actual influence of a

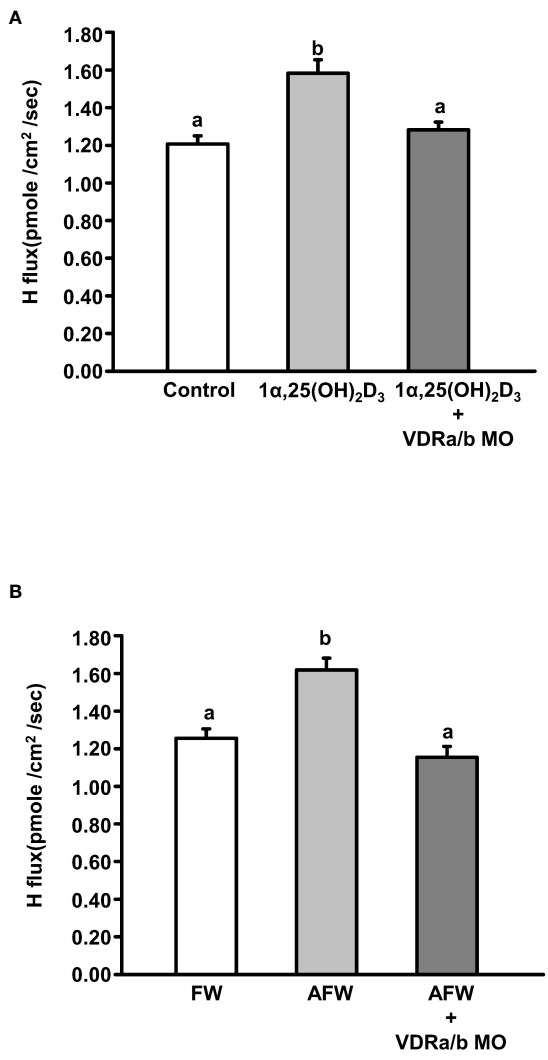

FIGURE 6
The effect of VDR MOs on proton secretion of acid-secreting ionocytes in 3 dpf zebrafish larvae treated with **(A)** 1α,25(OH)$_2$D$_3$ and **(B)** AFW. SIET was used to measure H^+ flux on the skin. Different letters indicate a significant difference ($p < 0.05$), as determined using one-way ANOVA followed by Tukey's multiple-comparison test. Values were expressed as the mean ± SEM (n = 12).

knocked-down VDR is neutralized. The transcription activity of VDR responsive element-containing expression constructs with medaka VDRα or VDRβ and steroid receptor coactivator co-expression is significantly induced by 1α,25(OH)$_2$D$_3$ treatment (Howarth et al., 2008). The present study demonstrated that the mRNA expression signals of both VDRa and VDRb were localized in HRCs. However, the knockdown of both VDRa and VDRb prevented 1α,25(OH)$_2$D$_3$- and AFW-induced acid secretion at each HRC. Under *in vivo* conditions, vitamin D may affect the mRNA expression of acid-secreting transporters and acid secretion *via* both VDRa and VDRb in zebrafish. However, a previous study has indicated that medaka VDRα and VDRβ respond differently to 1α,25(OH)$_2$D$_3$ treatment (Howarth et al., 2008). Lin C. H. et al., 2012 reported that zebrafish VDRa and VDRb show an amino acid sequence similarity of ~97% in the DNA binding domain and ~92% in the ligand binding domain. A slight change in the amino acid composition may result in a difference in transactivation (Lin C. H. et al., 2012). Thus, VDRa and VDRb may contribute differently to the regulation of acid secretion.

The present study demonstrated that VDRb knockdown increased VDRa mRNA expression. Lin C. H. et al., 2012 reported that VDRa but not VDRb knockdown is involved in the regulation of Ca^{2+} uptake and mRNA expression of Ca^{2+} transporters in zebrafish larvae. *vdra* and *vdrb* are universally expressed in tissues of zebrafish and the *vdra* expression is more dominant than *vdrb* expression in most tissues (Lin C. H. et al., 2012). The present data revealed that *vdrb* expression signals were present in HRCs. *vdrb* may not be expressed in Na^+/K^+-ATPase-rich cells, the subtype of ionocytes for Ca^{2+} absorption in zebrafish. Knockdown of *vdrb* caused increased *vdra* expression in most tissues but may not influence the *vdra* expression in Ca^{2+} absorption-related cells in zebrafish. Therefore, increased *vdra* mRNA expression following *vdrb* knockdown did not affect Ca^{2+} uptake. However, further evidence is required to support this hypothesis. Our results revealed that VDRa and VDRb are involved in acid regulation. Lin C. H. et al., 2012 reported that only VDRa is involved in Ca^{2+} absorption in zebrafish. VDRa and VDRb may have divergent roles in ion regulation in fishes. This is consistent with a previous study that reported that approximately 20%–50% of paralogous genes from gene duplication are conserved as one of the duplicates acquires a new function or subfunction (Lynch and Force, 2000).

Hormones can modulate the capacity for acid secretion through genomic and/or cytogenic pathways in fish (Guh et al., 2015; Yan and Hwang, 2019). For example, cortisol has been suggested to increase acid secretion at HRCs by stimulating the mRNA expression of acid-secreting transporters (Lin et al., 2015). Additionally, cortisol enhances ionocyte differentiation and elevates the systemic capacity for acid secretion (Cruz et al., 2013). Vitamin D and VDR have been shown to regulate the gene expression related to cellular differentiation (Gil et al., 2018). In the present study, treatment with 1α,25(OH)$_2$D$_3$ considerably stimulated the *gcm2* expression, but knockdown of VDRs prevented this increase. *gcm2* is a transcription factor specifically expressed in HRCs but not in Na^+/K^+-ATPase-rich cells or other ionocytes of zebrafish and is essential for the differentiation and functional regulation of HRCs (Chang et al., 2009). In addition, our preliminary result showed exogenous 1α,25(OH)$_2$D$_3$ treatment significantly increased HRCs density in 3 dpf zebrafish larvae (Supplemental Figure S2). Thus, vitamin D may increase the *gcm2* expression and promote HRC differentiation in zebrafish. A previous study indicated that PTH1 knockdown reduces the *gcm2* expression and the number of HRCs; however, when *gcm2* is overexpressed in zebrafish after

PTH1 knockdown, the decreased number of HRCs is recovered (Kwong and Perry, 2015). In mammals, parathyroid hormone is a major stimulator of vitamin D synthesis (Khundmiri et al., 2016). Therefore, PTH1 may increase vitamin D synthesis in zebrafish, and then, the increased vitamin D levels may enhance ionocyte differentiation. However, further studies are required to determine the role of vitamin D in ionocyte differentiation.

In summary, the present study reported that the synthesis and secretion levels of $1\alpha,25(OH)_2D_3$ were induced in zebrafish after AFW exposure, and the increased $1\alpha,25(OH)_2D_3$ levels may increase the expression of acid-secreting transporters in HRCs *via* VDRa and VDRb. This results in an increase in the acid secretion capacity of zebrafish larvae to prevent acidosis of body fluids. The present study is the first attempt to demonstrate a relationship between vitamin D and the acid-base balance in fish. This study extends our understanding of the role of vitamin D in ion regulation of fishes.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by NKUST Institutional Animal Care and Utilization Committee.

Author contributions

C-HL provided the conception and study design; C-HL, S-TL, Y-CW, Y-LT, and H-JH carried out the experiments and data collection; C-HL and S-TL performed the data analysis and interpretation; C-HL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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References

Binswanger, U., Helmle-Kolb, C., Forgo, J., Mrkic, B., and Murer, H. (1993). Rapid stimulation of Na^+/H^+ exchange by 1,25-dihydroxyvitamin D_3; interaction with parathyroid-hormone-dependent inhibition. *Pflügers Arch.* 424, 391–397. doi: 10.1007/BF00374899

Bobulescu, I. A., and Moe, O. W. (2006). Na^+/H^+ exchangers in renal regulation of acid-base balance. *Semin. Nephrol.* 26, 334–344. doi: 10.1016/j.semnephrol.2006.07.001

Chang, W. J., Horng, J. L., Yan, J. J., Hsiao, C. D., and Hwang, P. P. (2009). The transcription factor, glial cell missing 2, is involved in differentiation and functional regulation of h^+-ATPase-rich cells in zebrafish (Danio rerio). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 296, R1192–R1201. doi: 10.1152/ajpregu.90973.2008

Cheng, W., Guo, L., Zhang, Z., Soo, H. M., Wen, C., Wu, W., et al. (2006). HNF factors form a network to regulate liver-enriched genes in zebrafish. *Dev. Biol.* 294 (2), 482–496. doi: 10.1016/j.ydbio.2006.03.018

Chou, M. Y., Lin, C. H., Chao, P. L., Hung, J. C., Cruz, S. A., and Hwang, P. P. (2015). Stanniocalcin-1 controls ion regulation functions of ion-transporting epithelium other than calcium balance. *Int. J. Biol. Sci.* 11 (2), 122–132. doi: 10.7150/ijbs.10773

Craig, T. A., Sommer, S., Sussman, C. R., Grande, J. P., and Kumar, R. (2008). Expression and regulation of the vitamin d receptor in the zebrafish, *Danio rerio*. *J. Bone Miner. Res.* 23 (9), 1486–1496. doi: 10.1359/jbmr.080403

Cruz, S. A., Lin, C. H., Chao, P. L., and Hwang, P. P. (2013). Glucocorticoid receptor, but not mineralocorticoid receptor, mediates cortisol regulation of epidermal ionocyte development and ion transport in zebrafish (Danio rerio). *PloS One* 8, e77997. doi: 10.1371/journal.pone.0077997

du Bois, M. B., Milet, C., Garabedian, M., Guillozo, H., Martelly, E., Lopez, E., et al. (1988). Calcium-dependent metabolism of 25-hydroxycholecalciferol in silver eel tissues. *Gen. Comp. Endocrinol.* 71 (1) 1–9. doi: 10.1016/0016-6480(88)90288-2

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, andexcretion of nitrogenous waste. *Physiol. Rev.* 85, 97–177. doi: 10.1152/physrev.00050.2003

Gill, R., Nazir, T. M., Wali, R., Sitrin, M., Brasitus, T. A., Ramaswamy, K., et al. (2002). Regulation of rat ileal NHE3 by 1,25$(OH)_2$-vitamin D_3. *Dig. Dis. Sci.* 47 (5), 1169–1174. doi: 10.1023/a:1015071014584

Gil, Á., Plaza-Diaz, J., and Mesa, M. D. (2018). Vitamin d: Classic and novel actions. *Ann. Nutr. Metab.* 72 (2), 87–95. doi: 10.1159/000486536

Guh, Y. J., Lin, C. H., and Hwang, P. P. (2015). Osmoregulation in zebrafish: Ion transport mechanisms and functional regulation. *EXCLI J.* 14, 627–659. doi: 10.17179/excli2015-246

Guh, Y. J., Tseng, Y. C., Yang, C. Y., and Hwang, P. P. (2014). Endothelin-1 regulates H^+-ATPase-dependent transepithelial H^+ secretion in zebrafish. *Endocrinology* 155 (5), 1728–1737. doi: 10.1210/en.2013-1775

Hayes, M. E., Guilland-Cumming, D. F., Russell, R. G., and Henderson, I. W. (1986). Metabolism of 25-hydroxycholecalciferol in a teleost fish, the rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.* 64, 143–150. doi: 10.1016/0016-6480(86)90040-7

Holick, M. F. (2007). Vitamin d deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Horng, J. L., Lin, L. Y., and Hwang, P. P. (2009). Functional regulation of h^+-ATPase-rich cells in zebrafish embryos acclimated to an acidic environment. *Am. J. Physiol. Cell Physiol.* 296, C682–C692. doi: 10.1152/ajpcell.00576.2008

Howarth, D. L., Law, S. H., Barnes, B., Hall, J. M., Hinton, D. E., Moore, L., et al. (2008). Paralogous vitamin d receptors in teleosts: Transition of nuclear receptor function. *Endocrinology* 149 (5), 2411–2422. doi: 10.1210/en.2007-1256

Hsu, H. H., Lin, L. Y., Tseng, Y. C., Horng, J. L., and Hwang, P. P. (2014). A new model for fish ion regulation: Identification of ionocytes in freshwater- and seawater-acclimated medaka (Oryzias latipes). *Cell. Tissue. Res.* 357, 225–243. doi: 10.1007/s00441-014-1883-z

Hwang, P. P., Lee, T. H., and Lin, L. Y. (2011). Ion regulation in fish gills: Recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R28–R47. doi: 10.1152/ajpregu.00047.2011

Hwang, P. P., and Perry, S. F. (2010). "Ionic and acid-base regulation," in *Fish physiology vol. 29. zebrafish*. Eds. S. F. Perry, M. Ekker, A. P. Farrell and C. J. Brauner (San Diego, CA: Academic Press), p.311–p.343.

Karet, F. E. (2002). Inherited distal renal tubular acidosis. *J. Am. Soc Nephrol.* 13, 2178–2184. doi: 10.1097/01.ASN.0000023433.08833.88

Khundmiri, S. J., Murray, R. D., and Lederer, E. (2016). PTH and vitamin D. *Comp. Physiol.* 6 (2), 561–601. doi: 10.1002/cphy.c140071

Krasowski, M. D., Ai, N., Hagey, L. R., Kollitz, E. M., Kullman, S. W., Reschly, E. J., et al. (2011). The evolution of farnesoid X, vitamin d, and pregnane X receptors: Insights from the green-spotted pufferfish (*Tetraodon nigriviridis*) and other non-mammalian species. *BMC Biochem.* 12, 5. doi: 10.1186/1471-2091-12-5

Kumai, Y., Nesan, D., Vijayan, M. M., and Perry, S. F. (2012). Cortisol regulates na^+ uptake in zebrafish, *Danio rerio*, larvae *via* the glucocorticoid receptor. *Mol. Cell. Endocrinol.* 364, 113–125. doi: 10.1016/j.mce.2012.08.017

Kwong, R. W. M., and Perry, S. F. (2015). An essential role for parathyroid hormone in gill formation and differentiation of ion-transporting cells in developing zebrafish. *Endocrinology* 156 (7), 2384–2394. doi: 10.1210/en.2014-1968

Kwong, R. W. M., and Perry, S. F.. (2014). The physiology of fish at low pH: The zebrafish as a model system. *J. Exp. Biol.* 217, 651–62. doi: 10.1242/jeb.091603

Lee, Y. C., Yan, J. J., Cruz, S., Horng, J. L., and Hwang, P. P. (2011). Anion exchanger 1b, but not sodium-bicarbonate cotransporter 1b, plays a role in transport functions of zebrafish h+-ATPase-rich cells. *Am. J. Physiol. Cell Physiol.* 300, C295–C307. doi: 10.1152/ajpcell.00263.2010

Lin, L. Y., Horng, J. L., Kunkel, J. G., and Hwang, P. P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. *Am. J. Physiol. Cell Physiol.* 290, C371–C378. doi: 10.1152/ajpcell.00281.2005

Lin, C. H., Hu, H. J., Chuang, H. J., Tsou, Y. L., and Hwang, P. P. (2021). Cortisol and glucocorticoid receptor 2 regulate acid secretion in medaka (*Oryzias latipes*) larvae. *J. Comp. Physiol. B.* 191 (5), 855–864. doi: 10.1007/s00360-021-01390-w

Lin, C. H., and Hwang, P. P. (2016). The control of calcium metabolism in zebrafish (*Danio rerio*). *Int. J. Mol. Sci.* 17, 1783. doi: 10.3390/ijms17111783

Lin, C. C., Lin, L. Y., Hsu, H. H., Thermes, V., Prunet, P., Horng, J. L., et al. (2012). Acid secretion by mitochondrion-rich cells of medaka (*Oryzias latipes*) acclimated to acidic freshwater. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 302 (2), R283–R291. doi: 10.1152/ajpregu.00483.2011

Lin, C. H., Shih, T. H., Liu, S. T., Hsu, H. H., and Hwang, P. P. (2015). Cortisol regulates acid secretion of h^+-ATPase-richionocytes in zebrafish (*Danio rerio*) embryos. *Front. Physiol.* 6. doi: 10.3389/fphys.2015.00328

Lin, C. H., Su, C. H., Tseng, D. Y., Ding, F. C., and Hwang, P. P. (2012). Action of vitamin d and the receptor, VDRa, in calcium handling in zebrafish (*Danio rerio*). *PloS One* 7 (9), e45650. doi: 10.1371/journal.pone.0045650

Liu, S. T., Horng, J. L., Chen, P. Y., Hwang, P. P., and Lin, L. Y. (2016). Salt secretion is linked to acid-base regulation of ionocytes in seawater-acclimated medaka: New insights into the salt-secreting mechanism. *Sci. Rep.* 6, 31433. doi: 10.1038/srep31433

Lynch, M., and Force, A. (2000). The probability of duplicate gene preservation by subfunctionalization. *Genetics* 154 (1), 459–473. doi: 10.1093/genetics/154.1.459

Omdahl, J. L., Morris, H. A., and May, B. K. (2002). Hydroxylase enzymes of the vitamin d pathway: Expression, function, and regulation. *Annu. Rev. Nutr.* 22, 139–166. doi: 10.1146/annurev.nutr.22.120501.150216

Pan, W., Borovac, J., Spicer, Z., Hoenderop, J., Bindels, R., Shull, G., et al. (2012). The epithelial sodium/proton exchanger, NHE3, is necessary for renal and intestinal calcium (re)absorption. *Am. J. Physiol. Renal. Physiol.* 302 (8), F943–F956. doi: 10.1152/ajprenal.00504.2010

Perry, S. F., and Gilmour, K. M. (2006). Acid-base balance and CO_2 excretion in fish: unanswered questions and emerging models. *Respir. Physiol. Neurobiol.* 154, 199–215. doi: 10.1016/j.resp.2006.04.010

Reschly, E. J., Bainy, A. C., Mattos, J. J., Hagey, L. R., Bahary, N., Mada, S. R., et al. (2007). Functional evolution of the vitamin d and pregnane X receptors. *D BMC Evol. Biol.* 7, 222. doi: 10.1186/1471-2148-7-222

Shih, T. H., Horng, J. L., Hwang, P. P., and Lin, L. Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Am. J. Physiol. Cell Physiol.* 295, C1625–C1632. doi: 10.1152/ajpcell.00255.2008

Shih, T. H., Horng, J. L., Liu, S. T., Hwang, P. P., and Lin, L. Y. (2012). Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 302, R84–R93. doi: 10.1152/ajpregu.00318.2011

Stim, J. A., Bernardo, A. A., and Arruda, J. A. (1994). The role of parathyroid hormone and vitamin d in acid excretion and extrarenal buffer mobilization. *Miner. Electrolyte. Metab.* 20 (1-2), 60–71.

Sundell, K., Norman, A. W., and Björnsson, B. T. (1993). 1,25$(OH)_2$ vitamin D_3 increases ionized plasma calcium concentrations in the immature Atlantic cod. *Gadus morhua*. *Gen. Comp. Endocrinol.* 91 (3), 344–351. doi: 10.1006/gcen.1993.1135

Swarup, K., Das, V. K., and Norman, A. W. (1991). Dose-dependent vitamin D_3 and 1,25-dihydroxyvitamin D_3-induced hypercalcemia and hyperphosphatemia in male cyprinoid *Cyprinus carpio*. *Comp. Biochem. Physiol. A. Physiol.* 100 (2), 445–447. doi: 10.1016/0300-9629(91)90497-Z

Takeuchi, A., Okano, T., and Kobayashi, T. (1991). The existence of 25-hydroxyvitamin D_3–1 alpha-hydroxylase in the liver of carp and bastard halibut. *Life. Sci.* 48, 275–282. doi: 10.1016/0024-3205(91)90355-f

Taylor, J. S., Van de Peer, Y., Braasch, I., and Meyer, A. (2001). Comparative genomics provides evidence for an ancient genome duplication event in fish. philos. *Trans. R. Soc Lond. B. Biol. Sci.* 356, 1661–1679. doi: 10.1098/rstb.2001.0975

Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D., and Geibel, J. P. (2004). Renal vacuolar-ATPase. *Physiol. Rev.* 84, 1263–1314. doi: 10.1152/physrev.00045.2003

Wali, R. K., Baum, C. L., Bolt, M. J., Brasitus, T. A., and Sitrin, M. D. (1992). 1,25-dihydroxyvitamin D_3 inhibits na^+-h^+ exchange by stimulating membrane phosphoinositide turnover and increasing cytosolic calcium in caco-2 cells. *Endocrinology* 131 (3), 1125–1133. doi: 10.1210/endo.131.3.1324151

Yan, J. J., and Hwang, P. P. (2019). Novel discoveries in acid-base regulation and osmoregulation: A review of selected hormonal actions in zebrafish and medaka. *Gen. Comp. Endocrinol.* 277, 20–29. doi: 10.1016/j.ygcen.2019.03.007

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Mechanisms of Na^+ uptake from freshwater habitats in animals

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Life in fresh water is osmotically and energetically challenging for living organisms, requiring increases in ion uptake from dilute environments. However, mechanisms of ion uptake from freshwater environments are still poorly understood and controversial, especially in arthropods, for which several hypothetical models have been proposed based on incomplete data. One compelling model involves the proton pump V-type H^+ ATPase (VHA), which energizes the apical membrane, enabling the uptake of Na^+ (and other cations) *via* an unknown Na^+ transporter (referred to as the "Wieczorek Exchanger" in insects). What evidence exists for this model of ion uptake and what is this mystery exchanger or channel that cooperates with VHA? We present results from studies that explore this question in crustaceans, insects, and teleost fish. We argue that the Na^+/H^+ antiporter (NHA) is a likely candidate for the Wieczorek Exchanger in many crustaceans and insects; although, there is no evidence that this is the case for fish. NHA was discovered relatively recently in animals and its functions have not been well characterized. Teleost fish exhibit redundancy of Na^+ uptake pathways at the gill level, performed by different ion transporter paralogs in diverse cell types, apparently enabling tolerance of low environmental salinity and various pH levels. We argue that much more research is needed on overall mechanisms of ion uptake from freshwater habitats, especially on NHA and other potential Wieczorek Exchangers. Such insights gained would contribute greatly to our general understanding of ionic regulation in diverse species across habitats.

KEYWORDS

osmoregulation, ionic regulation, Arthropoda, fish, V-type H^+-ATPase, Na^+/K^+-ATPase, Na^+/H^+-antiporter

Introduction: The problem of ion uptake from fresh water

Marine to freshwater colonizations represent among the most dramatic evolutionary transitions in the history of life (Hutchinson, 1957; Little, 1983, 1990; Miller and Labandeira, 2002). Most animals evolved in the sea, and of the ~35 animal phyla, only 16 phyla contain representatives that have colonized freshwater habitats during the course of evolutionary history (Hutchinson, 1957; Little, 1983; Little, 1990; Lee and Bell, 1999; Miller and Labandeira, 2002). Marine and many estuarine animals, aside from most vertebrates, tend to possess body

fluids that resemble the surrounding seawater in ionic composition (Willmer et al., 2008). In contrast, freshwater animals tend to constantly lose their ions passively, mainly through their body surface or gills. Thus, active ion transport and the tightening of epithelia are essential to compensate for passive ionic losses across epithelia that are in direct or indirect contact with the freshwater environment.

Thus, living in dilute environments poses great challenges for acquiring essential ions against steep concentration gradients between body fluids and the environment (Beyenbach, 2001; Morris, 2001; Tsai and Lin, 2007; Lee et al., 2012). Freshwater animals cannot survive without maintaining elevated extracellular fluid (hemolymph or blood) osmolalities relative to the very dilute environment. In general, invertebrates tend to maintain a broader range of hemolymph osmolalities and often osmoconform to a relatively wide range of intermediate salinities. In contrast, teleost fish are strong regulators that maintain a much narrower range of blood osmotic concentrations.

For instance, invertebrates generally maintain a broad range of extracellular osmolalities in fresh water ranging from ca. 200 to 400 $mOsm.kg^{-1}$, with values as high as 600 $mOsm.kg^{-1}$ in the Chinese mitten crab *Eriocheir sinensis* and as low as 50 $mOsm.kg^{-1}$ in some mollusk species (Schmidt-Nielsen, 1997; Willmer et al., 2008; Charmantier et al., 2009; Evans and Claiborne, 2009). In contrast, teleost fish maintain more constant blood osmolalities, from around 260 to 380 $mOsm.kg^{-1}$ (Evans and Claiborne, 2009), with freshwater fish having lower blood osmolalities (i.e., 260 $mOsm/kg^{-1}$ in carps *Cyprinus carpio*; Holmes and Donaldson, 1969) than marine species (i.e., 360–380 $mOsm.kg^{-1}$ in the European sea bass *Dicentrarchus labrax;* L'Honoré et al., 2019). For fish under freshwater conditions, blood osmolality is generally maintained far above 250 $mOsm.kg^{-1}$, except in fish that are stressed or intolerant of fresh water (L'Honoré et al., 2019).

Overcoming the challenges of living in ion-poor environments through the evolution of body fluid regulation was critical for freshwater colonizations, which then provided key adaptations for the colonization of land (Wolcott, 1992; Anger, 2001; Morris, 2001; Glenner et al., 2006). Yet, basic questions regarding evolutionary adaptations during saline to freshwater transitions remain unresolved (Charmantier et al., 2009; Evans and Claiborne, 2009; Hwang et al., 2011; Dymowska et al., 2012). In particular, fundamental mechanisms of ionic regulation remain incompletely understood in most invertebrates, especially regarding ion uptake from very low salinities (Charmantier et al., 2009; McNamara and Faria, 2012). In teleost fish, ion uptake mechanisms are very diverse, possibly due to major evolutionary changes in genome architecture and diversification of ion transporter gene families (Desvignes et al., 2021). Numerous studies have described these diverse ion uptake mechanisms in fish, but only in a few species (Dymowska et al., 2012; Hwang and Chou, 2013; Zimmer and Perry, 2022).

A key toward understanding ion uptake mechanisms likely resides in the functions of *ionocytes* (Evans et al., 2005; Freire et al., 2008; Charmantier et al., 2009; Hwang and Perry, 2010; Hiroi and McCormick, 2012). Ionocytes, formerly called chloride cells or mitochondrion-rich cells (in fish gills), are cells rich in mitochondria and specialized for ion transport. These specialized cells perform ion uptake or excretion to regulate body fluid concentrations. These cells are ubiquitous across a wide range of osmoregulatory organs, including in crustacean gills, crustacean antennal and maxillary glands, insect Malpighian tubules and alimentary canal, fish gills, kidney, and intestine, as well as skin of fish larvae and embryos (Piermarini and Evans, 2000; Weihrauch et al., 2004; Varsamos et al., 2005; Patrick et al., 2006; Charmantier et al., 2009; Hiroi and McCormick, 2012). These cells possess deep basolateral infoldings and a suite of ion transporters and channels on their apical (outer) and basolateral (inner) membranes (Figure 1). Thus, the functioning of ion transporters and channels, and their cooperation within ionocytes, play critical roles for colonizations and migrations between different habitats. Yet, how ionocytes take up ions from environments is still not fully resolved in invertebrates, particularly from freshwater environments (Charmantier et al., 2009; McNamara and Faria, 2012). In fish, ion uptake mechanisms are well understood in only a few species, notably in their gills or embryonic skin (Dymowska et al., 2012; Kumai and Perry, 2012; Guh et al., 2015), and need to be investigated in other ecologically relevant species.

In addition to the functions of ionocytes, permeability between them (paracellular permeability) is crucial to consider when addressing ion transport. In freshwater osmoregulatory epithelia, intercellular adhesion complexes known as tight junctions control paracellular diffusion of ions and water (Tipsmark et al., 2008; Kolosov et al., 2013). While this mechanism of ionic regulation is important, this topic is beyond the scope of this particular paper.

In terms of driving ion uptake within ionocytes, Na^+/K^+-ATPase (NKA) was initially thought to provide the major limiting energetic driving force for ion uptake across all types of salinity environments (Towle, 1984). NKA was first shown to be basolaterally localized in fish gill ionocytes in the 1970s (Karnaky et al., 1976). NKA pumps Na^+ from the cytosol to the hemolymph in exchange for K^+ transported into the cell. The resulting accumulation of K^+ in the cytoplasm results in diffusion of K^+ back to hemolymph through basolateral K^+ channels or apical K^+ secretion (reviews in Kirschner, 2004; McNamara and Faria, 2012; Horng et al., 2017; Leone et al., 2017). As three Na^+ ions are exchanged for two K^+ ions, the cytosol becomes electronegative. NKA activity thus results in establishing and maintaining two gradients, a concentration gradient and an electrical gradient, both driving apical entry of Na^+ from the external medium into the cell. However, based on thermodynamic principles, NKA is insufficient to drive ion uptake below NaCl concentrations of ~1.0 mM (Larsen et al., 1996).

Thus, under very low salinity conditions, an additional energizing ion transporter is required. Since the 1990s, evidence has been mounting that an apically localized proton pump V-type H^+-ATPase (VHA) plays a crucial role in energizing ion uptake in low salinity environments (and from urine of terrestrial organisms) (Figure 1A, Model 1) (Lin and Randall, 1991). The role of an electrogenic proton pump in driving sodium uptake was originally discovered in frog skin (Ehrenfeld et al., 1985), and then subsequently hypothesized by Wieczorek et al. in insects (Wieczorek et al., 1991; Wieczorek et al., 1999; Chambrey et al., 2013) and by Avella and Bornancin in teleost fish (rainbow trout *Oncorhynchus mykiss*) (Avella and Bornancin, 1989). VHA localized on the apical (outer) membrane of the cell could generate an electrochemical potential by pumping H^+ out of the cell. This electrical gradient could then be used to take up ions, such as Na^+, *via* secondary transporters or channels. However, the identities of these secondary transporters responsible for Na^+ uptake have been unclear and controversial (see next sections; Charmantier et al., 2009; Evans and Claiborne, 2009; Kumai and Perry, 2012; McNamara and Faria, 2012).

Several secondary transporters and channels have been hypothesized to cooperate with VHA to transport Na^+ into ionocytes, such as a putative Na^+ channel (McNamara and Faria, 2012), the Na^+/H^+ exchanger (NHE, SCL9A) (Claiborne et al., 1999; Edwards et al., 1999; Towle and Weihrauch, 2001), or the Na^+/H^+ antiporter (NHA, SLC9B) (Xiang et al., 2012; Posavi et al., 2020; Stern and Lee, 2020). However, evidence for these secondary transporters working with VHA has been relatively limited (but see Xiang et al., 2012; Dymowska et al., 2014; Dymowska et al., 2015). Based on stoichiometry, Wieczorek et al. other hypothesized that an electrogenic antiporter that exchanges cations with H^+ must be cooperating with VHA (Wieczorek et al., 1991; Beyenbach and Wieczorek, 2006). This missing transporter had been dubbed the "Wieczorek exchanger" and its identity had remained a subject of debate.

This secondary Na^+ transporter that cooperates with VHA would have to be electrogenic, meaning that ion uptake would involve a net charge translocation across the membrane (e.g., by NHA or Na^+ channel, Figure 1A). For example, importing just Na^+ ion or exchanging two Na^+ for one H^+ would be electrogenic, whereas exchanging one Na^+ for one H^+ would be electroneutral (e.g., NHE in Figure 1B). Utilizing the electrical gradient (positive charge outside) generated by apical VHA would drive the secondary transporter to perform cation uptake, such as by NHA (SLC9B) or Na^+ channel (NaCh), and thus result in charge translocation. However, export of H^+ by VHA would not drive electroneutral Na^+ uptake from fresh water by NHE (SLC9A), because NHE exchange (of one H^+ out for one Na^+ in) would actually run against the H^+ gradient (chemical gradient, ΔpH) generated by VHA (Potts, 1994).

Starting in the late 1980s, kinetic Na^+ flux analyses suggested the presence of an electrogenic $2Na^+/1H^+$ antiporter in invertebrates that could potentially act as Wieczorek

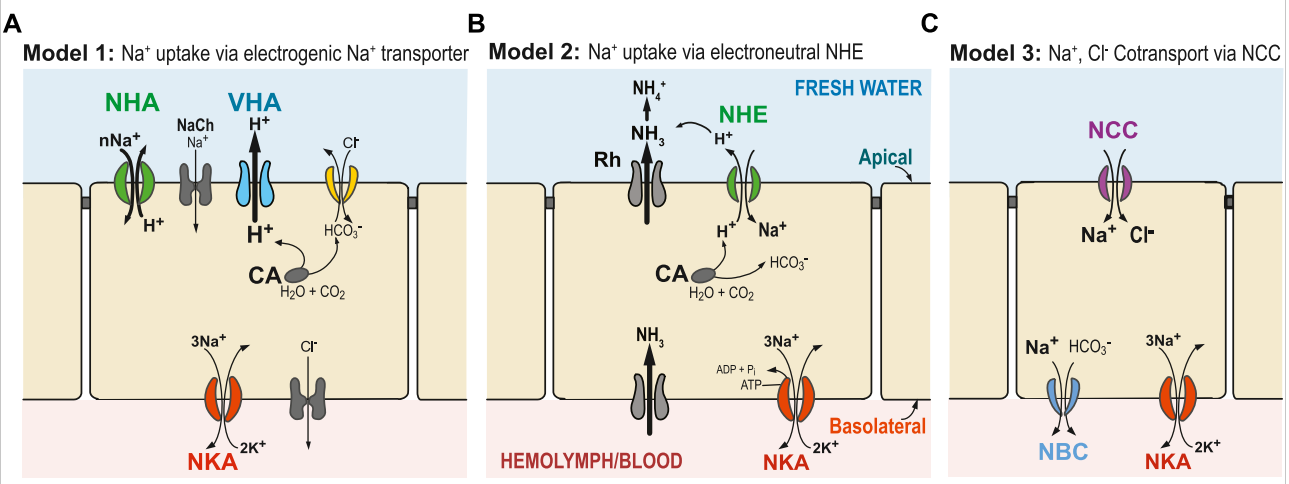

FIGURE 1
Generalized hypothetical models of ion uptake by ionocytes in aquatic animals under freshwater conditions. **(A)** Model 1 (Wieczorek's Model): VHA (blue) pumps out H^+ and creates an electrical gradient, through which Na^+ is transported into the cell through an electrogenic Na^+ transporter (potentially NHA or Na^+ channel, NaCh). **(B)** Model 2: Ammonia is transported out of the cell by an ammonia transporter (Rh protein), which then drives electroneutral NHE (green) to export H^+, and consequently import Na^+. **(C)** Model 3: Na^+ and Cl^- are co-transported to the cell by the NCC or NCC-like cotransporter. In these three models, transport of Na^+ across the basolateral membrane from the cell to the hemolymph or blood is performed by the primary transporter NKA (red) and, potentially, also by NBC (blue, in Model 3). Cytosolic carbonic anhydrase (CA) performs CO_2 hydration, supplying H^+ and HCO_3^- to apical or basolateral ion transporters (Henry 1996). Additionally, chloride uptake might occur through an Cl^-/HCO_3^- exchanger (yellow) or some other bicarbonate exchanger. Alternative models have also been proposed, and not all relevant ion transporters are shown.

exchangers; although, the genetic identity of these transporters had remained unknown (Ahearn and Clay, 1989; Shetlar and Towle, 1989; Ahearn and Pierette, 1991; Ahearn et al., 1994). More recently, a new ion transporter gene family was discovered for the first time in animals, identified as a putative electrogenic Na^+/H^+ antiporter (NHA or SLC9B, Figure 1A, green) and genetically distinct from the previously known electroneutral Na^+/H^+ exchanger (NHE or SLC9A, Figure 1B) (Brett et al., 2005; Rheault et al., 2007; Day et al., 2008; Xiang et al., 2012). These Na^+/H^+ antiporters (NHA), previously known in bacteria and yeast, were discovered and characterized in the fruit fly *Drosophila melanogaster* and mosquito *Anopheles gambiae* (Rheault et al., 2007; Day et al., 2008; Xiang et al., 2012). In particular, one type of NHA, found in apical membranes of larval mosquito Malpighian tubules, was analyzed in a heterologous yeast system and found to perform cation uptake in what appeared to be an electrogenic manner (nCations/$1H^+$) (Xiang et al., 2012). While this result provided support for Model 1, the evidence was not conclusive. While NHA does seem to be critical for ion homeostasis and response to salt stress, functional studies in animals have yielded divergent results, suggesting that its functions might vary among NHA paralogs, cell types, tissues, and taxa (Day et al., 2008; Xiang et al., 2012; Chintapalli et al., 2015).

Additional support for Model 1 was found relatively recently in fish gills (rainbow trout *Oncorhynchus mykiss* and zebrafish *Danio rerio*), but with the sodium channel ASIC4 (Dymowska et al., 2014; Dymowska et al., 2015). Acid-sensing ion channels (ASICs) are close relatives of the tetrapod epithelial sodium channels (ENaC) (Kellenberger and Schild, 2002) and were first analyzed in zebrafish neurons (Chen et al., 2010). ASIC4 was then localized in gill ionocytes of trout and zebrafish and investigated as potential Na^+ channels that would facilitate apical Na^+ uptake in fish acclimated to low salinity and low pH conditions. The apical coexpression of VHA and ASIC4.2 in zebrafish ionocytes indicates the presence of a Na^+ transporter coupled to VHA, supporting Model 1 in freshwater fish (Dymowska et al., 2014, 2015). At this point, no other evidence exists for ASIC4 expression in gills of other teleost fish species.

On the other hand, data linking ammonia excretion with Na^+ uptake, and the failure to find an electrogenic Na^+ transporter in several systems (e.g., particularly in fish), led to the proposal of a second model (Model 2, Figure 1B) (Wright and Wood, 2009; Dymowska et al., 2012; Ito et al., 2013). In this model, an electroneutral Na^+/H^+ exchanger (NHE) located on the apical membrane of ionocytes exchanges one Na^+ for one H^+. However, apical NHE cannot function adequately under neutral to low external pH (high H^+) and low external Na^+ (e.g., freshwater conditions), as this exchanger is driven by environmental and cellular concentration gradients of Na^+ and H^+ and not by membrane potential (Parks et al., 2008). Consequently, if NHE were functioning exclusively under completely freshwater conditions and pH < 8.0, it would work in the opposite direction, and Na^+ loss would dominate. To overcome these thermodynamic constraints that prevent NHE from functioning at lower external pH, an ammonia transporter has been proposed to transport NH_3 out of the cell to "trap" H^+ outside the cell. This exported NH_3 would react with H^+ and produce NH_4^+. Then the lowered external H^+ concentration would promote H^+ export out of the cell and facilitate Na^+ uptake through NHE activity (Wright and Wood, 2009; Wu et al., 2010; Shih et al., 2012). Incipient ideas for this model were first formulated in the 1930's, when Krogh found that Na^+ uptake is coupled to NH_4 excretion, without knowledge of the ion transporters/channels involved (Krogh, 1937).

In a third model, NaCl absorption occurs through an apical Na^+,Cl^- cotransporter (NCC -like, also called NCC2, SLC12A10) (Model 3, Figure 1C). This hypothesized model is based on mammalian kidney distal tubule cells, where the Na^+,Cl^- cotransporter (NCC, SLC12A3) is the major route promoting Na^+ absorption (Plotkin et al., 1996; Yang et al., 1996; Meneton et al., 2000). The fact that apical NCC promotes NaCl uptake in a specific cell type, the NCC cell, is now well-established in several fish species (Hiroi et al., 2008; Wang et al., 2009; Dymowska et al., 2012). Thus far, basolateral NKA is the driving force known to typically facilitate apical NCC2-mediated NaCl transport.

At extremely low salinities, Model 1 is considered more likely to operate than Model 2, as the transmembrane voltage gradient generated by VHA could enable cations to be taken up from extremely low concentrations (Figure 1A). In Model 2, NHE could overcome its aforementioned thermodynamic constraints at extremely low salinities and lower pH (Dymowska et al., 2015) when ion uptake is coupled with the action of Rh protein (Figure 1B). How Model 3 is operating under low ionic concentrations remains an open question. It is possible that each ion uptake mechanism might operate under different salinities and pH levels, with Model 1 more favored under very low salinities and low pH (Dymowska et al., 2014; Dymowska et al., 2015). The three models (Figure 1) do not represent all the mechanisms that have been proposed for Na^+ uptake, but they do represent plausible mechanisms, given the data (see next sections).

So, what is the current evidence that these mechanisms of ion uptake might be operating in animals residing in freshwater habitats? The next sections discuss the evidence for the three models described above (Figure 1) for selected taxa, namely, crustaceans, insects, and teleost fish. Members of these groups have served as the main models for exploring mechanisms of ion transport in aquatic habitats. Crustaceans and insects both belong to the arthropod subphylum Pancrustacea, with insects and related groups (hexapods) nested within the crustacean clade (Misof et al., 2014; Lozano-Fernandez et al., 2016). Thus, insects are essentially a lineage of crustaceans that have colonized land (Regier et al., 2005; Glenner et al., 2006). Fish are interesting examples of highly regulated systems with respect to osmotic and

ionic regulation, where their blood osmolality can be highly regulated regardless of the surrounding media (achieving greater homeostasis).

A key point to mention here is that ionocytes perform multiple functions, which include acid-based regulation and ion excretion, such as ammonia excretion, as well ion uptake. While this paper focuses on ion uptake from fresh water, it is important to note that ion uptake, acid-base regulation, and excretion of ammonia are inextricably linked (see reviews by Krogh, 1939; Evans, 2009; Evans, 2011, Hwang et al., 2011; Guh et al., 2015). For instance, ion uptake often occurs simultaneously with acid-base regulation and frequently involves the same ion transporters, such as VHA, Na^+/H^+ antiporter (NHA), Na^+/H^+ exchanger (NHE), Na^+,HCO_3^- cotransporter (NBC), and Cl^-/HCO_3^- exchanger (AE or some other anion exchanger). However, discussions of mechanisms of acid-base regulation are beyond the scope of this review.

This review is the first to explicitly discuss the evidence for the main proposed models of ion uptake in freshwater habitats (Figure 1) in a wide range of taxa. While these three models have been discussed in many studies, this study attempts to compare and contrast ion uptake mechanisms across three main groups (crustaceans, insects, and teleost fish), and also highlight gaps in our understanding that should be examined in future studies. This paper focuses on these three groups because most aquatic physiological studies have focused on model systems belonging to these groups. Research on many of these systems is still nascent and our knowledge is still incomplete and often without consensus. Nevertheless, we hope that this review serves as a useful reference for what is currently known regarding models of ion uptake (particularly sodium uptake) in freshwater habitats.

Ion uptake in crustaceans

The origin of the first "crustaceans" dates to the Lower Cambrian, more than 500 million years ago (Schram, 1982; Chen et al., 2001; Glenner et al., 2006; Lozano-Fernandez et al., 2016). Members of the paraphyletic crustacean clade have colonized a wide variety of habitats and exhibit a wide array of osmoregulatory patterns, from osmoconformers to strong osmoregulators. Among the ca. 67000 extant described species (Zhang et al., 2011), most live in aquatic habitats and 90% of them live in marine or brackish waters, such that freshwater crustaceans comprise only a small proportion of the group (Kawai and Cumberlidge, 2016). And, as in other freshwater animals, freshwater crustaceans are hyperosmoregulators (Anger, 2003; Anger, 2016). Therefore, a great challenge for freshwater crustaceans is taking up rare ions from the very dilute media of their environment.

Osmotic and ionic regulation have been extensively studied in crustaceans, resulting in many reviews (Potts and Parry, 1964; Mantel and Farmer, 1983; Péqueux, 1995; Harris and Aladin, 1997; Ahearn et al., 1999; Charmantier et al., 2009; Henry et al., 2012; McNamara and Faria, 2012; Larsen et al., 2014; Leone et al., 2017). Also, in addition to ion uptake from the environment, ion reabsorption from urine takes place in excretory organs, such as maxillary or antennal glands, in order to regulate hemolymph osmolality and conserve ions in freshwater environments. The topic of ion reabsorption from urine is covered extensively elsewhere (Harris and Micaleff, 1971; Kamemoto and Tullis, 1972; Peterson and Loizzi, 1974; Riegel, 1977; Mantel and Farmer, 1983; Henry and Wheatly, 1988; Ahearn and Franco, 1990; Sarver et al., 1994; Péqueux, 1995; Wheatly and Gannon, 1995; Vogt, 2002; Khodabandeh et al., 2005a; Khodabandeh et al., 2005b; Khodabandeh et al., 2005c; Freire et al., 2008; Charmantier et al., 2009). Given prior reviews covering various aspects of crustacean osmotic and ionic regulation, the goal here is not to provide a comprehensive review. Rather, the focus here is to present evidence supporting hypotheses of ion uptake, specifically Na^+ uptake, from very dilute environments (Figure 1) and discuss unresolved issues regarding those mechanisms.

Ion uptake in gills and extrabranchial organs of crustaceans

In crustaceans, ion uptake from the environment is performed in a wide range of organs, including in pleopods of isopods (Postel et al., 2000), epipodites (= epipods) of branchiopods (Aladin and Potts, 1995), swimming legs of copepods (Johnson et al., 2014; Gerber et al., 2016), and gills of decapod crustaceans (Onken and McNamara, 2002; Cieluch et al., 2005; Henry et al., 2012). In some smaller species, ionic and osmotic regulation are performed on surfaces of the body, such as integumental windows and dorsal organs, as in some syncarids and cladoceran branchiopods (reviewed in Charmantier et al., 2009; Loose et al., 2020). The structures and functions of crustacean iono- osmo-respiratory organs, especially those of decapods, have been reviewed elsewhere (Mantel and Farmer, 1983; Taylor and Taylor, 1992; Péqueux, 1995; Freire et al., 2008; Charmantier et al., 2009; Loose et al., 2020).

Interestingly, a wide array of crustacean iono- and osmoregulatory (and respiratory) organs, such as branchiopod epipodites and decapod gills, are thought to be homologous and developmentally derived from arthropod appendages (legs) (Franch-Marro et al., 2006; Boxshall and Jaume, 2009). The strong association of crustacean osmoregulatory organs with legs is supported by the discovery of ion transporters clustered in "Crusalis organs" of the swimming legs of the copepod *Eurytemora affinis* complex (Johnson et al., 2014; Gerber et al., 2016). Interestingly, development of crustacean epipodites and gills is controlled by orthologs of the master regulator transcription factors *trachealess* (*trh*) and *ventral veinless* (*vvl*) (Mitchell and Crews, 2002; Franch-Marro et al., 2006; Wang et al., 2012), which also control the development of respiratory trachea in insects (Franch-Marro et al., 2006; Chung et al., 2011) and lungs/trachea in vertebrates (Levesque et al., 2007; Zhou et al., 2009).

Support for model 1 in crustaceans

With respect to the hypotheses described above (Figure 1), available physiological, molecular, and histological data on hyperosmoregulating crustaceans in very dilute environments show predominant support for Model 1 (Figure 1A), or a more complex variant of this model (see e.g., Kirschner, 2004; Bianchini and Wood, 2008; Freire et al., 2008; Charmantier et al., 2009; McNamara and Faria, 2012). Here, we propose two models, either with a single cell or with two associated cells. In both models, the main driving force for ion transport is provided by the apical VHA and the basolateral NKA.

For ion uptake from fresh water, the involvement of VHA has been implicated in several groups of crustaceans (reviews in Charmantier et al., 2009; McNamara and Faria, 2012; Weihrauch and O'Donnell, 2015; Leone et al., 2017). These include crayfish (Zare and Greenaway, 1998), decapod crabs (Putzenlechner et al., 1992; Onken and Putzenlechner, 1995; Riestenpatt et al., 1995; Weihrauch et al., 2001; Genovese et al., 2005; Weihrauch et al., 2005; Tsai and Lin, 2007), palaemonid shrimps (Faleiros et al., 2010; Boudour-Boucheker et al., 2014, 2016; Lucena et al., 2015; McNamara et al., 2015; Faleiros et al., 2017), the amphipod *Gammarus fossarum* (Dayras et al., 2017), calanoid copepods (Lee C. E. et al., 2011; Johnson et al., 2014; Gerber et al., 2016), and neonates of the branchiopod *Daphnia magna* (Bianchini and Wood, 2008). In the copepod *Eurytemora affinis* complex, VHA activity shows both an evolutionary increase in recently derived freshwater populations, relative to saline populations, and an acclimatory increase under freshwater conditions for both saline and freshwater populations (Lee C. E. et al., 2011). This evolutionary increase in activity in freshwater populations suggests its important role in freshwater adaptation.

The first model for crustaceans, based mostly on studies of decapod crabs, involves a single ionocyte type (Figure 2A). In this model, the electrochemical gradient that drives ion uptake from the environment is generated by the combined actions of VHA and NKA (see previous section) (Figures 1A, 2A) (Charmantier et al., 2009; McNamara and Faria, 2012; Yang et al., 2019; Lee, 2021). This electrochemical gradient drives Na^+ uptake across the apical membrane (Larsen et al., 2014) by some unknown Na^+ transporter (i.e., the unknown "Wieczorek Exchanger; "see below). Na^+ is then transferred from cytoplasm to hemolymph through the basolateral NKA. Apical entry of Cl^- may be mediated by the Na^+,K^+,2Cl^- cotransporter (Riestenpatt et al., 1996; Towle, 1998; Weihrauch and Towle, 2000; Luquet et al., 2005) or by a Cl^-/HCO_3^- exchanger (Onken et al., 1991; Genovese et al., 2005) with basolateral transfer to the hemolymph performed by Cl^- channels (Bianchini et al., 1988; Siebers et al., 1990; Towle and Smith, 2006). Basolateral K^+ channels likely enable the recycling of K^+ used by NKA. The intracellular carbonic anhydrase supplies H^+ to the proton pump VHA, enabling the apical uptake of Na^+, and HCO_3^- ions to the apical Cl^-/HCO_3^- exchanger, in exchange for Cl^-.

A second model proposed in decapod palaemonid shrimps involves two morphologically distinct ionocytes that cooperate to perform ion uptake from the environment (Figure 2B) (reviewed in McNamara and Faria, 2012; Boudour-Boucheker et al., 2014; McNamara et al., 2015). The two distinct types of ionocytes include pillar cells, which have extensive apical flanges facing the environment, and septal cells, which are in contact with the hemolymph (McNamara and Faria, 2012; Boudour-Boucheker et al., 2014). As shown by their immunostaining, the two main enzymes driving ion transport are located in different cells, with VHA localized on the apical side of the pillar cell flange and NKA on the membranes of septal cells in contact with the hemolymph (Boudour-Boucheker et al., 2014; Lucena et al., 2015; Maraschi et al., 2015; Pinto et al., 2016). On the apical membrane of the pillar cell, VHA generates the electrical gradient that favors Na^+ uptake from the external medium, through an unknown Na^+ channel or some other electrogenic Na^+ transporter (McNamara and Faria, 2012; Boudour-Boucheker et al., 2014). Within the pillar cell, a cytoplasmic carbonic anhydrase (CA) delivers H^+ to VHA, to enable Na^+ uptake, and provides HCO_3^- to an apical Cl^-/HCO_3^- exchanger, permitting Cl^- uptake (McNamara and Faria, 2012). Subsequently, Na^+ and Cl^- are either transported directly to the hemolymph space or passed through a junctional complex to the adjacent septal cells. Freire and McNamara (1995) showed that septal and pillar cells of the shrimp *Macrobrachium olfersii* have areas of attachment through desmosomal contact. If passed to the septal cells, Na^+ would then be transported to the hemolymph by NKA on the membrane of the septal cell, with recycling of K^+ by hypothetical K^+ channels. The depletion in Na^+ content in the septal cell is what drives Na^+ from the adjacent pillar cell to the septal cell. Cl^- exits to the hemolymph through Cl^- channels, possibly from either pillar or septal cells (McNamara and Faria, 2012).

For freshwater crustaceans, there is considerable uncertainty regarding various features of the models of ion uptake (Figure 2). A key puzzle in these models regards which ion transporter is apically localized and responsible for transporting Na^+ into the cell from dilute environments. In crustaceans, three types of transporters have been proposed to mediate Na^+ entry under freshwater conditions, namely, a Na^+ channel (NaCh), the Na^+/H^+ exchanger (NHE, SCL9A), or the electrogenic Na^+/H^+ antiporter (NHA, SLC9B) (Towle and Weihrauch, 2001; Charmantier et al., 2009; McNamara and Faria, 2012; Stern and Lee, 2020). The Na^+,K^+,2Cl^- cotransporter has been proposed to perform Na^+ uptake under brackish water conditions (Riestenpatt et al., 1996; Weihrauch and Towle, 2001). As mentioned in the Introduction, the Na^+ transporter cooperating with VHA was hypothesized to be electrogenic (Wieczorek et al., 1999; Beyenbach and Wieczorek, 2006). As discussed in the following paragraphs, sufficient data are lacking regarding the identities of the secondary transporters that cooperate with VHA to perform the apical uptake of Na^+ in crustaceans and the topic remains controversial.

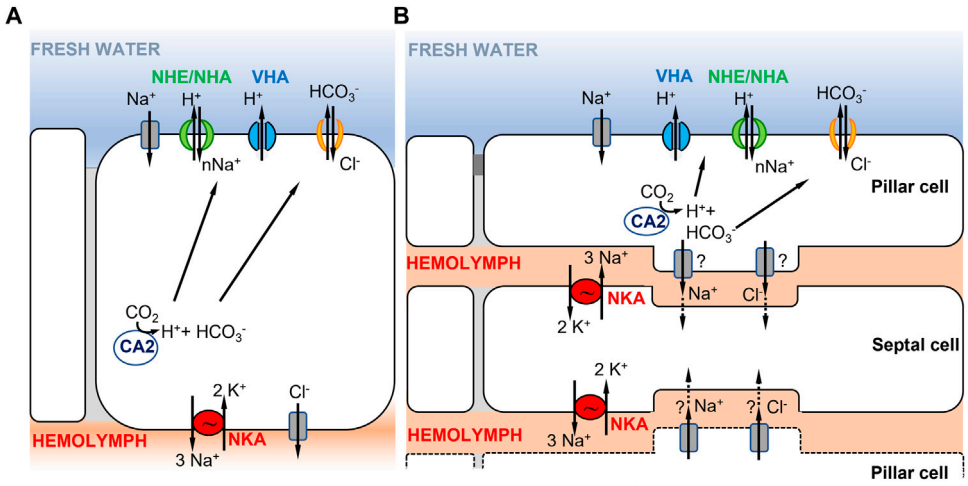

FIGURE 2
Hypothetical ionocyte models of ion uptake for crustaceans in freshwater habitats. **(A)** A single ionocyte, as found in decapods and particularly in brachyuran crabs (from Towle and Weihrauch, 2001; Charmantier et al., 2009). Under low salinity conditions, the apically localized VHA transports H^+ out of the cell, driving Na^+ entry into the cell through an unknown electrogenic Na^+ transporter. **(B)** Cooperating ionocytes, pillar and septal cells, as found in different species of palaemonid shrimps (adapted from McNamara and Faria, 2012; Boudour-Boucheker et al., 2014). On the apical membrane of pillar cells, VHA drives Na^+ uptake into the cell as in **(A)**. Na^+ is then passed to septal cells and then transported to the hemolymph by NKA located on the membrane of septal cells. In both models in **(A,B)**, the type of apical ion transporter responsible for transporting Na^+ from the environment into the cell is uncertain (see text), as well as the chloride bicarbonate exchangers or channels involved.

The two models (Figure 2) are based mostly on data from decapod crustaceans, which might not be representative of crustaceans as a whole. Within the order Decapoda, multiple independent instances of whole genome duplication have taken place (Lécher et al., 1995; Gutekunst et al., 2018), possibly leading to gene family expansions and the evolution of morphological complexity. Decapods are peculiar among crustaceans in possessing highly derived sets of gills, which are developmentally derived from legs (Franch-Marro et al., 2006). As mechanisms of ion transport have not been studied in most orders of crustaceans, the models based on decapods might not be generalizable to the diversity of mechanisms that could be operating across divergent crustacean taxa.

An apically localized Na^+ channel (NaCh) was proposed to utilize the voltage gradient generated by VHA to take up Na^+ by crustaceans under freshwater conditions (McNamara and Faria, 2012). An apical Na^+ channel was inferred to be involved in ion uptake in fresh water in gill ionocytes of the Chinese mitten crab *Eriocheir sinensis*, based on inhibition of Na^+ uptake using the pharmacological inhibitor amiloride (Zeiske et al., 1992). However, the use of amiloride cannot clearly distinguish between the effects of Na^+ channel and NHE (Masereel et al., 2003; Quijada-Rodriguez et al., 2017). Likewise, amiloride and ethylisopropyl-amiloride (EIPA) cannot distinguish between the two models of Na^+/H^+ exchange in the crayfish *Procambarus clarkii* (Kirschner, 2002). Thus, the identities of the ion transporters in these studies remain uncertain, as their DNA was not sequenced. Also, a subsequent study in split gill lamellae of the European green crab *Carcinus maenas* showed that most of the amiloride effect might be explained by its inhibition of ion fluxes through the cuticle, rather than *via* Na^+ channels in the apical membrane (Onken and Riestenpatt, 2002). The involvement of an epithelial Na^+ channel associated with VHA was inferred in whole-body Na^+ uptake by neonates of the branchiopod *Daphnia magna*, using the inhibitors phenamil and bafilomycin (Bianchini and Wood, 2008). However, thus far, there is no clear evidence that Na^+ channels are involved in ion uptake specifically in crustacean osmoregulatory organs, including in crustacean gills or epipodites. Thus, evidence for its role in Na^+ uptake in fresh water is still limited and inconclusive, requiring much more research.

Despite the presence of Na^+/H^+ exchanger (NHE) (SLC9A) in crustaceans, there is no clear evidence for Model 2 (Figure 1B) in freshwater crustaceans. As of yet, no study has conclusively established that NHE functions as an apical ion transporter performing Na^+ uptake in an electrogenic manner in crustaceans. In the literature on brackishwater decapods, Na^+ uptake across the apical membrane was claimed to be performed by an electrogenic Na^+/H^+ exchanger, where two Na^+ are exchanged for one H^+ (Shetlar and Towle, 1989). The stoichiometry of $2Na^+/1H^+$ exchange was detected using a fluorometric assay based on acridine orange. However, in this study, and other similar studies (Ahearn and Clay, 1989; Ahearn and Franco, 1990), the genetic identity of the Na^+ transporter

studied has been unclear. NHE was inferred to be involved in whole body Na^+ uptake in adults of the branchiopod *Daphnia magna* (Bianchini and Wood, 2008), but this result was determined using the inhibitor amiloride, which cannot discriminate between Na^+ channel and NHE (Masereel et al., 2003; Quijada-Rodriguez et al., 2017). Transcripts of the NHE gene have been found to be expressed in the gill tissue of carid shrimp *Macrobrachium amazonicum* (Boudour-Boucheker et al., 2016), the blue crab *Callinectes sapidus,* and the green shore crab *Carcinus maenas* (Towle et al., 1997; Towle and Weihrauch, 2001). However, conclusive links have not been made between expression of *NHE* in crab gills and its stoichiometry of Na^+ transport.

Rh proteins (RhCM) have been sequenced in crustacean species, notably crabs (Weihrauch et al., 2009), but they differ from vertebrate Rh proteins and have uncertain function. Moreover, the subcellular localization of RhCM in osmoregulatory posterior gills of crabs is not known. Importantly, no evidence is available on the presence of a functional link between ammonia excretion and Na^+ uptake, as has been described in fish gills (see *Support for Model 2 in teleost fish*, below).

Some empirical results are consistent with the argument that an electroneutral NHE might enable Na^+ uptake at brackish (low) salinities, and freshwater conditions (Parks et al., 2008; Dymowska et al., 2015). The estuarine carid shrimp *Macrobrachium amazonicum* showed higher gene expression of branchial *NHE* at lower (5 PSU) than at higher (25 PSU) salinity, suggesting a role in ion uptake at lower salinities (Boudour-Boucheker et al., 2016). Similarly, in the penaeid shrimp *Penaeus monodon*, expression of *NHE* was higher in fresh water than in saline media, in conjunction with higher expression of *NKA* and *VHA* (Rahi et al., 2021). However, In the copepod *Eurytemora affinis* complex, some *NHE* paralogs showed increased expression under saline conditions, whereas other paralogs displayed increased gene expression under freshwater conditions (Posavi et al., 2020), suggesting functional differentiation among NHE paralogs. Signatures of positive selection (based on dN/dS ratio) in the *NHE* gene were found in the lineage of the freshwater crab *Eriocheir sinensis*, relative to other decapod crab species, suggesting functional evolution (Wang et al., 2018).

The electrogenic Na^+/H^+ antiporter (NHA) (SLC9B) was originally thought to be an exclusively bacterial ion transporter until 2005, when the first DNA sequences of *NHA* were obtained for eukaryotes, including animals (Brett et al., 2005). Phylogenetically, *NHA* sequences were found to form a sister clade with *NHE* (SLC9A) (Brett et al., 2005). Animal NHAs were functionally analyzed only starting in 2007 in insects (Rheault et al., 2007; Day et al., 2008; Xiang et al., 2012). At this point, concrete functional information on NHA in arthropods comes almost exclusively from studies of insects (see next section).

Starting in the late 1980s, kinetic Na^+ flux analyses suggested the presence of an electrogenic $2Na^+/1H^+$ exchanger/antiporter in crustaceans that could potentially act as the missing "Wieczorek exchanger" (Shetlar and Towle, 1989; Ahearn et al., 1990; reviews in Ahearn, 1996; Ahearn et al., 2001; Towle and Weihrauch, 2001). Such putatively electrogenic Na^+/H^+ antiporters have been found in various decapod crustaceans, such the green crab *Carcinus maenas* (Shetlar and Towle, 1989), the American lobster *Homarus americanus* (Ahearn and Franco, 1990; Ahearn et al., 1994) and the shrimp *Macrobrachium rosenbergii* (Ahearn and Clay, 1989; Kimura et al., 1994; Ahearn et al., 1999). However, the genetic identities of these transporters are unclear, given that amiloride and other pharmacological inhibitors are poor at distinguishing among different sodium transporters (Masereel et al., 2003). Additional studies are needed to determine which Na^+ transporter was analyzed in these functional assays.

Several evolutionary genomic studies implicate NHA paralogs as important contributors to freshwater adaptation (Posavi et al., 2020; Stern and Lee, 2020; Lee, 2021; Stern et al., 2022). Population genomic studies of the copepod *Eurytemora affinis* species complex have found that gene paralogs of *NHA* exhibit signatures of natural selection between ancestral saline and recently freshwater invading populations (Stern and Lee, 2020; Lee, 2021; Stern et al., 2022). Often the same SNPs (single nucleotide polymorphisms) were under selection across repeated saline to freshwater invasion events, suggesting that the same functional sites are evolving within the ion transporter protein. Experimental evolution studies also revealed *NHA* paralogs as genetic targets of selection during rapid salinity decline in the laboratory (Stern et al., 2022). Of the *NHA* paralogs, the *E. affinis* paralogs *NHA7* and *NHA5* showed evolutionary shifts in gene expression between ancestral saline and recently derived freshwater populations, supporting the importance of NHA function during rapid salinity transitions (Posavi et al., 2020; Lee, 2021). These paralogs also showed acclimatory changes in expression with salinity change (Posavi et al., 2020). Additionally, crustacean species that are prone to crossing salinity boundaries exhibit striking *NHA* family expansions. While most insect genomes appear to possess only two paralogs of *NHA* (Xiang et al., 2012; Chintapalli et al., 2015), the genome of the copepod *E. affinis* complex contains 8 paralogs and that of the amphipod *Hyalella azteca* contains 4 (Poynton et al., 2018; Stern and Lee, 2020; Lee, 2021). The fact that *NHA* paralogs are genetic targets of selection during salinity shifts across multiple studies (Lee, 2021) suggest that they are critically important for freshwater adaptation.

We currently lack sufficient physiological data on NHA paralogs to adequately assess their roles in the models of ion uptake (Figure 1). First of all, functional characterization of ion transport by NHA is needed, such as whether ion transport is electrogenic and which specific ion(s) are being transported.

Additionally, we need to establish whether NHA is functionally linked to VHA. We still need to determine whether NHA is apically localized, with VHA, as hypothesized in the models of Na^+ uptake (Figures 1A, 2). Even for insect model systems, the functions of NHA paralogs are not resolved (Xiang et al., 2012; Chintapalli et al., 2015) (see next section). Detailed research is needed to uncover the functional differences among the different NHA paralogs across a variety of animal models.

The Na^+,K^+,$2Cl^-$ cotransporter (NKCC) (SLC12A) is thought to play an important role in Na^+ uptake in "weak hyperregulating" crustaceans in brackish water, but not under freshwater conditions (Towle and Weihrauch, 2001; McNamara and Faria, 2012; Moshtaghi et al., 2018). As of yet, there is no evidence that an apical NKCC performs Na^+ uptake under freshwater conditions. In brackishwater decapod crustaceans, an apical NKCC has been proposed to perform Na^+ uptake, in cooperation with K^+ channels, with this uptake energized by NKA at the basolateral membrane (Riestenpatt et al., 1996; Luquet et al., 2005). For instance, in the green shore crab *Carcinus maenas*, an apical NKCC was proposed to perform Na^+ uptake in brackish salinity (248 mmol/L NaCl, ~14.5 PSU) based on voltage clamp and ion flux studies on split gill lamella in an Ussing chamber (Riestenpatt et al., 1996). Likewise, an apical NKCC was hypothesized to perform Na^+ uptake in the epipodite of the lobster *Homarus americanus* under brackishwater conditions (240 mmol/L NaCl, ~14 PSU) (Lucu and Towle, 2010). Consistent with apical Na^+ uptake in brackish water, *NKCC* mRNA expression increased 10-22 fold after transfer from 30 PSU to 2 PSU salinity within 24 h in the posterior gills of the estuarine crab *Neohelice (Chasmagnathus) granulata* (Luquet et al., 2005). In addition, gill NKCC protein synthesis in several *Macrobrachium* species was associated with hyperosmoregulatory capacity at salinities close to 24 PSU, suggesting a role for NKCC in salt uptake at relatively high salinity (Maraschi et al., 2021). Synchronous patterns of gene expression of *NKCC* and other ion transporters (e.g. NHE, CA, NKA) in the posterior gills of the mud crab *Scylla paramamosain* across molt stages suggest that these ion transporters cooperate in function during larval development (Xu et al., 2017). However, more studies are needed to verify whether NKCC is apically localized and which ion transporters are cooperating with NKCC.

Conclusion and perspectives on models of ion uptake in crustaceans

At this point in time, many fundamental questions regarding mechanisms of ion uptake by crustaceans under freshwater conditions remain unresolved. In particular, the identity of the ion transporter(s) responsible for the apical uptake of Na^+ from fresh water remains uncertain. It is quite likely that NHA (SLC9B) is of widespread importance across crustaceans, as well as in many other taxa, but many more rigorous functional studies are needed. It is not clear where NHA is localized within ionocytes and which ions different NHA paralogs are transporting, even in model systems, such as *Drosophila melanogaster* (Rheault et al., 2007; Xiang et al., 2012; Chintapalli et al., 2015) (see next section).

Traditional physiological approaches for studying ion transport mechanisms in crustaceans have yielded inconclusive results. Many traditional physiological studies that explore ion transporter functions (often quite elegantly) have not genetically identified the ion transporters under study. Thus, the results on ion transporter function are often decoupled from identities of the actual ion transporters, contributing to uncertainties regarding the roles of Na^+ channel *vs.* NHE *vs.* NHA. Also, in many cases, it is unclear which ion transporter paralog (gene duplicates with DNA sequence variation) is being studied. Whole genome and transcriptome data are needed in order to identify the full complement of ion transporters that exist within genomes, including all gene duplicates and paralogs belonging to each ion transporter gene family. In many cases, different paralogs of an ion transporter could be performing different functions. Some of the confusion in the literature on the functions of particular ion transporters might arise from differences in function among gene paralogs (Xiang et al., 2012; Lee, 2021). It would also be important to identify the functions of alternative splice variants (isoforms).

In addition, traditional physiological approaches have often applied methods that lack the specificity to distinguish among ion transporters. Models of cellular ion transport have been based largely on biochemical and electrophysiological experiments on perfused gills or split gill lamellae, along with the use of pharmacological inhibitors to evaluate the impact of ion transporters on ion fluxes and transepithelial potential (Siebers et al., 1990; Onken et al., 1991; Zeiske et al., 1992; Onken and Putzenlechner, 1995; Riestenpatt et al., 1996; Postel et al., 2000; Onken and Riestenpatt, 2002). A key problem of these approaches is that pharmacological inhibitors are often insufficiently specific, inhibiting more than one ion transporter. For instance, amiloride cannot distinguish between Na^+ channels and NHE. Also, many inhibitors block the activity of only a subset of paralogs of a given ion transporter gene family, leading to erroneous conclusions regarding the function of an entire gene family (Masereel et al., 2003). Thus, while use of pharmacological inhibitors could provide useful initial results, such studies should be followed by more rigorous gene specific analyses, such as RNAi, or CRISPR/Cas gene editing approaches. In addition, heterologous gene expression assays, such as the expression of ion transporter genes in *Xenopus* oocytes or yeast, could be used to examine ion transporter function of specific gene paralogs (Piermarini et al., 2009; Piermarini et al., 2010).

Accurately localizing the ion transporters on the apical or basal membranes of ionocytes is a key step toward clarifying their functions, yet this information is lacking for many of the key

transporters. The actual localization of critical transporters, largely documented for NKA and to a lesser extent for VHA, is still largely missing for many ion transporters in most crustacean species, such as Na^+,K^+,$2Cl^-$ cotransporter, Cl^-/HCO_3^- exchanger, putative Na^+ channels, NHE, and NHA. Immunolocalization with specific antibodies has proven to serve as a powerful tool. Ideally, the antibodies would be specific to each individual paralog, given that different paralogs of an ion transporter might have different patterns of expression and localization, as well as divergent functions.

Additionally, the ion transporters, especially their paralogs, should ideally be studied in a phylogenetic context. Such an approach would uncover how the ion transporters are evolutionarily related to one another, as well as patterns and rates of evolution. For instance, applying a phylogenetic approach revealed the fact that NHA and NHE form sister clades, rather than belonging to the same gene family (Brett et al., 2005). Phylogenetic placement of the ion transporters allows the detection of the direction of evolutionary changes, such as the sequential order of gene duplications and mutations, identifying which paralogs are ancestral and which are derived. Uncovering the evolutionary history of ion transporters could provide insights into the evolutionary succession of their functional changes. For instance, it would be informative to identify mutational differences among paralogs and determine how the mutational changes affect the evolution of function.

Finally, additional studies are needed on the ontogeny of ion transporter localization and functions during development (Charmantier and Charmantier-Daures, 1994; Charmantier, 1998; Charmantier et al., 1998; Charmantier and Charmantier-Daures, 2001; Charmantier et al., 2001; Cieluch et al., 2004; Khodabandeh et al., 2005a; Khodabandeh et al., 2005c; Cieluch et al., 2007; Charmantier and Anger, 2011; Boudour-Boucheker et al., 2016). Most studies have focused on the adult stages, but adaptation to fresh water might take on different forms over the life cycle of aquatic organisms. Given the changes in body size and anatomy across life stages, physical constraints on ion uptake will change during development. Features of ion transporters that change during development might include the structure of ion transporters, their localization in ionocytes and tissues, their activity and expression, and the stoichiometry of ion transport. Constraints and requirements will vary across life stages, and natural selection in response to salinity stress might act differentially across different life history stages.

Ion uptake in insects

The Hexapoda, which include the insects, are essentially crustaceans that have colonized terrestrial habitats (Glenner et al., 2006). Phylogenomic analyses date the origin of insects to the Early Ordovician, approximately 479 million years ago (Misof et al., 2014); although, this date precedes earliest evidence of insects in the fossil record of ~400 million years ago (Engel and Grimaldi, 2004; Garrouste et al., 2012). Both the Hexapoda (i.e., Insecta, Collembola, Protura, Diplura) and the crustacean clades belong the subphylum Pancrustacea, where the Hexapoda are nested within the crustacean clades (Von Reumont et al., 2012; Misof et al., 2014). Thus, insects likely share some basic mechanisms of ion uptake with crustaceans, but with adaptations that reflect its evolutionary history of terrestrialization.

Life on land presents very different challenges from living in water, particularly due to the general lack of aqueous media on land. Physiological mechanisms to address the challenges of water retention include cuticular waterproofing, a tracheal system to reduce respiratory water loss, and the capacity to produce hyperosmotic excreta (Beyenbach and Piermarini, 2008; Bradley et al., 2009). These mechanisms are reviewed in detail elsewhere (Beyenbach and Piermarini, 2008; Bradley et al., 2009). Terrestrial insects no longer take up ions from the surrounding media, but from ingested fluids and food. In terrestrial insects, regulation of fluids and ions occurs mainly in the gut and Malpighian tubules (Beyenbach et al., 2010; Denholm, 2013). Subsequently, the regulation of urine concentration occurs through ion transport in the rectum.

This section focuses mainly on ion uptake mechanisms in aquatic insects, given that freshwater insects take up ions from their surrounding media, unlike terrestrial insects. Of particular relevance here is that larval stages of several insect taxa have secondarily colonized aquatic habitats multiple times independently (Grimaldi and Engel, 2005). For instance, mosquitoes (Diptera: Culicidae), which originated at least 226 million years ago (Reidenbach et al., 2009), have egg and larval stages that occur predominantly in freshwater habitats (95% of mosquito species) (Bradley, 1987; Bradley, 1994).

Mosquito larvae are able to survive in freshwater habitats by reducing drinking to a minimum, producing very dilute urine, and performing active ion uptake into the hemolymph from the external medium (Bradley, 1987, 1994; Bradley et al., 2009). The organs responsible for water elimination and ion conservation and uptake are the midgut, Malpighian tubules, rectum, and anal papillae. In the midgut, nutrients and ions enter the hemolymph from the midgut lumen by active transport, with water following passively. The Malpighian tubules, considered the "insect kidney," is where primary urine is produced in insects. Here, organic wastes, water, and ions are excreted as a fluid that is isosmotic with the hemolymph. The rectum is particularly important for ion and water uptake, as urine produced by the Malpighian tubules is modified in the rectum, with active transport of ions from the urine into the hemolymph when needed (Smith et al., 2008; Beyenbach, 2016). In the rectum, solutes such as NaCl and KCl that were secreted in the Malpighian tubules can be reabsorbed (Beyenbach, 2016). Under very low salinity, however, the urine cannot be made as dilute as the surrounding waters. In such cases, active ion

uptake takes place from the external environment, such as by specialized ionocytes in the anal papillae of freshwater mosquito larvae (Donini and O'Donnell, 2005; Donini et al., 2007; Del Duca et al., 2011; Beyenbach, 2016) or "gills" of mayfly (Bradley, 1994, 2009).

Several studies provide support for VHA as the apical driver for ion uptake in freshwater insects, working in conjunction with basolateral NKA. RT-PCR assays revealed that genes that encode VHA and NKA are expressed in all of the iono- and osmoregulatory tissues of the mosquito *Aedes aegypti* larvae (midgut, Malpighian tubules, rectum, and anal papillae) and adults (stomach, Malpighian tubules, anterior hindgut, and rectum) (Patrick et al., 2006; Durant et al., 2021). Various immunolocalization studies also found that VHA and NKA are expressed in key iono- and osmoregulatory tissues (Patrick et al., 2006; Okech et al., 2008; Smith et al., 2008; White et al., 2013). The critical enzyme carbonic anhydrase (Figure 1) was also found expressed in the rectum and anal papillae of larval mosquito (Smith et al., 2008; Durant et al., 2021).

Support for models 1 and 2 in mosquito larvae

In terms of sodium transporters, there is relatively good evidence that the Na^+/H^+ antiporter (NHA) plays an important role in ion uptake by mosquito larvae from freshwater habitats. Studies on mosquito larvae are notable in being the first to discover NHA in aquatic animals (Rheault et al., 2007). NHA is expressed throughout the ion regulatory organs of mosquito larvae, particularly in the chief sites of ion uptake, the rectum and anal papillae (White et al., 2013; Durant et al., 2021). Gene expression and immunolocalization in mosquito larvae (*Anopheles* and/or *Aedes aegypti*) revealed that NHA is localized in key ion regulatory tissues, such as the gastric caeca, anterior midgut, posterior midgut, proximal Malpighian tubules, rectum, and anal papillae (Rheault et al., 2007; Okech et al., 2008; Xiang et al., 2012; White et al., 2013; Durant et al., 2021). NHA is expressed along the luminal border of the rectum in larvae of the mosquitoes *Anopheles coluzzi* and *A. merus* (White et al., 2013), consistent with a role in ion uptake from the lumen of the rectum. Two paralogs of NHA are expressed in the anal papillae of the larvae of *Aedes aegypti* (Durant et al., 2021). In larvae of the mosquito *Anopheles gambiae*, AgNHA1 was found to be co-localized with VHA on the apical membrane of principal cells in the Malpighian tubules, whereas AgNHA2 was localized apically on the stellate cells in Malpighian tubules (Xiang et al., 2012). Similarly, NHA1 and NHA2 are localized on the apical membrane of epithelial cells in the Malpighian tubules of the fruit fly *D. melanogaster* (Day et al., 2008).

Xiang et al. (2012) proposed a model for ion transport in the Malpighian tubules, where mosquito paralogs NHA1 and NHA2 are both electrogenic and differentially localized in two different types of cells, namely, principal and stellate cells (Figure 3A). This model was based on immunolocalization of paralogs of NHA (AgNHA1 and AgNHA2) in the distal Malpighian tubule of larvae of the freshwater mosquito *Anopheles gambiae* and heterologous expression of AgNHA2 in yeast cells. The apical co-localization of AgNHA1 with VHA in the principal cells in the Malpighian tubules suggested that AgNHA1 function is voltage driven (Xiang et al., 2012). In contrast, AgNHA2 was localized on the apical membrane of stellate cells within Malpighian tubules. Yeast cells transformed with AgNHA2 became growth-inhibited when exposed to salts (LiCl, NaCl, and KCl), suggesting greater inward transport of cations (e.g. nNa^+ in to $1H^+$ out) due to the presence of AgNHA2 (Xiang et al., 2012).

In Xiang et al. (2012)'s model, the principal and stellate cells are interconnected and cooperate to perform ion transport and water secretion (Figure 3A). In the principal cells, VHA pumps H^+ out into the Malpighian tubule lumen, generating a voltage gradient across the apical membrane. This voltage gradient then drives apical NHA1 to transport nH^+ into the principal cells and Na^+ out into the lumen. Then NHA2, apically localized in the stellate cells, drives nNa^+ (or nK^+) from the lumen into the cell and H^+ out to the lumen. Na^+ is then transported to the hemolymph *via* a basolateral NKA (Xiang et al., 2012). A peculiar feature of this model is that Na^+ is transported out into the lumen of the Malpighian tubule by the principal cell, and then taken up again by the stellate cell. This mechanism recycles Na^+ back into the hemolymph to conserve ions from the urine excreted by the Malpighian tubule. Active ion uptake from the urine then takes place in the rectum (Smith et al., 2008). This model is based on immunolocalization of both NHA1 and NHA2, but on an assumed function of NHA1 and indirect inference of NHA2 function (see previous paragraph), such that the validity of this model is not clear. Another type of Na^+ transporter, the Na^+ amino acid cotransporters (NATs), might also be performing apical uptake of Na^+ in the Malpighian tubules. NATs perform amino acid and sodium uptake and have been apically localized in the salivary gland, cardia, gastric caeca, anterior midgut, posterior midgut, and Malpighian tubes of larval *A. gambiae* (Harvey et al., 2009).

In terms of active ion uptake from the external freshwater environment, results from functional ion flux and genome-wide gene expression analysis of anal papillae of mosquito larvae provide some support for Model 1, possibly in combination with Model 2 (Figures 1, 3B) (Del Duca et al., 2011; Durant et al., 2021). The anal papillae of mosquito larvae have important roles in both osmoregulation and ammonia excretion (Wigglesworth, 1933; Bradley, 1987, 1994). Ion flux studies using pharmacological inhibitors had shown that much of Na^+ uptake in the anal papillae is driven by H^+ secretion, likely by VHA (Del Duca et al., 2011). Genome-wide gene expression analysis (using RNA-seq) of anal papillae of the larval mosquito *Aedes aegypti* revealed high levels of expression of many subunits

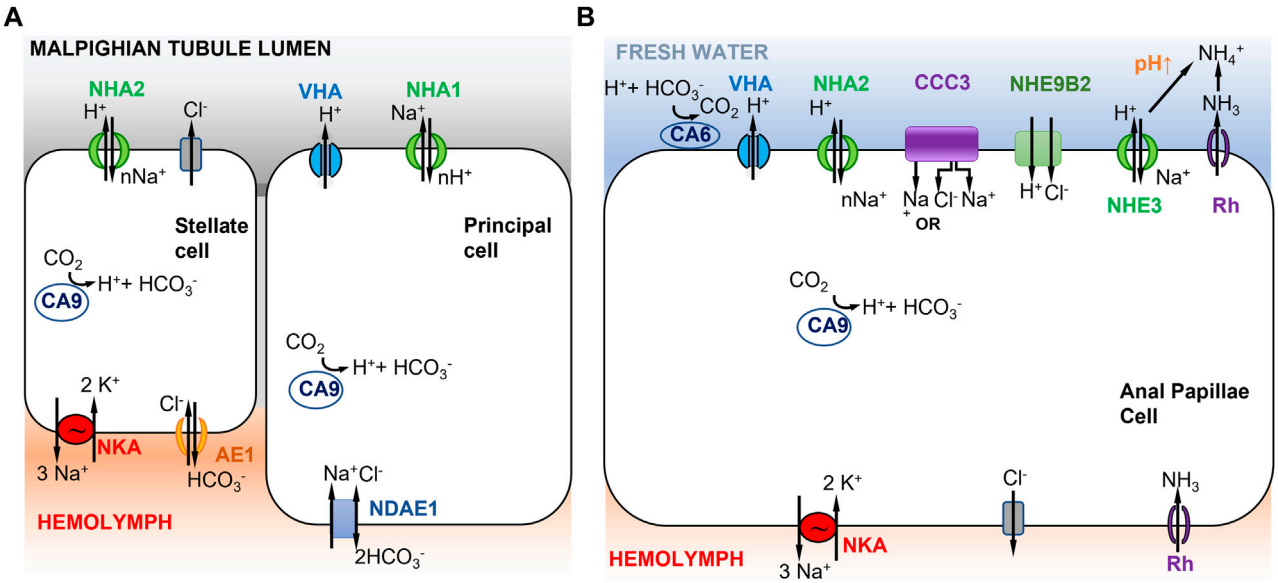

FIGURE 3
Models of ion uptake by mosquito larvae in freshwater habitats. **(A)** Model of Na^+ transport in distal Malpighian tubules of mosquito larvae. V-type H^+ ATPase (VHA) in the apical membrane of principal cells co-localizes with NHA1 ($1Na^+$ out to nH^+ in) and generates a positive voltage that energizes transport events across the apical membrane. The basolateral membrane of the principal cells contains Na^+ dependent anion exchanger 1 (NDAE1), which translocates $1Cl^-$ and $1Na^+$ into the cells and $2HCO_3^-$ out. NHA2 in the apical membrane of stellate cells has a proposed stoichiometry of nNa^+ in and $1H^+$ out. The basolateral membrane of the stellate cell contains Na^+/K^+-ATPase ($3Na^+$ out and $2K^+$ in) and anion exchanger AE1 ($1Cl^-$ in and $1HCO_3^-$ out). Adapted from Xiang et al. (2012). **(B)** Model of Na^+ uptake from the external freshwater environment in anal papillae of mosquito larvae. Both Model 1 (Figure 1A) and Model 2 (Figure 1B) are operating here (see text). CCC3 might function as a Na^+,Cl^- cotransporter or Na^+ transporter. NHE9B2 is homologous to *Drosophila* NHA1, which might function as a H^+,Cl^- cotransporter (Chintapalli et al., 2015) or as a Na^+/H^+ antiporter. Adapted from Durant et al. (2021).

of *VHA* in the anal papillae, as well as expression of three paralogs of *carbonic anhydrases* (*CA*) and two paralogs of *NHA* (Durant et al., 2021). Other ion transporters expressed in the anal papillae that could affect sodium transport include several paralogs each of *NKA*, *NHE*, Na^+-dependent cation-chloride cotransporters (*CCC*), and ammonia transporters (*AMT* and *Rh protein*) (Durant et al., 2021).

Based on these results, a combination of mechanisms could operate in the anal papillae to take up Na^+ from the freshwater environment (Figure 3B). Apical VHA would pump out H^+ and drive Na^+ uptake into the cell (Figure 3B, left) (Del Duca et al., 2011; Durant et al., 2021), consistent with Model 1 (Figure 1A). Na^+ would then be delivered to the hemolymph *via* NKA. CA would supply H^+ to VHA. In addition, given the anal papillae's additional role in ammonia excretion, Na^+ would also enter the cell during this process (Figure 3B, right), consistent with Model 2 (Figure 1B). Here, ammonia is transported out of the cell by an ammonia transporter (Rh protein). The exported NH_3 reacts with H^+ and is converted to NH_4^+. This consumption of H^+ would then drive electroneutral NHE (green) to export H^+, and consequently import Na^+. Additionally, the apical aeCCC3 might function as a Na^+,Cl^- cotransporter. However, aeCCC3 might possibly transport only Na^+, based on results from heterologous expression of a homolog (Kalsi et al., 2019). In either case, apical aeCCC3 would transport Na^+ inward using the gradient established by the basal NKA, which would keep cytosolic Na^+ levels very low. At this time, much of this model remains speculative, such that much additional analyses are required to confirm aspects of this model, including localization and co-localization of ion transporter proteins and functional analyses to determine the roles of individual ion transporters.

Functional assays of both NHA1 and NHA2 in the fruit fly *D. melanogaster* appear to tell a different story, based on heterologous expression in *Xenopus* oocytes. Two-electrode voltage clamping was used to measure H^+, Na^+, and Cl^- transport performed by NHA1 and NHA2 expressed in the oocytes (by measuring pH_i, aNa_i, and aCl_i of oocytes). These assays indicate that NHA1 possibly functions as an electroneutral H^+,Cl^- cotransporter, whereas NHA2 functions as an electroneutral Na^+/H^+-exchanger (Chintapalli et al., 2015). These results are not compatible with Xiang et al.'s (2012) two-cell model (previous paragraph), unless *Drosophila* NHA2 is electrogenic. As functional studies of NHA1 and NHA2 are still preliminary, it is still premature to draw any strong conclusions regarding these models of ion uptake. Moreover, given that *Drosophila* is terrestrial, results from this

model might be less relevant for ion uptake from freshwater habitats.

NHA functions are likely to be highly divergent among taxa, given recent gene duplications and gene family expansions and lack of sharing of paralogs among different taxa. Unlike *NHE*, which possesses ancient paralogs that had diverged prior to species splits, *NHA* paralogs typically arose after species splits and tend to not be shared (not orthologous) among distinct taxa (Brett et al., 2005; Rheault et al., 2007; Stern and Lee 2020; Stern et al., 2022). In particular, the NHA1 and NHA2 paralogs of the insect order Diptera (e.g., flies, mosquitoes) are not orthologous with "NHA1" and "NHA2" outside of dipterans, such that functional studies of these paralogs in mosquitoes and flies might not have broad relevance outside this particular insect order. Additionally, several arthropod species outside of insects possess more than two paralogs that are unique to a lineage, such as the 8 distinct and unique *NHA* paralogs in the genome of the copepod *E. affinis* complex (Stern and Lee 2020) and 4 unique *NHA* paralogs in the amphipod *Hyalella azteca* (Poynton et al., 2018). These distinct paralogs could potentially all differ in function from one another, lacking orthologs in other taxa.

Models of NHA function could become even more complex, given that there are cases where this class of ion transporters (i.e., the CPA2 superfamily) could function facultatively as ion channels (Fujisawa et al., 2007). In general, the strict dichotomy between ion channels and ion transporters often does not hold, as ion transporters could act as ion channels under certain conditions (DeFelice and Goswami, 2007; Barneaud-Rocca et al., 2011). For example, ancillary proteins modulate the cation flux activity of the cation/H^+ antiporters (CPAs) of bacteria, such that the transporters act as cation channels in the absence of their ancillary proteins (Fujisawa et al., 2007). Such results indicate the need to explore the conditions under which ion transport stoichiometry might become altered.

Conclusion on ion uptake in insects, focusing on NHA

Our understanding of ion transport mechanisms is still nascent, even in insects that serve as model systems. We require more comprehensive models of ion transport for all iono- and osmoregulatory organs and tissues of insects, including the alimentary canal, Malpighian tubules, and anal papillae. In particular, much more functional analyses are needed to elucidate the roles of specialized ionocytes of mosquito larvae and the ion transporters embedded within them. Of particular interest are the ionocytes of the anal papillae and rectum and the principal and stellate cells of Malpighian tubules. Also, the functions of different cell types and tissues could vary at different times and in different contexts.

While NHA has been studied most extensively in insects among animals, the functions of different insect NHA paralogs are still not resolved and require additional functional studies. Far more studies are needed to determine whether mosquito NHA1 and NHA2 are electrogenic or electroneutral, which ions are transported, and how their functions vary in different tissues and among taxa. In addition, it would be important to explore conditions under which functions of this ion transporter might become altered, given that this family of ion transporters (as well as other transporter families) have been found to function alternately as channels in bacteria (Fujisawa et al., 2007).

Additionally, it would be critical to extend the functional study of NHA beyond insects, given that NHA appears to be important for ion uptake at very low salinity. Aquatic insects are evolutionary constrained by being secondarily aquatic (from a terrestrial ancestor), such that they likely have many peculiarities that would not apply generally to other systems. It would be particularly interesting to explore the functions of the diverse NHA paralogs in crustaceans and other arthropod lineages. Exploring the roles of NHA during freshwater adaptation in diverse lineages would likely yield many insights into the evolution and functions of this intriguing ion transporter gene family.

Ion uptake in teleost fish

Teleost fish comprise around 25000 species, constituting the most evolutionary diverse group of vertebrates. At least three rounds of whole genome duplication events (Taylor et al., 2001, 2003; Jaillon et al., 2004), as well as increased complexity of regulatory networks controlling gene expression, have greatly influenced mechanisms of fish physiology, including freshwater adaptation (Meyer and Van de Peer, 2005). The third whole genome duplication event, called the "fish-specific genome duplication" (FSGD or 3R), occurred around 350 million years ago (mya) in teleost fish but not in terrestrial vertebrates (for more details on FSGD, see Meyer and Van de Peer, 2005). The salmonid genome duplication event that occurred afterward, about 50–100 mya, has been hypothesized to provide the genetic material for the evolution of anadromy, enabling salmonids to migrate between freshwater and marine habitats (Allendorf and Thorgaard, 1984). Regarding freshwater adaptation, these genome duplication events are thought to have contributed to the diversification of adaptive strategies among teleost species, or even among populations within a species, in order to cope with different salinity regimes (Dalziel et al., 2014).

Several excellent reviews on osmoregulation are available for teleost fish (Evans et al., 2005; Hwang and Lee, 2007; Hwang et al., 2011; Hiroi and McCormick, 2012; Takei et al., 2014; Zimmer and Perry, 2022), including those with a focus on freshwater environments (Kirschner, 2004; Evans 2011; Hwang, 2011; Dymowska et al., 2012; Zimmer et al., 2017). However, only a few species have been thoroughly investigated, among them zebrafish *Danio rerio*, Mozambique

tilapia *Oreochromis mossambicus*, killifish *Fundulus heteroclitus*, medaka *Oryzias latipes* (Hsu et al., 2014), and rainbow trout *Oncorhynchus mykiss* (Dymowska et al., 2012). For other ecologically important species, further investigations are required to construct models of ion uptake and determine the role of each ionocyte subtype according to habitat type.

The functions of organs involved in ionic and osmotic regulation in teleost fish are relatively well characterized (see references above). Under freshwater conditions, fish undergo passive osmotic influx of water and diffusive loss of ions (mainly Na^+ and Cl^-). Maintaining blood osmolality at relatively constant levels of around 260–380 mOsm/Kg, depending on the species, is accomplished in juvenile and adult fish by having (1) low integument and gill permeability to ions, (2) low water permeability of distal renal tubules, to avoid excess entry of water through the renal route, (3) reduced or even absent drinking rate and (4) development of elaborate mechanisms of ion transport through, essentially, the gills and the kidney. At the kidney level, these strategies lead to the production of large amounts of dilute urine (Hickman and Trump, 1969).

The gills of adults and skin of young larvae are the main sites of ion uptake in teleost fish. At the early life history stages (i.e., embryos and larvae) the tegument, including the yolk sac, is essential for transepithelial ion transport through integumental ionocytes (Hiroi et al., 2005; Varsamos et al., 2005; Inokuchi et al., 2022). In contrast, for juvenile and adult fish, the main osmoregulatory organs involved in ion uptake from fresh water are the gills (Varsamos et al., 2005; Evans, 2011). The kidney has been much less studied, but is also an important organ involved in the re-uptake of ions from the renal lumen (Hickman and Trump, 1969; Nebel et al., 2005; Madsen et al., 2020). The gut of freshwater-acclimated or freshwater fish does not play an important role in hyperosmoregulation. As water uptake is not required in freshwater habitats, the drinking rate is generally 10–50 times lower in freshwater than in marine fish (Fuentes and Eddy, 1997; Varsamos et al., 2004; Wilson, 2011). The diet can provide a source of ions, notably in species that have low capacities of branchial (gill) Cl^- uptake (i.e., killifish, eel, bluegill) or species living in ion-poor environments (reviewed in Wilson, 2011).

Ion uptake within gills of adult teleost fish and skin of young larvae is achieved mainly through active transport occurring in specialized ion-transporting cells, namely ionocytes. Ionocytes in the gills of fish are localized in filaments (interlamellar surfaces) and in lamellar surfaces (Evans et al., 2005). Their number, size, and specific position on the gill epithelium become altered according to salinity and other environmental factors, such as temperature (Mitrovic and Perry, 2009; Masroor et al., 2018), pH (Goss et al., 1998), oxygen levels (Sollid and Nilsson, 2006), and also according to species (Evans et al., 2005).

Concerted efforts have been made in the past decade to identify different ionocyte subtypes involved in ion uptake. Branchial (gill) NaCl uptake mechanisms have been shown to differ significantly between species (Hwang, 2011). Depending on the fish species, different nomenclatures are used to characterize ionocyte subtypes, such as peanut lectin agglutinin-positive (PNA^+, Figure 5C) or negative (PNA^-, Figure 4) ionocytes in rainbow trout (Galvez et al., 2002) *versus* VHA-rich cells (HR-cells, Figure 5B), NKA-rich cells (NaR; not shown), and Na^+,Cl^--cotransporter-expressing cells (NCC-type ionocytes; Figure 1C) in zebrafish skin and gills (Hwang and Lee, 2007). This diversity of ionocyte subtypes between species might reflect different ion uptake mechanisms in different environments. Fish evolving in divergent types of freshwater habitats (differing in pH, oxygen, ionic strength, etc.) and having different life history strategies, will have diversified ion uptake mechanisms. Moreover, ion transporters in these ionocytes are also involved in physiological functions other than osmoregulation, such as acid-base regulation and nitrogen excretion. Thus, the regulation and expression of a set of cooperating ion transporters depend not only on salinity, but also on other environmental factors, such that the transporters must accommodate and compromise their functions across their diverse roles (Evans et al., 2005).

Diverse ion uptake mechanisms have been reported in freshwater-acclimated euryhaline fish or freshwater species (notably zebrafish) (Galvez et al., 2002; Hwang and Lee, 2007; Hiroi et al., 2008), supporting Models 1, 2 and 3 described above (Figure 1). Ionocyte subtypes all express basolateral NKA, which is a crucial enzyme allowing ion transport to the blood, as previously shown in crustacean and insect models (Figures 2, 3). Some of the ionocytes also express VHA (apically or basally located, depending on the models), which can be functionally linked to Na^+ uptake (see Model 1, Figure 1A). Whether the expression of different cooperating ion transporters and channels are localized in basolateral or apical membranes determines their role in Na^+ uptake, Cl^- uptake, or acid-base regulation (through the transport of acid, H^+, and base, HCO_3^-, equivalents).

The study of freshwater adaptation and plasticity is now being greatly facilitated by the identification of new genes (including gene paralogs) involved in mechanisms of freshwater adaptation and acclimation. The availability of several new fish genomes in the last 20 years (Aparicio et al., 2002; Jaillon et al., 2004; Tine et al., 2014) (available on Ensembl, http://www.ensembl.org) has improved our capacity to identify relevant candidate genes, including different gene paralogs within the same gene family. With these genes, we can explore their functions involved in adaptation or acclimation using various approaches, such as quantifying their expression levels in ecologically important populations or species, performing gene knockdown studies, or examining genetic/genomic signatures of natural selection. Some of the recent studies that use mechanistic approaches are included in the examples below.

The following sections mainly address Na^+ uptake mechanisms in fish gills or skin (of embryos and larvae),

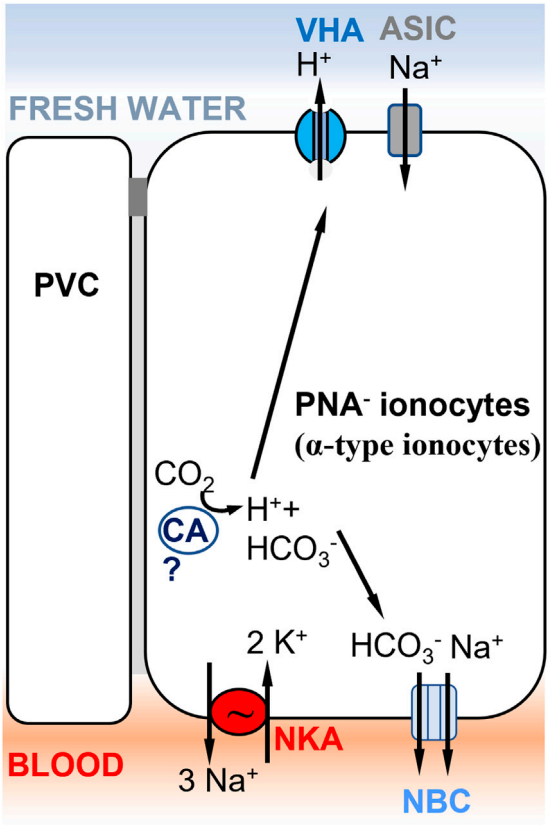

FIGURE 4
Ionocyte model supporting Model 1 (Figure 1A) in rainbow trout gills, involving ASIC as the Na^+ transporter. A PNA^- ionocyte model (Zimmer et al., 2017) with apical VHA (Lin et al., 1994) electrogenically coupled to a Na^+ channel, which has been identified as ASIC-4 by Dymowska et al. (2014). PNA^- ionocytes do not express NHE2/NHE3 and do not express Rh transporters. A basolateral electrogenic NBC transporter (3? HCO_3^-: 1 Na^+) has been shown to be present by Parks et al. (2007). The presence of a cytoplasmic carbonic anhydrase (CA) is hypothesized to support acid secretion in trout gills, but evidence of the specific CA paralog expressed in this cell type is lacking (Georgalis et al., 2006). PVC= pavement cells.

which have been the focus of most research at the cellular and molecular levels in fish. We include findings related to recently discovered ion transporter paralogs that could contribute to freshwater adaptation and acclimation. Based on available data, we describe models of ion uptake reported in teleost fish, in comparison with crustacean and insect models.

Diverse apical Na^+ uptake pathways in teleost fish gills

Studies show that within species, different Na^+ and Cl^- uptake pathways exist that involve different cell types. For instance, in zebrafish, five different ionocyte subtypes have been identified that express different sets of cooperating ion transporters (Guh and Hwang, 2017). Within one specific cell type (e.g., the HR cell-type in zebrafish), different Na^+ uptake pathways can be present, such as an apical Na^+ channel coupled to VHA (Model 1) and/or an apical NHE3 exchanger coupled to ammonium transport *via* apical Rhcg1 (Model 2). Various Na^+ uptake pathways coexist within a species, leading to the redundancy of Na^+ uptake systems. Each Na^+ uptake pathway seems to vary in its optimal environmental performance, leading to different expression patterns of different transporters depending on the environmental conditions (pH, Na^+ levels, etc.) (Dymowska et al., 2015).

Early studies have reported that the uptake of Na^+ *vs.* Cl^- are independent of each other. Their uptake occurs most likely through ion exchangers involving H^+/NH_4^+ exchanged with Na^+ and HCO_3^-/OH^- exchanged with Cl^-, such that electroneutrality is maintained across the gill epithelium (Smith, 1930; Krogh, 1937). This mechanism of Na^+ and Cl^- uptake has been verified in later investigations (García Romeu and Maetz, 1964; Avella and Bornancin, 1989) and also more recently (Weihrauch et al., 2009; Wright and Wood, 2009; Dymowska et al., 2014). Recent studies also consider elevated K^+ as a counter ion for Na^+ uptake in adult zebrafish under acid exposure (Clifford et al., 2022). The uptake of Na^+ in exchange for protons provides some support for Models 1 and 2 (Figures 1A,B, see next section). In some species, such as the eel *Anguilla anguilla* and bluegill *Lepomis macrochirus*, the gill epithelium is involved in active absorption of Na^+ with the lack of or minimal Cl^- uptake (Scott et al., 2005; Tomasso and Grosell, 2005). This pattern of uptake is consistent with the hypothesis of independent Na^+ and Cl^- uptake mechanisms in fish gills and precludes the need for the ionocyte type in Model 3 for coupled Na^+ and Cl^- uptake (Figure 1C) for the aforementioned species.

More recent findings include the presence of Na^+, Cl^- cotransporters (NCC) in gills of numerous fish species (reviewed in Hiroi and McCormick, 2012), such as European sea-bass (*Dicentrarchus labrax*) (Lorin-Nebel et al., 2006), Japanese sea-bass (*Lateolabrax japonicus*) (Inokuchi et al., 2017), Mozambique tilapia (*Oreochromis mossambicus*) (Hiroi et al., 2008; Inokuchi et al., 2008), Indian medaka (*Oryzias dancena)* (Kang et al., 2010), killifish (*Fundulus heteroclitus)* (Katoh et al., 2008), sailfin molly (*Poecilia latipinna)* (Yang et al., 2011), and zebrafish (Wang et al., 2009). In some of the species mentioned above, a non-specific heterologous antibody (called T4) that recognizes NKCC1, NKCC2 and NCC was used in *in situ* localization studies. The apical staining detected in gill ionocytes of these species was later shown to be NCC2 (reviewed in Hiroi and McCormick, 2012). Subsequent studies have used specific NCC antibodies and confirmed its apical localization (Horng et al., 2009; Hsu et al., 2014). These findings indicate that in some specific ionocyte subtypes of several species, Na^+ and Cl^- transport might be partially linked. Interestingly, salmonid species, and in general migratory species analyzed thus far, do not appear to express the branchial NCC (reviewed in Hiroi and McCormick., 2012).

Thus, Na^+ uptake models have become increasingly complex and diversified with the discovery and molecular characterization of new ion transporters and their paralogs. Accumulating evidence suggests the presence of three different Na^+ uptake mechanisms at the apical membrane of branchial ionocytes across different teleost fish taxa, consistent with all three models depicted in Figure 1, namely, (1) Na^+ uptake coupled to VHA (Model 1), (2) Na^+/H^+ exchange (through electroneutral NHE2 or NHE3) linked to apical ammonium excretion through Rhcg (Model 2), (3) Na^+,Cl^- cotransport (NCC-like) coupled to basolateral NKA (Model 3) (Hwang and Lee, 2007; Evans and Claiborne, 2009; Wright and Wood, 2009; Evans, 2011; Dymowska et al., 2014).

Apical Na^+ uptake, a long debate regarding models 1 versus 2

Pathways of apical Na^+ uptake, whether they involve Na^+ channels (Figure 1A, Model 1) or NHE2/NHE3 (Figure 1B, Model 2), have been debated for numerous years (for details, see Evans, 2011; Dymowska et al., 2012). Na^+/H^+ exchange through NHE had been suggested as early as the 1970's (Kerstetter et al., 1970); however, Na^+ concentration of freshwater environments is considered too low for NHE to function properly, notably at low pH (Parks et al., 2008). Thermodynamic constraints on the function of NHEs exist at low Na^+ concentrations ($Na^+ < 0.1$ mmol L^{-1}) and low pH (pH < 5) levels (Parks et al., 2008), despite the contribution of basolateral NKA to lower intracellular Na^+ and drive apical Na^+ uptake (Kirschner, 2004; Parks et al., 2008). For this reason, an alternative Na^+ uptake mechanism involving an epithelial Na^+ channel coupled to apical VHA, rather than NHE, has been considered far more plausible (Parks et al., 2008). Although, NHE could function to import Na^+ under low Na^+ conditions in the context of a Na^+/NH_4^+ exchange complex (such as with Rh in Model 2, see below).

Support for model 1 in teleost fish

Among diverse functions reported for VHA in aquatic organisms (Tresguerres, 2016), H^+ excretion coupled to Na^+ uptake is crucial in some freshwater-acclimated species or

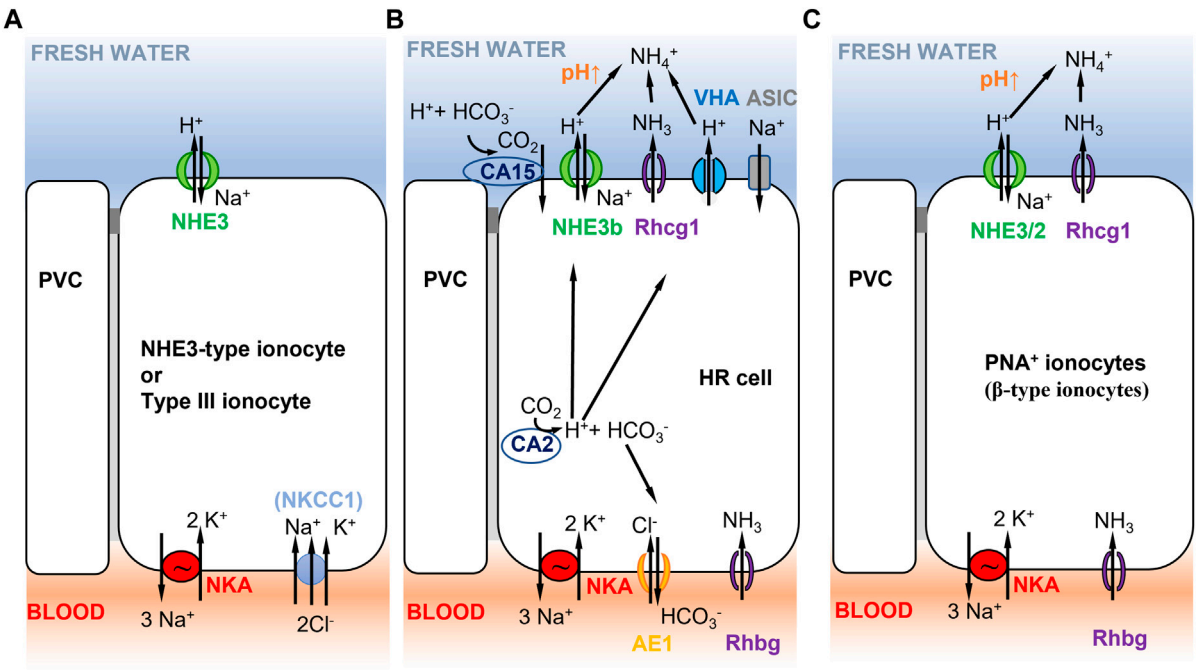

FIGURE 5
Selected ionocyte models consistent with Model 2 (Figure 1B) in different teleost fish species. **(A)** Tilapia type III ionocyte, and Japanese and European sea bass *D. labrax* NHE3-type cell. Type III ionocyte in tilapia involves apical NHE3 and basolateral NKA, and NKCC1 in fish that have been recently transferred to freshwater (Hiroi et al., 2005, 2008; Inokuchi et al., 2008). NHE-3 cells show similar characteristics and are present in gill lamellae of Japanese (Inokuchi et al., 2017) and European sea bass (Blondeau-Bidet et al., 2019). **(B)** zebrafish HR cell. Na^+/NH_4^+ exchange complex in zebrafish HR cells (Hwang et al., 2011) with basolateral NKA (low expression, mainly *ATP1a1a.5* and *ATP1b1b* paralogous genes, Liao et al., 2009), AE1 (*SLC4a1b*, Lee Y. C. et al., 2011), and Rhbg. HR cells express apical NHE3b, VHA (Yan et al., 2007), Rhcg1, and the acid-sensitive ion channel ASIC (ASIC4.2 in adults, Dymowska et al., 2015; ASIC4b in larvae, Zimmer et al., 2018). Cytoplasmic CA2 contributes to H^+ production that is apically excreted *via* NHE3b or VHA. Extracellular CA15 (or CA4-like) contributes to lower apical H^+, resulting in increased pH that favors NHE3b activity. No NBC1 cotransporter has been reported in this cell type (Lee Y. C. et al., 2011). **(C)** Rainbow trout PNA^+ ionocyte. PNA^+ ionocyte model with apical NHE3/2 (*slc9a2*, *slc9a3*; Ivanis et al., 2008) and Rhcg1 coupled to basolateral NKA.

strictly freshwater fish, like zebrafish (Goss et al., 1998; Perry et al., 2003; Hwang and Lee, 2007). The role of VHA appears crucial, given the steep concentration gradient between the water and the blood (estimated at around 1800 fold for Na^+ in Hwang, 2011). Branchial VHA is considered to be mainly apically localized in ionocytes (PNA^- ionocytes, see Figure 4) of rainbow trout, mudskipper, and zebrafish HR cells (Figure 5B) (Lin et al., 1994; Wilson et al., 2000b; Lin et al., 2006). In some species, however, VHA has not been localized, because no commercial antibodies are available. VHA is highly expressed in rainbow trout PNA^- ionocytes (Figure 4) and zebrafish HR cells (Figure 5B). Both cell types are involved in Na^+ uptake and acid excretion (Reid et al., 2003; Lin et al., 2006). Several studies have also identified in these cells the importance of carbonic anhydrase (CA) paralogs, which perform the hydration of CO_2 to H^+ and HCO_3^- (as in *CA2-like a* and *CA15a* in zebrafish HR-cells, Lin et al., 2008) (Figure 5B). H^+ is then exported through VHA or NHE, whereas HCO_3^- is exchanged with Cl^- through the basolateral anion exchanger (AE1) (Figure 5B). Contrary to zebrafish HR cells (Figure 5B), VHA of trout (in PNA^- ionocytes) does not colocalize with NHE2/3 (Figure 4), which is present in another cell type (the PNA^+ ionocyte) (Figure 5C) (Ivanis et al., 2008).

In teleost fish, as epithelial Na^+ channels (ENaCs) have not been discovered, they are unlikely to serve as the Na^+ transporter that cooperates with VHA to transport Na^+ into ionocytes. However, some inconclusive data suggest its presence in teleost fish, though not well substantiated. For instance, in trout PNA^- cells, the pharmacological inhibitors phenamil (considered a specific Na^+ channel blocker (Garty and Palmer, 1997)) and bafilomycin (a VHA blocker) reduced acid-stimulated Na^+ influx (Reid et al., 2003). Other studies showed phenamil-sensitive Na^+ transport in teleost species (Bury and Wood, 1999; Grosell and Wood, 2002; Parks et al., 2007), but without any molecular evidence of the presence of ENaCs. A single study using immunolocalization found the apical presence of ENaC in ionocytes and PVCs of trout, with colocalization with VHA (Wilson et al., 2000a). However, this study used a heterologous antibody that was not specifically raised against trout ENaC. As molecular evidence is lacking that ENaC is the phenamil-sensitive Na^+ channel and, more generally, ENaC is absent in published teleost genomes (Hwang and Lee, 2007), ENaCs are no longer considered to be the Na^+ transporter that cooperates with VHA in teleost ionocytes.

Rather than ENaC, the acid-sensing ion channel (ASIC) could be the missing apical Na^+ channels in freshwater-type ionocytes of teleost gills (Montalbano et al., 2021) (Figure 4). Investigations combining the use of pharmacological inhibitors, immunocytochemistry, and mRNA expression identified ASIC channels in trout and zebrafish gills (Dymowska et al., 2014; Dymowska et al., 2015). ASIC is a member of the H^+-gated Na^+ channel subfamily, belonging to the amiloride-sensitive ENaC/DEG (Degenerin)/ASIC superfamily of ion channels (Waldmann et al., 1997). Interestingly, ASIC4 is expressed in apical cell membranes of trout NKA-rich ionocytes (called PNA^- ionocytes) (Dymowska et al., 2014) (Figure 4) and zebrafish HR cells (Figure 5B) (Dymowska et al., 2015). In those cell types, VHA is believed to export H^+, leading to acidification and opening of ASICs for enhanced Na^+ uptake. In juvenile rainbow trout, ASIC-specific pharmacological inhibitors (Chen et al., 2010), diminazene and DAPI, decreased Na^+ uptake rates in a dose-dependent manner (Dymowska et al., 2014). Some evidence in adult zebrafish supports the role of ASICs in Na^+ uptake, dependent on external Na^+ concentrations (Dymowska et al., 2015).

More recently, knockdown studies of *ASIC4b* in zebrafish embryos and the use of DAPI have, however, shown no effect on whole-animal Na^+ uptake (Zimmer et al., 2018). *ASIC4b* knockdown has led to significant increases in *NHEb* and *NCC* mRNA expression, suggesting that Na^+ uptake was rescued through other pathways. Interestingly, *ASIC4b* knockdown stimulated Na^+ uptake in acidic water, suggesting other unidentified Na^+ uptake mechanisms at low pH (Clifford et al., 2022).

ASICs have been analyzed in only a few species (i.e., rainbow trout and zebrafish) (Zimmer et al., 2018; Clifford et al., 2022). Gene expression levels of *ASIC4* in European sea-bass exposed to fresh water were undetectable in gills, making ASIC4 an unlikely candidate for branchial Na^+ uptake in this marine species (Blondeau-Bidet et al., 2019). Additional studies on ASIC ion channels in freshwater species, but also in marine and euryhaline species (as a comparison), are required to fully support the role of ASIC as the Na^+ transporter in Model 1 (Figures 1A, 4).

Support for coexistence of models 1 and 2 in ionocytes of some teleost fish

Two Na^+ uptake mechanisms coexist in the zebrafish HR cell (Figure 5B), supporting both Models 1 and 2 (Figures 1A,B). HR cells express apical VHA coupled to ASIC-4 (see previous section) and also express an apical NHE3b exchanger (see next section for more details). NHE is considered electroneutral in mammalian vertebrates, as well as in fish (Demaurex et al., 1995; Ito et al., 2014). Numerous studies showed that both Na^+ uptake mechanisms are present in zebrafish HR cells, including those using pharmacological inhibitors (Fenwick et al., 1999; Ito et al., 2014), zebrafish *NHE3b* expression in *Xenopus* oocytes (Ito et al., 2014), and *VHA* knockdown approaches (Horng et al., 2007). The paucity of NKA in HR-cells in zebrafish embryos emphasizes the importance of VHA in providing the energy for Na^+ uptake (Lin et al., 2006; Esaki et al., 2007; Horng et al., 2007).

Interestingly, the predominance of individual Na^+ uptake pathways (either NHE3 or VHA coupled to a Na^+ transporter) seems to depend on environmental conditions. In low-Na^+ freshwater, for example, mRNA of *NHE3* is upregulated, whereas *VHA* is downregulated in zebrafish gills. An opposite

trend in the expression pattern is observed in acidic fresh water (Yan et al., 2007). It thus seems that the partitioning of each Na^+ uptake pathway depends on the environmental condition in which zebrafish reside, with Model 1 (Figure 1A) being favored in acidic environments and Model 2 (Figure 1B) being favored in low-Na^+ environments.

Support for model 2 in teleost fish

Several lines of evidence support the presence of a Na^+/NH_4^+exchange complex (shown in Model 2, Figure 1B), partially supporting the model initially proposed by Krogh (1937). This model combines Na^+ uptake, acid secretion, and ammonia excretion. The model was developed for gills of zebrafish (Figure 5B), mangrove killifish (*Kryptolebias marmoratus*) (Wright and Wood, 2009), and medaka larvae (Wu et al., 2010) based on the discovery that Rhesus (Rh) glycoproteins are involved in ammonia transport in aquatic animals (Weihrauch et al., 2004; Hung et al., 2007; Nakada et al., 2007; Nawata et al., 2007). There is also evidence for this model from other freshwater teleost or euryhaline fish transferred to low salinity (Tsui et al., 2009; Weihrauch et al., 2009; Wood and Nawata, 2011; Hsu et al., 2014).

In this model (Figures 5B,C), apical NHE (2 or 3) (and VHA, if present) transports H^+ out of the cell, resulting in acidification directly outside the apical membrane. This acid-trapping mechanism facilitates NH_3 (base) excretion *via* an ammonia transporter (Rh protein) (basolateral transport from blood to the cell *via* Rhbg, followed by apical transport from the cell to the external water *via* Rhcg1 or 2). Extracellular NH_3 then reacts with H^+ to form NH_4^+ in the mucus covering the apical cell membrane. An extracellular carbonic anhydrase (*zCA15a* in zebrafish HR cells) likely contributes to H^+ production *via* CO_2 hydration (Lin et al., 2008; Weihrauch et al., 2009). Production of NH_4^+, from excreted NH_3 (*via* Rhcg1 or 2) and H^+ (*via* VHA or NHE), locally increases the pH, favoring NHE activity to take up Na^+ and excrete H^+. Intracellular Na^+ then enters the blood stream *via* basolateral NKA and/or a Na^+,HCO_3^- (NBC1) cotransporter.

Support for this model is substantial, as various Rh genes have been sequenced in gills of numerous fish species (Huang and Peng, 2005; Nakada et al., 2007; Nawata and Wood, 2008) and have been localized to branchial ionocytes and PVC in apical (Rhcg1+2) and basolateral (Rhbg) membranes. For instance, morpholino gene knockdown and immunolocalization in zebrafish showed the importance of apical Rhcg1 in ammonia excretion in HR cells (Figure 5B) (Nakada et al., 2007; Shih et al., 2008). Localization of Rhcg1 and Rgbg in the same cell expressing NHE was also reported in ionocytes of medaka fish, with increased expression levels upon low Na^+ acclimation (Wu et al., 2010). *Rhcg2* mRNA upregulation has also been reported in cultured branchial cells of rainbow trout in low-Na^+ media (Tsui et al., 2009), but seems to be specific to pavement cells (Zimmer et al., 2017). Zimmer et al. (2017) also showed colocalization of Rhcg1 with NHE2 and NHE3b in PNA^+ ionocytes of rainbow trout (Figure 5C). In European sea bass, *Rhcg2* mRNA expression is high in gills of freshwater compared to seawater acclimated fish (Blondeau-Bidet et al., 2019), but there is no information about Rhcg1 subcellular localization in ionocytes.

There is also considerable support for apical Na^+ uptake through NHE2 or NHE3 with data from numerous teleost fish species of freshwater and marine origins, with species-specific differences (Figure 5). In several non-model euryhaline species, such as the Japanese sea bass (Inokuchi et al., 2017), European sea bass (Blondeau-Bidet et al., 2019), and Mozambique tilapia (Hiroi et al., 2005; Inokuchi et al., 2008), NHE3 is apically localized in ionocytes that express basolateral NKA (Figure 5A). Euryhaline species that move naturally between marine and freshwater habitats must be able to switch the function of their branchial epithelium between excretory and absorptive roles. There is increasing evidence that during freshwater acclimation, seawater-type ionocytes expressing apical NHE3 and high levels of basolateral NKCC1 are able to differentiate into freshwater-type ionocytes expressing high levels of NHE3 and decreasing levels of NKCC1 (Hiroi et al., 2005; Inokuchi et al., 2008) (Figure 5A). This plasticity of ionocytes to shift from an ion excreting to an ion absorptive function is remarkable and has been observed in only a few non-model species that are considered to be truly euryhaline (Inokuchi et al., 2017; Blondeau-Bidet et al., 2019). Thus, NHE3 seems essential in both seawater and freshwater environments for H^+ excretion. The presence of NHE-type cells could be advantageous for species facing environmental challenges, such as fluctuating environmental CO_2 (Montgomery et al., 2022). Determining the expression patterns of cooperating transporters that potentially form a functional Na^+/NH_4^+exchange complex (see section above) in this NHE3-type cell would help us understand the role of NHE2 and NHE3 in effective Na^+ uptake. This finding would suggest the presence of ammonia excretion and provides additional evidence for Model 2.

Support for model 3 in teleost fish

In teleost fish, there is evidence for coupled NaCl uptake mediated by an apical Na^+, Cl^- cotransporter (NCC2 [or NCC-like], SLC12A10 in gills; NCC1, SLC12A3 in kidney), providing support for Model 3 (Figure 1C) (Hiroi et al., 2008; Wang et al., 2009; Blondeau-Bidet et al., 2019; Breves et al., 2021). This mechanism is contrary to earlier assumptions of independent Na^+ and Cl^- transport across fish gills (Krogh, 1938). Ionocytes with apical (in fresh water) and basolateral (in seawater) NKCC/NCC immunolocalization were first identified in Mozambique tilapia (Wu et al., 2003), using a heterologous antibody that recognizes NKCC1, NKCC2, and NCC cotransporters (Wu et al., 2003). It is likely that this antibody recognized apical NCC2 rather than an apical NKCC2 in fish gills. This

localization of NCC/NKCC was then confirmed in several additional species (see review by Hiroi and McCormick, 2012 and sections above). Apical NCC2 is in fact highly expressed in gills of freshwater-acclimated fish, rather than NKCC2, which is expressed in intestine and kidney (review in Hiroi and McCormick, 2012). In apical NCC2 expressing ionocytes in fish gills, basolateral NKA and NBC1 are thought to facilitate Na^+ extrusion to the blood (Hiroi et al., 2005; Hwang, 2009) and a basolateral chloride channel (potentially ClC2/3) is involved in chloride export to the blood (Tang and Lee, 2011; Bossus et al., 2013). NKA-positive cells with apical NCC2 (called type II ionocytes in tilapia, corresponding to Model 3, Figure 1C) have only been identified in strictly freshwater fish or euryhaline freshwater-acclimatized fish, and are thus clearly identified as freshwater-type ionocytes (Hiroi et al., 2008; Inokuchi et al., 2008, 2017; Hsu et al., 2014; Blondeau-Bidet et al., 2019).

When euryhaline fish are transferred from seawater to fresh water, NCC-type cells are newly synthesized, within 7–14 days of acclimation (Inokuchi et al., 2017; Blondeau-Bidet et al., 2019). Their localization within the gill epithelium (lamellar or filamentary) can differ from NHE3-type cells, as is the case for Japanese and European sea bass (Inokuchi et al., 2017; Blondeau-Bidet et al., 2019). NHE3-type cells (Model 2, Figure 1B), on the contrary, exist in freshwater and seawater-acclimated fish due to their dual roles of ion uptake and acid secretion (Dymowska et al., 2012).

NCC-type ionocytes are not present in all teleost fish species. For instance, the absence of NCC-type ionocytes in several diadromous species (salmonids, eels) is intriguing and requires further investigation. NCC-type cells are also absent in gills of seawater-acclimated teleost fish.

NCC cells (Model 3, Figure 1C) are essential for NaCl uptake in freshwater environments for certain fish species, particularly under low Cl^- and/or potentially low pH conditions. In Mozambique tilapia maintained under low Cl^- or a combination of low Cl^- and low Na^+, the apical surface of NCC cells is much larger than in control fish (Inokuchi et al., 2009). These morphological changes at the cellular level suggest an important role of NCC cells in Cl^- poor environments. Gill mRNA expression of zebrafish *NCC2* (*SLC12a10.2*) and Mozambique tilapia *NCC* (the NCC paralog was not indicated) (Inokuchi et al., 2009) was induced by a low-Cl^- environment, but not by a low-Na^+ environment (Inokuchi et al., 2009; Wang et al., 2009). Also, NCC protein expression was induced in low Cl^- conditions, suggesting an important role in Cl^- uptake. Finally, following acute acidosis (low pH), whole body Na^+ and Cl^- content decreased in larval zebrafish, indicating that low pH induces ion loss and temporary inhibition of Na^+ uptake (Kwong and Perry, 2016). In these fish, compensatory Na^+ and Cl^- uptake resulted exclusively from increased function of NCC-cells (Kwong and Perry, 2016). Studies in additional species are necessary to determine the functional role of NCC in Na^+ uptake in acidic environments.

In addition, NCC functions are redundant with NHE3 for Na^+ uptake in certain fish. But, as mentioned above, NCC function is favored under low Cl^- and/or potentially low pH conditions, whereas NHE3 is not. Na^+ uptake *via* NHE3 is more challenging in an acidic environment, notably in the absence of interaction with Rh proteins (see Model 2). In zebrafish larvae, knockdown of *NHE3* caused an increase in NCC-expressing ionocytes, whereas knockdown of *NCC* increased the number of NHE3-expressing cells (HR cells) (Wang et al., 2009; Chang et al., 2013). In each knockdown experiment, whole-animal Na^+ uptake was recovered due to the compensatory regulation of either NCC or NHE3. The presence of both Na^+ uptake mechanisms in some fish gill epithelia (Models 2 and 3) enables them to maintain whole-animal Na^+ homeostasis under different environmental conditions, depending on water pH (Hirata et al., 2003), ionic strength, and potentially other factors, such as water hardness (Wood et al., 1998; Wilson et al., 1999) and possibly temperature (Kwong et al., 2014).

Interestingly, gills of the killifish *Fundulus heteroclitus* are known to possess only one ionocyte subtype, with apical NCC and NHE and basolateral VHA coexpressed with NKA (Katoh et al., 2003; Breves et al., 2020). The basal localization of VHA with NKA, based on *in situ* immunolocalization, suggests an alternative Na^+ uptake mechanism in this species (Katoh et al., 2003). This configuration of apical NCC and NHE (Katoh et al., 2003; Breves et al., 2020) differs drastically from other species, where NHE and NCC are expressed in different cell types (see the sections above). Intriguingly, in killifish no discernible Cl^- uptake has been measured in gills, suggesting that NCC is involved in Na^+ uptake, but not Cl^- uptake. Acclimation from brackish to freshwater conditions increases the mRNA expression of *carbonic anhydrase 2 (CA2), VHA, Na^+/H^+ exchanger 2 (NHE2)*, as well as *Na^+,HCO_3^- cotransporter 1 (NBC1)* (Scott et al., 2005). These results suggest that both basolateral pumps VHA and NKA would generate an electrical gradient (negatively charged cells) driving apical Na^+ through NHE-like proteins (possibly NHE2, Edwards et al., 2005) or NCC (see above; Dymowska et al., 2012). More functional data are required to validate this alternative model, in which basolateral VHA is involved in Na^+ uptake.

Conclusions on ion uptake in teleost fish, focusing on Na^+ uptake

A striking feature of ion uptake mechanisms in teleost fish is the diversity of ionocyte types and the variety of configurations of ion transporters within ionocytes. There is evidence for the presence of all three hypothesized classes of models (Figure 1) in teleost fish. Several studies have shown that environmental conditions, such as pH, Na^+, and Cl^- levels, are key factors that determine which Na^+

uptake model is more prevalent. However, we have no understanding of whether there is a phylogenetic pattern in the occurrence of these models among taxa. Too few fish models have been studied in detail to determine whether more closely related fish have more similar mechanisms of ion uptake.

The understanding of ion uptake mechanisms in fish gills have been enhanced by the availability of fish genomes and complementary approaches to identify the presence and function of ion transporters (i.e., non-invasive scanning ion-selective electrode technology (SIET) to measure ion transport at the cellular level). Novel molecular approaches such as single-cell RNA sequencing would help identify new ionocyte cell types and elucidate ion transport mechanisms.

Relative to insects and crustaceans, an additional model (Model 3) of ion uptake is available in some fish species, notably involving the NCC-like (or NCC2) cotransporter in gills (Hiroi and McCormick, 2012). This NCC-like cotransporter provides some evidence for coupled NaCl uptake, rather than the canonical independent transport of Na^+ and Cl^-. This type of cotransport had previously been demonstrated in mammalian renal distal convoluted tubules (Jacquillet et al., 2011).

Substantial evidence supports the presence of Model 2 involving NHE2 or NHE3 along with Rh proteins working together as a metabolon. NHE3 or NHE2-type cells are observed in numerous fish species. However, whether Rh proteins are also present in these cell types remains to be investigated in non-model fish species.

There is still some debate regarding the unknown Na^+ uptake mechanisms in fish to support Model 1 (Figure 1A), notably in harsh environments, such as very low pH. Recently, Clifford et al. (2022) has identified another K^+-dependent Na^+ uptake mechanism at low pH in zebrafish, but more evidence is necessary for this mechanism in other teleost species. Also, ASICs have been investigated only in a few teleost species and need to be further explored in non-model species. No evidence is available for the role of an NHA antiporter involved in Na^+ uptake, despite the presence of *NHA* in vertebrate genomes, including in teleost fish (Brett et al., 2005). The potential involvement of NHA in gills and other osmoregulatory organs should be investigated, notably in renal and intestinal epithelial cells. In addition, there has been much less effort devoted to investigating Na^+ re-uptake mechanisms in the fish kidney, which is a main organ contributing to Na^+ homeostasis in fish.

VHA is mainly localized apically in fish gill ionocytes. However, in numerous non-model fish species, VHA has not yet been localized. The presence of basolateral VHA in gill ionocytes of killifish is intriguing and requires further investigation. Ionocytes with basolateral VHA potentially provide alternative models for Na^+ uptake in species that have been less extensively investigated thus far.

Concluding remarks on Na^+ uptake in aquatic organisms

Studies support sharply different models of ion uptake from fresh water between crustaceans/insects and teleost fish. However, it is still premature to make strong conclusions on the details of these models and their prevalence within and between these groups. We have not sampled enough taxa to know how widespread each model is among taxa and whether there is a phylogenetic pattern in their occurrence. For both arthropods and teleost fish, information on mechanisms of ion transport is completely lacking for many (if not most) lineages.

As of yet, ionocyte models for crustaceans are often hypothetical, where functional studies are often not linked to molecular evidence of specific ion transporters. For crustaceans and insects living in very dilute environments, available physiological, molecular, and histological data show predominant support for Model 1 (Figure 1A), or more complex variants of this model involving one or two cells (Figures 2, 3). In all models, the main driving force for ion transport is provided by the apical VHA and the basolateral NKA.

However, sufficient data are lacking to make any strong conclusions involving the sodium transporters involved. In particular, a major problem is that functional analyses do not exist in crustaceans for the top candidate Na^+ transporter, the Na^+/H^+ antiporter (NHA), while only a few studies exist for insects (e.g., Xiang et al., 2012; Chintapalli et al., 2015). Also, it is not clear in crustacean models whether different Na^+ uptake models operate under different environmental conditions (as is the case for teleost fish). Future studies should focus on analyzing the stoichiometry of ion transport for NHA paralogs in crustaceans and aquatic insects. In addition, the presence and roles of NCC proteins or ASICs in arthropod taxa still require investigation.

In teleost fish, there is some support for all three models of ion uptake from fresh water (Figure 1) and some data suggest the presence of novel and unique models of Na^+ uptake. In most teleost species, more than one ionocyte subtype has been identified in gills, with up to five ionocytes subtypes occurring in zebrafish. The prevalence of a particular Na^+ uptake pathway in gills or skin depends on environmental conditions, such as pH, Na^+, or Cl^- levels. Future studies should also focus on non-model teleost species to gain a better understanding of the diversity of ion uptake mechanisms in fish. Our understanding of ion uptake mechanisms in gills needs to be completed in conjunction with studies on ion uptake mechanisms in other osmoregulatory organs, notably the kidney (Takvam et al., 2021).

Whole-genome analyses have the potential to reveal novel ion transporter gene families and paralogs across a wide variety of taxa. However, analyses of ion transporter gene families are still in their infancy for most crustacean taxa, even as more crustacean genomes are becoming available. Genomic studies of the copepod *E. affinis* complex have been valuable in revealing strong selection acting on the *NHA* gene family, as well as other ion transporter families, and

implicating NHA paralogs in freshwater adaptation (Posavi et al., 2020; Stern and Lee, 2020; Stern et al., 2022). Single-cell RNA sequencing could greatly improve our knowledge on the diverse ionocyte subtypes in fish and would potentially help identify new Na^+ uptake pathways in specific cell types (Leguen et al., 2015; Xue et al., 2015). As more genomes become available for non-model species, comparative genomic studies could facilitate invaluable discoveries on novel and diverse ion transport systems.

At this point, relatively little cross communication occurs among investigators working on ion transport mechanisms in different taxa (e.g., crustaceans versus fish). A key problem is that the nomenclature for ion transporters varies wildly across taxa, causing much confusion on which ion transporters are being studied and which ion transporters are homologous between different taxa. Performing many more phylogenetic studies of ion transporter gene families that include a broad range of taxa, including arthropods and vertebrates, would be helpful in determining the homology of ion transporters across taxa. Such clarification of nomenclature and homologous relationships of ion transporter gene families will make it far easier to compare results from studies across a broader range of taxonomic groups, broadening our inferences on the prevalence and peculiarities of ion transport mechanisms in nature.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Ahearn, G. A., and Clay, L. P. (1989). Kinetic analysis of 2 Na^+-1H^+ antiport in crustacean hepatopancreas. *Am. J. Physiol.* 257, R484–R493. doi:10.1152/ajpregu.1989.257.3.R484

Ahearn, G. A., Duerr, J. M., Zhuang, Z., Brown, R. J., Aslamkhan, A., and Killebrew, D. A. (1999). Ion transport processes of crustacean epithelial cells. *Physiol. Biochem. Zool.* 72, 1–18. doi:10.1086/316643

Ahearn, G. A., and Franco, P. (1990). Sodium and calcium share the electrogenic $2Na^+/1H^+$ antiporter in crustacean antennal glands. *Am. J. Physiol.* 59, F758–F767. doi:10.1152/ajprenal.1990.259.5.F758

Ahearn, G. A., Franco, P., and Clay, L. P. (1990). Electrogenic 2 Na /1 H exchange in crustaceans. *J. Membr. Biol.* 116 (3), 215–226. doi:10.1007/BF01868461

Ahearn, G. A., Mandal, P. K., and Mandal, A. (2001). Biology of the $2Na^+/1H^+$ antiporter in invertebrates. *J. Exp. Zool.* 289, 232–244. doi:10.1002/1097-010x(20010401/30)289:4<232::aid-jez4>3.0.co;2-t

Ahearn, G. A., and Pierette, F. (1991). Electrogenic $2Na^+/H^+$ antiport in echinoderm gastrointestinal epithelium. *J. Exp. Biol.* 158, 495–507. doi:10.1242/jeb.158.1.495

Ahearn, G. A. (1996). The invertebrate electrogenic $2Na^+/1H^+$ exchanger: Polyfunctional workstation. *Physiology* 11, 31–35. doi:10.1152/physiologyonline.1996.11.1.31

Ahearn, G. A., Zhuang, Z., Duerr, J., and Pennington, V. (1994). Role of the invertebrate electrogenic $2Na^+/1H^+$ antiporter in monovalent and divalent cation transport. *J. Exp. Biol.* 196, 319–335. doi:10.1242/jeb.196.1.319

Aladin, N. V., and Potts, T. W. (1995). Osmoregulatory capacity of the cladocera. *J. Comp. Physiol. B* 164, 671–683. doi:10.1007/bf00389810

Allendorf, F., and Thorgaard, G. H. (1984). "Tetraploidy and the evolution of salmonid fishes," in *Evolutionary genetics of fishes*. Editor B. Turner (New York: Plenum publishing), 1–53.

Anger, K. (2016). "Adaptation to life in fresh water by decapod crustaceans: Evolutionary challenges in the early life-history stages," in *A global overview of the conservation of freshwater decapod Crustaceans*. Editors T. Kawai and N. Cumberlidge (Cham, Switzerland: Springer), 127–168.

Anger, K. (2003). Salinity as a key parameter in the larval biology of decapod crustaceans. *Invertebr. Reprod. Dev.* 43, 29–45. doi:10.1080/07924259.2003.9652520

Anger, K. (2001). The biology of decapod crustacean larvae. *Crustac. Issues* 14, 1–420.

Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J.-M., Dehal, P., et al. (2002). Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science* 297, 1301–1310. doi:10.1126/science.1072104

Avella, M., and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* 142, 155–175. doi:10.1242/jeb.142.1.155

Barneaud-Rocca, D., Borgese, F., and Guizouarn, H. (2011). Dual transport properties of anion exchanger 1: The same transmembrane segment is involved in anion exchange and in a cation leak. *J. Biol. Chem.* 286, 8909–8916. doi:10.1074/jbc.M110.166819

Beyenbach, K. W. (2001). Energizing epithelial transport with the vacuolar H^+-ATPase. *News Physiol. Sci.* 16, 145–151. doi:10.1152/physiologyonline.2001.16.4.145

Beyenbach, K. W., and Piermarini, P. M. (2008). "Osmotic and ionic regulation in Insects," in *Osmotic and ionic regulation: Cells and animals*. Editor D. H. Evans (Boca Raton: CRC Press), 231–293.

Beyenbach, K. W., Skaer, H., and Dow, J. A. T. (2010). The developmental, molecular, and transport biology of Malpighian tubules. *Annu. Rev. Entomol.* 55, 351–374. doi:10.1146/annurev-ento-112408-085512

Beyenbach, K. W. (2016). The plasticity of extracellular fluid homeostasis in insects. *J. Exp. Biol.* 219, 2596–2607. doi:10.1242/jeb.129650

Beyenbach, K. W., and Wieczorek, H. (2006). The V-type H^+-ATPase: Molecular structure and function, physiological roles and regulation. *J. Exp. Biol.* 209, 577–589. doi:10.1242/jeb.02014

Bianchini, A., Péqueux, A., and Gilles, R. (1988). Effects of TAP and DPC on the transepithelial potential difference of isolated perfused gills on the freshwater acclimated crab, *Eriocheir sinensis*. *Comp. Biochem. Physiology Part A Physiology* 90, 315–319. doi:10.1016/0300-9629(88)91122-x

Bianchini, A., and Wood, C. M. (2008). Sodium uptake in different life stages of crustaceans: The water flea *Daphnia magna* strauss. *J. Exp. Biol.* 211, 539–547. doi:10.1242/jeb.009175

Blondeau-Bidet, E., Hiroi, J., and Lorin-Nebel, C. (2019). Ion uptake pathways in European sea bass *Dicentrarchus labrax*. *Gene* 692, 126–137. doi:10.1016/j.gene.2019.01.006

Bossus, M., Charmantier, G., Blondeau-Bidet, E., Valletta, B., Boulo, V., and Lorin-Nebel, C. (2013). The ClC-3 chloride channel and osmoregulation in the European sea bass, *Dicentrarchus labrax*. *J. Comp. Physiol. B* 183, 641–662. doi:10.1007/s00360-012-0737-9

Boudour-Boucheker, N., Boulo, V., Charmantier-Daures, M., Anger, K., Charmantier, G., and Lorin-Nebel, C. (2016). Osmoregulation in larvae and juveniles of two recently separated *Macrobrachium* species: Expression patterns of ion transporter genes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 195, 39–45. doi:10.1016/j.cbpa.2016.02.005

Boudour-Boucheker, N., Boulo, V., Charmantier-Daures, M., Grousset, E., Anger, K., Charmantier, G., et al. (2014). Differential distribution of V-type H^+-ATPase and Na^+/K^+-ATPase in the branchial chamber of the palaemonid shrimp *Macrobrachium amazonicum*. *Cell Tissue Res.* 357, 195–206. doi:10.1007/s00441-014-1845-5

Boxshall, G. A., and Jaume, D. (2009). Exopodites, epipodites and gills in Crustaceans. *Arthropod Syst. phylo.* 67, 229–254.

Bradley, T. J. (2009). *Animal osmoregulation*. Oxford: Oxford University Press.

Bradley, T. J. (1987). Physiology of osmoregulation in mosquitoes. *Annu. Rev. Entomol.* 32, 439–462. doi:10.1146/annurev.en.32.010187.002255

Bradley, T. J. (1994). "The role of physiological capacity, morphology, and phylogeny in determining habitat use in mosquitoes," in *Ecological morphology: Integrative organismal biology*. Editors P. C. Wainwright and S. M. Reilly (Chicago: University of Chicago Press).

Bradley, T. J., Briscoe, A. D., Brady, S. G., Contreras, H. L., Danforth, B. N., Dudley, R., et al. (2009). Episodes in insect evolution. *Integr. Comp. Biol.* 49, 590–606. doi:10.1093/icb/icp043

Brett, C. L., Donowitz, M., and Rao, R. (2005). Evolutionary origins of eukaryotic sodium/proton exchangers. *Am. J. Physiol. Cell Physiol.* 288, C223–C239. doi:10.1152/ajpcell.00360.2004

Breves, J. P., Nelson, N. N., Koltenyuk, V., Petro-Sakuma, C. K., Celino-Brady, F. T., and Seale, A. P. (2021). Enhanced expression of ncc1 and clc2c in the kidney and urinary bladder accompanies freshwater acclimation in Mozambique tilapia. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 260, 111021. doi:10.1016/j.cbpa.2021.111021

Breves, J. P., Starling, J. A., Popovski, C. M., Doud, J. M., and Tipsmark, C. K. (2020). Salinity-dependent expression of ncc2 in opercular epithelium and gill of mummichog (*Fundulus heteroclitus*). *J. Comp. Physiol. B* 190, 219–230. doi:10.1007/s00360-020-01260-x

Bury, N. R., and Wood, C. M. (1999). Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. *Am. J. Physiol.* 277, R1385–R1391. doi:10.1152/ajpregu.1999.277.5.R1385

Chambrey, R., Kurth, I., Peti-Peterdi, J., Houillier, P., Purkerson, J. M., Leviel, F., et al. (2013). Renal intercalated cells are rather energized by a proton than a sodium pump. *Proc. Natl. Acad. Sci. U. S. A.* 110, 7928–7933. doi:10.1073/pnas.1221496110

Chang, W.-J., Wang, Y.-F., Hu, H.-J., Wang, J.-H., Lee, T.-H., and Hwang, P.-P. (2013). Compensatory regulation of Na^+ absorption by Na^+/H^+ exchanger and Na^+-Cl^- cotransporter in zebrafish (*Danio rerio*). *Front. Zool.* 10, 46. doi:10.1186/1742-9994-10-46

Charmantier, G., and Anger, K. (2011). Ontogeny of osmoregulatory patterns in the South American shrimp *Macrobrachium amazonicum*: Loss of hypo-regulation in a land-locked population indicates phylogenetic separation from estuarine ancestors. *J. Exp. Mar. Biol. Ecol.* 396, 89–98. doi:10.1016/j.jembe.2010.10.013

Charmantier, G., Charmantier-Daures, M., and Anger, K. (1998). Ontogeny of osmoregulation in the grapsid crab *Armases miersii* (Crustacea, Decapoda). *Mar. Ecol. Prog. Ser.* 164, 285–292. doi:10.3354/meps164285

Charmantier, G., and Charmantier-Daures, M. (1994). Ontogeny of osmoregulation and salinity tolerance in the isopod crustacean *Sphaeroma serratum*. *Mar. Ecol. Prog. Ser.* 114, 93–102. doi:10.3354/meps114093

Charmantier, G., and Charmantier-Daures, M. (2001). Ontogeny of osmoregulation in crustaceans: The embryonic phase. *Am. Zool.* 41, 1078–1089. doi:10.1093/icb/41.5.1078

Charmantier, G., Charmantier-Daures, M., and Towle, D. (2009). "Osmotic and ionic regulation in aquatic arthropods," in *Osmotic and ionic regulation. Cells and animals*. Editor D. H. Evans (Boca Raton, FL, Oxford, New York: CRC Press), 165–230.

Charmantier, G., Haond, C., Lignot, J. H., and Charmantier-Daures, M. (2001). Ecophysiological adaptation to salinity throughout a life cycle: A review in homarid lobsters. *J. Exp. Biol.* 204, 967–977. doi:10.1242/jeb.204.5.967

Charmantier, G. (1998). Ontogeny of osmoregulation in crustaceans: A review. *Invertebr. Reprod. Dev.* 33, 177–190. doi:10.1080/07924259.1998.9652630

Chen, J. Y., Vannier, J., and Huang, D. Y. (2001). The origin of crustaceans: New evidence from the early cambrian of China. *Proc. Biol. Sci.* 268, 2181–2187. doi:10.1098/rspb.2001.1779

Chen, X., Qiu, L., Li, M., Dürrnagel, S., Orser, B. A., Xiong, Z.-G., et al. (2010). Diarylamidines: High potency inhibitors of acid-sensing ion channels. *Neuropharmacology* 58, 1045–1053. doi:10.1016/j.neuropharm.2010.01.011

Chintapalli, V. R., Kato, A., Henderson, L., Hirata, T., Woods, D. J., Overend, G., et al. (2015). Transport proteins NHA1 and NHA2 are essential for survival, but have distinct transport modalities. *Proc. Natl. Acad. Sci. U. S. A.* 112, 11720–11725. doi:10.1073/pnas.1508031112

Chung, S., Chavez, C., and Andrew, D. J. (2011). Trachealess (Trh) regulates all tracheal genes during *Drosophila* embryogenesis. *Dev. Biol.* 360, 160–172. doi:10.1016/j.ydbio.2011.09.014

Cieluch, U., Anger, K., Aujoulat, F., Buchholz, F., Charmantier-Daures, M., and Charmantier, G. (2004). Ontogeny of osmoregulatory structures in the green crab *Carcinus maenas* (Crustacea, Decapoda). *J. Exp. Biol.* 207, 325–336. doi:10.1242/jeb.00759

Cieluch, U., Charmantier, G., Grousset, E., Charmantier-Daures, M., and Anger, K. (2005). Osmoregulation, immunolocalization of Na^+/K^+-ATPase, and ultrastructure of branchial epithelia in the developing brown shrimp, Crangon crangon (Decapoda, Caridea). *Physiol. Biochem. Zool.* 78 (6), 1017–1025. doi:10.1086/432856

Cieluch, U., Anger, K., Charmantier-Daures, M., and Charmantier, G. (2007). Osmoregulation and immunolocalization of Na^+/K^+-ATPase during the ontogeny of the mitten crab, *Eriocheir sinensis* (Decapoda, Grapsoidea). *Mar. Ecol. Prog. Ser.* 329, 169–178. doi:10.3354/meps329169

Claiborne, J. B., Blackston, C. R., Choe, K. P., Dawson, D. C., Harris, S. P., Mackenzie, L. A., et al. (1999). A mechanism for branchial acid excretion in marine fish: Identification of multiple Na^+/H^+ antiporter (NHE) isoforms in gills of two seawater teleosts. *J. Exp. Biol.* 202, 315–324. doi:10.1242/jeb.202.3.315

Clifford, A. M., Tresguerres, M., Goss, G. G., and Wood, C. M. (2022). A novel K^+-dependent Na^+ uptake mechanism during low pH exposure in adult zebrafish (*Danio rerio*): New tricks for old dogma. *Acta Physiol.* 234, e13777. doi:10.1111/apha.13777

Dalziel, A. C., Bittman, J., Mandic, M., Ou, M., and Schulte, P. M. (2014). Origins and functional diversification of salinity-responsive Na , K ATPase alpha1 paralogs in salmonids. *Mol. Ecol.* 11, 12828. doi:10.1111/mec.12828

Day, J. P., Wan, S., Allan, A. K., Davies, S. A., Gray, J. V., Dow, J. A., et al. (2008). Identification of two partners from the bacterial Kef exchanger family for the apical plasma membrane V-ATPase of Metazoa. *J. Cell Sci.* 121, 2612–2619. doi:10.1242/jcs.033084

Dayras, P., Charmantier, G., Chaumot, A., Vigneron, A., Coquery, M., Quéau, H., et al. (2017). Osmoregulatory responses in reference and historically metal contaminated *Gammarus fossarum* (Crustacea, Amphipoda) populations. *Chemosphere* 180, 412–422. doi:10.1016/j.chemosphere.2017.04.016

DeFelice, L. J., and Goswami, T. (2007). Transporters as channels. *Annu. Rev. Physiol.* 69, 87–112. doi:10.1146/annurev.physiol.69.031905.164816

Del Duca, O., Nasirian, A., Galperin, V., and Donini, A. (2011). Pharmacological characterisation of apical Na^+ and Cl^- transport mechanisms of the anal papillae in the larval mosquito *Aedes aegypti*. *J. Exp. Biol.* 214, 3992–3999. doi:10.1242/jeb.063719

Demaurex, N., Orlowski, J., Brisseau, G., Woodside, M., and Grinstein, S. (1995). The mammalian Na^+/H^+ antiporters NHE-1, NHE-2, and NHE-3 are electroneutral and voltage independent, but can couple to an H^+ conductance. *J. Gen. Physiol.* 106, 85–111. doi:10.1085/jgp.106.1.85

Denholm, B. (2013). Shaping up for action: The path to physiological maturation in the renal tubules of *Drosophila*. *Organogenesis* 9, 40–54. doi:10.4161/org.24107

Desvignes, T., Sydes, J., Montfort, J., Bobe, J., and Postlethwait, J. H. (2021). Evolution after whole-genome duplication: Teleost MicroRNAs. *Mol. Biol. Evol.* 38, 3308–3331. doi:10.1093/molbev/msab105

Donini, A., Gaidhu, M. P., Strasberg, D. R., and O'Donnell, M. J. (2007). Changing salinity induces alterations in hemolymph ion concentrations and Na^+ and Cl^- transport kinetics of the anal papillae in the larval mosquito, *Aedes aegypti*. *J. Exp. Biol.* 210, 983–992. doi:10.1242/jeb.02732

Donini, A., and O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: Application of self-referencing ion-selective microelectrodes. *J. Exp. Biol.* 208, 603–610. doi:10.1242/jeb.01422

Durant, A. C., Grieco Guardian, E., Kolosov, D., and Donini, A. (2021). The transcriptome of anal papillae of *Aedes aegypti* reveals their importance in xenobiotic detoxication and adds signicant knowledge on ion, water and ammonia transport mechanisms. *J. Insect Physiol.* 132, 104269. doi:10.1016/j.jinsphys.2021.104269

Dymowska, A. K., Boyle, D., Schultz, A. G., and Goss, G. G. (2015). The role of acid-sensing ion channels in epithelial Na^+ uptake in adult zebrafish (*Danio rerio*). *J. Exp. Biol.* 218, 1244–1251. doi:10.1242/jeb.113118

Dymowska, A. K., Hwang, P.-P., and Goss, G. G. (2012). Structure and function of ionocytes in the freshwater fish gill. *Respir. Physiol. Neurobiol.* 184, 282–292. doi:10.1016/j.resp.2012.08.025

Dymowska, A. K., Schultz, A. G., Blair, S. D., Chamot, D., and Goss, G. G. (2014). Acid-sensing ion channels are involved in epithelial Na^+ uptake in the rainbow trout *Oncorhynchus mykiss*. *Am. J. Physiol. Cell Physiol.* 307, C255–C265. doi:10.1152/ajpcell.00398.2013

Edwards, S. L., Tse, C. M., and Toop, T. (1999). Immunolocalisation of NHE3-like immunoreactivity in the gills of the rainbow trout (*Oncorhynchus mykiss*) and the blue-throated wrasse (*Pseudolabrus tetrious*). *J. Anat.* 195, 465–469. doi:10.1046/j.1469-7580.1999.19530465.x

Edwards, S. L., Wall, B. P., Morrison-Shetlar, A., Sligh, S., Weakley, J. C., and Claiborne, J. B. (2005). The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). *J. Exp. Zool. A, Comp. Exp. Biol.* 303, 464–475. doi:10.1002/jez.a.175

Ehrenfeld, J., Garcia Romeu, F., and Harvey, B. J. (1985). Electrogenic active proton pump in *Rana esculenta* skin and its role in sodium ion transport. *J. Physiol.* 359, 331–355. doi:10.1113/jphysiol.1985.sp015588

Engel, M. S., and Grimaldi, D. A. (2004). New light shed on the oldest insect. *Nature* 427, 627–630. doi:10.1038/nature02291

Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K., and Hirose, S. (2007). Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R470–R480. doi:10.1152/ajpregu.00200.2006

Evans, D. H., and Claiborne, J. B. (2009). "Osmotic and ionic regulation in fishes," in *Osmotic and ionic regulation. Cells and animals*. Editor D. H. Evans (Boca Raton, FL, Oxford, New York: CRC Press), 295–366.

Evans, D. H. (2011). Freshwater fish gill ion transport: August Krogh to morpholinos and microprobes. *Acta Physiol.* 202, 349–359. doi:10.1111/j.1748-1716.2010.02186.x

Evans, D. H. (2009). *Osmotic and ionic regulation. Cells and animals*. Boca Raton, FL, Oxford, New York: CRC Press.

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97–177. doi:10.1152/physrev.00050.2003

Faleiros, R. O., Furriel, R. P. M., and McNamara, J. C. (2017). Transcriptional, translational and systemic alterations during the time course of osmoregulatory acclimation in two palaemonid shrimps from distinct osmotic niches. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 212, 97–106. doi:10.1016/j.cbpa.2017.07.014

Faleiros, R. O., Goldman, M. H. S., Furriel, R. P. M., and McNamara, J. C. (2010). Differential adjustment in gill Na^+/K^+- and V-ATPases activities and transporter mRNA expression during osmoregulatory acclimation in the cinnamon shrimp *Macrobrachium amazonicum* (Decapoda, Palaemonidae). *J. Exp. Biol.* 213, 3894–3905. doi:10.1242/jeb.046870

Fenwick, J. C., Wendelaar Bonga, S. E., and Flik, G. (1999). *In vivo* bafilomycin-sensitive $Na^{(+)}$ uptake in young freshwater fish. *J. Exp. Biol.* 202, 3659–3666. doi:10.1242/jeb.202.24.3659

Franch-Marro, X., Martín, N., Averof, M., and Casanova, J. (2006). Association of tracheal placodes with leg primordia in *Drosophila* and implications for the origin of insect tracheal systems. *Development* 133, 785–790. doi:10.1242/dev.02260

Freire, C. A., and McNamara, J. C. (1995). Fine structure of the gills of the freshwater shrimp *Macrobrachium olfersii* (Decapoda): Effect of acclimation to high salinity medium and evidence for involvement of the lamellar septum in ion uptake. *J. Crustac. Biol.* 15, 103–116. doi:10.2307/1549015

Freire, C. A., Onken, H., and McNamara, J. C. (2008). A structure-function analysis of ion transport in crustacean gills and excretory organs. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 151, 272–304. doi:10.1016/j.cbpa.2007.05.008

Fuentes, J., and Eddy, F. B. (1997). "Drinking in marine, euryhaline and freshwater teleost fish," in *Ionic regulation in animals: A tribute to professor W.T.W. Potts*. Editors N. Hazon, F. B. Eddy, and G. Flik (Berlin, Heidelberg: Springer Berlin Heidelberg), 135–149.

Fujisawa, M., Ito, M., and Krulwich, T. A. (2007). Three two-component transporters with channel-like properties have monovalent cation/proton antiport activity. *Proc. Natl. Acad. Sci. U. S. A.* 104, 13289–13294. doi:10.1073/pnas.0703709104

Galvez, F., Reid, S. D., Hawkings, G., and Goss, G. (2002). Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 282, R658–R668. doi:10.1152/ajpregu.00342.2001

García Romeu, F., and Maetz, J. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus* : I. Evidence for an independent uptake of sodium and chloride ions. *J. Gen. Physiol.* 47, 1195–1207. doi:10.1085/jgp.47.6.1195

Garrouste, R., Clément, G., Nel, P., Engel, M. S., Grandcolas, P., D'Haese, C., et al. (2012). A complete insect from the Late Devonian period. *Nature* 488, 82–85. doi:10.1038/nature11281

Garty, H., and Palmer, L. G. (1997). Epithelial sodium channels: Function, structure, and regulation. *Physiol. Rev.* 77, 359–396. doi:10.1152/physrev.1997.77.2.359

Genovese, G., Ortiz, N., Urcola, M. R., and Luquet, C. M. (2005). Possible role of carbonic anhydrase, V-H^+-ATPase and Cl^-/HCO_3^- exchanger in electrogenic ion transport across the gills of the euryhaline crab *Chasmagnathus granulatus*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 142, 362–369. doi:10.1016/j.cbpa.2005.08.024

Georgalis, T., Perry, S. F., and Gilmour, K. M. (2006). The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 209, 518–530. doi:10.1242/jeb.02018

Gerber, L., Lee, C. E., Grousset, E., Blondeau-Bidet, E., Boudour-Boucheker, N., Charmantier-Daures, M., et al. (2016). The legs have it: *In situ* expression of ion transporters H^+-ATPase and Na^+/K^+-ATPase in the osmoregulatory leg organs of the invading copepod *Eurytemora affinis*. *Physiol. Biochem. Zool.* 89, 233–250. doi:10.1086/686323

Glenner, H., Thomsen, P. F., Hebsgaard, M. B., Sørensen, M. V., and Willerslev, E. (2006). Evolution. The origin of insects. *Science* 314, 1883–1884. doi:10.1126/science.1129844

Goss, G. G., Perry, S. F., Fryer, J. N., and Laurent, P. (1998). Gill morphology and acid-base regulation in freshwater fishes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 119, 107–115. doi:10.1016/s1095-6433(97)00401-7

Grimaldi, D. A., and Engel, M. S. (2005). *Evolution of the insects*. New York: Cambridge University Press.

Grosell, M., and Wood, C. M. (2002). Copper uptake across rainbow trout gills: Mechanisms of apical entry. *J. Exp. Biol.* 205, 1179–1188. doi:10.1242/jeb.205.8.1179

Guh, Y. J., and Hwang, P. P. (2017). Insights into molecular and cellular mechanisms of hormonal actions on fish ion regulation derived from the zebrafish model. *Gen. Comp. Endocrinol.* 251, 12–20. doi:10.1016/j.ygcen.2016.08.009

Guh, Y. J., Lin, C. H., and Hwang, P. P. (2015). Osmoregulation in zebrafish: Ion transport mechanisms and functional regulation. *EXCLI J.* 14, 627–659. doi:10.17179/excli2015-246

Gutekunst, J., Andriantsoa, R., Falckenhayn, C., Hanna, K., Stein, W., Rasamy, J., et al. (2018). Clonal genome evolution and rapid invasive spread of the marbled crayfish. *Nat. Ecol. Evol.* 2, 567–573. doi:10.1038/s41559-018-0467-9

Harris, R. R., and Aladin, N. V. (1997). "The ecophysiology of osmoregulation in Crustacea," in *Ionic regulation in animals: A tribute to professor W.T.W. Potts*. Editors N. Hazon, F. B. Eddy, and G. Flik (Berlin: Springer), 1–25.

Harris, R. R., and Micaleff, H. (1971). Osmotic and ionic regulation in *Potamon edulis*, a fresh-water crab from Malta. *Comp. Biochem. Physiology Part A Physiology* 38, 769–776. doi:10.1016/0300-9629(71)90218-0

Harvey, W. R., Boudko, D. Y., Rheault, M. R., and Okech, B. A. (2009). Nhevnat: An H^+ V-ATPase electrically coupled to a Na^+:nutrient amino acid transporter (NAT) forms an Na^+/H^+ exchanger (NHE). *J. Exp. Biol.* 212, 347–357. doi:10.1242/jeb.026047

Henry, R. P., Lucu, C., Onken, H., and Weihrauch, D. (2012). Multiple functions of the crustacean gill: Osmotic/ionic regulation, acid-base balance, ammonia excretion and bioaccumulation of toxic metals. *Front. Physiol.* 3, 431–463. doi:10.3389/fphys.2012.00431

Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu. Rev. Physiol.* 58, 523–538. doi:10.1146/annurev.ph.58.030196.002515

Henry, R. P., and Wheatly, M. G. (1988). Dynamics of salinity adaptations in the euryhaline crayfish *Pacifastacus leniusculus*. *Physiol. Zool.* 61, 260–271. doi:10.1086/physzool.61.3.30161239

Hickman, C., and Trump, B. (1969). "The kidney," in *Fish physiology*. Editors W. S. Hoar and D. J. Randall (New York: Academic Press), 91–239.

Hirata, T., Kaneko, T. O., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., et al. (2003). Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284, R1199–R1212. doi:10.1152/ajpregu.00267.2002

Hiroi, J., and McCormick, S. D. (2012). New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. *Respir. Physiol. Neurobiol.* 184, 257–268. doi:10.1016/j.resp.2012.07.019

Hiroi, J., McCormick, S. D., Ohtani-Kaneko, R., and Kaneko, T. (2005). Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel. *J. Exp. Biol.* 208, 2023–2036. doi:10.1242/jeb.01611

Hiroi, J., Yasumasu, S., McCormick, S. D., Hwang, P.-P., and Kaneko, T. (2008). Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. *J. Exp. Biol.* 211, 2584–2599. doi:10.1242/jeb.018663

Holmes, W. N., and Donaldson, E. M. (1969). in *The body compartments and the distribution of electrolytes" in: Fish Physiology*. Editors W. S. Hoar and D. J. Randall (New York: Academic Press), 1–89.

Horng, J.-L., Hwang, P.-P., Shih, T.-H., Wen, Z.-H., Lin, C.-S., and Lin, L.-Y. (2009). Chloride transport in mitochondrion-rich cells of euryhaline tilapia (*Oreochromis mossambicus*) larvae. *Am. J. Physiol. Cell Physiol.* 292, C845–C854. doi:10.1152/ajpcell.00218.2009

Horng, J.-L., Lin, L.-Y., Huang, C.-J., Katoh, F., Kaneko, T., and Hwang, P.-P. (2007). Knockdown of V-ATPase subunit A (*atp6v1a*) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292, R2068–R2076. doi:10.1152/ajpregu.00578.2006

Horng, J.-L., Yu, L. L., Liu, S. T., Chen, P.-Y., and Lin, L.-Y. (2017). Potassium regulation in medaka (*Oryzias latipes*) larvae acclimated to fresh water: Passive uptake and active secretion by the skin cells. *Sci. Rep.* 7, 16215. doi:10.1038/s41598-017-16381-7

Hsu, H.-H., Lin, L.-Y., Tseng, Y.-C., Horng, J.-L., and Hwang, P.-P. (2014). A new model for fish ion regulation: Identification of ionocytes in freshwater- and seawater-acclimated medaka (*Oryzias latipes*). *Cell Tissue Res.* 357, 225–243. doi:10.1007/s00441-014-1883-z

Huang, C.-H., and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci. U. S. A.* 102, 15512–15517. doi:10.1073/pnas.0507886102

Hung, C. Y. C., Tsui, K. N. T., Wilson, J. M., Nawata, M., Wood, C. M., and Wright, P. A. (2007). Molecular cloning, characterization and tissue distribution of the Rhesus glycoproteins Rhbg, Rhcg1 and Rhcg2 in the mangrove killifish Rivulus marmoratus exposed to elevated environmental ammonia levels. *J. Exp. Biol.* 210, 2419–2429.

Hutchinson, G. E. (1957). *A treatise on limnology*. New York: John Wiley.

Hwang, P.-P., and Chou, M. Y. (2013). Zebrafish as an animal model to study ion homeostasis. *Pflugers Arch.* 465, 1233–1247. doi:10.1007/s00424-013-1269-1

Hwang, P.-P. (2009). Ion uptake and acid secretion in zebrafish (*Danio rerio*). *J. Exp. Biol.* 212, 1745–1752. doi:10.1242/jeb.026054

Hwang, P.-P., and Lee, T.-H. (2007). New insights into fish ion regulation and mitochondrion-rich cells. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 148, 479–497. doi:10.1016/j.cbpa.2007.06.416

Hwang, P.-P., Lee, T. H., and Lin, L. Y. (2011). Ion regulation in fish gills: Recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301, R28–R47. doi:10.1152/ajpregu.00047.2011

Hwang, P.-P. (2011). "Osmotic, ionic and nitrogenous-waste balance : Mechanisms of ion transport in freshwater fishes," in *Encyclopedia of fish physiology,: From genome to environment*. Editors A. P. Farrell and Amsterdam (Academic Press), 1359–1365.

Hwang, P.-P., and Perry, S. F. (2010). "Ionic and acid–base regulation," in *Fish physiology*. Editors S. F. Perry, M. Ekker, A. P. Farrell, and C. J. Brauner (Academic Press), 29, 311–344.

Inokuchi, M., Hiroi, J., and Kaneko, T. (2022). Why can Mozambique tilapia acclimate to both freshwater and seawater? Insights from the plasticity of ionocyte functions in the euryhaline teleost. *Front. Physiol.* 13, 914277. doi:10.3389/fphys.2022.914277

Inokuchi, M., Hiroi, J., Watanabe, S., Hwang, P.-P., and Kaneko, T. (2009). Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. *J. Exp. Biol.* 212, 1003–1010. doi:10.1242/jeb.025957

Inokuchi, M., Hiroi, J., Watanabe, S., Lee, K. M., and Kaneko, T. (2008). Gene expression and morphological localization of NHE3, NCC and NKCC1a in branchial mitochondria-rich cells of Mozambique tilapia (*Oreochromis mossambicus*) acclimated to a wide range of salinities. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 151, 151–158. doi:10.1016/j.cbpa.2008.06.012

Inokuchi, M., Nakamura, M., Miyanishi, H., Hiroi, J., and Kaneko, T. (2017). Functional classification of gill ionocytes and spatiotemporal changes in their distribution after transfer from seawater to freshwater in Japanese seabass. *J. Exp. Biol.* 220, 4720–4732. doi:10.1242/jeb.167320

Ito, Y., Kato, A., Hirata, T., Hirose, S., and Romero, M. F. (2014). Na^+/H^+ and $Na^+/NH4^+$ activities of zebrafish NHE3b expressed in *Xenopus* oocytes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 306, R315–R327. doi:10.1152/ajpregu.00363.2013

Ito, Y., Kobayashi, S., Nakamura, N., Miyagi, H., Esaki, M., Hoshijima, K., et al. (2013). Close association of carbonic anhydrase (CA2a and CA15a), $Na^{(+)}/H^{(+)}$ exchanger (Nhe3b), and ammonia transporter Rhcg1 in zebrafish ionocytes responsible for $Na^{(+)}$ uptake. *Front. Physiol.* 4, 59. doi:10.3389/fphys.2013.00059

Ivanis, G., Esbaugh, A. J., and Perry, S. F. (2008). Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 211, 2467–2477. doi:10.1242/jeb.017491

Jacquillet, G., Rubera, I., and Unwin, R. J. (2011). Potential role of serine proteases in modulating renal sodium transport *in vivo*. *Nephron. Physiol.* 119, 22–29. doi:10.1159/000328926

Jaillon, O., Aury, J. M., Brunet, F., Petit, J. L., Stange-Thomann, N., Mauceli, E., et al. (2004). Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* 431, 946–957. doi:10.1038/nature03025

Johnson, K. E., Perreau, L., Charmantier, G., Charmantier-Daures, M., and Lee, C. E. (2014). Without gills: Localization of osmoregulatory function in the copepod *Eurytemora affinis*. *Physiol. Biochem. Zool.* 87, 310–324. doi:10.1086/674319

Kalsi, M., Gillen, C., and Piermarini, P. M. (2019). Heterologous expression of *Aedes aegypti* cation chloride cotransporter 2 (aeCCC2) in *Xenopus laevis* Oocytes Induces an enigmatic Na^+/Li^+ conductance. *Insects* 10, 1–11. doi:10.3390/insects10030071

Kamemoto, F. I., and Tullis, R. E. (1972). Hydromineral regulation in decapod crustacea. *Gen. Comp. Endocrinol.* 3, 299–307. doi:10.1016/0016-6480(72)90159-1

Kang, C.-K., Tsai, H.-J., Liu, C.-C., Lee, T.-H., and Hwang, P.-P. (2010). Salinity-dependent expression of a Na^+, K^+, $2Cl^-$ cotransporter in gills of the brackish medaka *Oryzias dancena*: A molecular correlate for hyposmoregulatory endurance. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 157, 7–18. doi:10.1016/j.cbpa.2010.05.013

Karnaky, K. J., Jr., Kinter, L. B., Kinter, W. B., and Stirling, C. E. (1976). Teleost chloride cell. II. Autoradiographic localization of gill Na, K-ATPase in killifish *Fundulus heteroclitus* adapted to low and high salinity environments. *J. Cell Biol.* 70, 157–177. doi:10.1083/jcb.70.1.157

Katoh, F., Cozzi, R. R. F., Marshall, W. S., and Goss, G. G. (2008). Distinct $Na^+/K^+/2Cl^-$ cotransporter localization in kidneys and gills of two euryhaline species, rainbow trout Cell, and killifish. *Cell Tiss. Res.* 334, 265–281. doi:10.1007/s00441-008-0679-4

Katoh, F., Hyodo, S., and Kaneko, T. (2003). Vacuolar-type proton pump in the basolateral plasma membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment. *J. Exp. Biol.* 206, 793–803. doi:10.1242/jeb.00159

Kawai, T., and Cumberlidge, N. (2016). *A global overview of the conservation of freshwater decapod Crustaceans*. Cham, Switzerland: Springer.

Kellenberger, S., and Schild, L. (2002). Epithelial sodium channel/degenerin family of ion channels: A variety of functions for a shared structure. *Physiol. Rev.* 82, 735–767. doi:10.1152/physrev.00007.2002

Kerstetter, T. H., Kirschner, L. B., and Rafuse, D. (1970). On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). *J. Gen. Physiol.* 56, 342–359. doi:10.1085/jgp.56.3.342

Khodabandeh, S., Charmantier, G., Blasco, C., Grousset, E., and Charmantier-Daures, M. (2005a). Ontogeny of the antennal glands in the crayfish *Astacus leptodactylus* (Crustacea, Decapoda): Anatomical and cell differentiation. *Cell Tissue Res.* 319, 153–165. doi:10.1007/s00441-004-0982-7

Khodabandeh, S., Charmantier, G., and Charmantier-Daures, M. (2005c). Ultrastructural studies and Na^+K^+-ATPase immunolocalization in the antennal urinary glands of the lobster *Homarus gammarus* (Crustacea, Decapoda). *J. Histochem. Cytochem.* 53, 1203–1214. doi:10.1369/jhc.4A6540.2005

Khodabandeh, S., Kutnik, S., Aujoulat, F., Charmantier, G., and Charmantier-Daures, M. (2005b). Ontogeny of the antennal glands in the crayfish *Astacus leptodactylus* (Crustacea, Decapoda). Immunolocalization of Na^+K^+-ATPase. *Cell Tissue Res.* 319, 167–174. doi:10.1007/s00441-004-0970-y

Kimura, C., Ahearn, G. A., Busquets-Turner, L., Haley, S. R., Nagao, C., and De Couet, H. G. (1994). Immunolocalization of an antigen associated with the invertebrate electrogenic $2Na^+$-$1H^+$ antiporter. *J. Exp. Biol.* 189, 85–104. doi:10.1242/jeb.189.1.85

Kirschner, L. B. (2002). Sodium-proton exchange in crayfish. *Biochim. Biophys. Acta* 1566, 67–71. doi:10.1016/s0005-2736(02)00585-0

Kirschner, L. B. (2004). The mechanism of sodium chloride uptake in hyperregulating aquatic animals. *J. Exp. Biol.* 207, 1439–1452. doi:10.1242/jeb.00907

Kolosov, D., Bui, P., Chasiotis, H., and Kelly, S. P. (2013). Claudins in teleost fishes. *Tissue Barriers* 1, e25391. doi:10.4161/tisb.25391

Krogh, A. (1939). *Osmotic regulation in aquatic animals*. Cambridge: Cambridge University Press.

Krogh, A. (1937). Osmotic regulation in fresh water fishes by active absorption of chloride ions. *Z. Vergl. Physiol.* 24, 656–666. doi:10.1007/bf00592303

Krogh, A. (1938). The active absorption of ions in some freshwater animals. *Z. F. Vergl. Physiol.* 25, 335–350. doi:10.1007/bf00339641

Kumai, Y., and Perry, S. F. (2012). Mechanisms and regulation of Na^+ uptake by freshwater fish. *Respir. Physiol. Neurobiol.* 184, 249–256. doi:10.1016/j.resp.2012.06.009

Kwong, R. W. M., Kumai, Y., and Perry, S. F. (2014). The physiology of fish at low pH: The zebrafish as a model system. *J. Exp. Biol.* 217, 651–662. doi:10.1242/jeb.091603

Kwong, R. W. M., and Perry, S. F. (2016). A role for sodium-chloride cotransporters in the rapid regulation of ion uptake following acute environmental acidosis: New insights from the zebrafish model. *Am. J. Physiol. Cell Physiol.* 311, C931–C941. doi:10.1152/ajpcell.00180.2016

Larsen, E. H. B., Christoffersen, C., Jensen, L. J., Sorensen, J. B., and Willumsen, N. J. (1996). Role of mitochondria-rich cells in epithelial chloride uptake. *Exp. Physiol.* 81, 525–534. doi:10.1113/expphysiol.1996.sp003955

Larsen, E. H., Deaton, L. E., Onken, H., O'Donnell, M., Grosell, M., Dantzler, W. H., et al. (2014). Osmoregulation and excretion. *Compr. Physiol.* 4, 405–573. doi:10.1002/cphy.c130004

Lécher, P., Defaye, D., and Noel, P. (1995). Chromosomes and nuclear DNA of crustacea. *Invertebr. Reprod. Dev.* 27, 85–114. doi:10.1080/07924259.1995.9672440

Lee, C. E., and Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. *Trends Ecol. Evol.* 14, 284–288. doi:10.1016/S0169-5347(99)01596-7

Lee, C. E. (2021). Ion transporter gene families as physiological targets of natural selection during salinity transitions in a copepod. *Physiology* 36, 335–349. doi:10.1152/physiol.00009.2021

Lee, C. E., Kierkegaard, M., Gelembiuk, G. W., Eads, B. D., and Posavi, M. (2011). Pumping ions: Rapid parallel evolution of ionic regulation following habitat invasions. *Evolution* 65, 2229–2244. doi:10.1111/j.1558-5646.2011.01308.x

Lee, C. E., Posavi, M., and Charmantier, G. (2012). Rapid evolution of body fluid regulation following independent invasions into freshwater habitats. *J. Evol. Biol.* 25, 625–633. doi:10.1111/j.1420-9101.2012.02459.x

Lee, Y. C., Yan, J. J., Cruz, S. A., Horng, J. L., and Hwang, P.-P. (2011). Anion exchanger 1b, but not sodium-bicarbonate cotransporter 1b, plays a role in transport functions of zebrafish H^+-ATPase-rich cells. *Am. J. Physiol. Cell Physiol.* 300, C295–C307. doi:10.1152/ajpcell.00263.2010

Leguen, I., Le Cam, A., Montfort, J., Peron, S., and Fautrel, A. (2015). Transcriptomic analysis of trout gill ionocytes in fresh water and sea water using laser capture microdissection combined with microarray analysis. *PLoS One* 10, e0139938. doi:10.1371/journal.pone.0139938

Leone, F. A., Lucena, M. L., Garçon, D. P., Pinto, M. R., and McNamara, J. C. (2014). "Modulation by K+ plus NH4+ of microsomal (Na+, K+)-ATPase activity in selected ontogenetic stages of the diadromous river shrimp Macrobrachium amazonicum (Decapoda, Palaemonidae). *PLoS One* 9, e89625. doi:10.1371/journal.pone.0089625

Leone, F. A., Garçon, D. P., Lucena, M. L., Faleiros, R. O., Azevedo, S. V., Pinto, M., et al. (2015). Gill-specific (Na+, K+)-ATPase activity and α-subunit mRNA expression during low-salinity acclimation of the ornate blue crab Callinectes ornatus (Decapoda, Brachyura). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 186, 59–67. doi:10.1016/j.cbpb.2015.04.010

Leone, F. A., Lucena, M. L., Garçon, D. P., Pinto, M. R., and McNamara, J. C. (2017). "Gill ion transport ATPases and ammonia excretion in aquatic crustaceans," in *Acid-base balance and nitrogen excretion in invertebrates*. Editors D. Weihrauch and M. O'Donnell M (Switzerland: Springer), 61–107.

Leone, F. A., Masui, D. C., Bezerra, T. M. S., Garçon, D. P., Valenti, V. C., Augusto, A. S., et al. (2021). Kinetic analysis of gill (Na+, K+)-ATPase activity in selected ontogenetic stages of the Amazon River shrimp Macrobrachium amazonicum (Decapoda Palaemonidae): Interactions at ATP- and cationbinding sites.. *J. Membr. Biol.* 45, 201–215. doi:10.1007/s00232-012-9431-9

Levesque, B. M., Zhou, S., Shan, L., Johnston, P., Kong, Y., Degan, S., et al. (2007). NPAS1 regulates branching morphogenesis in embryonic lung. *Am. J. Respir. Cell Mol. Biol.* 36, 427–434. doi:10.1165/rcmb.2006-0314OC

L'Honoré, T., Farcy, E., Chatain, B., Gros, R., Ruelle, F., Hermet, S., et al. (2019). Are European sea bass as euryhaline as expected? Intraspecific variation in freshwater tolerance. *Mar. Biol.* 166, 102. doi:10.1007/s00227-019-3551-z

Liao, B.-K., Chen, R.-D., and Hwang, P.-P. (2009). Expression regulation of Na^+-K^+-ATPase α1-subunit subtypes in zebrafish gill ionocytes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 296, R1897–R1906. doi:10.1152/ajpregu.00029.2009

Lin, H., Pfeiffer, D., Vogl, A., Pan, J., and Randall, D. (1994). Immunolocalization of H^+-ATPase in the gills epithelia of rainbow trout. *J. Exp. Biol.* 195, 169–183. doi:10.1242/jeb.195.1.169

Lin, H., and Randall, D. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J. Exp. Biol.* 161, 119–134. doi:10.1242/jeb.161.1.119

Lin, L.-Y., Horng, J.-L., Kunkel, J. G., and Hwang, P.-P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. *Am. J. Physiol. Cell Physiol.* 290, C371–C378. doi:10.1152/ajpcell.00281.2005

Lin, T. Y., Liao, B. K., Horng, J. L., Yan, J. J., Hsiao, C. D., and Hwang, P.-P. (2008). Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na^+ uptake in zebrafish H^+-ATPase-rich cells. *Am. J. Physiol. Cell Physiol.* 294, C1250–C1260. doi:10.1152/ajpcell.00021.2008

Little, C. (1983). *The colonisation of land: Origins and adaptations of terrestrial animals*. Cambridge, New York: Cambridge University Press.

Little, C. (1990). *The terrestrial invasion: An ecophysiological approach to the origins of land animals*. Cambridge, New York: Cambridge University Press.

Loose, G., Vogt, G., Charmantier-Daures, M., Charmantier, G., and Harzsch, S. (2020). "Organogenesis," in The natural History of the Crustacea. *Vol. 7 developmental biology and larval ecology*. Editors K. Anger, S. Harzsch, and M. Thiel (Oxford, New York: University Press), 79–111.

Lorin-Nebel, C., Boulo, V., Bodinier, C., and Charmantier, G. (2006). The Na^+/K^+/$2Cl^-$ cotransporter in the sea-bass *Dicentrarchus labrax* during ontogeny: Involvement in osmoregulation. *J. Exp. Biol.* 209, 4908–4922. doi:10.1242/jeb.02591

Lozano-Fernandez, J., Carton, R., Tanner, A. R., Puttick, M. N., Blaxter, M., Vinther, J., et al. (2016). A molecular palaeobiological exploration of arthropod terrestrialization. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 371, 20150133. doi:10.1098/rstb.2015.0133

Lucena, M. N., Pinto, M. R., Garçon, D. P., McNamara, J. C., and Leone, F. A. (2015). A kinetic characterization of the gill $V(H^+)$-ATPase in juvenile and adult *Macrobrachium amazonicum*, a diadromous palaemonid shrimp. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 181, 15–25. doi:10.1016/j.cbpb.2014.11.002

Lucu, C., and Towle, D. W. (2010). Characterization of ion transport in the isolated epipodite of the lobster *Homarus americanus*. *J. Exp. Biol.* 213, 418–425. doi:10.1242/jeb.033860

Luquet, C. M., Weihrauch, D., Senek, M., and Towle, D. W. (2005). Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*. *J. Exp. Biol.* 208, 3627–3636. doi:10.1242/jeb.01820

Madsen, S. S., Bollinger, R. J., Brauckhoff, M., and Engelund, M. B. (2020). Gene expression profiling of proximal and distal renal tubules in Atlantic salmon (*Salmo salar*) acclimated to fresh water and seawater. *Am. J. Phys.* 319, F380–F393. doi:10.1152/ajprenal.00557.2019

Mantel, L. H., and Farmer, L. L. (1983). "Osmotic and ionic regulation," in *The biology of Crustacea. Internal anatomy and physiological regulation*. Editor L. H. Mantel (New York: Academic Press), 53–161.

Maraschi, A. C., Faria, S. C., and McNamara, J. C. (2021). Salt transport by the gill Na^+-K^+-$2Cl^-$ symporter in palaemonid shrimps: Exploring physiological, molecular and evolutionary landscapes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 257, 110968. doi:10.1016/j.cbpa.2021.110968

Maraschi, A. C., Freire, C. A., and Prodocimo, V. (2015). Immunocytochemical localization of V-H^+ -ATPase, Na^+/K^+-ATPase, and carbonic anhydrase in gill

lamellae of adult freshwater euryhaline shrimp *Macrobrachium acanthurus* (Decapoda, Palaemonidae). *J. Exp. Zool. A* 323, 414–421.

Masereel, B., Pochet, L., and Laeckmann, D. (2003). An overview of inhibitors of Na^+/H^+ exchanger. *Eur. J. Med. Chem.* 38, 547–554. doi:10.1016/s0223-5234(03)00100-4

Masroor, W., Farcy, E., Gros, R., and Lorin-Nebel, C. (2018). Effect of combined stress (salinity and temperature) in European sea bass *Dicentrarchus labrax* osmoregulatory processes. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 215, 45–54. doi:10.1016/j.cbpa.2017.10.019

McNamara, J. C., and Faria, S. C. (2012). Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: A review. *J. Comp. Physiol. B* 182, 997–1014. doi:10.1007/s00360-012-0665-8

McNamara, J. C., Freire, C. A., Torres, A. H., and Faria, S. C. (2015). The conquest of fresh water by the palaemonid shrimps: An evolutionary history scripted in the osmoregulatory epithelia of the gills and antennal glands. *Biol. J. Linn. Soc. Lond.* 114, 673–688. doi:10.1111/bij.12443

Meneton, P., Ichikawa, I., Inagami, T., and Schnermann, J. (2000). Renal physiology of the mouse. *Am. J. Physiol. Renal. Physiol.* 278 (3), F339–F351. doi:10.1152/ajprenal.2000.278.3.F339

Meyer, A., and Van de Peer, Y. (2005). From 2R to 3R: Evidence for a fish-specific genome duplication (FSGD). *Bioessays* 27, 937–945. doi:10.1002/bies.20293

Miller, M. F., and Labandeira, C. C. (2002). Slow crawl across the salinity divide: Delayed colonization of freshwater ecosystems by invertebrates. *Gsa Today* 12, 4–10. doi:10.1130/1052-5173(2002)012<0004:scatsd>2.0.co;2

Misof, B., Liu, S., Meusemann, K., Peters, R. S., Donath, A., Mayer, C., et al. (2014). Phylogenomics resolves the timing and pattern of insect evolution. *Science* 346, 763–767. doi:10.1126/science.1257570

Mitchell, B., and Crews, S. T. (2002). Expression of the *Artemia* trachealess gene in the salt gland and epipod. *Evol. Dev.* 4, 344–353. doi:10.1046/j.1525-142x.2002.02023.x

Mitrovic, D., and Perry, S. F. (2009). The effects of thermally induced gill remodeling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *J. Exp. Biol.* 212, 843–852. doi:10.1242/jeb.025999

Montalbano, G., Levanti, M., Mhalhel, K., Abbate, F., Laurà, R., Guerrera, M. C., et al. (2021). Acid-sensing ion channels in zebrafish. *Animals.* 11, 2471. doi:10.3390/ani11082471

Montgomery, D. W., Kwan, G. T., Davison, W. G., Finlay, J., Berry, A., Simpson, S. D., et al. (2022). Rapid blood acid-base regulation by European sea bass (*Dicentrarchus labrax*) in response to sudden exposure to high environmental CO_2. *J. Exp. Biol.* 225, 242735. doi:10.1242/jeb.242735

Morris, S. (2001). Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. *J. Exp. Biol.* 204, 979–989. doi:10.1242/jeb.204.5.979

Moshtaghi, A., Rahi, M. L., Mather, P. B., and Hurwood, D. A. (2018). An investigation of gene expression patterns that contribute to osmoregulation in *Macrobrachium australiense*: Assessment of adaptive responses to different osmotic niches. *Gene Rep.* 13, 76–83. doi:10.1016/j.genrep.2018.09.002

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K., and Hirose, S. (2007). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 293, R1743–R1753. doi:10.1152/ajpregu.00248.2007

Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A., and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): Evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol. Genomics.* 31, 463–474. doi:10.1152/physiolgenomics.00061.2007

Nawata, C. M., and Wood, C. M. (2008). The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* 211, 3226–3236. doi:10.1242/jeb.020396

Nebel, C., Nègre-Sadargues, G., Blasco, C., and Charmantier, G. (2005). Morphofunctional ontogeny of the urinary system of the European sea bass *Dicentrarchus labrax*. *Anat. Embryol.* 209, 193–206. doi:10.1007/s00429-004-0438-6

Okech, B. A., Boudko, D. Y., Linser, P. J., and Harvey, W. R. (2008). Cationic pathway of pH regulation in larvae of *Anopheles gambiae*. *J. Exp. Biol.* 211, 957–968. doi:10.1242/jeb.012021

Onken, H., Graszynski, K., and Zeiske, W. (1991). Na^+-independent, electrogenic Cl^- uptake across the posterior gills of the Chinese crab (*Eriocheir sinensis*): Voltage-clamp and microelectrode studies. *J. Comp. Physiol. B* 161, 293–301. doi:10.1007/bf00262311

Onken, H., and McNamara, J. C. (2002). Hyperosmoregulation in the red crab *Dilocarcinus pagei* (Brachyura, trichodactylidae): Structural and functional asymmetries of the posterior gills. *J. Exp. Biol.* 205, 167–175. doi:10.1242/jeb.205.2.167

Onken, H., and Putzenlechner, M. A. (1995). A V-ATPase drives active, electrogenic and Na^+-independent Cl^- absorption across the gills of *Eriocheir sinensis*. *J. Exp. Biol.* 198, 767–774. doi:10.1242/jeb.198.3.767

Onken, H., and Riestenpatt, S. (2002). Ion transport across posterior gills of hyperosmoregulating shore crabs (*Carcinus maenas*): Amiloride blocks the cuticular Na^+ conductance and induces current noise. *J. Exp. Biol.* 205, 523–531. doi:10.1242/jeb.205.4.523

Parks, S. K., Tresguerres, M., and Goss, G. G. (2007). Interactions between Na^+ channels and Na^+-HCO_3^- cotransporters in the freshwater fish gill MR cell: A model for transepithelial Na^+ uptake. *Am. J. Physiol. Cell Physiol.* 292, C935–C944. doi:10.1152/ajpcell.00604.2005

Parks, S. K., Tresguerres, M., and Goss, G. G. (2008). Theoretical considerations underlying Na^+ uptake mechanisms in freshwater fishes. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* 148, 411–418. doi:10.1016/j.cbpc.2008.03.002

Patrick, M. L., Aimanova, K., Sanders, H. R., and Gill, S. S. (2006). P-type Na^+/K^+-ATPase and V-type H^+-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. *J. Exp. Biol.* 209, 4638–4651. doi:10.1242/jeb.02551

Péqueux, A. (1995). Osmotic regulation in crustaceans. *J. Crustac. Biol.* 15, 1–60. doi:10.2307/1549010

Perry, S. F., Furimsky, M., Bayaa, M., Georgalis, T., Shahsavarani, A., Nickerson, J. G., et al. (2003). Integrated responses of Na^+/HCO_3^- cotransporters and V-type H^+-ATPases in the fish gill and kidney during respiratory acidosis. *Biochim. Biophys. Acta* 1618, 175–184. doi:10.1016/j.bbamem.2003.09.015

Peterson, D. R., and Loizzi, R. F. (1974). Biochemical and cytochemical investigations of (Na^+, K^+)-ATPase in the crayfish kidney. *Comp. Biochem. Physiol. A Comp. Physiol.* 49, 763–773. doi:10.1016/0300-9629(74)90903-7

Piermarini, P. M., and Evans, D. H. (2000). Effects of environmental salinity on Na^+/K^+-ATPase in the gills and rectal gland of a euryhaline elasmobranch (*Dasyatis sabina*). *J. Exp. Biol.* 203, 2957–2966. doi:10.1242/jeb.203.19.2957

Piermarini, P. M., Grogan, L. F., Lau, K., Wang, L., and Beyenbach, K. W. (2010). A SLC4-like anion exchanger from renal tubules of the mosquito (*Aedes aegypti*): Evidence for a novel role of stellate cells in diuretic fluid secretion. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 298, R642–R660. doi:10.1152/ajpregu.00729.2009

Piermarini, P. M., Weihrauch, D., Meyer, H., Huss, M., and Beyenbach, K. W. (2009). NHE8 is an intracellular cation/H^+ exchanger in renal tubules of the yellow fever mosquito *Aedes aegypti*. *Am. J. Physiol. Ren. Physiol.* 296, F730–F750. doi:10.1152/ajprenal.90564.2008

Pinto, M. R., Lucena, M. N., Faleiros, R. O., Almeida, E. A., McNamara, J. C., and Leone, F. A. (2016). Effects of ammonia stress in the amazon river shrimp *Macrobrachium amazonicum* (Decapoda, palaemonidae). *Aquat. Toxicol.* 170, 13–23. doi:10.1016/j.aquatox.2015.10.021

Plotkin, M. D., Kaplan, M. R., Verlander, J. W., Lee, W. S., Brown, D., Poch, E., et al. (1996). Localization of the thiazide sensitive Na-Cl cotransporter, rTSC1 in the rat kidney. *Kidney Int.* 50, 174–183. doi:10.1038/ki.1996.300

Posavi, M., Gulisija, D., Munro, J. B., Silva, J. C., and Lee, C. E. (2020). Rapid evolution of genome-wide expression and plasticity during saline to freshwater invasions by the copepod *Eurytemora affinis* species complex. *Mol. Ecol.* 29, 4835–4856. doi:10.1111/mec.15681

Postel, U., Becker, W., Brandt, A., Luck-Kopp, S., Riestenpatt, S., Weihrauch, D., et al. (2000). Active osmoregulatory ion uptake across the pleopods of the isopod *Idotea baltica* (pallas): Electrophysiological measurements on isolated split endo- and exopodites mounted in a micro-ussing chamber. *J. Exp. Biol.* 203, 1141–1152. doi:10.1242/jeb.203.7.1141

Potts, W. T. W. (1994). Kinetics of sodium uptake in freshwater animals: A comparison of ion-exchange and proton pump hypotheses. *Am. J. Physiol.* 266, R315–R320. doi:10.1152/ajpregu.1994.266.2.R315

Potts, W. T. W., and Parry, G. (1964). *Osmotic and ionic regulation*. Oxford: Pergamon Press.

Poynton, H. C., Hasenbein, S., Benoit, J. B., Sepulveda, M. S., Poelchau, M. F., Hughes, D. S. T., et al. (2018). The toxicogenome of *Hyalella azteca*: A model for sediment ecotoxicology and evolutionary toxicology. *Environ. Sci. Technol.* 52, 6009–6022. doi:10.1021/acs.est.8b00837

Putzenlechner, M., Onken, H., Klein, U., and Graszynski, K. (1992). Electrogenic Cl^- uptake across the gill epithelium of *Eriocheir sinensis*; energized by a V-type ATPase? *Verh. Dtsch. Zool. Ges.* 1, 85–160.

Quijada-Rodriguez, A. R., Schultz, A. G., Wilson, J. M., He, Y., Allen, G. J. P., Goss, G. G., et al. (2017). Ammonia-independent sodium uptake mediated by Na^+ channels and NHEs in the freshwater ribbon leech *Nephelopsis obscura*. *J. Exp. Biol.* 220, 3270–3279. doi:10.1242/jeb.159459

Rahi, M. L., Azad, K. N., Tabassum, M., Irin, H. H., Hossain, K. S., Aziz, D., et al. (2021). Effects of salinity on physiological, biochemical and gene expression parameters of black tiger shrimp (*Penaeus monodon*): Potential for farming in low-salinity environments. *Biology* 10, 1220. doi:10.3390/biology10121220

Regier, J. C., Shultz, J. W., and Kambic, R. E. (2005). Pancrustacean phylogeny: Hexapods are terrestrial crustaceans and maxillopods are not monophyletic. *Proc. Biol. Sci.* 272, 395–401. doi:10.1098/rspb.2004.2917

Reid, S. D., Hawkings, G. S., Galvez, F., and Goss, G. G. (2003). Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. *J. Exp. Biol.* 206, 551–559. doi:10.1242/jeb.00109

Reidenbach, K. R., Cook, S., Bertone, M. A., Harbach, R. E., Wiegmann, B. M., and Besansky, N. J. (2009). Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. *BMC Evol. Biol.* 9, 298. doi:10.1186/1471-2148-9-298

Rheault, M. R., Okech, B. A., Keen, S. B., Miller, M. M., Meleskevitch, E. A., Linser, P. J., et al. (2007). Molecular cloning, phylogeny and localization of AgNHA1: The first Na^+/H^+ antiporter (NHA) from a metazoan, *Anopheles gambiae*. *J. Exp. Biol.* 210, 3848–3861. doi:10.1242/jeb.007872

Riegel, J. A. (1977). "Fluid movement through the crayfish antennal gland," in *Transport of ions and water in animals*. Editors B. L. Gupta, R. B. Moreton, J. L. Oschman, B. J. Wall, and London (Academic Press), 613–631.

Riestenpatt, S., Onken, H., and Siebers, D. (1996). Active absorption of Na^+ and CL^- across the gill epithelium of the shore crab *Carcinus maenas*: Voltage-clamp and ion-fluxes studies. *J. Exp. Biol.* 199, 1545–1554. doi:10.1242/jeb.199.7.1545

Riestenpatt, S., Petrausch, G., and Siebers, D. (1995). CL^- influx across posterior gills of the Chinese crab (*Eriocheir sinensis*): Potential energization by a V-type H^+-ATPase. *Comp. Biochem. Physiology Part A Physiology* 110, 235–241. doi:10.1016/0300-9629(94)00169-t

Sarver, G. L., Flynn, M. A., and Holliday, C. W. (1994). Renal Na, K-ATPase and osmoregulation in the crayfish *Procambarus clarkii*. *Comp. Biochem. Physiology Part A Physiology* 107, 349–356. doi:10.1016/0300-9629(94)90392-1

Schmidt-Nieslen, K. (1997). *Animal physiology. Adaptation and environment*. Cambridge, New York: Cambridge University Press.

Schram, F. R. (1982). "The fossil record and evolution of Crustacea," in *The biology of Crustacea. Systematics, the fossil record and biogeography*. Editor L. G. Abele (New York: Academic Press), 93–147.

Scott, G. R., Claiborne, J. B., Edwards, S. L., Schulte, P. M., and Wood, C. M. (2005). Gene expression after freshwater transfer in gills and opercular epithelia of killifish: Insight into divergent mechanisms of ion transport. *J. Exp. Biol.* 208, 2719–2729. doi:10.1242/jeb.01688

Shetlar, R. E., and Towle, D. W. (1989). Electrogenic sodium-proton exchange in membrane vesicles from crab (*Carcinus maenas)* gill. *Am. J. Physiol.* 257, R924–R931. doi:10.1152/ajpregu.1989.257.4.R924

Shih, T.-H., Horng, J.-L., Hwang, P.-P., and Lin, L. Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Am. J. Physiol. Cell Physiol.* 295, C1625–C1632. doi:10.1152/ajpcell.00255.2008

Shih, T.-H., Horng, J.-L., Liu, S.-T., Hwang, P.-P., and Lin, L.-Y. (2012). Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 302, R84–R93. doi:10.1152/ajpregu.00318.2011

Siebers, D., Böttcher, K., Petrausch, G., and Hamann, A. (1990). Effects of some chloride channel blockers on potential differences and ion fluxes in isolated perfused gills of shore crabs *Carcinus maenas*. *Comp. Biochem. Physiology Part A Physiology* 97, 9–15. doi:10.1016/0300-9629(90)90714-4

Smith, H. W. (1930). The absorption and excretion of water and salts by marine teleosts. *Am. J. Physiology-Legacy Content* 93, 480–505. doi:10.1152/ajplegacy.1930.93.2.480

Smith, K. E., VanEkeris, L. A., Okech, B. A., Harvey, W. R., and Linser, P. J. (2008). Larval anopheline mosquito recta exhibit a dramatic change in localization patterns of ion transport proteins in response to shifting salinity: A comparison between anopheline and culicine larvae. *J. Exp. Biol.* 211, 3067–3076. doi:10.1242/jeb.019299

Sollid, J., and Nilsson, G. E. (2006). Plasticity of respiratory structures - adaptive remodeling of fish gills induced by ambient oxygen and temperature. *Respir. Physiol. Neurobiol.* 154, 241–251. doi:10.1016/j.resp.2006.02.006

Stern, D. B., Anderson, N. W., Juanita, D., and Lee, C. E. (2022). Genome-wide signatures of synergistic epistasis during parallel adaptation in a Baltic Sea copepod. *Nat. Commun.* 13, 4024. doi:10.1038/s41467-022-31622-8

Stern, D. B., and Lee, C. E. (2020). Evolutionary origins of genomic adaptations in an invasive copepod. *Nat. Ecol. Evol.* 4, 1084–1094. doi:10.1038/s41559-020-1201-y

Takei, Y., Hiroi, J., Takahashi, H., and Sakamoto, T. (2014). Diverse mechanisms for body fluid regulation in teleost fishes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307, R778–R792. doi:10.1152/ajpregu.00104.2014

Takvam, M., Wood, C. M., Kryvi, H., and Nilsen, T. O. (2021). Ion transporters and osmoregulation in the kidney of teleost fishes as a function of salinity. *Front. Physiol.* 12, 664588. doi:10.3389/fphys.2021.664588

Tang, C. H., and Lee, T. H. (2011). Ion-deficient environment induces the expression of basolateral chloride channel, ClC-3-like protein, in gill mitochondrion-rich cells for chloride uptake of the tilapia *Oreochromis mossambicus*. *Physiol. Biochem. Zool.* 84, 54–67. doi:10.1086/657161

Taylor, H. H., and Taylor, E. W. (1992). "Gills and lungs: The exchanges of gases and ions," in *Microscopic anatomy of invertebrates*. Editors F. W. Harrison and A. G. Humes (New York: Wiley-Liss), 203–343.

Taylor, J. S., Braasch, I., Frickey, T., Meyer, A., and Van de Peer, Y. (2003). Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res.* 13, 382–390. doi:10.1101/gr.640303

Taylor, J. S., Van de Peer, Y., Braasch, I., and Meyer, A. (2001). Comparative genomics provides evidence for an ancient genome duplication event in fish. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 356, 1661–1679. doi:10.1098/rstb.2001.0975

Tine, M., Kuhl, H., Gagnaire, P.-A., Louro, B., Desmarais, E., Martins, R. S. T., et al. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nat. Commun.* 5, 5770. doi:10.1038/ncomms6770

Tipsmark, C. K., Baltzegar, D. A., Ozden, O., Grubb, B. J., and Borski, R. J. (2008). Salinity regulates claudin mRNA and protein expression in the teleost gill. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294, R1004–R1014. doi:10.1152/ajpregu.00112.2007

Tomasso, J. R., Jr., and Grosell, M. (2005). Physiological basis for large differences in resistance to nitrite among freshwater and freshwater-acclimated euryhaline fishes. *Environ. Sci. Technol.* 39, 98–102. doi:10.1021/es048900t

Towle, D. W. (1998). Cloning and sequencing a $Na^+/K^+/2Cl^-$ cotransporter from gills of the euryhaline blue crab *Callinectes sapidus*. *Am. J. Physiol.* 38, 114A.

Towle, D. W. (1984). Membrane-bound ATPases in arthropod ion transporting tissues. *Am. Zool.* 24, 177–185. doi:10.1093/icb/24.1.177

Towle, D. W. (1997). Molecular approaches to understanding salinity adaptation of estuarine animals. *Am. Zool.* 37, 575–584. doi:10.1093/icb/37.6.575

Towle, D. W., Rushton, M. E., Heidysch, D., Magnani, J. J., Rose, M. J., Amstutz, A., et al. (1997). Sodium/proton antiporter in the euryhaline crab *Carcinus maenas* : Molecular cloning, expression and tissue distribution. *J. Exp. Biol.* 200, 1003–1014. doi:10.1242/jeb.200.6.1003

Towle, D. W., and Smith, C. M. (2006). Gene discovery in *Carcinus maenas* and *Homarus americanus* via expressed sequence tags. *Integr. Comp. Biol.* 46, 912–918. doi:10.1093/icb/icl002

Towle, D. W., and Weihrauch, D. (2001). Osmoregulation by gills of euryhaline crabs: Molecular analysis of transporters. *Am. Zool.* 41, 770–780. doi:10.1093/icb/41.4.770

Tresguerres, M. (2016). Novel and potential physiological roles of vacuolar-type H^+-ATPase in marine organisms. *J. Exp. Biol.* 219 (14), 2088–2097. doi:10.1242/jeb.128389

Tsai, J. R., and Lin, H. C. (2007). V-type H^+-ATPase and Na^+, K^+-ATPase in the gills of 13 euryhaline crabs during salinity acclimation. *J. Exp. Biol.* 210, 620–627. doi:10.1242/jeb.02684

Tsui, T. K. N., Hung, C. Y. C., Nawata, C. M., Wilson, J. M., Wright, P. A., and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: The importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. *J. Exp. Biol.* 212, 878–892. doi:10.1242/jeb.021899

Varsamos, S., Nebel, C., and Charmantier, G. (2005). Ontogeny of osmoregulation in post-embryonic fish: A review. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 141A, 401–429. doi:10.1016/j.cbpb.2005.01.013

Varsamos, S., Wendelaar Bonga, S. E., Charmantier, G., and Flik, G. (2004). Drinking and Na^+/K^+ ATPase activity during early development of European sea bass, *Dicentrarchus labrax*: Ontogeny and short-term regulation following acute salinity changes. *J. Exp. Mar. Biol. Ecol.* 311, 189–200. doi:10.1016/j.jembe.2004.04.019

Vogt, G. (2002). "Functional anatomy," in *Biology of freshwater crayfish*. Editor D. M. Holdich (Oxford: Blackwell), 53–151.

Von Reumont, B. M., Jenner, R. A., Wills, M. A., Dell'ampio, E., Pass, G., Ebersberger, I., et al. (2012). Pancrustacean phylogeny in the light of new phylogenomic data: Support for remipedia as the possible sister group of Hexapoda. *Mol. Biol. Evol.* 29, 1031–1045. doi:10.1093/molbev/msr270

Waldmann, R., Champigny, G., Bassilana, F., Heurteaux, C., and Lazdunski, M. (1997). A proton-gated cation channel involved in acid-sensing. *Nature* 386, 173–177. doi:10.1038/386173a0

Wang, J. Q., Hou, L., Yi, N., Zhang, R. F., Zou, X. Y., Xiao, Q., et al. (2012). Molecular cloning and its expression of trachealess gene (As-trh) during development in brine shrimp, *Artemia sinica*. *Mol. Biol. Rep.* 39, 1659–1665. doi:10.1007/s11033-011-0905-0

Wang, Y. F., Tseng, Y. C., Yan, J. J., Hiroi, J., and Hwang, P.-P. (2009). Role of SLC12A10.2, a Na-Cl cotransporter-like protein, in a Cl uptake mechanism in zebrafish (*Danio rerio*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 296, R1650–R1660. doi:10.1152/ajpregu.00119.2009

Wang, Z., Bai, Y., Zhang, D., and Tang, B. (2018). Adaptive evolution of osmoregulatory-related genes provides insight into salinity adaptation in Chinese mitten crab, *Eriocheir sinensis*. *Genetica* 146, 303–311. doi:10.1007/s10709-018-0021-3

Weihrauch, D., McNamara, J. C., Towle, D. W., and Onken, H. (2005). Ion-motive ATPases and active transbranchial NaCl uptake in the red freshwater crab, *Dilocarcinus pagei* (Decapoda, Trichodactylidae). *J. Exp. Biol.* 207, 4623–4631. doi:10.1242/jeb.01333

Weihrauch, D., Morris, S., and Towle, D. W. (2004). Ammonia excretion in aquatic and terrestrial crabs. *J. Exp. Biol.* 207, 4491–4504. doi:10.1242/jeb.01308

Weihrauch, D., and O'Donnell, M. J. (2015). Links between osmoregulation and nitrogen-excretion in insects and crustaceans. *Integr. Comp. Biol.* 55, 816–829. doi:10.1093/icb/icv013

Weihrauch, D., and Towle, D. W. (2000). Na^+/H^+ exchanger and $Na^+/K^+/2Cl^-$ cotransporter are expressed in gills of the euryhaline Chinese crab *Eriocheir sinensis*. *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 126 B, 158. doi:10.1016/s1095-6433(00)80314-1

Weihrauch, D., and Towle, D. W. (2001). Osmoregulation by gills of euryhaline crabs: Molecular analysis of transporters. *Am. Zool.* 41, 770–780. doi:10.1093/icb/41.4.770

Weihrauch, D., Wilkie, M. P., and Walsh, P. J. (2009). Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J. Exp. Biol.* 212, 1716–1730. doi:10.1242/jeb.024851

Weihrauch, D., Ziegler, A., Siebers, D., and Towle, D. W. (2001). Molecular characterization of V-type H^+-ATPase (b-subunit) in gills of euryhaline crabs and its physiological role in osmoregulatory ion uptake. *J. Exp. Biol.* 204, 25–37. doi:10.1242/jeb.204.1.25

Wheatly, M. G., and Gannon, A. T. (1995). Ion regulation in crayfish: Freshwater adaptations and the problem of molting. *Am. Zool.* 35, 49–59. doi:10.1093/icb/35.1.49

White, B. J., Kundert, P. N., Turissini, D. A., Van Ekeris, L., Linser, P. J., and Besansky, N. J. (2013). Dose and developmental responses of *Anopheles merus* larvae to salinity. *J. Exp. Biol.* 216, 3433–3441. doi:10.1242/jeb.087189

Wieczorek, H., Brown, D., Grinstein, S., Ehrenfeld, J., and Harvey, W. R. (1999). Animal plasma membrane energization by proton-motive V-ATPases. *BioEssays* 21, 637–648. doi:10.1002/(SICI)1521-1878(199908)21:8<637::AID-BIES3>3.0.CO;2-W

Wieczorek, H., Putzenlechner, R. M., Zeiske, W., and Klein, U. (1991). A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J. Biol. Chem.* 266, 15340–15347. doi:10.1016/s0021-9258(18)98621-7

Wigglesworth, V. B. (1933). The function of the anal gills of the mosquito larva. *J. Exp. Biol.* 10, 16–26. doi:10.1242/jeb.10.1.16

Willmer, P., Stone, G., and Johnston, I. (2008). *Environmental physiology of animals*. Malden, Oxford: Blackwell.

Wilson, J. M., Laurent, P., Tufts, B. L., Benos, D. J., Donowitz, M., Vogl, A. W., et al. (2000a). NaCl uptake by the branchial epithelium in freshwater teleost fish: An immunological approach to ion-transport protein localization. *J. Exp. Biol.* 203, 2279–2296. doi:10.1242/jeb.203.15.2279

Wilson, J. M., Randall, D. J., Donowitz, M., Vogl, A. W., and Ip, A. K. (2000b). Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). *J. Exp. Biol.* 203, 2297–2310. doi:10.1242/jeb.203.15.2297

Wilson, R. W. (2011). "Role of the gut. Gut ion, osmotic and acid-base regulation," in *Encyclopedia of fish physiology*. Editor A. P. Farrell (Academic Press), 1419–1428.

Wilson, R. W., Wood, C. M., Gonzalez, R. J., Patrick, M. L., Bergman, H. L., Narahara, A., et al. (1999). Ion and acid–base balance in three species of Amazonian fish during gradual acidification of extremely soft water. *Physiol. Biochem. Zool.* 72, 277–285. doi:10.1086/316672

Wolcott, T. G. (1992). Water and solute balance in the transition to land. *Am. Zool.* 32, 428–437. doi:10.1093/icb/32.3.428

Wood, C. M., and Nawata, C. M. (2011). A nose-to-nose comparison of the physiological and molecular responses of rainbow trout to high environmental ammonia in seawater *versus* freshwater. *J. Exp. Biol.* 214, 3557–3569. doi:10.1242/jeb.057802

Wood, C. M., Wilson, R. W., Gonzalez, R. J., Patrick, M. L., Bergman, H. L., Narahara, A., et al. (1998). Responses of an Amazonian teleost, the tambaqui (*Colossoma macropomum*), to low pH in extremely soft water. *Physiol. Zool.* 71, 658–670. doi:10.1086/515977

Wright, P. A., and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: Role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* 212, 2303–2312. doi:10.1242/jeb.023085

Wu, S.-C., Horng, J.-L., Liu, S.-T., Hwang, P.-P., Wen, Z.-H., Lin, C.-S., et al. (2010). Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Am. J. Physiol. Cell Physiol.* 298, C237–C250. doi:10.1152/ajpcell.00373.2009

Wu, Y.-C., Lin, L.-Y., and Leen, T.-H. (2003). Na^+, K^+, $2Cl^-$-cotransporter: A novel marker for identifying freshwater and seawater-type mitochondria-rich cells in gills of the euryhaline tilapia. *Oreochromis Mossambicus. Zool. Stud.* 42, 186–192.

Xiang, M. A., Linser, P. J., Price, D. A., and Harvey, W. R. (2012). Localization of two Na^+- or K^+-H^+ antiporters, AgNHA1 and AgNHA2, in *Anopheles gambiae* larval Malpighian tubules and the functional expression of AgNHA2 in yeast. *J. Insect Physiol.* 58, 570–579. doi:10.1016/j.jinsphys.2011.12.009

Xu, B.-P., Tu, D.-D., Yan, M.-C., Shu, M.-A., and Shao, Q.-J. (2017). Molecular characterization of a cDNA encoding $Na^+/K^+/2Cl^-$ cotransporter in the gill of mud crab (*Scylla paramamosain*) during the molt cycle: Implication of its function in osmoregulation. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 203, 115–125. doi:10.1016/j.cbpa.2016.08.019

Xu, Z., Gan, L., Li, T., Xu, C., Chen, K., Wang, X., et al. (2021). Transcriptome profiling and molecular pathway analysis of genes in association with salinity adaptation in Nile tilapia Oreochromis niloticus.. *PLoS One* 10, e0136506. doi:10.1371/journal.pone.0136506

Xue, R., Li, R., and Bai, F. (2015). Single cell sequencing: Technique, application, and future development. *Sci. Bull. (Beijing).* 60, 33–42. doi:10.1007/s11434-014-0634-6

Yan, J. J., Chou, M. Y., Kaneko, T., and Hwang, P.-P. (2007). Gene expression of Na^+/H^+ exchanger in zebrafish H^+-ATPase-rich cells during acclimation to low-Na^+ and acidic environments. *Am. J. Physiol. Cell Physiol.* 293, C1814–C1823. doi:10.1152/ajpcell.00358.2007

Yang, T., Huang, Y. G., Singh, I., Schnermann, J., and Briggs, J. P. (1996). Localization of bumetanide- and thiazide-sensitive Na-K-Cl cotransporters along the rat nephron. *Am. J. Physiol.* 271, F931–F939. doi:10.1152/ajprenal.1996.271.4.F931

Yang, W.-K., Kang, C.-K., Chen, T.-Y., Chang, W.-B., and Lee, T.-H. (2011). Salinity-dependent expression of the branchial $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+-ATPase in the sailfin molly correlates with hypoosmoregulatory endurance. *J. Comp. Physiol. B* 181, 953–964. doi:10.1007/s00360-011-0568-0

Yang, Z., Zhou, J., Wei, B., Cheng, Y., Zhang, L., and Zhen, X. (2019). Comparative transcriptome analysis reveals osmotic-regulated genes in the gill of Chinese mitten crab (*Eriocheir sinensis*). *PLoS ONE* 14, e0210469. doi:10.1371/journal.pone.0210469

Zare, S., and Greenaway, P. (1998). The effect of molting and sodium depletion on sodium transport and the activities of Na^+K^+-ATPase and V ATPase in the freshwater crayfish *Cherax destructor* (Crustacea: Parastacidae). *Comp. Biochem. Physiology Part A Mol. Integr. Physiology* 119, 739–745. doi:10.1016/s1095-6433(98)01029-0

Zeiske, W., Onken, H., Schwarz, H.-J., and Grazinsky, K. (1992). Invertebrate epithelial Na^+ channels: Amiloride-induced current-noise in crab gill. *Biochim. Biophys. Acta* 1105, 245–252. doi:10.1016/0005-2736(92)90201-v

Zhang, Z. Q., Chen, W. B., Chen, W. Y., Guo, Y. C., Fu, Y., Yang, Z. F., et al. (2011). Phylum Arthropoda von Siebold, 1848 In: Zhang, Z.-Q. (Ed.) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. *Zootaxa* 3148, 99–103. doi:10.11646/zootaxa.3148.1.14

Zhou, S., Degan, S., Potts, E. N., Foster, W. M., and Sunday, M. E. (2009). NPAS3 is a trachealess homolog critical for lung development and homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* 106, 11691–11696. doi:10.1073/pnas.0902426106

Zimmer, A. M., Dymowska, A. K., Kumai, Y., Goss, G. G., Perry, S. F., and Kwong, R. W. M. (2018). Assessing the role of the acid-sensing ion channel ASIC4b in sodium uptake by larval zebrafish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 226, 1–10. doi:10.1016/j.cbpa.2018.06.012

Zimmer, A. M., and Perry, S. F. (2022). Physiology and aquaculture: A review of ion and acid-base regulation by the gills of fishes. *Fish. Fish.* 00, 1–25.

Zimmer, A. M., Wilson, J. M., Wright, P. A., Hiroi, J., and Wood, C. M. (2017). Different mechanisms of Na^+ uptake and ammonia excretion by the gill and yolk sac epithelium of early life stage rainbow trout. *J. Exp. Biol.* 220, 775–786. doi:10.1242/jeb.148429

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Recent progress and debates in molecular physiology of Na^+ uptake in teleosts

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How teleosts take up Na^+ from the surrounding freshwater (FW) as well as the underlying mechanisms associated with this process have received considerable attention over the past 85 years. Owing to an enormous ion gradient between hypotonic FW and fish body fluids, teleosts gills have to actively absorb Na^+ (via ionocytes) to compensate for the passive loss of Na^+. To date, three models have been proposed for Na^+ uptake in teleost ionocytes, including Na^+/H^+ exchanger (NHE)-mediated, acid-sensing ion channel (ASIC)-mediated, Na^+-Cl^- co-transporter (NCC)-mediated pathways. However, some debates regarding these models and unclear mechanisms still remain. To better understand how teleosts take up Na^+ from FW, this mini-review summarizes the main progress and related regulatory mechanisms of Na^+ uptake, and discusses some of the challenges to the current models.

KEYWORDS

ionocyte, NHE, ASIC, NCC, Na^+ uptake, teleost

Introduction

Body fluid Na^+ homeostasis is pivotal for maintaining proper cell activities and physiological processes. In teleosts, the principal organs for ion exchange are the gills (and larval skin), which function *via* the large surface of the epithelium that is directly exposed to water. Regulation of ion transport functions and the epithelial permeability is key for precisely controlling internal osmolality and ion concentrations within a narrow range. In hypotonic freshwater (FW), teleosts actively take up Na^+ *via* ionocytes and reduce passive Na^+ loss by regulating epithelial permeability (Evans et al., 2005). To date, Na^+ uptake mechanisms have become a highly discussed issue in osmoregulatory and evolutionary physiology (Wichmann and Althaus, 2020; Tseng et al., 2022). Although they have been widely studied in different species, several unclear mechanisms and controversial models still need to be clarified in FW teleosts.

Compared to salt excreting pathways in seawater (SW) teleosts, Na^+ uptake mechanisms in FW ones are more diverse and sophisticated in terms of ionocyte subtypes and related transporters (Evans et al., 2005; Yan and Hwang, 2019). Currently, there are three proposed pathways for Na^+ uptake in FW teleosts, including Na^+/H^+ exchanger (NHE)-mediated, acid-

sensing ion channel (ASIC)-mediated, and Na^+-Cl^- co-transporter (NCC)-mediated Na^+ absorption models. Among these, the NHE and NCC models were established with solid and convincing molecular/physiological evidence in FW teleosts, and therefore have become widely accepted concepts about Na^+ uptake (Evans, 2011; Guh and Hwang, 2017; Lewis and Kwong, 2018). However, these models were recently challenged, and an alternative pathway was proposed (Zimmer and Perry, 2020; Zimmer et al., 2020; Clifford et al., 2022). To better understand how teleosts absorb Na^+, this mini-review not only summarizes the major progress in the studies of the three models and the related regulatory mechanisms, but also describes and clarifies the debates on the current models.

Main progress in the studies of Na^+ uptake pathways

Krogh's pioneering Na^+/NH_4^+ exchange idea was the first concept proposed for fish Na^+ uptake and was based on the correlation of decreasing Na^+ and increasing NH_4^+ in the water containing fish (Krogh, 1938). Several decades later, it was indicated that Na^+ is actually exchanged for H^+, not NH_4^+, *via* Na^+/H^+ exchangers (NHEs) (Kerstetter et al., 1970; Kirschner et al., 1973). Thus, until 2009, an idea of metabolon was proposed that apical Rhcg and NHE in ionocytes function together to achieve Na^+/NH_4^+ (Wright and Wood, 2009). In zebrafish and medaka, knockdown/pharmacological experiments and *in situ* proximity ligation assays demonstrated a coupling function of apical Na^+/H^+ exchange (via NHE3) and NH_3 excretion (via Rhcg2) in ionocytes (Wu et al., 2010; Shih et al., 2012; Ito et al., 2013). Intracellular H^+ and NH_3 (NH_4^+ deprotonated by Rhcg2) respectively facilitate apical Na^+/H^+ exchange and NH_3 excretion, and excreted H^+ and NH_3 further convert into NH_4^+ in the external water. Soon after these experiments, zebrafish NHE3b was surprisingly reported to exhibit Na^+/NH_4^+ activity (even under ion-poor conditions). NHE3b-expressing *Xenopus* oocytes exposed to NH_3/NH_4^+-containing medium showed decreased intracellular Na^+ and increased intracellular NH_4^+ activities (Ito et al., 2014). Together, in the current model of NHE3-expressing ionocytes (Figure 1), basolateral Rhbg transports NH_3/NH_4^+ (and NKA probably transports NH_4^+) from the interstitial fluid to the cytosol (Nakada et al., 2007; Wu et al., 2010). The NH_4^+ could either be deprotonated by apical Rhcg for the Na^+/H^+ activity, or directly provide a chemical gradient for the Na^+/NH_4^+ activity of apical NHE3. In addition, carbonic anhydrases (CAs) are involved in extracellular regeneration and intracellular deprotonation of CO_2, which also elevates Na^+/H^+ activity of NHE3 (Ito et al., 2013; Ito et al., 2014; Yan and Hwang, 2019).

On the other hand, a model focusing on the epithelial Na^+ channel (ENaC) coupled vacuolar-type H^+-ATPase (VHA), derived from the model in frog skin (Harvey, 1992), was proposed as an alternative pathway for fish Na^+ uptake (Avella and Bornancin, 1989; Bury and Wood, 1999). In fact, teleosts have lost ENaC genes and thus lack the trait of VHA-driven ENaC that is needed to absorb Na^+ (Waldmann and Lazdunski, 1998). However, several studies have provided functional evidence that bafilomycin (a VHA inhibitor) decreased Na^+ uptake in FW tilapia, carp, zebrafish, and trout (Fenwick et al., 1999; Reid et al., 2003; Esaki et al., 2007), raising the possibility of other VHA-driven Na^+ channels. The long-sought after candidate turned out to be the acid-sensing ion channel (ASIC, belonging to ENaC/degenerin superfamily) which was found in teleost genomes (Paukert et al., 2004; Holzer, 2009). ASIC4b was found to be expressed in trout ionocytes and zebrafish H^+-ATPase-rich (HR) ionocytes (Dymowska et al., 2014; Dymowska et al., 2015). Unfortunately, the ASIC model may not fit all FW teleosts. Medaka express VHA in the basolateral membrane of ionocytes, and tilapia did not show VHA expressed in ionocytes at all (Hiroi et al., 1998; Hsu et al., 2014). Actually, only zebrafish and very limited stenohaline FW species were reported to show apical VHA in gill ionocytes

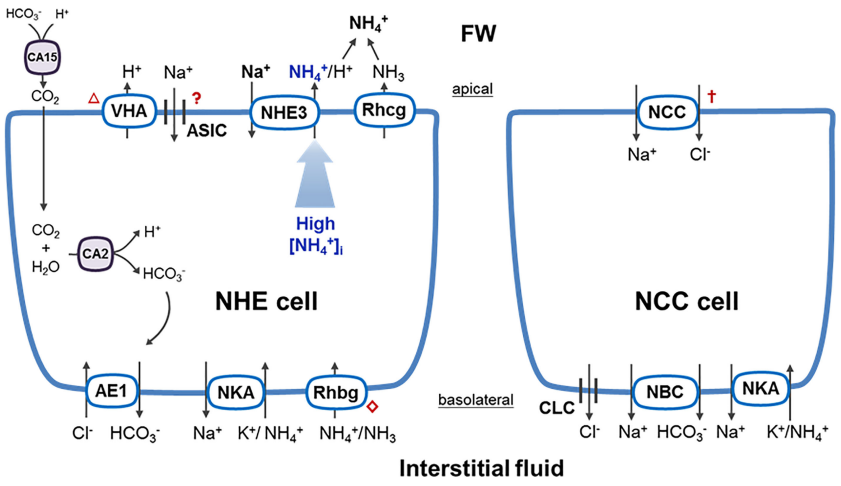

FIGURE 1
General model of ionocytes for teleost Na^+ uptake. Details refer to the text. AE1, anion exchanger 1; ASIC, acid-sensing ion channel; CA15, membrane-bound carbonic anhydrase 15; CA2, cytosolic carbonic anhydrase 2; CLC, Cl^- channel; NBC, Na^+-HCO_3^- co-transporter; NCC, Na^+-Cl^- co-transporter; NCC cell, NCC-expressing ionocyte; NHE3, Na^+/H^+ exchanger 3; NHE cell, NHE3-expressing ionocyte; NKA, Na^+/K^+-ATPase; Rhbg, rhesus B glycoprotein; Rhcg, rhesus C glycoprotein; VHA, vacuolar-type H^+-ATPase; question mark (?), uptake function with controversial evidence; triangle mark (△), apical localization only in zebrafish and limited species; cross mark (†), unclear driving force for NCC. Rhombus mark (◇), transporter expressed in ionocytes or in pavement cells/keratinocytes (Nakada et al., 2007; Wu et al., 2010; Shih et al., 2013). Model size does not represent a relative cell size for NHE and NCC cells.

(Tseng et al., 2020). Functionally, it does not seem possible to take up Na^+ from FW *via* ASIC, owing to its gating kinetics. ASIC is constitutively inactivated and only opens transiently when encountering external acidification, but prolonged acidification desensitizes ASIC and makes it closed (Gründer and Pusch, 2015; Yoder et al., 2018; Wichmann and Althaus, 2020). Altogether, it seems that ASIC may not play a role in Na^+ uptake of ionocytes. This is probably the reason why ASIC inhibitor treatments or knockdown of ASIC4b did not decrease Na^+ influxes in zebrafish larvae (Zimmer et al., 2018). Overall, whether the ASIC model is applicable to teleost ionocytes remains controversial.

An early concept that stood for almost 70 years suggested that the Na^+ uptake pathway was uncoupled with Cl^- transport in fish (Krogh, 1937; Maetz and Garcia Romeu, 1964). However, a direct linkage between Na^+ and Cl^- uptake was functionally observed in tilapia and goldfish (Chang et al., 2003; Preest et al., 2005). Subsequently, the Na^+-Cl^- co-transporter (NCC) was discovered to apically localize in gill ionocytes of FW tilapia (Hiroi et al., 2005; Hiroi et al., 2008). Na^+ and Cl^- uptake functions of NCC-expressing ionocytes were also examined using metolazone (a NCC inhibitor) or specific morpholino knockdown in the larvae of tilapia and zebrafish (Horng et al., 2009; Wang et al., 2009). In the current model of NCC-expressing ionocytes (Figure 1), apical uptake of Na^+ and Cl^- is achieved through NCC, and basolateral absorptions of Na^+ and Cl^- are considered to be achieved through the $Na^+ - HCO_3^-$ co-transporter (NBC)/NKA and Cl^- channel (CLC), respectively (Evans, 2011; Wang et al., 2015; Yan and Hwang, 2019).

Thermodynamic considerations and driving forces underlying Na^+ uptake mechanisms

Thermodynamic principles and the driving force behind Na^+ uptake are the pressing issues yet to be addressed in membrane ion transport. In the NHE model, NHE3, an electroneutral transporter, extrudes H^+/NH_4^+ to bring Na^+ into ionocytes across the apical membrane down the chemical gradient between the environment and the cytosol. The Na^+ concentration (< 1 mM) in FW is much lower than the intracellular concentration of Na^+ in gill ionocytes (6.4-15 mM, data from opercular ionocytes in tilapia), suggesting that NHE must rely on H^+ and/or NH_4^+ gradients against unfavorable Na^+ gradients. Indeed, the intracellular NH_4^+ concentration in teleost gill cells (626-963 μM) is much higher than that in FW (<0.6 μM) (Li et al., 1997; Tseng et al., 2022). High intracellular NH_4^+ could provide a great chemical gradient of NH_4^+ (or H^+, dissociated from NH_4^+) to inwardly drive Na^+ transport. Although short-term acid (pH< 5) or low-Na^+ (Na^+< 0.1 mM) exposure may suddenly increase the thermodynamic constraint (Parks et al., 2008), most FW teleosts are able to increase NHE3/Rhcg/Rhbg expression and the number of NHE3-expressing ionocytes after long-term acclimation, as well as elevate NH_4^+ excretion (Hirata et al., 2003; Wu et al., 2010; Furukawa et al., 2011; Lin et al., 2012; Tseng et al., 2020). Taken together, the contribution of intracellular NH_4^+ and apical NHE-mediated Na^+/NH_4^+ exchange are key factors to be reckoned with. Interestingly, during the evolution of FW adaptation, the NHE model may have developed as the dominant way for teleosts to take up Na^+ (see our next section).

On the other hand, in the NCC model, basolateral NBC, CLC, and NKA of ionocytes do not seem capable of inwardly driving Na^+ and Cl^- uptake *via* apical NCC, owing to high Na^+ and Cl^- concentrations in teleost blood (130 mM and 125mM respectively) (Evans et al., 2005). Although the transport function of NCC has been examined *in vivo* using morpholinos and inhibitors (Horng et al., 2009; Wang et al., 2009), the driving force for apical uptake *via* NCC is still an open question.

Reliance on NHE-mediated Na^+/NH_4^+ exchange for Na^+ uptake

As ammonotelic animals, teleosts mainly produce ammonia as nitrogen wastes and directly excrete ammonia (including ~2% NH_3 and ~98% NH_4^+ under normal physiological pH) into the surrounding water, which saves more energy than further converting ammonia into urea or uric acid before excretion. It is physiologically reasonable that NHE-mediated Na^+/NH_4^+ exchange would be an efficient and energy-saving pathway for excreting acid (H^+) and nitrogen wastes (ammonia), as well as taking up Na^+ from FW. Because most FW teleosts show a high NHE3 expression in a specific subtype of gill/skin ionocytes (NHE3-expressing ionocytes), an evolutionary hypothesis has been recently proposed. During the evolution of FW adaptation, teleosts likely relied on NHE-mediated Na^+/NH_4^+ exchange for a large amount of Na^+ uptake (Tseng et al., 2020; Tseng et al., 2022). Of note, teleosts generally exhibit a relative high NH_4^+ excretion rate up to almost 2500 μmole/kg/h in FW, compared to that of non-teleost fishes such as stenohaline lamprey (Cyclostomata) (50-100 μmole/kg/h in FW), skate (Chondrichthyes) (~130 μmole/kg/h in SW), and sturgeon (Condrostei) (208-724 μmole/kg/h in FW) (Gershanovich and Pototskij, 1995; Altinok and Grizzle, 2004; Steele et al., 2005; Tseng et al., 2022). Moreover, convincing physiological evidence was also found in two model species of teleosts, euryhaline medaka and stenohaline zebrafish. Acute exposure to high ammonia FW decreased Na^+ uptake in skin ionocytes of larval medaka by around 70%; a treatment of NHE inhibitor (5-ethylisopropyl amiloride, EIPA) caused similar declines (65-70%) in Na^+ uptake and NH_4^+ excretion (Tseng et al., 2022). Similarly in larval skin of zebrafish acclimated to low-Na^+ FW, both high ammonia exposure and knockdown of NHE3b impaired over 50% of Na^+ uptake and NH_4^+ excretion (Shih et al., 2012). In the gills of zebrafish and medaka, NHE3 expression was also stimulated by Na^+-deficient FW (Shih et al., 2012; Tseng et al., 2022). These findings reinforce the notion of a considerable reliance on NHE-mediated Na^+/NH_4^+ exchange by FW teleosts.

Functional regulation of Na^+ uptake

Differentially expressed in two subtypes of ionocyte, NHE3 and NCC work in collaboration to take up Na^+ (Yan and Hwang, 2019; Inokuchi et al., 2022). It is widely accepted that NHE3 is a major transporter and NCC is a minor transporter for Na^+ uptake in FW teleosts, based on the evidence that Na^+ is mainly accumulated in zebrafish HR ionocytes (NHE3b-expressing cells), and the density of NHE3-expressing ionocytes is higher than that of NCC-expressing

ionocytes in larval skin (Esaki et al., 2007; Hiroi et al., 2008; Shih et al., 2021). Besides, compensatory regulation on Na^+ uptake by NHE3b and NCC was also revealed in larval zebrafish (Chang et al., 2013).

Several reviews have comprehensively summarized how hormones act on Na^+ uptake regulation in teleosts (Guh and Hwang, 2017; Lewis and Kwong, 2018; Yan and Hwang, 2019). Here, we focused on describing the cases in which the regulation of Na^+ uptake is also dependent on water chemistry. Acidic or low-Na^+ FW results in the alteration of transporter expression and ionocyte number. Long-term exposure to acidic FW triggers the expression of NHE3 (and Rhcg) in most FW teleosts such as dace, tilapia, medaka, carp, and goldfish (Hirata et al., 2003; Tseng et al., 2020). They adopt NHE3 to excrete more H^+/NH_4^+ against acidic environments and simultaneously absorb Na^+. Meanwhile, very few teleosts (zebrafish, for example) mainly up-regulate apical VHA instead of NHE3 for the enhancement of acid excretion (Yan et al., 2007; Tseng et al., 2020). Zebrafish gills showed a down-regulated NHE3b expression with an increased number of NCC2b-expressing ionocytes after acid acclimation for 7 days (Chang et al., 2013). That is, zebrafish utilize NCC2b as a backup transporter for maintaining Na^+ homeostasis under acidic FW, although this fact still cannot exclude the possibility that other NHE isoforms may compensate for the loss of NHE3b. Long-term exposure to Na^+-deficient FW stimulates mRNA expression of NHE3 (and Rhcg) and the number of NHE3-expressing ionocytes in FW teleosts (Inokuchi et al., 2009; Wu et al., 2010; Shih et al., 2012; Tseng et al., 2022). However, studies from zebrafish and medaka revealed that branchial mRNA expression of NCC was down-regulated in low-Na^+ FW (with low-Cl^-) (Wang et al., 2009; Hsu et al., 2014). In tilapia gills, low-Na^+ FW (with normal- or low-Cl^-) did not affect the mRNA expression of NCC, while low-Cl^- FW (with normal Na^+) increased the mRNA expression of NCC and the density of NCC-expressing ionocytes (Inokuchi et al., 2009). These findings suggest that up-regulation of NHE3 is the major pathway for functional enhancement of Na^+ uptake under Na^+-deficient situations, but the regulation of NCC is depending on both Na^+/Cl^- levels in FW and probably varies in different species.

Debates on the roles of NHE and NCC in Na^+ uptake

Debates on the current models of Na^+ uptake pathways originated from the thermodynamic considerations for NHE and NCC. Some studies have proposed that the Na^+ uptake function of NHEs is only favored when a ratio of intracellular and FW concentration of Na^+ is smaller than that of a ratio of H^+, which is not feasible under acidic or Na^+-poor situations (Dymowska et al., 2014; Dymowska et al., 2015; Clifford et al., 2022). Obviously, their concern probably neglected the Na^+/NH_4^+ activity of NHEs. As we described above, apical Na^+/NH_4^+ exchange of NHEs could be driven down the NH_4^+ gradients in ionocytes. But for NCC, how to drive Na^+/Cl^- into ionocytes against the thermodynamic limitations indeed remains a mystery. Based on these debates, recent studies generated *nhe3b*- and *rhcg2*-knockout zebrafish (using CRISPR/Cas9) to reassess the contribution of NHE3b, Rhcg2, and NCC to Na^+ uptake in larvae (Zimmer and Perry, 2020; Zimmer et al., 2020). They found that knockout of *nhe3b* or *rhcg2* did not reduce whole-body Na^+ uptake and Na^+ content, and Na^+ or Cl^- influxes were not respectively affected by Cl^--free or low-Na^+ FW in NHE3b mutants, thereby concluding that larval zebrafish do not require NHE3b and Rhcg2 to sustain whole-body Na^+ uptake, nor do they adopt an NCC-mediated pathway to compensate for the loss of NHE3b function. The finding that zebrafish could survive even lacking the major transporter (NHE3b) for Na^+ uptake is unexpected and does suggest the possibility of unknown back-up pathways for Na^+ compensatory regulation in teleosts. However, the knockout results are not necessary to overrule the previous knockdown/pharmacological evidence that supported the crucial role of NHE3b and NCC in Na^+ uptake (Esaki et al., 2007; Wang et al., 2009; Shih et al., 2012; Chang et al., 2013; Ito et al., 2014). In fact, it is quite reasonable to observe different results among gene knockout and knockdown experiments. Knockdown and knockout probably induced distinct compensatory mechanisms and thereby resulted in inconsistent phenotypes (Rossi et al., 2015). That said, further and more comprehensive explorations of the compensatory mechanisms activated in those knockout mutants (Zimmer and Perry, 2020; Zimmer et al., 2020) are awaited. Loss- (or gain-) of-function experiments using pharmacology, knockdown, or knockout approaches are powerful, but could link misleading information to related issues without the appropriate and careful characterizations of the methodology effectiveness and related compensatory mechanisms.

A new pathway for Na^+ uptake, derived from the same debates around thermodynamics, was recently proposed in adult zebrafish. Clifford and his colleagues found that Na^+ uptake was constitutively lower at 0 h of acid exposure but recovered after 8-10 h of acid exposure. They considered this recovery of Na^+ uptake to be linked to the environmental K^+ concentration, not the NHE- and NCC-mediated pathways (Clifford et al., 2022), and thus proposed an alternative pathway for zebrafish coping with short-term acidification. However, inconsistent results in a previous study reported that acute acid exposure (0 h) did not reduce Na^+ uptake in adult zebrafish (Kumai et al., 2011), which implies further confirmation of the methodology or a detailed description of experimental designs would be necessary in advance. Furthermore, Na^+ uptake did not change during the initial 96 h acid exposure, and instead, a great degree of increase in Na^+ uptake was observed after 120 h acid exposure (Kumai et al., 2011). These results highlight the variable physiological responses that could be observed during the acclimation period. A reasonable comparison of mechanisms or hypothetical differences between studies should base on a similar or comparable experimental time period. On the other hand, Clifford proposed K^+-dependent Na^+/Ca^{2+} exchangers (NCKXs) to be the candidates that mediate the K^+-associated Na^+ uptake function (Clifford et al., 2022). It has been noted that NCKX3 was found to localize to the basolateral layer of mice DCT and involved in Ca^{2+} transport (Lee et al., 2009), but how NCKXs work and even cellular localization of NCKXs in teleost gills are unknown. Further characterization of the molecular identity of the newly-proposed transport pathway is needed.

Concluding remarks

FW teleosts absorb Na^+ *via* NHE-mediated and NCC-mediated Na^+ uptake pathways in gill/skin ionocytes, and they rely on NHE for a majority of Na^+ uptake probably due to a powerful force (NH_4^+

gradient) that efficiently drives NHE (Figure 1). Although some unclear mechanisms still remain, powerful techniques (e.g., single cell transcriptome analysis and a scanning ion-selective electrode technique) have been developed and recently applied to fish gills (Pan et al., 2022; Shih et al., 2022), which may shed some light on fish osmoregulation and the transport mechanisms of Na^+ and other ions.

Author contributions

S-WS and P-PH conceived the idea. J-JY and M-YC provided suggestions to this review. S-WS wrote the manuscript draft. P-PH supervised and finalized the manuscript. All authors approved the manuscript for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Altinok, I., and Grizzle, J. M. (2004). Excretion of ammonia and urea by phylogenetically diverse fish species in low salinities. *Aquaculture* 238, 499–507. doi: 10.1016/j.aquaculture.2004.06.020

Avella, M., and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* 142, 155–175. doi: 10.1242/jeb.142.1.155

Bury, N. R., and Wood, C. M. (1999). Mechanism of branchial apical silver uptake by rainbow trout is *via* the proton-coupled na^+ channel. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 277, R1385–R1391. doi: 10.1152/ajpregu.1999.277.5.R1385

Chang, W.-J., Wang, Y.-F., Hu, H.-J., Wang, J.-H., Lee, T.-H., and Hwang, P.-P. (2013). Compensatory regulation of na^+ absorption by Na^+/H^+ exchanger and na^+-cl^- cotransporter in zebrafish (*Danio rerio*). *Front. zoology* 10, 1–12. doi: 10.1186/1742-9994-10-46

Chang, I.-C., Wei, Y.-Y., Chou, F.-I., and Hwang, P.-P. (2003). Stimulation of cl^- uptake and morphological changes in gill mitochondria-rich cells in freshwater tilapia (*Oreochromis mossambicus*). *Physiol. Biochem. Zoology* 76, 544–552. doi: 10.1086/375438

Clifford, A. M., Tresguerres, M., Goss, G. G., and Wood, C. M. (2022). A novel k^+-dependent na^+ uptake mechanism during low pH exposure in adult zebrafish (*Danio rerio*): New tricks for old dogma. *Acta Physiologica* 234, e13777. doi: 10.1111/apha.13777

Dymowska, A. K., Boyle, D., Schultz, A. G., and Goss, G. G. (2015). The role of acid-sensing ion channels in epithelial na^+ uptake in adult zebrafish (*Danio rerio*). *J. Exp. Biol.* 218, 1244–1251. doi: 10.1242/jeb.113118

Dymowska, A. K., Schultz, A. G., Blair, S. D., Chamot, D., and Goss, G. G. (2014). Acid-sensing ion channels are involved in epithelial na^+ uptake in the rainbow trout *Oncorhynchus mykiss*. *Am. J. Physiology-Cell P hysiology* 307, C255–C265. doi: 10.1152/ajpcell.00398.2013

Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K., and Hirose, S. (2007). Visualization in zebrafish larvae of na^+ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. *Am. J. Physiology-Regulatory Integr. Comp. Physiol.* 292, R470–R480. doi: 10.1152/ajpregu.00200.2006

Evans, D. (2011). Freshwater fish gill ion transport: August krogh to morpholinos and microprobes. *Acta Physiologica* 202, 349–359. doi: 10.1111/j.1748-1716.2010.02186.x

Evans, D. H., Piermarini, P. M., and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 85, 97–177. doi: 10.1152/physrev.00050.2003

Fenwick, J., Wendelaar Bonga, S., and Flik, G. (1999). *In vivo* bafilomycin-sensitive na^+ uptake in young freshwater fish. *J. Exp. Biol.* 202, 3659–3666. doi: 10.1242/jeb.202.24.3659

Furukawa, F., Watanabe, S., Inokuchi, M., and Kaneko, T. (2011). Responses of gill mitochondria-rich cells in Mozambique tilapia exposed to acidic environments (pH 4.0) in combination with different salinities. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 158, 468–476. doi: 10.1016/j.cbpa.2010.12.003

Gershanovich, A., and Pototskij, I. (1995). The peculiarities of non-faecal nitrogen excretion in sturgeons (Pisces; acipenseridae)–2. effects of water temperature, salinity and pH. *Comp. Biochem. Physiol. Part A: Physiol.* 111, 313–317. doi: 10.1016/0300-9629(94)00201-4

Gründer, S., and Pusch, M. (2015). Biophysical properties of acid-sensing ion channels (ASICs). *Neuropharmacology* 94, 9–18. doi: 10.1016/j.neuropharm.2014.12.016

Guh, Y.-J., and Hwang, P.-P. (2017). Insights into molecular and cellular mechanisms of hormonal actions on fish ion regulation derived from the zebrafish model. *Gen. Comp. Endocrinol.* 251, 12–20. doi: 10.1016/j.ygcen.2016.08.009

Harvey, B. J. (1992). Energization of sodium absorption by the h^+-ATPase pump in mitochondria-rich cells of frog skin. *J. Exp. Biol.* 172, 289–309. doi: 10.1242/jeb.172.1.289

Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., et al. (2003). Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am. J. Physiology-Regulatory Integr. Comp. Physiol.* 284, R1199–R1212. doi: 10.1152/ajpregu.00267.2002

Hiroi, J., Kaneko, T., Uchida, K., Hasegawa, S., and Tanaka, M. (1998). Immunolocalization of vacuolar-type h-ATPase in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) larvae. *Zoological Sci.* 15, 447–453. doi: 10.2108/0289-0003(1998)15[447:IOVHIT]2.0.CO;2

Hiroi, J., McCormick, S. D., Ohtani-Kaneko, R., and Kaneko, T. (2005). Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and CFTR anion channel. *J. Exp. Biol.* 208, 2023–2036. doi: 10.1242/jeb.01611

Hiroi, J., Yasumasu, S., McCormick, S. D., Hwang, P.-P., and Kaneko, T. (2008). Evidence for an apical Na–cl cotransporter involved in ion uptake in a teleost fish. *J. Exp. Biol.* 211, 2584–2599. doi: 10.1242/jeb.018663

Holzer, P. (2009). Acid-sensitive ion channels and receptors. *Sensory nerves* 194, 283–332. doi: 10.1007/978-3-540-79090-7_9

Horng, J.-L., Hwang, P.-P., Shih, T.-H., Wen, Z.-H., Lin, C.-S., and Lin, L.-Y. (2009). Chloride transport in mitochondrion-rich cells of euryhaline tilapia (*Oreochromis mossambicus*) larvae. *Am. J. Physiology-Cell Physiol.* 297, C845–C854. doi: 10.1152/ajpcell.00218.2009

Hsu, H.-H., Lin, L.-Y., Tseng, Y.-C., Horng, J.-L., and Hwang, P.-P. (2014). A new model for fish ion regulation: identification of ionocytes in freshwater-and seawater-acclimated medaka (*Oryzias latipes*). *Cell Tissue Res.* 357, 225–243. doi: 10.1007/s00441-014-1883-z

Inokuchi, M., Hiroi, J., and Kaneko, T. (2022). Why can Mozambique tilapia acclimate to both freshwater and seawater? insights from the plasticity of ionocyte functions in the euryhaline teleost. *Front. Physiol.* 13. doi: 10.3389/fphys.2022.914277

Inokuchi, M., Hiroi, J., Watanabe, S., Hwang, P.-P., and Kaneko, T. (2009). Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. *J. Exp. Biol.* 212, 1003–1010. doi: 10.1242/jeb.025957

Ito, Y., Kato, A., Hirata, T., Hirose, S., and Romero, M. F. (2014). Na^+/H^+ and Na^+/NH_4^+ exchange activities of zebrafish NHE3b expressed in xenopus oocytes. *Am. J. Physiology-Regulatory Integr. Comp. Physiol.* 306, R315–R327. doi: 10.1152/ajpregu.00363.2013

Ito, Y., Kobayashi, S., Nakamura, N., Miyagi, H., Esaki, M., Hoshijima, K., et al. (2013). Close association of carbonic anhydrase (CA2a and CA15a), Na^+/H^+ exchanger (Nhe3b), and ammonia transporter Rhcg1 in zebrafish ionocytes responsible for na^+ uptake. *Front. Physiol.* 4, 59. doi: 10.3389/fphys.2013.00059

Kerstetter, T. H., Kirschner, L. B. , and Rafuse, D. D. (1970). On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). *J. Gen. Physiol.* 56, 342–359. doi: 10.1085/jgp.56.3.342

Kirschner, L. B., Greenwald, L., and Kerstetter, T. H. (1973). Effect of amiloride on sodium transport across body surfaces of freshwater animals. *Am. J. Physiology-Legacy Content* 224, 832–837. doi: 10.1152/ajplegacy.1973.224.4.832

Krogh, A. (1937). Osmotic regulation in fresh water fishes by active absorption of chloride ions. *Z. für vergleichende Physiologie* 24, 656–666. doi: 10.1007/BF00592303

Krogh, A. (1938). The active absorption of ions in some freshwater animals. *Z. für vergleichende Physiologie* 25, 335–350. doi: 10.1007/BF00339641

Kumai, Y., Bahubeshi, A., Steele, S., and Perry, S. F. (2011). Strategies for maintaining na^+ balance in zebrafish (*Danio rerio*) during prolonged exposure to acidic water. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 160, 52–62. doi: 10.1016/j.cbpa.2011.05.001

Lee, G.-S., Choi, K.-C., and Jeung, E.-B. (2009). K^+-dependent Na^+/Ca^{2+} exchanger 3 is involved in renal active calcium transport and is differentially expressed in the mouse kidney. *Am. J. Physiology-Renal Physiol.* 297, F371–F379. doi: 10.1152/ajprenal.90615.2008

Lewis, L., and Kwong, R. W. (2018). Zebrafish as a model system for investigating the compensatory regulation of ionic balance during metabolic acidosis. *Int. J. Mol. Sci.* 19, 1087. doi: 10.3390/ijms19041087

Li, J., Eygensteyn, J., Lock, R., Bonga, S., and Flik, G. (1997). Na^+ and Ca^{2+} homeostatic mechanisms in isolated chloride cells of the teleost oreochromis mossambicus analysed by confocal laser scanning microscopy. *J. Exp. Biol.* 200, 1499–1508. doi: 10.1242/jeb.200.10.1499

Lin, C.-C., Lin, L.-Y., Hsu, H.-H., Thermes, V., Prunet, P., Horng, J.-L., et al. (2012). Acid secretion by mitochondrion-rich cells of medaka (*Oryzias latipes*) acclimated to acidic freshwater. *Am. J. Physiology-Regulatory Integr. Comp. Physiol.* 302, R283–R291. doi: 10.1152/ajpregu.00483.2011

Maetz, J., and Garcia Romeu, F. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, carassius auratus: II. evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. *J. Gen. Physiol.* 47, 1209–1227. doi: 10.1085/jgp.47.6.1209

Nakada, T., Westhoff, C. M., Kato, A., and Hirose, S. (2007). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J.* 21, 1067–1074. doi: 10.1096/fj.06-6834com

Pan, W., Godoy, R. S., Cook, D. P., Scott, A. L., Nurse, C. A., and Jonz, M. G. (2022). Single-cell transcriptomic analysis of neuroepithelial cells and other cell types of the gills of zebrafish (*Danio rerio*) exposed to hypoxia. *Sci. Rep.* 12, 1–17. doi: 10.1038/s41598-022-13693-1

Parks, S. K., Tresguerres, M., and Goss, G. G. (2008). Theoretical considerations underlying na+ uptake mechanisms in freshwater fishes. *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol.* 148, 411–418. doi: 10.1016/j.cbpc.2008.03.002

Paukert, M., Sidi, S., Russell, C., Siba, M., Wilson, S. W., Nicolson, T., et al. (2004). A family of acid-sensing ion channels from the zebrafish: widespread expression in the central nervous system suggests a conserved role in neuronal communication. *J. Biol. Chem.* 279, 18783–18791. doi: 10.1074/jbc.M401477200

Preest, M. R., Gonzalez, R. J., and Wilson, R. W. (2005). A pharmacological examination of na^+ and cl^- transport in two species of freshwater fish. *Physiol. Biochem. Zoology* 78, 259–272. doi: 10.1086/427058

Reid, S. D., Hawkings, G., Galvez, F., and Goss, G. (2003). Localization and characterization of phenamil-sensitive na^+ influx in isolated rainbow trout gill epithelial cells. *J. Exp. Biol.* 206, 551–559. doi: 10.1242/jeb.00109

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M., et al. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* 524, 230–233. doi: 10.1038/nature14580

Shih, T.-H., Horng, J.-L., Lai, Y.-T., and Lin, L.-Y. (2013). Rhcg1 and rhbg mediate ammonia excretion by ionocytes and keratinocytes in the skin of zebrafish larvae: H^+-ATPase-linked active ammonia excretion by ionocytes. *Am. J. Physiology-Regulatory Integr. Comp. Physiol.* 304, R1130–R1138. doi: 10.1152/ajpregu.00550.2012

Shih, T.-H., Horng, J.-L., Liu, S.-T., Hwang, P.-P., and Lin, L.-Y. (2012). Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae acclimated to low-sodium water. *Am. J. Physiology-Regulatory Integr. Comp. Physiol.* 302, R84–R93. doi: 10.1152/ajpregu.00318.2011

Shih, S.-W., Yan, J.-J., Tsou, Y.-L., Lu, S.-W., Wang, M.-C., Chou, M.-Y., et al. (2022). *In vivo* functional assay in fish gills: Exploring branchial acid-excreting mechanisms in zebrafish. *Int. J. Mol. Sci.* 23, 4419. doi: 10.3390/ijms23084419

Shih, S.-W., Yan, J.-J., Wang, Y.-H., Tsou, Y.-L., Chiu, L., Tseng, Y.-C., et al. (2021). Estrogen-related receptor γ2 controls NaCl uptake to maintain ionic homeostasis. *J. Endocrinol.* 251, 149–159. doi: 10.1530/JOE-21-0112

Steele, S. L., Yancey, P. H., and Wright, P. A. (2005). The little skate raja erinacea exhibits an extrahepatic ornithine urea cycle in the muscle and modulates nitrogen metabolism during low-salinity challenge. *Physiol. Biochem. Zoology* 78, 216–226. doi: 10.1086/427052

Tseng, Y. C., Yan, J. J., Furukawa, F., Chen, R. D., Lee, J. R., Tsou, Y. L., et al. (2022). Teleostean fishes may have developed an efficient na^+ uptake for adaptation to the freshwater system. *Front. Physiol.* 13, 947958. doi: 10.3389/fphys.2022.947958

Tseng, Y. C., Yan, J. J., Furukawa, F., and Hwang, P. P. (2020). Did acidic stress resistance in vertebrates evolve as Na^+/H^+ exchanger-mediated ammonia excretion in fish? *BioEssays* 42, 1900161. doi: 10.1002/bies.201900161

Waldmann, R., and Lazdunski, M. (1998). H^+-gated cation channels: neuronal acid sensors in the NaC/DEG family of ion channels. *Curr. Opin. Neurobiol.* 8, 418–424. doi: 10.1016/S0959-4388(98)80070-6

Wang, Y.-F., Tseng, Y.-C., Yan, J.-J., Hiroi, J., and Hwang, P.-P. (2009). Role of SLC12A10. 2, a Na-cl cotransporter-like protein, in a cl uptake mechanism in zebrafish (*Danio rerio*). *Am. J. Physiology-Regulatory Integr. Comp. Physiol.* 296, R1650–R1660. doi: 10.1152/ajpregu.00119.2009

Wang, Y.-F., Yan, J.-J., Tseng, Y.-C., Chen, R.-D., and Hwang, P.-P. (2015). Molecular physiology of an extra-renal cl^- uptake mechanism for body fluid cl^- homeostasis. *Int. J. Biol. Sci.* 11, 1190. doi: 10.7150/ijbs.11737

Wichmann, L., and Althaus, M. (2020). Evolution of epithelial sodium channels: current concepts and hypotheses. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 319, R387–R400. doi: 10.1152/ajpregu.00144.2020

Wright, P. A., and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of rhesus (Rh) glycoproteins. *J. Exp. Biol.* 212, 2303–2312. doi: 10.1242/jeb.023085

Wu, S.-C., Horng, J.-L., Liu, S.-T., Hwang, P.-P., Wen, Z.-H., Lin, C.-S., et al. (2010). Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *Am. J. Physiology-Cell Physiol.* 298, C237–C250. doi: 10.1152/ajpcell.00373.2009

Yan, J.-J., Chou, M.-Y., Kaneko, T., and Hwang, P.-P. (2007). Gene expression of Na^+/H^+ exchanger in zebrafish h^+-ATPase-rich cells during acclimation to low-na^+ and acidic environments. *Am. J. Physiology-Cell Physiol.* 293, C1814–C1823. doi: 10.1152/ajpcell.00358.2007

Yan, J.-J., and Hwang, P.-P. (2019). Novel discoveries in acid-base regulation and osmoregulation: a review of selected hormonal actions in zebrafish and medaka. *Gen. Comp. Endocrinol.* 277, 20–29. doi: 10.1016/j.ygcen.2019.03.007

Yoder, N., Yoshioka, C., and Gouaux, E. (2018). Gating mechanisms of acid-sensing ion channels. *Nature* 555, 397–401. doi: 10.1038/nature25782

Zimmer, A. M., Dymowska, A. K., Kumai, Y., Goss, G. G., Perry, S. F., and Kwong, R. W. (2018). Assessing the role of the acid-sensing ion channel ASIC4b in sodium uptake by larval zebrafish. *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 226, 1–10. doi: 10.1016/j.cbpa.2018.06.012

Zimmer, A. M., and Perry, S. F. (2020). The rhesus glycoprotein rhcgb is expendable for ammonia excretion and na^+ uptake in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol.* 247, 110722. doi: 10.1016/j.cbpa.2020.110722

Zimmer, A. M., Shir-Mohammadi, K., Kwong, R. W., and Perry, S. F. (2020). Reassessing the contribution of the Na^+/H^+ exchanger Nhe3b to na^+ uptake in zebrafish (*Danio rerio*) using CRISPR/Cas9 gene editing. *J. Exp. Biol.* 223, jeb215111. doi: 10.1242/jeb.234062

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Insights into the control and consequences of breathing adjustments in fishes-from larvae to adults

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Adjustments of ventilation in fishes to regulate the volume of water flowing over the gills are critically important responses to match branchial gas transfer with metabolic needs and to defend homeostasis during environmental fluctuations in O_2 and/or CO_2 levels. In this focused review, we discuss the control and consequences of ventilatory adjustments in fish, briefly summarizing ventilatory responses to hypoxia and hypercapnia before describing the current state of knowledge of the chemoreceptor cells and molecular mechanisms involved in sensing O_2 and CO_2. We emphasize, where possible, insights gained from studies on early developmental stages. In particular, zebrafish (*Danio rerio*) larvae have emerged as an important model for investigating the molecular mechanisms of O_2 and CO_2 chemosensing as well as the central integration of chemosensory information. Their value stems, in part, from their amenability to genetic manipulation, which enables the creation of loss-of-function mutants, optogenetic manipulation, and the production of transgenic fish with specific genes linked to fluorescent reporters or biosensors.

KEYWORDS

gill, ventilation, chemoreceptor, zebrafish, *Danio rerio*, ontogeny

1 Introduction

Arguably, the most thorough review of the control of breathing in fishes was published in 1986 in the *Handbook of Physiology* (Shelton et al., 1986) as part of an exhaustive tome that covered the regulation of ventilation in all ectothermic vertebrates. In the ensuing years, numerous reviews synthesizing aspects of the control of breathing in fishes have appeared (Burleson et al., 1992; Glass, 1992; Gilmour, 2001; Burleson and Milsom, 2003; Gilmour and Perry, 2007; Sundin et al., 2007; Perry et al., 2009a; Perry et al., 2009b; Perry and Gilmour, 2010; Perry, 2011; Perry and Abdallah, 2012; Jonz et al., 2015a; Perry et al., 2016; Perry and Tzaneva, 2016; Tresguerres et al., 2019; Pan and Perry, 2020), including a recent sequel to the 1986 *Handbook* classic (Milsom et al., 2022). The topics covered in these reviews span all levels of respiratory control from chemoreception of the respiratory gases (O_2, CO_2 and ammonia) and central integration of afferent sensory input to efferent motor output to the muscles controlling water- or air-breathing. Additionally, several of these reviews include sections on the physiological significance of ventilatory adjustments associated with environmental change or increased physical activity (Perry et al., 2009b; Perry, 2011; Milsom et al., 2022). Thus, one might reasonably question the need for yet another review of piscine respiratory control. Frankly, there is probably no need for another **comprehensive** synthesis. However, in light of recent findings, particularly pertaining to early developmental stages, several emerging themes merit discussion in a brief and focused review. These themes include the ontogeny of chemoreception and the associated respiratory reflexes as well as the

molecular and cellular mechanisms underlying the sensing of the two well-studied respiratory gases, O_2 and CO_2.

The current review will focus exclusively on ventilation in water-breathing fishes and highlight research on zebrafish (*Danio rerio*) with a specific focus on the early stages of development. The emphasis on zebrafish reflects recent advances in transgenic and loss-of-function (e.g., knockdown, knockout) approaches (Zimmer et al., 2019) that were developed to study the control of breathing in this model species. The review begins with a brief discussion of the ventilatory responses associated with environmental changes in O_2 and CO_2, which is followed by more detailed descriptions of the mechanisms underlying the sensing of these respiratory gases. In a final section, we propose areas for future studies that would use genetic techniques amenable to larval zebrafish.

2 Ventilatory adjustments to environmental changes in respiratory gases

The volume of water ventilating the gills is a key determinant of O_2 consumption, hence aerobic metabolic rate. Thus, adjustments to ventilation (in concert with cardiac changes) are used to match O_2 consumption with metabolic needs. To modify O_2 uptake rates independently of metabolic rate, ventilation volumes also are controlled by environmental cues linked to modulation of chemoreceptors. Although less well-studied, the excretion rates of CO_2 and NH_3 (water-breathers) also are governed, in part, by ventilation volumes. Over a relatively narrow range of ventilation volumes (~60–150 mL min^{-1}), Iwama et al. (1987) reported a significant correlation between O_2 uptake and CO_2 excretion yielding a slope of 1.0. The effects of hyperventilation on branchial ammonia excretion are complex and vary according to the prevailing diffusion limitations (Randall and Ip, 2006). Indeed, under so-called resting conditions, the rate of ammonia excretion was unaffected by water flow (Eom et al., 2020). However, under conditions of reduced diffusion limitations (e.g. in fish experiencing elevated plasma ammonia levels and increased expression of Rh ammonia channels), there was a clear correlation between ventilation and ammonia excretion. Owing to the limited data available on mechanisms of ammonia sensing, the following discussion focuses on the ventilatory effects of changes in environmental O_2 and CO_2 levels. However, readers interested in the ventilatory effects of the third respiratory gas, NH_3, are directed to the following publications (Zhang and Wood, 2009; Zhang et al., 2011; Zhang et al., 2013; De Boeck and Wood, 2015; Zhang et al., 2015; Eom et al., 2019; Eom et al., 2020; Eom and Wood, 2021; Porteus et al., 2021).

2.1 Environmental O_2 levels

Numerous aquatic habitats vary spatially and temporally in levels of dissolved O_2, ranging from hypoxia [a reduction in O_2 partial pressure (PO_2) below that of air-equilibrated water] to hyperoxia (an increase in PO_2). Although such gradients in ambient PO_2 have existed through "geological time" (Diaz, 2001), their severity and zones of occurrence are increasing steadily owing to human activities. In response to hyperoxia, fish lower ventilation (hypoventilate) (Wood and Jackson, 1980; Heisler et al., 1988; Kinkead and Perry, 1991; Reid et al., 2005; Vulesevic et al., 2006; Tzaneva and Perry, 2014; Porteus et al., 2015; Porteus et al., 2021). Assuming that the cost of gill ventilation is a significant component of the overall energy budget (Jones and Schwarzfeld, 1974; Steffensen and Lomholt, 1983), hyperoxic hypoventilation may confer some energetic savings. The associated reduction in gas transfer efficiency is of no consequence to blood O_2 transport because the arterial PO_2 will remain well in excess of that needed for full saturation of haemoglobin. However, the retention of CO_2 associated with hypoventilation elicits respiratory acidosis (a lowering of pH caused by an increase in PCO_2) (Wood and Jackson, 1980), an obvious detrimental consequence of hyperoxia exposure that could impact fish health in commercial aquaculture settings that add supplemental O_2 to the water (Zimmer and Perry, 2022).

The effects of hypoxia on ventilation (Randall and Shelton, 1963) have received far more attention than those of hyperoxia (Wood and Jackson, 1980). The reader is referred to several extensive reviews that summarise the ventilatory effects linked to hypoxia (Randall, 1982; Shelton et al., 1986; Burleson et al., 1992; Gilmour, 2001; Perry and Gilmour, 2002; Gilmour and Perry, 2007; Perry et al., 2009b; Milsom, 2012). In brief, fish hyperventilate during hypoxia by increasing respiratory frequency (f_V) and/or amplitude (V_{AMP}), depending on species. Collectively, these changes in breathing are termed the hypoxic ventilatory response (HVR). Hyperventilation enhances oxygenation of the blood flowing through the gills and therefore serves to minimize the extent of the reduction in arterial PO_2 (PaO_2), and hence haemoglobin O_2 saturation, that is an inevitable consequence of ambient hypoxia (e.g., Holeton and Randall, 1967). The vast majority of studies that have examined the piscine HVR were performed on adults. In recent years, however, the HVR during early development has received renewed and increasing attention. Studies on zebrafish, in particular, have provided insight into the maturation of the HVR and its developmental plasticity while shedding light on the physiological significance of the HVR in larvae lacking fully developed gills. Given its underdeveloped gills, a high surface-to-volume ratio, thin integument and immature autonomic nervous system, the larva should not be considered a miniature version of the adult although some elements of the HVR are conserved.

2.2 Ontogeny of the hypoxic ventilatory response

The scarcity of studies on early life stages may reflect the generally held view that the developing gills of larvae do not contribute significantly to whole body O_2 uptake until they have developed an adult-like morphology (Rombough, 1988; Rombough, 2007). In zebrafish (Rombough, 2002) and rainbow trout (*Oncorhynchus mykiss*) (Fu et al., 2010), the gills do not assume a predominant role in O_2 uptake until 14 and 27 days post-hatch, respectively. However, despite the apparently minor role of the gills in O_2 uptake during early stages of development, zebrafish (Jonz and Nurse, 2005) and rainbow trout (Holeton, 1971) exhibit an obvious HVR as early as 3 and 1 day post fertilization (dpf), respectively. Similar hyperventilatory responses before gill maturation were reported for Arctic char (*Salvelinus alpinus*; McDonald and McMahon, 1977) and gar (*Atractosteus tropicus*; Burggren et al., 2016) larvae, although Atlantic salmon (*Salmo salar*) larvae at 50 dpf do not exhibit a HVR (Polymeropoulos et al., 2014).

Developing fishes ventilate their buccal and opercular cavities with increasing regularity as they mature. Additionally, they possess a finely tuned HVR before the gills are fully formed, a time when the skin is thought to be the dominant site of O_2 uptake. Thus, the physiological significance of the HVR in larvae prior to gill development is not immediately obvious. One possibility is that this seemingly precocious HVR may be present to ensure that O_2-sensing pathways are operational by the time the larvae become dependent on branchial gas transfer (Jonz and Nurse, 2005). It is also plausible that O_2 extraction from the buccal cavity, while not significant during normoxia, assumes an increasingly important role in maintaining routine O_2 consumption during hypoxia. That is while the gills may not yet exhibit the extensive lamellae that confer large surfaces for gas transfer, the vascularized primordial filaments, lamellar buds and the lining of the buccal cavity, itself, are potential gas transfer surfaces. In support of this idea, the critical PO_2 (P_{crit}; the PO_2 of the water during progressive hypoxia at which MO_2 can no longer be maintained) was increased in 7 dpf zebrafish larvae prevented from ventilating their buccal cavity (Pan et al., 2019). Clearly despite the absence of a fully developed gill, the HVR in zebrafish larvae older than 7 dpf benefits O_2 uptake during hypoxia. It is important to note that the elevated ventilation during hypoxia not only raises water flow through the buccal and opercular cavities, but also increases water flow across cutaneous surfaces. This increase in convection over the skin associated with hyperventilation and increased fin movements (see below) likely increases the trans-cutaneous O_2 diffusion gradient while reducing physical boundary layers; both effects are expected to increase O_2 uptake across the skin during hypoxia.

The ventilatory responses of larval fish to hypoxia are temporally complex and influenced by age and the severity of the hypoxia (Turesson et al., 2006; Mandic et al., 2019; Pan et al., 2019). For example, the HVR of larval zebrafish exposed to a PO_2 of 55 mmHg was transient, lasting only 5 min, at 4 dpf, but persisting for the duration of a 30 min exposure at 10 dpf (Pan et al., 2019). Thus, the HVR might easily go undetected if ventilation was measured only at a limited number of time points after beginning hypoxia. The overall HVR consists of several phases even when the hyperventilation persists. The initial response is a rapid increase in f_V that typically peaks within 5 min of the onset of hypoxia (Mandic et al., 2019; Pan et al., 2019). The next phase of the HVR consists of a decline in f_V to normoxic values or to a level that is intermediate between resting and peak f_V. The likelihood of f_V remaining elevated increases with age and the severity of hypoxia (Mandic et al., 2019; Pan et al., 2019). Similar to adults, the period of increased ventilation in larvae <15 dpf is accompanied by stable O_2 consumption (Mandic et al., 2020). The limited data on larvae indicate that the increased f_V begins to decline prior to P_{crit} whereas in adults, the peak HVR is sustained slightly beyond P_{crit} (Mandic et al., 2020). Future studies may benefit from longer exposures (e.g., days to weeks), which would enable comparisons of hypoxic acclimation in larvae with data from adults (Vulesevic et al., 2006).

2.2.1 Synchrony of buccal ventilation and fin movements

In early larval stages, breathing is infrequent and irregular (Holeton, 1971; McDonald and McMahon, 1977; Jonz and Nurse, 2005). For example, breathing movements in zebrafish larvae post hatch are rare under normoxic conditions; the HVR is first observed at 3 dpf but even during hypoxia, the buccal movements are irregular until about 8 dpf (Jonz and Nurse, 2005). Qualitatively similar results were observed for rainbow trout and Arctic char, although the transition from irregular to regular breathing movements occurred over a longer time course, corresponding with their slower rate of development (Holeton, 1971; McDonald and McMahon, 1977). During these early developmental stages, the buccal/opercular movements are synchronized with rapid pectoral fin movements, a behaviour presumed to aid gas transfer across the skin by increasing water flow over the body (Rombough, 1988). The synchrony between gill ventilation and pectoral fin movements during hypoxia in zebrafish larvae was studied by Zimmer et al. (Zimmer et al., 2020). Their data demonstrated that the synchronous movements, which are obvious at 4 dpf, are absent by 21 dpf. It was suggested that the loss of synchrony reflects the transition from reliance on cutaneous gas transfer to reliance on branchial gas transfer. To our knowledge, the sensory mechanisms underlying the fin movements during hypoxia have not been investigated (see Zimmer et al., 2020).

2.2.2 Developmental plasticity

The ventilatory responses of adult fish to hypoxia can be modified by the status of the environment during early development. For example, zebrafish embryos treated with hyperoxic water for 7 days (0–7 dpf) exhibited a blunted HVR when exposed as adults to acute hypoxia (Vulesevic and Perry, 2006). However, there was no effect of subjecting larvae to hypoxia or hypercapnia on the responses of adult fish to hypoxia. The plasticity exhibited by zebrafish exposed to hyperoxia during early development (Vulesevic and Perry, 2006) agrees with the results of studies on mammals showing that exposure to hyperoxia during a critical prenatal period may permanently impair the phrenic response to hypoxia (Ling et al., 1997; Donnelly, 2000; Carroll, 2003). The mechanisms responsible for blunting of the HVR in adult zebrafish subjected to hyperoxia as larvae are unknown but as in mammals, may reflect changes to the O_2 chemoreceptors or afferent neurons (Donnelly, 2000).

Not only can the HVR be shaped by developmental plasticity (Vulesevic and Perry, 2006), but it is possible that the HVR is modified epigenetically in offspring of parents that were exposed to hypoxia. Notably, zebrafish larvae of one (male) or both parents exposed to hypoxia exhibited significant differences in their hypoxia tolerance compared to offspring of parents that had never experienced hypoxia (Ho and Burggren, 2012; Ragsdale et al., 2022). Although no breathing data were obtained (Ho and Burggren, 2012; Ragsdale et al., 2022), it is tempting to speculate that the transgenerational changes in hypoxia tolerance are related to changes in the HVR. Assessing possible epigenetic effects on the HVR is an area that warrants further attention.

2.2.3 Aquatic surface respiration

In naturally hypoxic aquatic habitats, a thin layer of water at the surface tends to be enriched with O_2 relative to the bulk water (Burggren, 1982). Fish exploit this microenvironment with a behavioural adaptation termed aquatic surface respiration (ASR) (Kramer and Mehegan, 1981). During ASR, fish rise to the surface and skim the uppermost layer to ventilate their gills with O_2-enriched water (Gee et al., 1978; Kramer and McClure, 1982). Aquatic surface respiration has been studied almost exclusively in adult fishes (Chapman and McKenzie, 2009) where it has been shown to increase blood oxygenation during severe hypoxia (Burggren, 1982) as well as survival (Kramer and McClure, 1982). To our knowledge,

ASR has been examined rarely in juveniles (Weber and Kramer, 1983; Sloman et al., 2008) and in larvae of a single species, zebrafish (Abdallah et al., 2015b; Mandic et al., 2022), in which ASR increased as the severity of hypoxia was increased. In zebrafish, the age of onset of ASR during acute hypoxia is 5 dpf (Abdallah et al., 2015b). At earlier developmental stages, ASR may be constrained by the lack of swimbladder inflation that is needed to control buoyancy (Abdallah et al., 2015b). Similar to adult zebrafish, ASR improves survival during severe hypoxia in larvae older than 5 dpf (Mandic et al., 2022). Indeed, when exposed to a level of hypoxia of 16 mmHg, the survival of larvae (>5 dpf) performing ASR was similar to that of normoxic larvae at close to 100%. Larvae (>5 dpf) spent approximately 30% of their time engaged in ASR, which was similar to the situation in adults experiencing the same level of hypoxia (16 mmHg) (Mandic et al., 2022). The energetic costs associated with extended periods of ASR in zebrafish larvae are unknown but are likely to be low given their state of neutral buoyancy (Lindsey et al., 2010). Conversely, longer time spent at the surface is likely to increase the risk of predation.

2.3 Environmental CO_2 levels

Elevated levels of dissolved CO_2 in aquatic habitats (interchangeably termed hypercapnia or hypercarbia) arise naturally from biological respiration and microbial decomposition of organic matter, especially in tropical waters with high biomass including the so-called "blackwaters" of the Amazon basin (Furch and Junk, 1997; Rasera et al., 2013). Additionally, eutrophication is a major cause of hypercapnia that is worsening globally owing to pollution, the warming of aquatic environments and increasing levels of atmospheric CO_2.

As with hypoxia, most studies that assessed the ventilatory effects of hypercapnia were conducted on adult fish. Although PCO_2 thresholds of onset vary widely, a ubiquitous response to hypercapnia is hyperventilation (Gilmour, 2001; Gilmour and Perry, 2007; Perry et al., 2009a; Milsom, 2012; Perry and Abdallah, 2012; Tresguerres et al., 2019) that, in contrast to hypoxia, typically results from increases in V_{AMP} rather than f_V (Milsom et al., 2022). The regulation of ventilation by ambient CO_2 or associated changes in the internal acid-base status, while important, is secondary to the dominant role of O_2 status in the control of breathing in fishes. In adult zebrafish, episodic breathing is a relatively common occurrence under resting conditions (e.g., 20% of individuals examined) (Vulesevic et al., 2006). Unlike hypoxia, which alters breathing patterns from episodic to continuous, episodic breathing is unchanged by hypercapnia (Vulesevic et al., 2006; Milsom et al., 2022). Currently, there is no explanation for the different patterns of ventilatory responses to hypoxia and hypercapnia, which are presumably to be triggered by the same chemoreceptors (see below).

2.4 Ontogeny of the hypercapnic ventilatory response

Little is known about the respiratory reflexes associated with hypercapnia in developing fish. Indeed, the only species that has been studied during larval stages is zebrafish. Unlike adults, which increase ventilation during hypercapnia exclusively by adjusting V_{AMP} (see above), larval zebrafish hyperventilate by markedly increasing f_V when exposed to elevated ambient CO_2 (Kunert et al., 2022). To date, there are no reliable methods to measure V_{AMP} in zebrafish larvae; thus, it is conceivable that V_{AMP} increases in concert with changes in f_V. Regardless, it would be useful to determine the time of development when the ventilatory response to hypercapnia shifts from altering f_V to altering V_{AMP}. As in adult fishes, the breathing response of zebrafish larvae to hypercapnia is caused specifically by an increase in ambient PCO_2 rather than the associated acidification of the water (Kunert et al., 2022). The benefit, if any, of the hypercapnic hyperventilation in larvae, which occurs concurrently with tachycardia (Miller et al., 2014), is unknown.

3 Sensory and molecular mechanisms of chemoreception

The reflex ventilatory responses to hypoxia and hypercapnia described above are thought to be triggered by the activation of peripheral chemoreceptors. However, much remains to be learned about the molecular mechanisms of gas sensing as well as the afferent sensory pathways and neural circuitry that process chemosensory signals. As with reflex ventilatory responses, most studies of sensory mechanisms have focused on adult fish or chemosensory cells isolated from adult fish. It is only recently that the power of larval zebrafish as a model for investigating chemoreceptor function *in vivo* has begun to be realized. Below, the evidence that neuroepithelial cells (NECs) serve as O_2 and CO_2 chemoreceptors is briefly surveyed and our current understanding of the molecular mechanisms of chemoreception is discussed.

3.1 Hypoxia

3.1.1 Peripheral chemoreceptors

The O_2 chemoreceptors in fishes are believed to be NECs that were first described by Dunel-Erb et al. (1982). In adult fish, they are found on the tips of gill filaments and in larvae, they are located on the skin prior to gill maturation (Jonz and Nurse, 2006; Coccimiglio and Jonz, 2012). NECs also may be present on cutaneous surfaces in adults of some species including the mangrove rivulus (*Kryptolebias marmoratus*) (Regan et al., 2011; Rossi et al., 2020) and giant mudskipper (*Periophthalmodon schlosseri*) (Zaccone et al., 2017).

Gill NECs are characterized by the presence of dense-cored vesicles containing serotonin (5-HT) and their innervation is derived from a plexus of nerve fibres (Dunel-Erb et al., 1982; Bailly et al., 1989; Bailly et al., 1992; Jonz and Nurse, 2003). Initial suggestions of gill NECs being O_2 chemoreceptors were based on their morphology, location and innervation patterns (Dunel-Erb et al., 1982; Bailly et al., 1992; Jonz and Nurse, 2003; Bailly, 2009). Substantial indirect evidence supports a role for the NECs as peripheral O_2 chemoreceptors, largely based on their morphological changes (hypertrophy, hyperplasia and increased numbers of neuron-like processes) during exposure to prolonged hypoxia (Jonz et al., 2004; Burleson et al., 2006; Regan et al., 2011; Shakarchi et al., 2013; Rossi et al., 2020; Pan et al., 2021a). Direct evidence that gill NECs are O_2 sensitive was provided by whole-cell patch-clamp recordings *in vitro* from NECs isolated from adult gill filaments. Specifically, patch clamp experiments demonstrated

hypoxia-induced membrane depolarization in isolated gill NECs under current-clamp conditions in zebrafish (Jonz et al., 2004) and channel catfish (*Ictalurus punctatus*) (Burleson et al., 2006). Additional direct evidence was provided by measuring intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and synaptic vesicle activity in isolated goldfish (*Carassius auratus*) NECs (Zachar et al., 2017).

There is less evidence supporting the notion that skin NECs are O_2 chemoreceptors. In zebrafish, skin NECs exhibiting innervation are evident in embryos at 1 dpf, before the onset of behavioral responses to hypoxia (Coccimiglio and Jonz, 2012). A population of skin NECs also expresses synaptic vesicle protein, suggesting a capacity for the secretion of neurotransmitters (Coccimiglio and Jonz, 2012). However, all evidence of hypoxia sensitivity in skin NECs is indirect, based on their increasing density and/or size under hypoxic conditions (Regan et al., 2011; Coccimiglio and Jonz, 2012; Rossi et al., 2020), or elimination of the HVR following their partial chemical denervation (Coccimiglio and Jonz, 2012). In contrast, however, NEC development in larval mangrove rivulus is largely unaffected by environmental O_2 levels (Cochrane et al., 2021). Thus, the current evidence supports hypoxia sensitivity of isolated gill NECs *in vitro*, whereas direct evidence for hypoxia sensitivity in skin NECs is lacking. Additional research, especially *in vivo*, is required to establish both gill and skin NECs as piscine O_2 chemoreceptors.

3.1.2 Molecular mechanisms and pathways of O_2 sensing

The sensing of O_2 begins with the detection of PO_2 changes by an O_2 sensor within the chemoreceptor, which leads to the inhibition of K^+ channels and subsequent membrane depolarization. Cytosolic Ca^{2+} levels increase, through either extracellular Ca^{2+} entry *via* voltage-dependant Ca^{2+} channels or the release of Ca^{2+} from intracellular stores, thereby facilitating vesicle fusion and the release of neurotransmitters. This neurosecretion activates the afferent nerve fibers innervating the chemoreceptors, resulting in the signal being transmitted to the central nervous system (CNS) where it is processed to elicit downstream responses (Gonzalez et al., 2010; Zachar and Jonz, 2012; Prabhakar, 2013; Lopez-Barneo et al., 2016; Ortega-Sáenz et al., 2020).

Regardless of vertebrate class, the molecular mechanisms underlying acute O_2 sensing remain elusive (Rakoczy and Wyatt, 2018). The earliest ideas for molecular O_2 sensing were based on mitochondrial inhibition. In this scheme, inhibition of the mitochondrial electron transport chain under hypoxic conditions leads to decreased ATP production that is sensed by ATP-sensitive K^+ channels, resulting in depolarization and neurotransmitter release (Duchen and Biscoe, 1992a; Duchen and Biscoe, 1992b; Wyatt and Buckler, 2004; Varas et al., 2007). In addition, the decrease in ATP may cause AMP to rise, which could activate AMP-activated protein kinase (AMPK), further phosphorylating membrane ion channels and leading to depolarization; thus, AMPK is a candidate O_2 sensor (Evans, 2004). Furthermore, disruption of mitochondrial complex I on the electron transport chain could increase production of reactive oxygen species (ROS), changing the redox status of membrane ion channels and thus initiating excitation (Fernandez-Aguera et al., 2015; Gao et al., 2017). The gasotransmitters CO and H_2S also have been proposed as components of the molecular mechanism for O_2 sensing (Li et al., 2010; Buckler, 2012; Yuan et al., 2015). Lactate is one of the newest candidates to be proposed as a member of the O_2 sensing pathway. Chang et al. (2015) demonstrated that the olfactory receptor encoded by *Olfr78* is sensitive to lactate, which may accumulate during hypoxia. *Olfr78* knockout mice did not exhibit a HVR but responded normally to hypercapnia. However, the idea of lactate being involved in O_2 sensing is not universally accepted because the original results obtained using *Olfr78* knockout mice were not fully reproducible (Chang et al., 2018; Torres-Torrelo et al., 2018). It is likely that additional molecular mechanisms for O_2 chemoreception will be proposed. Indeed, it is likely that ultimately multiple mechanisms will be identified for O_2 sensing within the chemoreceptors (Kumar and Bin-Jaliah, 2007).

In fishes, the molecular mechanisms of O_2 chemoreception have received less attention, and only H_2S and lactate have been examined in the context of cellular (NEC) O_2 sensing. In trout branchial tissues where gill NECs are located, genes for the H_2S-synthesizing enzymes, cystathionine *ß*-synthase and cystathionine γ-lyase, are expressed (Olson et al., 2008). Further, gill homogenates produce H_2S enzymatically, a process which is inhibited by high levels of O_2 (Olson et al., 2008). Behaviourally, intrabuccal injection of H_2S in anaesthetized trout produced increases in f_V and V_{AMP} similar to those of the HVR (Olson et al., 2008). Similar results were obtained in zebrafish larvae in which H_2S elicited hyperventilation that was blocked by preventing endogenous H_2S synthesis throughout the body, including in the cystathionine γ-lyase-containing skin NECs (Porteus et al., 2014). Additionally, H_2S exposure increased $[Ca^{2+}]_i$ in NECs isolated from zebrafish gill and resulted in membrane depolarization similar to that observed under hypoxia (Olson et al., 2008; Perry et al., 2016). Lactate can also elevate $[Ca^{2+}]_i$ in isolated killifish (*Fundulus heteroclitus*) NECs (Leonard et al., 2022). In addition, lactate also elevates gill ventilation in a dose-dependent manner independent of pH changes in both the striped catfish (*Pangasianodon hypophthalmus*) and trout, with this response being attenuated following denervation of the first gill arch (Thomsen et al., 2017; Thomsen et al., 2019). Thus the existing evidence suggests that both H_2S and lactate have the potential to participate in O_2 sensing within NECs in fish. Two other gasotransmitters, CO and NO, also can modify ventilatory responses in fishes, with CO inhibiting ventilation in goldfish (Tzaneva and Perry, 2014) and zebrafish (Tzaneva and Perry, 2016). Interestingly, the effects of NO on ventilation are not only dependent on developmental age–NO stimulates breathing in zebrafish larvae while inhibiting breathing in adults (Porteus et al., 2015)–but also on HIF-1α, as NO is unable to contribute to the HVR in mutant larvae lacking HIF-1 α. However, there is no direct evidence that CO or NO participates in O_2 sensing specifically at the level of NECs (Olson et al., 2012; Mandic et al., 2019). A recent single-cell transcriptomic analysis of the zebrafish gill (Pan et al., 2022) revealed high expression of genes encoding for NADH dehydrogenase and cytochrome c oxidase in NECs, genes related to mitochondrial function that have been implicated in O_2 sensing in mammals.

Under voltage-clamp conditions, isolated gill NECs from adult zebrafish (Jonz et al., 2004) and channel catfish (Burleson et al., 2006) respond with a decrease in K^+ current that is insensitive to voltage-dependent K^+ channel blockers but sensitive to the background K^+ channel blocker, quinidine (Jonz et al., 2004). In addition, hypoxia caused a reversible depolarization that was associated with a conductance decrease in isolated zebrafish gill NECs under current-clamp conditions; this response was reduced in the presence of quinidine (Jonz et al., 2004). In isolated gill NECs from goldfish,

hypoxia exposure resulted in increases in $[Ca^{2+}]_i$ and vesicular activity that were blocked by the Ca^{2+} channel blocker, cadmium, and the L-type Ca^{2+} channel blocker, nifedipine (Zachar et al., 2017). These data suggest that hypoxia sensing in gill NECs is mediated by inhibition of a background K^+ channel leading to depolarization, an increase in $[Ca^{2+}]_i$, and possibly neurosecretion, similar to that observed in glomus cells of the mammalian carotid body (Lopez-Barneo et al., 2016).

Although there is evidence that trout NECs appear "degranulated" after hypoxia exposure (Dunel-Erb et al., 1982), and that NECs possess the synaptic vesicle protein SV2 indicative of neurosecretion potential (Jonz et al., 2004), there is no direct evidence of any neurotransmitter being released by NECs under hypoxic conditions. Serotonin (5-HT) is the only neurotransmitter directly and routinely identified in fish NECs, but there is an array of neurotransmitters that may enable signal transduction between NECs and the afferent neurons, and which have been shown to modulate breathing. The neuroendocrine control of breathing in fish was recently reviewed (Reed and Jonz, 2022) and thus, only a brief summary will be provided here. Using isolated gill arches from rainbow trout, application of acetylcholine resulted in potent stimulation of afferent neural activity, while 5-HT and dopamine caused brief, small bursts in neural activity followed by mild inhibition (Burleson and Milsom, 1995). Adrenaline and noradrenaline did not elicit any neural responses (Burleson and Milsom, 1995). However, when neurotransmitters were administered through intra-vascular injection or applied externally, 5-HT, adrenaline, noradrenaline, acetylcholine and purines generally stimulated ventilation, whereas dopamine inhibited ventilation (Burleson and Milsom, 1995). A similar inhibitory effect of dopamine on ventilation was reported in larval zebrafish (Shakarchi et al., 2013). In addition, single-cell transcriptomic analysis of the zebrafish gill showed that NECs and neurons express genes encoding transmembrane receptors for serotonergic, cholinergic and dopaminergic neurotransmission (Pan et al., 2022). These results are broadly consistent with the mammalian situation in which acetylcholine and purines are excitatory carotid body neurotransmitters whereas dopamine is inhibitory (Leonard et al., 2018). However, unlike in mammals in which neurotransmitter secretion from the carotid body was demonstrated, studies in fish, while establishing a direct link between neurotransmitters and ventilation, have not yet revealed the specific site of action. Future studies are needed to directly examine neurosecretion from NECs under hypoxic conditions.

Upon activation of the chemoreceptors and the presumed release of neurotransmitters, the signal is transmitted to the CNS leading to downstream ventilatory responses. Existing data suggest that cranial nerves IX (glossopharyngeal) and X (vagus) are the afferent neurons innervating the gill chemoreceptors. Thus, total gill denervation of cranial nerves IX and X decreased the magnitude of the HVR in nine species over a wide range of taxonomic diversity (Milsom, 2012) whereas rhythmic stimulation of cranial nerve X entrains the respiratory rhythm in carp (*Cyprinus carpio*) (De Graaf and Roberts, 1991). Using genetically encoded calcium sensors GCaAMP6s under control of the pan-neuronal promoter *elavl3*, Rosales et al. (2019) showed that in zebrafish, the average magnitude of Ca^{2+} transients increased within the sensory ganglia of cranial nerve X upon exposure to hypoxia, providing arguably the first direct *in vivo* evidence for any of the O_2 sensing components in a fish. Central projections from the ganglia of cranial nerves IX and X enter the hindbrain at presumptive rhombomere 6 and *via* a series of nerve roots to form a 'plexus' (Kucenas et al., 2006). Given that the preBötzinger complex, which is the origin of respiratory motor output in mammals, arises from rhombomeres 6 and 7 (Mellen and Thoby-Brisson, 2012), and also that rhombomere 7 is essential for gill and buccal bursts in bullfrog tadpoles (Duchcherer et al., 2013), it is reasonable to hypothesize that O_2 signals originating in the chemoreceptors are conveyed to the respiratory motor output centres in the hindbrain *via* cranial nerves IX and X.

3.2 Hypercapnia

3.2.1 Peripheral chemoreceptors

In water-breathing adult fishes, evidence for a primarily branchial location of CO_2 chemosensors has come from studies in which CO_2-stimulated ventilatory responses were eliminated by denervation or extirpation of one or more gill arches (Burleson and Smatresk, 2000; Reid et al., 2000; Sundin et al., 2000; McKendry et al., 2001; Perry and Reid, 2002; Florindo et al., 2004; Bojink et al., 2010). These studies also revealed a predominant role for the first gill arch in eliciting CO_2-stimulated ventilatory responses in some species [rainbow trout (Perry and Reid, 2002) and jeju *Hoplerythrinus unitaeniatus* (Bojink et al., 2010), but see also (Sundin et al., 2000)], and they identified cranial nerves IX and X as those responsible for transmitting branchial CO_2 chemoreceptor activity to the brain. In the tambaqui *Colossoma macropomum*, total gill denervation did not completely eliminate ventilatory responses to CO_2, suggesting that extra-branchial CO_2 chemoreceptors may be present in some species (Milsom et al., 2002; Florindo et al., 2004). These extra-branchial receptors are likely to be associated with the orobranchial cavity or other peripheral sites because the balance of evidence does not support the presence of central CO_2/pH chemoreceptors in strictly water-breathing fishes (for discussion, see Tresguerres et al., 2019; Milsom et al., 2022).

The branchial chemoreceptors appear to respond primarily to water CO_2 rather than to water pH or to changes in blood CO_2/pH (reviewed by Gilmour and Perry, 2007; Milsom, 2012). Evidence to support this consensus has come from studies that have attempted to independently manipulate water *versus* blood CO_2 *versus* pH. For example, injection of CO_2-equilibrated water into the buccal cavity elicits hyperventilation, whereas injection of isocapnic water adjusted to the pH corresponding to that of the CO_2-equilibrated water has little or no effect (Reid et al., 2000; Sundin et al., 2000; Perry and McKendry, 2001; Gilmour et al., 2005; Bojink et al., 2010). These data argue for the importance of water CO_2 rather than water pH as the proximate factor controlling ventilation during ambient hypercapnia. A comparison between injection of CO_2-enriched water into the buccal cavity and injection of CO_2-enriched saline into the vasculature similarly argues for the importance of water CO_2, with injection of CO_2-enriched saline being without effect on ventilation (Perry and McKendry, 2001; Perry and Reid, 2002; Gilmour et al., 2005; Bojink et al., 2010). In addition, treatments that raise internal (but not water) CO_2 levels do not elicit hyperventilatory responses. For example, arterial CO_2 tension more than doubled in tambaqui treated with the carbonic anhydrase inhibitor acetazolamide (to inhibit CO_2 excretion), yet ventilation did not change until the fish were exposed to aquatic hypercapnia (Gilmour et al., 2005). Where questions remain is with the possibility of internally-oriented receptors that detect acid-base status. Although injections of acidified saline into the vasculature

(Reid et al., 2000; Sundin et al., 2000; Bojink et al., 2010) or acetazolamide treatment (Gilmour et al., 2005) generally have not evoked ventilatory responses, several studies have reported results that are consistent with ventilation being adjusted by blood acid-base status (for detailed discussion, see Gilmour and Perry, 2007; Tresguerres et al., 2019). In particular, a close correspondence between ventilation and blood acid-base status during recovery from exhaustive exercise was observed in rainbow trout. Treatment of trout with carbonic anhydrase to enhance CO_2 excretion alleviated not only the acid-base disturbance, but also the accompanying hyperventilation (Wood and Munger, 1994). Thus, the door remains open to the possibility that ventilation may be adjusted according to internal acid-base status, with further research needed to refute or confirm this possibility.

Taken as a whole, the studies of ventilatory responses to hypercapnia suggest that CO_2 chemoreceptors are located in the gill of (adult) fishes and respond primarily to changes in water CO_2 levels. As with hypoxia, NECs located in the gill of adult fishes are thought to serve as the CO_2 chemoreceptors. In comparison with hypoxia, however, very few studies have investigated ventilatory responses to CO_2 in larvae, and there are no data that directly link cutaneous NECs to CO_2-stimulated ventilatory responses. Changes in NEC abundance, size and transcriptome in response to changes in environmental O_2 levels have provided indirect evidence of NEC involvement in O_2 chemoreception (see above). For CO_2 chemoreception, however, the data are both sparse and mixed. Acclimation to hypercapnic conditions for 28 d had no impact on the density of branchial (serotonin-positive) NECs in adult zebrafish (Vulesevic et al., 2006). On the other hand, increases in branchial NEC density were reported in mangrove rivulus acclimated to hypercapnia or to acidic water, although whether there were functional consequences of the changes in NEC density was not explored (Robertson et al., 2015). Overall, the paucity of data makes it difficult to draw any firm conclusions. However, there is evidence that branchial NECs detect and respond to hypercapnia in a manner that is consistent with their involvement in CO_2-evoked reflexes.

Direct evidence that branchial NECs can serve as CO_2 chemoreceptors was obtained from two studies of cellular responses to CO_2/pH in NECs isolated from the gills of adult zebrafish. Qin et al. (2010) reported that exposure to hypercapnia caused depolarization in a subset of the NECs that were tested, with the magnitude of the response increasing with increasing partial pressure of CO_2. The NECs that responded to increases in CO_2 also depolarized in response to lowering of O_2 levels, indicating that at least some NECs are dual sensors of O_2 and CO_2. Subsequently, an increase in $[Ca^{2+}]_i$ in response to hypercapnia was reported by Abdallah et al. (2015a). Although both responses were elicited by hypercapnia, the sensory mechanisms involved appear to differ, with a CO_2-stimulated change in intracellular pH being required for depolarization of the NEC (Qin et al., 2010), but a fall in extracellular pH that is independent of CO_2 being required for the increase in $[Ca^{2+}]_i$ (Abdallah et al., 2015a). Further research is needed to resolve this difference, and to reconcile the apparent discrepancy between sensory mechanisms at the cellular level (i.e. where extracellular pH is an adequate stimulus for a change in $[Ca^{2+}]_i$) and the stimuli that elicit ventilatory responses at the whole-animal level (i.e. changes in water CO_2 but not pH; see above). In addition, there is a need to link activation of CO_2-sensing NECs directly to the occurrence of CO_2-stimulated ventilatory reflexes.

3.2.2 Molecular mechanisms and pathways of CO_2 sensing

Current understanding of the signalling associated with NEC CO_2 sensing is based on the two studies mentioned above on NECs isolated from adult zebrafish gills (Qin et al., 2010; Abdallah et al., 2015a), and from studies in which cardiorespiratory responses to CO_2 in larval zebrafish were investigated (Miller et al., 2014; Koudrina et al., 2020; Kunert et al., 2022). As with O_2 sensing (Jonz et al., 2004), the initial cellular response to CO_2 is depolarization, which is caused by the inhibition of K^+ flux through background K^+ channels (Qin et al., 2010). It is likely that multiple K^+ channels are present in NECs, with evidence supporting the involvement of TASK-2 (Koudrina et al., 2020), a member of the tandem-pore domains in a weak inward rectifying K^+ channel (TWIK)-related acid-sensitive K^+ (TASK) channel family. Functional studies carried out on zebrafish TASK-2 expressed in HEK-293 cells revealed that it is inhibited by intracellular or extracellular acidification as well as by increases in CO_2 (Peña-Münzenmayer et al., 2013). Notably, the inhibition of TASK-2 by CO_2 included both a contribution of intracellular acidification and a direct effect of CO_2 that was not dependent on changes in intracellular pH. Therefore, the properties of TASK-2 channels are consistent with the requirements for CO_2 sensing in NECs. Immunohistochemistry was used to demonstrate that TASK-2 is expressed by serotonin-positive NECs in the skin of larvae and the gills of adult zebrafish (Koudrina et al., 2020). A functional role for TASK-2 was identified using antisense oligonucleotide morpholinos to knock down TASK-2 and/or its paralog TASK-2b, which attenuated the hyperventilatory responses to hypercapnia in 4 dpf zebrafish larvae (Koudrina et al., 2020). Interestingly, the genes that encode for TASK-2 (*kcnk5a*) and TASK-2b (*kcnk5b*) were not detected in a single-cell transcriptomic analysis of zebrafish NECs (Pan et al., 2022) (nor was *ca17a*, see below). However, the focus of Pan et al. (2022) was on NECs that respond to hypoxia. If only a subset of NECs serve as bimodal sensors of O_2 and CO_2 (Qin et al., 2010), then it could be difficult to detect differential expression of transcripts specific to CO_2 sensing.

CO_2-induced depolarization of NECs is expected to cause an increase in $[Ca^{2+}]_i$. Consistent with this pathway, Abdallah et al. (2015a) documented increases in $[Ca^{2+}]_i$ in isolated zebrafish NECs exposed to hypercapnia, with Ca^{2+} being derived primarily from intracellular stores. The increase in $[Ca^{2+}]_i$, in turn, is expected to lead to neurosecretion, transmission of the sensory signal to the brain and activation of the appropriate ventilatory response. However, currently there are no data to support this pathway, nor has any specific neurotransmitter been identified as being secreted. Serotonin is viewed as likely to be involved owing to its status as the predominant neurochemical in NECs (Porteus et al., 2012) and the ability of serotonin receptor agonists to elicit hyperventilatory responses in zebrafish larvae (Jonz et al., 2015b; for reviews see Pan and Perry, 2020; Reed and Jonz, 2022). Although, as alluded to above (section 3.1.2), the fact that certain neurochemicals evoke hyperventilation does not necessarily indicate that they are secreted by NECs.

The CO_2 sensing mechanism likely involves carbonic anhydrase (CA), specifically the cytosolic isoform Ca17a (Ferreira-Martins et al., 2016). Immunohistochemistry supports the presence of CA or Ca17a specifically in larval cutaneous and adult branchial NECs of zebrafish (Qin et al., 2010; Miller et al., 2014; Kunert et al., 2022). Pharmacological inhibition of CA, as well as specific knockdown or knockout of Ca17a, blunts the cardiorespiratory responses to hypercapnia in zebrafish larvae (Miller et al., 2014; Kunert et al.,

2022). These data argue that Ca17a contributes to CO_2 sensing, yet its specific role at the cellular level remains unclear. Inhibition of CA both slowed the rate and reduced the magnitude of CO_2-stimulated depolarization in isolated branchial NECs (Qin et al., 2010). In conjunction with the observation that extracellular acidification was not needed to elicit depolarization, these data suggest that depolarization occurs in response to CO_2 entry into the cell and its CA-catalyzed hydration to H^+, resulting in intracellular acidification. However, intracellular acidification was not sufficient to elicit a $[Ca^{2+}]_i$ response, nor did inhibition of CA alter the $[Ca^{2+}]_i$ response to hypercapnia even though intracellular acidification was slowed (Abdallah et al., 2015a). Indeed, extracellular acidification alone was sufficient to elicit a $[Ca^{2+}]_i$ response (Abdallah et al., 2015a). Resolving these discrepancies likely will provide insight into the role of Ca17a in CO_2 sensing.

4 Knowledge gaps and perspectives

Despite decades of research focused on unravelling the mechanisms of O_2 sensing in fish, there remain several significant unanswered questions. What are the molecular O_2 sensors in O_2 chemosensing cells? What is/are the neurotransmitter(s) secreted by O_2 chemoreceptors? How are peripheral signals from the O_2 chemoreceptors integrated within the CNS to evoke the HVR? Similar questions apply to the mechanisms of CO_2 sensing, which have received much less attention. With respect to CO_2 sensing, additional questions surround the nature of the critical stimulus that activates the CO_2 sensor–is molecular CO_2 itself sensed, or is the necessary stimulus a change in pH, and if so, is it extracellular or intracellular pH? A combination of CO_2 and a resultant pH change is also possible. Further, for both O_2 and CO_2 sensing, there is a need to gather additional empirical evidence to demonstrate convincingly that NECs function as respiratory chemoreceptors *in vivo*. In our opinion, the absence of direct *in vivo* data to support the idea that NECs function as O_2/CO_2 chemoreceptors to regulate ventilation is THE limiting factor constraining progress in the field of piscine breathing control.

Providing the empirical evidence to answer these questions likely will be constrained by the technical challenges associated with identifying chemoreceptors *in vivo* or culturing chemoreceptor-nerve complexes *in vitro*. With recent advances in genetic manipulation, especially Tol2 based transgenesis and CRISPR/Cas9 based knockout (Zimmer et al., 2019), the zebrafish is emerging as an important model species to examine O_2 and CO_2 chemoreception *in vivo*. First and foremost, the promoters of *tph1a* and *vmat2* have been identified to be able to drive transgene expression in zebrafish skin and gill NECs (Pan et al., 2021a; Pan et al., 2021b; Pan et al., 2022). Given the transparency of zebrafish larvae and the ability to render adult zebrafish transparent by using the *Casper* line (White et al., 2008), a wide array of *in vivo* imaging techniques can now be applied. For example, calcium activity within NECs in response to changing levels of PO_2 can be examined through *in vivo* calcium imaging using genetically encoded calcium indicators (Chen et al., 2013; Dana et al., 2016). Or, neurotransmitters being released by NECs under hypoxia can be examined by expressing specific neurotransmitter sensors (Wang et al., 2018) in NECs or the cranial sensory nerves that projects into the gill region (Kucenas et al., 2006).

An alternate approach that allows for experimental manipulation of sensory pathways is to express transgenes for cell activation, inhibition or ablation uniquely in NECs. Although genes such as *tph1a* and *vmat2* are expressed in NECs, they are also found in cells within the CNS that are critical for the regulation of breathing. Thus, driving transgenes with *tph1a* or *vmat2* promoters also would introduce transgene activity within the CNS, complicating data interpretation. It will be important, therefore, to identify genes that are expressed exclusively in NECs. Once achieved, NECs could be activated specifically with light or capsaicin by expressing channel rhodopsin (Antinucci et al., 2020) or rat TRPV1 channels (Matty et al., 2016). Similarly, NECs could be inhibited with light by expressing transient receptor potential cation channels (Antinucci et al., 2020) or ablated with metronidazole treatment in fish with NECs expressing nitroreductase (Sharrock et al., 2022). With such experiments, it would be possible to establish a direct link between NEC activity and ventilation. In summary, combining gene manipulation with physiological measurements offers a powerful approach to advance the field of chemoreception and control of breathing in fishes.

Author contributions

SFP wrote Section 1 (introduction) and Section 2 (ventilatory adjustments to environmental changes in respiratory gases). YKP wrote Section 3.1 (hypoxia) and KMG wrote Section 3.2 (hypercapnia). All authors contributed to Section 4 (knowledge gaps and perspectives) and final editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abdallah, S., Jonz, M. G., and Perry, S. F. (2015a). Extracellular H^+ induces Ca^{2+} signals in respiratory chemoreceptors of zebrafish. *Pflügers Arch.* 467, 399–413. doi:10.1007/s00424-014-1514-2

Abdallah, S. J., Thomas, B. S., and Jonz, M. G. (2015b). Aquatic surface respiration and swimming behaviour in adult and developing zebrafish exposed to hypoxia. *J. Exp. Biol.* 218, 1777–1786. doi:10.1242/jeb.116343

Antinucci, P., Dumitrescu, A., Deleuze, C., Morley, H. J., Leung, K., Hagley, T., et al. (2020). A calibrated optogenetic toolbox of stable zebrafish opsin lines. *Elife* 9, e54937. doi:10.7554/eLife.54937

Bailly, Y., Dunel-Erb, S., Geffard, M., and Laurent, P. (1989). The vascular and epithelial serotonergic innervation of the actinopterygian gill filament with special reference to the trout, *Salmo gairdneri*. *Cell Tissue Res.* 258, 349–363. doi:10.1007/bf00239455

Bailly, Y., Dunel-Erb, S., and Laurent, P. (1992). The neuroepithelial cells of the fish gill filament: Indolamine-immunocytochemistry and innervation. *Anat. Rec.* 233, 143–161. doi:10.1002/ar.1092330118

Bailly, Y. (2009). "Serotonergic neuroepithelial cells in fish gills: Cytology and innervation," in *Airway chemoreceptors in the vertebrates*. Editor G. Zaccone (Boca Raton: CRC Press), 61–98.

Bojink, C. L., Florindo, L. H., Leite, C. a. C., Kalinin, A. L., Milsom, W. K., and Rantin, F. T. (2010). Hypercarbic cardiorespiratory reflexes in the facultative air-breathing fish jeju (*Hoplerythrinus unitaeniatus*): The role of branchial CO_2 chemoreceptors. *J. Exp. Biol.* 213, 2797–2807. doi:10.1242/jeb.040733

Buckler, K. J. (2012). Effects of exogenous hydrogen sulphide on calcium signalling, background (TASK) K channel activity and mitochondrial function in chemoreceptor cells. *Pflügers Arch.* 463, 743–754. doi:10.1007/s00424-012-1089-8

Burggren, W. W. (1982). Air gulping" improves blood oxygen transport during aquatic hypoxia in the goldfish *Carassius auratus*. *Physiol. Zool.* 55, 327–334. doi:10.1086/physzool.55.4.30155860

Burggren, W. W., Bautista, G. M., Coop, S. C., Couturier, G. M., Delgadillo, S. P., García, R. M., et al. (2016). Developmental cardiorespiratory physiology of the air-breathing tropical gar, *Atractosteus tropicus*. *Am. J. Physiol.* 311, R689–R701. doi:10.1152/ajpregu.00022.2016

Burleson, M. L., Mercer, S. E., and Wilk-Blaszczak, M. A. (2006). Isolation and characterization of putative O_2 chemoreceptor cells from the gills of channel catfish (*Ictalurus punctatus*). *Brain Res.* 1092, 100–107. doi:10.1016/j.brainres.2006.03.085

Burleson, M. L., and Milsom, W. K. (1995). Cardio-ventilatory control in rainbow trout: I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. *Respir. Physiol.* 100, 231–238. doi:10.1016/0034-5687(95)91595-x

Burleson, M. L., and Milsom, W. K. (2003). "Comparative aspects of O_2 chemoreception. Anatomy, physiology, and environmental applications," in *Oxygen sensing. Responses and adaptation to hypoxia*. Editors S. Lahiri, G. L. Semenza, and N. R. Prabhakar (New York: Marcel Dekker), 685–707.

Burleson, M. L., and Smatresk, N. J. (2000). Branchial chemoreceptors mediate ventilatory responses to hypercapnic acidosis in channel catfish. *Comp. Biochem. Physiol.* 125A, 403–414. doi:10.1016/s1095-6433(00)00167-7

Burleson, M. L., Smatresk, N. J., and Milsom, W. K. (1992). "Afferent inputs associated with cardioventilatory control in fish," in *Fish Physiology v12B the cardiovascular system*. Editors W. S. Hoar, D. J. Randall, and A. P. Farrell (San Diego: Academic Press), 389–423.

Carroll, J. L. (2003). Developmental plasticity in respiratory control. *J. Appl. Physiol.* 94, 375–389. doi:10.1152/japplphysiol.00809.2002

Chang, A. J., Kim, N. S., Hireed, H., De Arce, A. D., Ortega, F. E., Riegler, J., et al. (2018). Chang et al. reply. *Nature* 561, E41. doi:10.1038/s41586-018-0547-7

Chang, A. J., Ortega, F. E., Riegler, J., Madison, D. V., and Krasnow, M. A. (2015). Oxygen regulation of breathing through an olfactory receptor activated by lactate. *Nature* 527, 240–244. doi:10.1038/nature15721

Chapman, L. J., and McKenzie, D. J. (2009). "Behavioral responses and ecological consequences," in *Fish Physiology v27 hypoxia*. Editors J. G. Richards, A. P. Farrell, and C. J. Brauner (USA: Elsevier), 25–77.

Chen, T.-W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., et al. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499, 295–300. doi:10.1038/nature12354

Coccimiglio, M. L., and Jonz, M. G. (2012). Serotonergic neuroepithelial cells of the skin in developing zebrafish: Morphology, innervation and oxygen-sensitive properties. *J. Exp. Biol.* 215, 3881–3894. doi:10.1242/jeb.074575

Cochrane, P. V., Jonz, M. G., and Wright, P. A. (2021). The development of the O_2-sensing system in an amphibious fish: Consequences of variation in environmental O_2 levels. *J. Comp. Physiol. B* 191, 681–699. doi:10.1007/s00360-021-01379-5

Dana, H., Mohar, B., Sun, Y., Narayan, S., Gordus, A., Hasseman, J. P., et al. (2016). Sensitive red protein calcium indicators for imaging neural activity. *Elife* 5, e12727. doi:10.7554/eLife.12727

De Boeck, G., and Wood, C. M. (2015). Does ammonia trigger hyperventilation in the elasmobranch, *Squalus acanthias suckleyi*? *J. Exp. Biol.* 206, 25–35. doi:10.1016/j.resp.2014.11.009

De Graaf, P., and Roberts, B. (1991). Effects of vagal sensory input on the breathing rhythm of the carp. *J. Exp. Biol.* 155, 77–91. doi:10.1242/jeb.155.1.77

Diaz, R. J. (2001). Overview of hypoxia around the world. *J. Environ. Qual.* 30, 275–281. doi:10.2134/jeq2001.302275x

Donnelly, D. F. (2000). Developmental aspects of oxygen sensing by the carotid body. *J. Appl. Physiol.* 88, 2296–2301. doi:10.1152/jappl.2000.88.6.2296

Duchcherer, M., Baghdadwala, M. I., Paramonov, J., and Wilson, R. J. (2013). Localization of essential rhombomeres for respiratory rhythm generation in bullfrog tadpoles using a binary search algorithm: Rhombomere 7 is essential for the gill rhythm and suppresses lung bursts before metamorphosis. *Dev. Neurobiol.* 73, 888–898. doi:10.1002/dneu.22108

Duchen, M., and Biscoe, T. (1992a). Mitochondrial function in type I cells isolated from rabbit arterial chemoreceptors. *J. Physiol.* 450, 13–31. doi:10.1113/jphysiol.1992.sp019114

Duchen, M., and Biscoe, T. (1992b). Relative mitochondrial membrane potential and $[Ca^{2+}]_i$ in type I cells isolated from the rabbit carotid body. *J. Physiol.* 450, 33–61. doi:10.1113/jphysiol.1992.sp019115

Dunel-Erb, S., Bailly, Y., and Laurent, P. (1982). Neuroepithelial cells in fish gill primary lamellae. *J. Appl. Physiol.* 53, 1342–1353. doi:10.1152/jappl.1982.53.6.1342

Eom, J., Fehsenfeld, S., and Wood, C. M. (2020). Is ammonia excretion affected by gill ventilation in the rainbow trout *Oncorhynchus mykiss*? *Respir. Physiol. Neurobiol.* 275, 103385. doi:10.1016/j.resp.2020.103385

Eom, J., Giacomin, M., Clifford, A. M., Goss, G. G., and Wood, C. M. (2019). Ventilatory sensitivity to ammonia in the Pacific hagfish (*Eptatretus stoutii*), a representative of the oldest extant connection to the ancestral vertebrates. *J. Exp. Biol.* 222, jeb199794. doi:10.1242/jeb.199794

Eom, J., and Wood, C. M. (2021). Brain and gills as internal and external ammonia sensing organs for ventilatory control in rainbow trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol. A* 254, 110896. doi:10.1016/j.cbpa.2021.110896

Evans, A. M. (2004). "Hypoxia, cell metabolism, and cADPR accumulation," in *Hypoxic pulmonary vasoconstriction*. Editor X. J. J. Yuan, (New York: Springer), 313–338.

Fernandez-Aguera, M. C., Gao, L., Gonzalez-Rodriguez, P., Pintado, C. O., Arias-Mayenco, I., Garcia-Flores, P., et al. (2015). Oxygen sensing by arterial chemoreceptors depends on mitochondrial complex I signaling. *Cell. Metab.* 22, 825–837. doi:10.1016/j.cmet.2015.09.004

Ferreira-Martins, D., McCormick, S. D., Campos, A., Lopes-Marques, M., Osório, H., Coimbra, J., et al. (2016). A cytosolic carbonic anhydrase molecular switch occurs in the gills of metamorphic sea lamprey. *Sci. Rep.* 6, 33954. doi:10.1038/srep33954

Florindo, L. H., Reid, S. G., Kalinin, A. L., Milsom, W. K., and Rantin, F. T. (2004). Cardiorespiratory reflexes and aquatic surface respiration in the neotropical fish tambaqui (*Colossoma macropomum*): Acute responses to hypercarbia. *J. Comp. Physiol. B* 174, 319–328. doi:10.1007/s00360-004-0417-5

Fu, C., Wilson, J. M., Rombough, P. J., and Brauner, C. J. (2010). Ions first: Na^+uptake shifts from the skin to the gills before O_2 uptake in developing rainbow trout, *Oncorhynchus mykiss*. *Proc. R. Soc. Lond. B* 277, 1553–1560. doi:10.1098/rspb.2009.1545

Furch, K., and Junk, W. J. (1997). "Physicochemical conditions in the floodplains," in *The central Amazon floodplain*. Editor W. J. Junk (Berlin, Heidelberg: Springer), 69–108.

Gao, L., González-Rodríguez, P., Ortega-Sáenz, P., and López-Barneo, J. (2017). Redox signaling in acute oxygen sensing. *Redox Biol.* 12, 908–915. doi:10.1016/j.redox.2017.04.033

Gee, J. H., Tallman, R. F., and Smart, H. J. (1978). Reactions of some great plains fishes to progressive hypoxia. *Can. J. Zool.* 56, 1962–1966. doi:10.1139/z78-263

Gilmour, K. M., Milsom, W. K., Rantin, F. T., Reid, S. G., and Perry, S. F. (2005). Cardiorespiratory responses to hypercarbia in tambaqui (*Colossoma macropomum*): Chemoreceptor orientation and specificity. *J. Exp. Biol.* 208, 1095–1107. doi:10.1242/jeb.01480

Gilmour, K. M., and Perry, S. F. (2007). "Branchial chemoreceptor regulation of cardiorespiratory function," in *Fish Physiology v25 sensory systems neuroscience*. Editors T. J. Hara and B. Zielinski (USA: Elsevier), 97–151.

Gilmour, K. M. (2001). The CO_2/pH ventilatory drive in fish. *Comp. Biochem. Physiol.* 130A, 219–240. doi:10.1016/s1095-6433(01)00391-9

Glass, M. L. (1992). "Ventilatory responses to hypoxia in ectothermic vertebrates," in *Physiological adaptations in vertebrates, respiration, circulation, and metabolism*. Editors S. C. Wood, R. E. Weber, A. R. Hargens, and R. W. Millard (New York: Marcel Dekker), 97–118.

Gonzalez, C., Agapito, M. T., Rocher, A., Gomez-Nino, A., Rigual, R., Castaneda, J., et al. (2010). A revisit to O_2 sensing and transduction in the carotid body chemoreceptors in the context of reactive oxygen species biology. *Respir. Physiol. Neurobiol.* 174, 317–330. doi:10.1016/j.resp.2010.09.002

Heisler, N., Toews, D. P., and Holeton, G. F. (1988). Regulation of ventilation and acid-base status in the elasmobranch *Scyliorhinus stellaris* during hyperoxia induced hypercapnia. *Respir. Physiol.* 71, 227–246. doi:10.1016/0034-5687(88)90018-7

Ho, D. H., and Burggren, W. W. (2012). Parental hypoxic exposure confers offspring hypoxia resistance in zebrafish (*Danio rerio*). *J. Exp. Biol.* 215, 4208–4216. doi:10.1242/jeb.074781

Holeton, G. F., and Randall, D. J. (1967). The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. *J. Exp. Biol.* 46, 317–327. doi:10.1242/jeb.46.2.317

Holeton, G. F. (1971). Respiratory and circulatory responses of rainbow trout larvae to carbon monoxide and to hypoxia. *J. Exp. Biol.* 55, 683–694. doi:10.1242/jeb.55.3.683

Iwama, G. K., Boutilier, R. G., Heming, T. A., Randall, D. J., and Mazeaud, M. (1987). The effects of altering gill water flow on gas transfer in rainbow trout. *Can. J. Zool.* 65, 2466–2470. doi:10.1139/z87-372

Jones, D. R., and Schwarzfeld, T. (1974). The oxygen cost to the metabolism and efficiency of breathing in trout (*Salmo gairdneri*). *Respir. Physiol.* 21, 241–254. doi:10.1016/0034-5687(74)90097-8

Jonz, M. G., Buck, L. T., Perry, S. F., Schwerte, T., and Zaccone, G. (2015a). Sensing and surviving hypoxia in vertebrates. *Ann. N.Y. Acad. Sci.* 1365, 43–58. doi:10.1111/nyas.12780

Jonz, M. G., Fearon, I. M., and Nurse, C. A. (2004). Neuroepithelial oxygen chemoreceptors of the zebrafish gill. *J. Physiol.* 560, 737–752. doi:10.1113/jphysiol.2004.069294

Jonz, M. G., and Nurse, C. A. (2005). Development of oxygen sensing in the gills of zebrafish. *J. Exp. Biol.* 208, 1537–1549. doi:10.1242/jeb.01564

Jonz, M. G., and Nurse, C. A. (2003). Neuroepithelial cells and associated innervation of the zebrafish gill: A confocal immunofluorescence study. *J. Comp. Neurol.* 461, 1–17. doi:10.1002/cne.10680

Jonz, M. G., and Nurse, C. A. (2006). Ontogenesis of oxygen chemoreception in aquatic vertebrates. *Respir. Physiol. Neurobiol.* 154, 139–152. doi:10.1016/j.resp.2006.01.004

Jonz, M. G., Zachar, P. C., Da Fonte, D. F., and Mierzwa, A. S. (2015b). Peripheral chemoreceptors in fish: A brief history and a look ahead. *Comp. Biochem. Physiol. A* 186, 27–38. doi:10.1016/j.cbpa.2014.09.002

Kinkead, R., and Perry, S. F. (1991). The effects of catecholamines on ventilation in rainbow trout during hypoxia or hypercapnia. *Respir. Physiol.* 84, 77–92. doi:10.1016/0034-5687(91)90020-j

Koudrina, N., Perry, S. F., and Gilmour, K. M. (2020). The role of TASK-2 channels in CO_2 sensing in zebrafish (*Danio rerio*). *Am. J. Physiol.* 319, R329–R342. doi:10.1152/ajpregu.00132.2020

Kramer, D. L., and McClure, M. (1982). Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. *Env. Biol. Fish.* 7, 47–55. doi:10.1007/bf00011822

Kramer, D. L., and Mehegan, J. P. (1981). Aquatic surface respiration, an adaptive response to hypoxia in the guppy, *Poecilia reticulata* (Pisces, Poeciliidae). *Env. Biol. Fish.* 6, 299–313. doi:10.1007/bf00005759

Kucenas, S., Soto, F., Cox, J., and Voigt, M. (2006). Selective labeling of central and peripheral sensory neurons in the developing zebrafish using P2X3 receptor subunit transgenes. *Neurosci* 138, 641–652. doi:10.1016/j.neuroscience.2005.11.058

Kumar, P., and Bin-Jaliah, I. (2007). Adequate stimuli of the carotid body: More than an oxygen sensor? *Respir. Physiol. Neurobiol.* 157, 12–21. doi:10.1016/j.resp.2007.01.007

Kunert, E., Joyce, W., Pan, Y. K., Chen, A., Perry, S. F., and Gilmour, K. M. (2022). The role of cytosolic carbonic anhydrase Ca17a in cardiorespiratory responses to CO_2 in developing zebrafish (*Danio rerio*). *Am. J. Physiol.* 323, R532–R546. doi:10.1152/ajpregu.00050.2022

Leonard, E. M., Salman, S., and Nurse, C. A. (2018). Sensory processing and integration at the carotid body tripartite synapse: Neurotransmitter functions and effects of chronic hypoxia. *Front. Physiol.* 9, 225. doi:10.3389/fphys.2018.00225

Leonard, E. M., Weaver, F. E., and Nurse, C. A. (2022). Lactate sensing by neuroepithelial cells isolated from the gills of killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* 225, jeb245088. doi:10.1242/jeb.245088

Li, Q., Sun, B., Wang, X., Jin, Z., Zhou, Y., Dong, L., et al. (2010). A crucial role for hydrogen sulfide in oxygen sensing via modulating large conductance calcium-activated potassium channels. *Antioxid. Redox Signal.* 12, 1179–1189. doi:10.1089/ars.2009.2926

Lindsey, B. W., Smith, F. M., and Croll, R. P. (2010). From inflation to flotation: Contribution of the swimbladder to whole-body density and swimming depth during development of the zebrafish (*Danio rerio*). *Zebrafish* 7, 85–96. doi:10.1089/zeb.2009.0616

Ling, L., Olson, E. B., Vidruk, E. H., and Mitchell, G. S. (1997). Developmental plasticity of the hypoxic ventilatory response. *Respir. Physiol.* 110, 261–268. doi:10.1016/s0034-5687(97)00091-1

Lopez-Barneo, J., Gonzalez-Rodriguez, P., Gao, L., Fernandez-Aguera, M. C., Pardal, R., and Ortega-Saenz, P. (2016). Oxygen sensing by the carotid body: Mechanisms and role in adaptation to hypoxia. *Am. J. Physiol.* 310, C629–C642. doi:10.1152/ajpcell.00265.2015

Mandic, M., Flear, K., Qiu, P., Pan, Y. K., Perry, S. F., and Gilmour, K. M. (2022). Aquatic surface respiration significantly improves survival of zebrafish (*Danio rerio*) lacking hypoxia inducible factor 1-α. *Proc. R. Soc. Lond. B* 289, 20211863. doi:10.1098/rspb.2021.1863

Mandic, M., Pan, Y. K., Gilmour, K. M., and Perry, S. F. (2020). Relationships between the peak hypoxic ventilatory response and critical O_2 tension in larval and adult zebrafish (*Danio rerio*). *J. Exp. Biol.* 223, jeb213942. jeb.213942. doi:10.1242/jeb.213942

Mandic, M., Tzaneva, V., Careau, V., and Perry, S. F. (2019). Hif-1α paralogs play a role in the hypoxic ventilatory response of larval and adult zebrafish (*Danio rerio*). *J. Exp. Biol.* 222, jeb195198. doi:10.1242/jeb.195198

Matty, M. A., Beerman, R. W., and Tobin, D. M. (2016). Drug-inducible, cell-specific manipulation of intracellular calcium in Zebrafish through mammalian TRPV1 expression. *Zebrafish* 13, 374–375. doi:10.1089/zeb.2016.29004.mat

McDonald, D. G., and McMahon, B. R. (1977). Respiratory development in Arctic char *Salvelinus alpinus* under conditions of normoxia and chronic hypoxia. *Can. J. Zool.* 55, 1461–1467. doi:10.1139/z77-189

McKendry, J. E., Milsom, W. K., and Perry, S. F. (2001). Branchial CO_2 receptors and cardiorespiratory adjustments during hypercarbia in Pacific spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.* 204, 1519–1527. doi:10.1242/jeb.204.8.1519

Mellen, N. M., and Thoby-Brisson, M. (2012). Respiratory circuits: Development, function and models. *Curr. Opin. Neurobiol.* 22, 676–685. doi:10.1016/j.conb.2012.01.001

Miller, S., Pollack, J., Bradshaw, J., Kumai, Y., and Perry, S. F. (2014). Cardiac responses to hypercapnia in larval zebrafish (*Danio rerio*): The links between CO_2 chemoreception, catecholamines and carbonic anhydrase. *J. Exp. Biol.* 217, 3569–3578. doi:10.1242/jeb.107987

Milsom, W. K., Gilmour, K. M., Perry, S. F., Gargaglioni, L., Hedrick, M., Kinkead, R., et al. (2022). Control of breathing in ectothermic vertebrates. *Compr. Physiol.* 12, 3869–3988. doi:10.1002/cphy.c210041

Milsom, W. K. (2012). New insights into gill chemoreception: Receptor distribution and roles in water and air breathing fish. *Respir. Physiol. Neurobiol.* 184, 326–339. doi:10.1016/j.resp.2012.07.013

Milsom, W. K., Reid, S. G., Rantin, F. T., and Sundin, L. (2002). Extrabranchial chemoreceptors involved in respiratory reflexes in the neotropical fish *Colossoma macropomum* (the tambaqui). *J. Exp. Biol.* 205, 1765–1774. doi:10.1242/jeb.205.12.1765

Olson, K. R., Donald, J. A., Dombkowski, R. A., and Perry, S. F. (2012). Evolutionary and comparative aspects of nitric oxide, carbon monoxide and hydrogen sulfide. *Respir. Physiol. Neurobiol.* 184, 117–129. doi:10.1016/j.resp.2012.04.004

Olson, K. R., Healy, M., Zhaohong, Q., Vulesevic, B., Duff, D. W., Whitfield, N., et al. (2008). Hydrogen sulfide as an oxygen sensor in trout gill chemoreceptors. *Am. J. Physiol.* 295, R669–R680. doi:10.1152/ajpregu.00807.2007

Ortega-Sáenz, P., Moreno-Domínguez, A., Gao, L., and López-Barneo, J. (2020). Molecular mechanisms of acute oxygen sensing by arterial chemoreceptor cells. Role of Hif2α. *Front. Physiol.* 11, 614893. doi:10.3389/fphys.2020.614893

Pan, W., Godoy, R. S., Cook, D. P., Scott, A. L., Nurse, C. A., and Jonz, M. G. (2022). Single-cell transcriptomic analysis of neuroepithelial cells and other cell types of the gills of zebrafish (*Danio rerio*) exposed to hypoxia. *Sci. Rep.* 12, 10144. doi:10.1038/s41598-022-13693-1

Pan, W., Scott, A. L., Nurse, C. A., and Jonz, M. G. (2021a). Identification of oxygen-sensitive neuroepithelial cells through an endogenous reporter gene in larval and adult transgenic zebrafish. *Cell Tissue Res.* 384, 35–47. doi:10.1007/s00441-020-03307-5

Pan, Y. K., Jensen, G., and Perry, S. F. (2021b). Disruption of tph1 genes demonstrates the importance of serotonin in regulating ventilation in larval zebrafish (*Danio rerio*). *Respir. Physiol. Neurobiol.* 285, 103594. doi:10.1016/j.resp.2020.103594

Pan, Y. K., Mandic, M., Zimmer, A. M., and Perry, S. F. (2019). Evaluating the physiological significance of hypoxic hyperventilation in larval zebrafish (*Danio rerio*). *J. Exp. Biol.* 222, jeb204800. doi:10.1242/jeb.204800

Pan, Y. K., and Perry, S. F. (2020). Neuroendocrine control of breathing in fish. *Mol. Cell. Endocrinol.* 509, 110800. doi:10.1016/j.mce.2020.110800

Peña-Münzenmayer, G., Niemeyer, M. I., Sepúlveda, F. V., and Cid, L. P. (2013). Zebrafish and mouse TASK-2 K^+ channels are inhibited by increased CO_2 and intracellular acidification. *Pflügers Arch.* 466, 1317–1327. doi:10.1007/s00424-013-1365-2

Perry, S. F., and Abdallah, S. J. (2012). Mechanisms and consequences of carbon dioxide sensing in fish. *Respir. Physiol. Neurobiol.* 184, 309–315. doi:10.1016/j.resp.2012.06.013

Perry, S. F., Esbaugh, A., Braun, M., and Gilmour, K. M. (2009a). "Gas transport and gill function in water-breathing fish," in *Cardio-respiratory control in vertebrates: Comparative and evolutionary aspects*. Editors M. L. Glass and S. C. Wood (Heidelberg: Springer), 5–42.

Perry, S. F., and Gilmour, K. M. (2010). "Oxygen uptake and transport in water breathers," in *Respiratory Physiology of vertebrates: Life with and without oxygen*. Editor G. E. Nilsson (Cambridge: Cambridge University Press), 49–94.

Perry, S. F., and Gilmour, K. M. (2002). Sensing and transfer of respiratory gases at the fish gill. *J. Exp. Zool.* 293, 249–263. doi:10.1002/jez.10129

Perry, S. F., Jonz, M. G., and Gilmour, K. M. (2009b). "Oxygen sensing and the hypoxic ventilatory response," in *Fish Physiology v27 hypoxia*. Editors J. G. Richards, C. J. Brauner, and A. P. Farrell (Amsterdam: Academic Press), 193–251.

Perry, S. F., and Mckendry, J. E. (2001). The relative roles of external and internal CO_2 *versus* H^+ in eliciting the cardiorespiratory responses of *Salmo salar* and *Squalus acanthias* to hpercarbia. *J. Exp. Biol.* 204, 3963–3971. doi:10.1242/jeb.204.22.3963

Perry, S. F., and Reid, S. G. (2002). Cardiorespiratory adjustments during hypercarbia in rainbow trout *Oncorhynchus mykiss* are initiated by external CO_2 receptors on the first gill arch. *J. Exp. Biol.* 205, 3357–3365. doi:10.1242/jeb.205.21.3357

Perry, S. F. (2011). "Respiratory responses to hypoxia in fishes," in *Encyclopaedia of fish Physiology: From genome to environment*. Editor A. P. Farrell (San Diego: Elsevier), 1751–1756.

Perry, S. F., and Tzaneva, V. (2016). The sensing of respiratory gases in fish: Mechanisms and signalling pathways. *Respir. Physiol. Neurobiol.* 224, 71–79. doi:10.1016/j.resp.2015.06.007

Perry, S., Kumai, Y., Porteus, C., Tzaneva, V., and Kwong, R. (2016). An emerging role for gasotransmitters in the control of breathing and ionic regulation in fish. *J. Comp. Physiol. B* 186, 145–159. doi:10.1007/s00360-015-0949-x

Polymeropoulos, E. T., Plouffe, D., Leblanc, S., Elliott, N. G., Currie, S., and Frappell, P. B. (2014). Growth hormone transgenesis and polyploidy increase metabolic rate, alter the cardiorespiratory response and influence HSP expression in response to acute hypoxia in Atlantic salmon (*Salmo salar*) yolk-sac alevins. *J. Exp. Biol.* 217, 2268–2276. doi:10.1242/jeb.098913

Porteus, C., Abdallah, S. J., Pollack, J., Kumai, Y., Kwong, R. W. M., Yew, H. M., et al. (2014). The role of hydrogen sulphide in the control of breathing in hypoxic zebrafish (*Danio rerio*). *J. Physiol.* 592, 3075–3088. doi:10.1113/jphysiol.2014.271098

Porteus, C., Kumai, Y., Abdallah, S. J., Yew, H. M., Kwong, R. W. M., Pan, Y. K., et al. (2021). Respiratory responses to external ammonia in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. A* 251, 110822. doi:10.1016/j.cbpa.2020.110822

Porteus, C. S., Brink, D. L., and Milsom, W. K. (2012). Neurotransmitter profiles in fish gills: Putative gill oxygen chemoreceptors. *Respir. Physiol. Neurobiol.* 184, 316–325. doi:10.1016/j.resp.2012.06.019

Porteus, C. S., Pollack, J., Tzaneva, V., Kwong, R. W. M., Kumai, Y., Abdallah, S. J., et al. (2015). A role for nitric oxide in the control of breathing in zebrafish (*Danio rerio*). *J. Exp. Biol.* 218, 3746–3753. doi:10.1242/jeb.127795

Prabhakar, N. R. (2013). Sensing hypoxia: Physiology, genetics and epigenetics. *J. Physiol.* 591, 2245–2257. doi:10.1113/jphysiol.2012.247759

Qin, Z., Lewis, J. E., and Perry, S. F. (2010). Zebrafish (*Danio rerio*) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO_2. *J. Physiol.* 588, 861–872. doi:10.1113/jphysiol.2009.184739

Ragsdale, A., Ortega-Recalde, O., Dutoit, L., Besson, A. A., Chia, J. H. Z., King, T., et al. (2022). Paternal hypoxia exposure primes offspring for increased hypoxia resistance. *BMC Biol.* 20, 185. doi:10.1186/s12915-022-01389-x

Rakoczy, R. J., and Wyatt, C. N. (2018). Acute oxygen sensing by the carotid body: A rattlebag of molecular mechanisms. *J. Physiol.* 596, 2969–2976. doi:10.1113/JP274351

Randall, D. J., and Ip, Y. K. (2006). Ammonia as a respiratory gas in water and air-breathing fishes. *Respir. Physiol. Neurobiol.* 154, 216–225. doi:10.1016/j.resp.2006.04.003

Randall, D. J., and Shelton, G. (1963). The effects of changes in environmental gas concentrations on the breathing and heart rate of a teleost fish. *Comp. Biochem. Physiol.* 9, 229–239. doi:10.1016/0010-406x(63)90046-x

Randall, D. J. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol.* 100, 275–288. doi:10.1242/jeb.100.1.275

Rasera, M. D. F. L., Krusche, A. V., Richey, J. E., Ballester, M. V. R., and Victória, R. L. (2013). Spatial and temporal variability of pCO_2 and CO_2 efflux in seven Amazonian Rivers. *Biogeochem* 116, 241–259. doi:10.1007/s10533-013-9854-0

Reed, M., and Jonz, M. G. (2022). Neurochemical signalling associated with gill oxygen sensing and ventilation: A receptor focused mini-review. *Front. Physiol.* 13, 940020. doi:10.3389/fphys.2022.940020

Regan, K. S., Jonz, M. G., and Wright, P. A. (2011). Neuroepithelial cells and the hypoxia emersion response in the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* 214, 2560–2568. doi:10.1242/jeb.056333

Reid, S. G., Perry, S. F., Gilmour, K. M., Milsom, W. K., and Rantin, F. T. (2005). Reciprocal modulation of O_2 and CO_2 cardiorespiratory chemoreflexes in the tambaqui. *Respir. Physiol. Neurobiol.* 146, 175–194. doi:10.1016/j.resp.2004.12.008

Reid, S. G., Sundin, L., Kalinin, A. L., Rantin, F. T., and Milsom, W. K. (2000). Cardiovascular and respiratory reflexes in the tropical fish, traira (*Hoplias malabaricus*): CO_2/pH chemoresponses. *Respir. Physiol.* 120, 47–59. doi:10.1016/s0034-5687(99)00100-0

Robertson, C. E., Turko, A. J., Jonz, M. G., and Wright, P. A. (2015). Hypercapnia and low pH induce neuroepithelial cell proliferation and emersion behaviour in the amphibious fish *Kryptolebias marmoratus*. *J. Exp. Biol.* 218, 2987–2990. doi:10.1242/jeb.123133

Rombough, P. (2002). Gills are needed for ionoregulation before they are needed for O_2 uptake in developing zebrafish, *Danio rerio*. *J. Exp. Biol.* 205, 1787–1794. doi:10.1242/jeb.205.12.1787

Rombough, P. J. (1988). "Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life," in *Fish Physiology vXIA*. Editors W. S. Hoar and D. J. Randall (San Diego: Academic Press), 59–161.

Rombough, P. (2007). The functional ontogeny of the teleost gill: Which comes first, gas or ion exchange? *Comp. Biochem. Physiol. A* 148, 732–742. doi:10.1016/j.cbpa.2007.03.007

Rosales, K., Yeh, C.-M., How, J. J., Villa-Real, R., Depasquale, E., Groisman, A., et al. (2019). Neural pathways linking hypoxia with pectoral fin movements in *Danio rerio*. bioRxiv, doi:10.1101/655084

Rossi, G. S., Cochrane, P. V., and Wright, P. A. (2020). Fluctuating environments during early development can limit adult phenotypic flexibility: Insights from an amphibious fish. *J. Exp. Biol.* 223, jeb228304. doi:10.1242/jeb.228304

Shakarchi, K., Zachar, P. C., and Jonz, M. G. (2013). Serotonergic and cholinergic elements of the hypoxic ventilatory response in developing zebrafish. *J. Exp. Biol.* 216, 869–880. doi:10.1242/jeb.079657

Sharrock, A. V., Mulligan, T. S., Hall, K. R., Williams, E. M., White, D. T., Zhang, L., et al. (2022). Ntr 2.0: A rationally engineered prodrug-converting enzyme with substantially enhanced efficacy for targeted cell ablation. *Nat. Methods* 19, 205–215. doi:10.1038/s41592-021-01364-4

Shelton, G., Jones, D. R., and Milsom, W. K. (1986). "Control of breathing in ectothermic vertebrates," in *Handbook of Physiology, section 3. The respiratory system*. Editors N. S. Cherniak and J. G. Widdicombe (Bethesda: American Physiological Society), 2, 857–909. Control of Breathing.

Sloman, K. A., Mandic, M., Todgham, A. E., Fangue, N. A., Subrt, P., and Richards, J. G. (2008). The response of the tidepool sculpin, *Oligocottus maculosus*, to hypoxia in laboratory, mesocosm and field environments. *Comp. Biochem. Physiol. A* 149, 284–292. doi:10.1016/j.cbpa.2008.01.004

Steffensen, J. F., and Lomholt, J. P. (1983). Energetic cost of active branchial ventilation in the sharksucker, *Echeneis naucrates*. *J. Exp. Biol.* 103, 185–192. doi:10.1242/jeb.103.1.185

Sundin, L., Burleson, M. L., Sanchez, A. P., Amin-Naves, J., Kinkead, R., Gargaglioni, L. H., et al. (2007). Respiratory chemoreceptor function in vertebrates comparative and evolutionary aspects. *Integr. Comp. Biol.* 47, 592–600. doi:10.1093/icb/icm076

Sundin, L., Reid, S. G., Rantin, F. T., and Milsom, W. K. (2000). Branchial receptors and cardiorespiratory reflexes in the neotropical fish, Tambaqui (*Colossoma macropomum*). *J. Exp. Biol.* 203, 1225–1239. doi:10.1242/jeb.203.7.1225

Thomsen, M. T., Lefevre, S., Nilsson, G. E., Wang, T., and Bayley, M. (2019). Effects of lactate ions on the cardiorespiratory system in rainbow trout (*Oncorhynchus mykiss*). *Am. J. Physiol.* 316, R607–R620. doi:10.1152/ajpregu.00395.2018

Thomsen, M. T., Wang, T., Milsom, W. K., and Bayley, M. (2017). Lactate provides a strong pH-independent ventilatory signal in the facultative air-breathing teleost *Pangasianodon hypophthalmus*. *Sci. Rep.* 7, 6378–6379. doi:10.1038/s41598-017-06745-4

Torres-Torrelo, H., Ortega-Sáenz, P., Macías, D., Omura, M., Zhou, T., Matsunami, H., et al. (2018). The role of Olfr78 in the breathing circuit of mice. *Nature* 561, E33–E40. doi:10.1038/s41586-018-0545-9

Tresguerres, M., Milsom, W. K., and Perry, S. F. (2019). "CO_2 and acid-base sensing," in *Fish Physiology v37 carbon dioxide*. Editors M. Grosell, P. L. Munday, A. P. Farrell, and C. J. Brauner (USA: Elsevier), 33–68.

Turesson, J., Schwerte, T., and Sundin, L. (2006). Late onset of NMDA receptor-mediated ventilatory control during early development in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. A* 143, 332–339. doi:10.1016/j.cbpa.2005.12.008

Tzaneva, V., and Perry, S. F. (2014). Heme oxygenase-1 (HO-1) mediated respiratory responses in the goldfish, *Carassius auratus*. *Resp. Physiol. Neurobiol.* 199, 1–8. doi:10.1016/j.resp.2014.04.006

Tzaneva, V., and Perry, S. F. (2016). Role of endogenous carbon monoxide in the control of breathing in zebrafish (*Danio rerio*). *Am. J. Physiol.* 311, R1262–R1270. doi:10.1152/ajpregu.00094.2016

Varas, R., Wyatt, C. N., and Buckler, K. J. (2007). Modulation of TASK-like background potassium channels in rat arterial chemoreceptor cells by intracellular ATP and other nucleotides. *J. Physiol.* 583, 521–536. doi:10.1113/jphysiol.2007.135657

Vulesevic, B., McNeill, B., and Perry, S. F. (2006). Chemoreceptor plasticity and respiratory acclimation in the zebrafish, *Danio rerio*. *J. Exp. Biol.* 209, 1261–1273. doi:10.1242/jeb.02058

Vulesevic, B., and Perry, S. F. (2006). Developmental plasticity of ventilatory control in zebrafish, *Danio rerio*. *Respir. Physiol. Neurobiol.* 154, 396–405. doi:10.1016/j.resp.2006.01.001

Wang, H., Jing, M., and Li, Y. (2018). Lighting up the brain: Genetically encoded fluorescent sensors for imaging neurotransmitters and neuromodulators. *Curr. Opin. Neurobiol.* 50, 171–178. doi:10.1016/j.conb.2018.03.010

Weber, J.-M., and Kramer, D. L. (1983). Effects of hypoxia and surface access on growth, mortality, and behavior of juvenile guppies, *Poecilia reticulata*. *Can. J. Fish. Aquat. Sci.* 40, 1583–1588. doi:10.1139/f83-183

White, R. M., Sessa, A., Burke, C., Bowman, T., Leblanc, J., Ceol, C., et al. (2008). Transparent adult zebrafish as a tool for *in vivo* transplantation analysis. *Cell Stem Cell* 2, 183–189. doi:10.1016/j.stem.2007.11.002

Wood, C. M., and Jackson, E. B. (1980). Blood acid-base regulation during environmental hyperoxia in the rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* 42, 351–372. doi:10.1016/0034-5687(80)90125-5

Wood, C. M., and Munger, R. S. (1994). Carbonic anhydrase injection provides evidence for the role of blood acid-base status in stimulating ventilation after exhaustive exercise in rainbow trout. *J. Exp. Biol.* 194, 225–253. doi:10.1242/jeb.194.1.225

Wyatt, C. N., and Buckler, K. J. (2004). The effect of mitochondrial inhibitors on membrane currents in isolated neonatal rat carotid body type I cells. *J. Physiol.* 556, 175–191. doi:10.1113/jphysiol.2003.058131

Yuan, G., Vasavda, C., Peng, Y.-J., Makarenko, V. V., Raghuraman, G., Nanduri, J., et al. (2015). Protein kinase G–regulated production of H_2S governs oxygen sensing. *Sci. Signal.* 8, ra37. doi:10.1126/scisignal.2005846

Zaccone, G., Lauriano, E. R., Kuciel, M., Capillo, G., Pergolizzi, S., Alesci, A., et al. (2017). Identification and distribution of neuronal nitric oxide synthase and neurochemical markers in the neuroepithelial cells of the gill and the skin in the giant mudskipper, *Periophthalmodon schlosseri*. *Periophthalmodon Schlosseri. Zool.* 125, 41–52. doi:10.1016/j.zool.2017.08.002

Zachar, P. C., and Jonz, M. G. (2012). Neuroepithelial cells of the gill and their role in oxygen sensing. *Respir. Physiol. Neurobiol.* 184, 301–308. doi:10.1016/j.resp.2012.06.024

Zachar, P. C., Pan, W., and Jonz, M. G. (2017). Characterization of ion channels and O_2 sensitivity in gill neuroepithelial cells of the anoxia-tolerant goldfish (*Carassius auratus*). *J. Neurophysiol.* 118, 3014–3023. doi:10.1152/jn.00237.2017

Zhang, L., Nawata, C. M., and Wood, C. M. (2013). Sensitivity of ventilation and brain metabolism to ammonia exposure in rainbow trout, *Oncorhynchus mykiss*. *J. Exp. Biol.* 216, 4025–4037. doi:10.1242/jeb.087692

Zhang, L., Nawata, M., De Boeck, G., and Wood, C. M. (2015). Rh protein expression in branchial neuroepithelial cells, and the role of ammonia in ventilatory control in fish. *Comp. Biochem. Physiol.* 186A, 39–51. doi:10.1016/j.cbpa.2014.10.004

Zhang, L., Nurse, C. A., Jonz, M. G., and Wood, C. M. (2011). Ammonia sensing by neuroepithelial cells and ventilatory responses to ammonia in rainbow trout. *J. Exp. Biol.* 214, 2678–2689. doi:10.1242/jeb.055541

Zhang, L., and Wood, C. M. (2009). Ammonia as a stimulant to ventilation in rainbow trout *Oncorhynchus mykiss*. *Respir. Physiol. Neurobiol.* 168, 261–271. doi:10.1016/j.resp.2009.07.011

Zimmer, A. M., Mandic, M., Rourke, K. M., and Perry, S. F. (2020). Breathing with fins: Do the pectoral fins of larval fishes play a respiratory role? *Am. J. Physiol.* 318, R89–R97. doi:10.1152/ajpregu.00265.2019

Zimmer, A. M., Pan, Y. K., Chandrapalan, T., Kwong, R. W. M., and Perry, S. F. (2019). Loss-of-function approaches in comparative physiology: Is there a future for knockdown experiments in the era of genome editing? *J. Exp. Biol.* 222, jeb175737. doi:10.1242/jeb.175737

Zimmer, A. M., and Perry, S. F. (2022). Physiology and aquaculture: A review of ion and acid-base regulation by the gills of fishes. *Fish. Fish.* 23, 874–898. doi:10.1111/faf.12659

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Metabolic cost of osmoregulation by the gastro-intestinal tract in marine teleost fish

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Introduction: Although dozens of studies have attempted to determine the metabolic cost of osmoregulation, mainly by comparing standard metabolic rates (SMR) in fish acclimated to different salinities, consensus is still lacking.

Methods: In the present study, using the Gulf toadfish, *Opsanus beta*, we aimed to determine the metabolic cost of esophageal and intestinal osmoregulatory processes by estimating ATP consumption from known ion transport rates and pathways and comparing these estimates with measurements on isolated tissues. Further, we performed whole animal respirometry on fish acclimated to 9, 34 and 60 ppt.

Results and Discussion: Our theoretical estimates of esophageal and intestinal osmoregulatory costs were in close agreement with direct measurements on isolated tissues and suggest that osmoregulation by these tissues amounts to ~2.5% of SMR. This value agrees well with an earlier attempt to estimate osmoregulation cost from ion transport rates and combined with published measurements of gill osmoregulatory costs suggests that whole animal costs of osmoregulation in marine teleosts is ~7.5% of SMR. As in many previous studies, our whole animal measurements were variable between fish and did not seem suited to determine osmoregulatory costs. While the esophagus showed constant metabolic rate regardless of acclimation salinity, the intestine of fish acclimated to higher salinities showed elevated metabolic rates. The esophagus and the intestine had 2.1 and 3.2-fold higher metabolic rates than corresponding whole animal mass specific rates, respectively. The intestinal tissue displays at least four different Cl^- uptake pathways of which the Na^+:Cl^-:2 K^+ (NKCC) pathway accounts for 95% of the Cl^- uptake and is the most energy efficient. The remaining pathways are *via* apical anion exchange and seem to primarily serve luminal alkalinization and the formation of intestinal $CaCO_3$ which is essential for water absorption.

KEYWORDS

intestinal ion absorption, esophagus, water absorption, tissue respirometry, standard metabolic rate

1 Introduction

Marine teleost fish live in a strongly desiccating environment as they maintain their internal osmotic pressure (310–350 mOsm) much below that of their surroundings. The osmoregulatory process facilitating marine teleost survival has been the subject of study for nearly a century (Smith, 1930) and more recent efforts have aimed to determine the metabolic cost of this vital process. While we have a good understanding of many

physiological processes involved in salt and water balance, the question of metabolic cost remains to be answered with any degree of certainty.

Marine teleost fish drink seawater to compensate for diffusive water loss (Smith, 1930). Early studies found that ingested seawater is desalinized by the water-impermeable esophagus and that solute-coupled water absorption occurs in the intestine with excretion of the excess Na^+ and Cl^- gain across the gill (Shehadeh and Gordon, 1969; Parmelee and Renfro, 1983). The stomach of unfed fish likely plays little, if any, role in osmoregulation (Larsen et al., 2014). More recent studies have revealed that alkalinization of the intestinal lumen is essential for precipitation of $CaCO_3$ (ichthyocarbonates) and thereby reduction in luminal osmotic pressure to promote water absorption (Wilson et al., 2002; Grosell et al., 2009b; Genz et al., 2011). Although the intestine is largely impermeable to divalent ions, some are assimilated and ultimately cleared by the kidney in low volumes of isosmotic urine (reviewed in (Larsen et al., 2014)).

The esophageal and intestinal osmoregulatory processes are both ATP–demanding, with basolateral Na^+/K^+ pumps and apical proton pumps (in the intestine) facilitating Na^+ and Cl^- uptake (Guffey et al., 2011). Several studies have demonstrated upregulation of intestinal Na^+/K^+ pumps as well as apical proton pumps as fish acclimate to higher salinities (Jampol and Epstein, 1970; Colin et al., 1985; Madsen et al., 1994; Fuentes et al., 1997; Kelly et al., 1999; Seidelin et al., 2000; Guffey et al., 2011), suggesting increased metabolic demands in these tissues. Recent and elegant studies have demonstrated increased blood flow to the gastro-intestinal tract of seawater compared to freshwater-acclimated rainbow trout, supporting the suggestion that osmoregulation in hyperosmotic environments imposes a metabolic demand on the intestinal tissue (Brijs et al., 2015; Brijs et al., 2016). However, no study to date has directly determined the metabolic costs of esophageal and intestinal osmoregulatory processes.

Metabolic demands of osmoregulation have been the subject of much interest for decades, with dozens of papers published on the subject (reviewed in (Ern et al., 2014)). Most of these studies have attempted to determine the cost of osmoregulation from differences in whole animal metabolic rates (oxygen consumption) between fish acclimated to freshwater, intermediate salinities, and seawater. Generally, the expectation was that fish held in salinities near that of their body fluids would display limited osmoregulatory costs and lower standard metabolic rates (SMRs; metabolic rate in fully resting, unfed fish acclimated to their environment). The differences between oxygen consumption invoked by freshwater or seawater acclimation, compared to those at isosmotic salinities, are thought to reflect the metabolic costs of osmoregulation at those salinities. In short, there is no consensus in findings among these studies and estimates of osmoregulatory costs vary from few % to >30% of standard metabolic rate (Ern et al., 2014; Christensen et al., 2018; Christensen et al., 2019). While interspecies differences may account for some of this variation, interindividual differences in non-osmoregulatory organismal responses, such as spontaneous activity and/or stress, likely also contribute to the lack of consensus in the field (Chabot et al., 2016).

Surprisingly, a second approach of using isolated osmoregulatory tissues to estimate the metabolic costs of osmoregulation has not been frequently employed, even though it avoids the problems of confounding organismal responses. One study using isolated branchial arches from cutthroat trout (*Oncorhynchus clarki*) and blockers of relevant ATPases determined the branchial cost of osmoregulation to be 3.9% and 2.4% of BMR following acclimation to freshwater and seawater, respectively (Morgan and Iwama, 1999). A third approach to estimate osmoregulatory metabolic costs is utilizing known ion transport rates and ATP demands per mole of ions transported, which offers the same advantages as measurements of metabolic rates on isolated tissues. This approach has also only been applied once and the estimated metabolic cost of osmoregulation was in the order of 7%–17% of SMR in marine teleosts (Kirschner, 1993).

To date, no attempts have been made to measure metabolic rates in the esophagus of teleost fish, to estimate metabolic costs based on ion transport rates for this tissue, or to measure the metabolic cost of intestinal transport processes related to osmoregulation. In the present study, we aimed to investigate the metabolic costs of osmoregulation by comparing the three approaches discussed above; whole animal respirometry, isolated tissue respirometry, and metabolic estimates calculated from known ion transport rates. We performed whole animal and isolated-tissue (esophagus and intestine) respirometry in gulf toadfish (*Opsanus beta*) acclimated to 9, 34 and 60 ppt. In addition, we took advantage of the available literature on ion transport across the esophageal and intestinal tissue of this teleost to compare predicted osmoregulatory costs across the three methods. The two latter methods showed strong agreement and are supported by earlier estimates of osmoregulatory costs in the intestinal tracts of other marine teleosts (Kirschner, 1993).

2 Methods

2.1 Animal husbandry

Gulf toadfish (*Obsanus beta*) were obtained from commercial shrimp fishermen trawling Biscayne Bay, Miami, from October 2016 to January 2017. On arrival, toadfish were treated for ecto-parasites (McDonald and Crosell. 2006) and sorted by size into 40-L tanks (8–10 per tank) with aerated, sand-filtered, flow-through seawater from Bear Cut (21°C–26°C, 30–35 ppt salinity). Pieces of polyvinylchloride tubing were used as shelters to reduce stress and decrease aggressive behavior. Toadfish were fed squid to satiation weekly but fasted for at least 144 h before experimentation. Toadfish were held in ambient seawater for at least 2 weeks prior to experimental treatments. All general animal care and animal sacrifice protocols were carried out in accordance with relevant guidelines for experiments on teleost provided by University of Miami IACUC (Institutional Animal Care and Use Committee) and experimental protocols were approved by University of Miami IACUC (15–019). University of Miami's IACUC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC). Toadfish were collected with the approval and in accordance with guidelines outlined by the Florida Fish and Wildlife Conservation Commission (SAL-16-0729-SR).

TABLE 1 Esophageal and intestinal ion transport pathways and their contribution to Cl^- uptake, ATP and O_2 consumption as well as their relative contribution to standard metabolic rate (SMR). See text for further detail.

Route of Cl^- uptake	Cl^- absorption (μmol kg^{-1} h^{-1})	Fraction of total (%)	ATP: Cl^-	ATP consumed (μmol kg^{-1} h^{-1})	O_2 consumed (μmol kg^{-1} h^{-1})	Fraction of SMR (%)
Esophageal absorption						
NHE2-AE mediated	575	100	0.33	94.8	19.0	1.22
Intestinal absorption						
NBC mediated	17.0	2.7	0.22	3.7	0.75	0.04
CAc-NHE mediated	15.3	2.4	0.66	10.1	2.02	0.12
CAc-VHA mediated	1.7	0.3	0.5	1.7	0.34	0.02
NKCC	599.8	94.6	0.17	102	20.39	1.21
Total	633.8	100		117.5	23.5	1.40

2.1.1 Salinity acclimation

Toadfish (range: 24.0–43.7 g for whole-animal respirometry; 25–45 g for intestinal preps; and 54–130 g for esophageal preps) were housed in 20-L tanks with aerated, sand-filtered seawater adjusted to 9 ppt (seawater diluted with reverse osmosis purified water), 34 ppt (seawater), or 60 ppt (seawater supplemented with Instant Ocean marine salt, Spectrum Brands, Blacksburg, VA, United States of America) for a minimum of 10 days. Common to all experiments, toadfish assigned to the 9 ppt acclimation groups were introduced to 20 ppt seawater day 1, followed by 17.5 ppt day 3, 15 ppt day 5, 12.5 ppt day 7, and 9 ppt day 9. Toadfish assigned to 34 ppt and 60 ppt acclimation treatments were transferred immediately to their respective salinities. Tank water was maintained at 23°C ± 1°C with 75% tank water changed every second day. Toadfish were maintained at their target salinity for a minimum of 10 days and were fasted for 6–13 days prior to experimental procedures to avoid influence of feeding status on metabolic rates.

2.1.2 Whole-animal respirometry

Toadfish (n = 10–14) were housed individually in 20 L tanks with aerated, sand-filtered seawater at 22°C. Following acclimation to constant salinity, intermittent flow respirometry was performed using Brett-style swim respirometers (Loligo Systems, Denmark) as previously described (Mager et al., 2014; Stieglitz et al., 2016) but with constant and low water velocity to ensure adequate mixing (< 0.5 body lengths per second). Fish were sedentary on the bottom under these conditions. SMR was determined by fitting a double Gaussian curve to $\dot{M}O_2$ measurements with an $r^2 > 0.80$. Elevated values of $\dot{M}O_2$ were excluded using the first (higher) normal distribution, whereas the second normal distribution was used as the best estimate of SMR (Steffensen et al., 1994; Herskin, 1999; Jordan and Steffensen, 2007; Svendsen et al., 2012).

2.1.3 Theoretical calculations for MO_2 in esophageal and intestinal epithelia

Gulf toadfish held in seawater display drinking rates of 2.6 mL kg^{-1} h^{-1} (Genz et al., 2008) which corresponds to an intake of 1,271 μmol Cl^- kg^{-1} h^{-1} (Grosell, 2014). Assuming no water absorption by the esophagus (Hirano and Mayer-Gostan, 1976; Parmelee and Renfro, 1983) and an average gastric Cl^- concentration in unfed marine teleosts fish of 268 mM (n = 11) (Grosell, 2014), esophageal Cl^- absorption can be estimated to be (1,271 μmol Cl^- kg^{-1} h^{-1}—(2.6 mL^{-1} kg^{-1} h-1 X 268 mM Cl^-)) 575 μmol Cl^- kg^{-1} h^{-1} leaving 697 μmol Cl^- kg^{-1} h^{-1} to be transferred through the pyloric sphincter into the anterior intestine (Table 1). Rectal fluids voided at low rates contain little Cl^-, resulting in a limited rectal excretion of 63 μmol Cl^- kg^{-1} h^{-1} (Genz et al., 2008), which is a product of an intestinal absorption of (697–63 μmol Cl^- kg^{-1} h^{-1}) 634 μmol Cl^- kg^{-1} h^{-1} (Table 1).

Cl^- (and Na^+) is absorbed across the esophageal epithelium by both passive (paracellular) and active (transcellular) paths of approximate equal quantities in the flounder (*Pseudopleuronectes americanus*) (Parmelee and Renfro, 1983). Assuming the same is true for toadfish, active absorption of (575/2) 287 μmol Cl^- kg^{-1} h^{-1} takes place across the esophageal epithelium. For toadfish, the active esophageal salt absorption is mediated by apical anion and Na^+/H^+ (NHE2) exchangers deriving energy for H^+ extrusion and Na^+ uptake from the basolateral Na^+/K^+-ATPase (Esbaugh and Grosell, 2014). Assuming an active 1:1 Cl^-:Na^+ absorption rate by the esophageal epithelium and a 3Na^+/ATP stoichiometry of the Na^+/K^+-ATPase (NKA), Cl^- absorption can be estimated to occur at the cost of 0.33 mol ATP/mol Cl^-. A further assumption of 5 mol ATP produced per mol of O_2 consumed (Hinkle, 2005) allow for estimation of O_2 consumption arising from esophageal active absorption of NaCl (Table 1).

Multiple Cl^- uptake pathways operate in parallel in the intestine of marine teleosts (Grosell, 2006) (Figure 2). In the toadfish, rectal HCO_3^- excretion in the order of 68 μmol kg^{-1} h^{-1} (Genz et al., 2008) allows for quantification of intestinal Cl^- uptake by Cl^-/HCO_3^- exchange. This anion exchange fraction of intestinal Cl^- absorption occurs *via* SLC26a6 which is an electrogenic anion exchanger operating by secreting 2 (or more) HCO_3^- ions in exchange for absorption of 1 Cl^- ion across the apical membrane (Kurita et al., 2008; Grosell et al., 2009b). Assuming a 2:1 HCO_3^-/Cl^- exchange ratio toadfish intestinal Cl^- absorption *via* anion exchange (Kurita et al., 2008; Grosell et al., 2009b) amounts to 5.4% of total intestinal Cl^- uptake with the rest (94.6%) attributed to Na^+/K^+/2 Cl^- co-transport *via* apical NKCC2 driven by the basolateral NKA. We have no evidence for the presence of Na^+:Cl^- cotransport in the gulf toadfish intestine but cannot rule out a role for this transporter in other species. Considering the stoichiometry of NKCC2 (2 mol of Cl^- absorbed per mole of Na^+) and NKA (3 mol of Na^+ transported per mole of ATP consumed), absorption

TABLE 2 Saline composition (mM).

	Serosal	Mucosal (9 ppt)	Mucosal (33 ppt)	Mucosal (60 ppt)
NaCl	151	135	69	20
KCl	3	5	5	5
$MgSO_4$	0.88	77.5	77.5	80
MgCl2	-	22.5	22.5	50
Na_2HPO_4	0.5	-	-	-
KH_2PO_4	0.5	-	-	-
$CaCl_2$	1	3	3	3
$NaHCO_3$	5	-	-	-
HEPES free acid	11	-	-	-
HEPES Na^+ salt	11	-	-	-
Urea	4.5	-	-	-
-Glucose	5	-	-	-

of Cl^- via NKCC2 consumes 0.17 mol ATP/mol Cl^- absorbed. With this ratio, the intestinal Cl^- absorption rate via NKCC2 and the above assumption of ATP production per mol of O_2 consumed, O_2 consumption via the NKCC2 Cl^- absorption pathway can be estimated (Table 1). We are aware of three distinct pathways associated with Cl^- absorption via anion exchange in the toadfish. Approximately 50% of anion exchange is fueled by transepithelial HCO_3^- movement mediated by basolateral Na^+/HCO_3^- co-transport (NBC) and apical SLC26a6 anion exchange (Grosell and Genz, 2006). The remaining 50% of Cl^- absorption mediated by SLC26a6 is fueled by hydration of endogenous CO_2, a reaction mediated by carbonic anhydrase (CAc) (Grosell and Genz, 2006; Sattin et al., 2010). The CAc mediated Cl^- absorption relies on secretion of H^+ which occurs largely *via* basolateral Na^+/H^+ exchange (90% of the CAc mediated Cl^- uptake) (Grosell and Genz, 2006) but also *via* apical V-type H^+ ATPase (VHA) (10% of the CAc mediated Cl^- uptake) (Grosell et al., 2009b; Guffey et al., 2011).

Considering first the metabolic cost of the NBC-SLC26a6 pathway, we assumed a stoichiometry of 3 HCO_3^-:1Na^+ (Chang et al., 2012) and the usual 3Na^+:ATP for NKA and 2HCO_3^-:Cl^- for SCL26a6. Under these assumptions, the Cl^- absorption taking place *via* this pathway occurs at a cost of 0.22 mol of ATP/mol Cl^-. With the above assumption of 5 mol ATP produced per mol of O_2 consumed we estimated the oxygen consumption for Cl^- absorption attributable to the NBC-SLC26a6 pathway (Table 1). The stoichiometry of the only marine fish NBC transporter examined, the euryhaline pufferfish, *Takifugu obscurus*, is dependent on extracellular Na^+ and HCO_3^- and appears to be higher than for human NBC (Chang et al., 2012). Since the pufferfish NBC stoichiometry was determined in *xenopus* oocytes, it was not measured under fully physiological conditions but likely falls somewhere between 1.93 and 4.06 at extracellular Na^+ and HCO_3^- concentrations relevant for marine teleosts (Chang et al., 2012).

Considering next the two carbonic anhydrase (CAc) dependent Cl^- absorption pathways, NHE-SCL26a6 and VHA-SLC26a6, we assumed a 1Na^+:1H^+ ratio for NHE and a 0.5 mol ATP/mol H^+ ratio for VHA. Under these assumptions Cl^- absorption by NHE-SLC26a6 and VHA-SLCa6 pathways occur at a cost of 0.66 and 1 mol of ATP/mol of Cl^-, respectively, and again under the assumption of 5 mol ATP produced per mol of O_2, oxygen consumption attributable to both CAc-dependent pathways were calculated (Table 1). The stoichiometry of VHA is variable (Tomashek and Brusilow, 2000; Maxson and Grinstein, 2014) but generally assumed to be 2H^+/ATP, or higher (Tomashek and Brusilow, 2000; Maxson and Grinstein, 2014; Anandakrishnan and Zuckerman, 2017).

2.2 Empirical measures of MO_2 in isolated esophageal and intestinal tissue preparations

Toadfish acclimated to 9 ppt (N = 7 for esophageal; N = 6 for intestinal), 34 ppt (N = 7 for esophageal; N = 6 for intestinal) and 60 ppt (N = 6 for esophageal; N = 6 for intestinal) as described above were sacrificed using a lethal dose of 0.2 g/L MS-222 buffered with 0.4 g/L $NaHCO_3$. MO_2 for isolated anterior intestine and esophagus were measured using a custom-designed epithelial respirometer (Loligo Systems, Tjele, Denmark) described elsewhere (Taylor and Grosell, 2009; Secor et al., 2012). Intestinal and esophageal tissues were dissected, weighed, and mounted so that 0.87 cm^2 of tissue was exposed to two half-chambers (2.80 mL each), with mucosal saline on the gut side, and serosal saline on the blood-side. Serosal saline compositions (Table 2) were the same between esophageal and intestinal MO_2 preparations, and were adjusted to pH 7.8 and 330 mmol/kg osmolality with mannitol, and sterile filtered. Mucosal salines for intestinal MO_2 measurements (Table 2) were made to approximate the composition of anterior intestinal fluids of toadfish acclimated to 9, 34, and 60 ppt (McDonald and Grosell, 2006) and were adjusted to 330 mmol/kg osmolality with mannitol, and sterile filtered. Mucosal solution for esophageal measurements was sterile-filtered seawater of the acclimation salinity. All salines were pre-gassed with air, rather than custom O_2 mixes, so that MO_2 could be compared to whole-animal measurements. Note that intestinal O_2 consumption is not limited by O_2 levels in air (Taylor and Grosell, 2009).

Salines in half chambers were continuously mixed by micromagnetic glass-coated Teflon stir bars (Loligo Systems), and a

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Teflon tissue mount ensured that the system was gas-tight (Taylor and Grosell, 2009). Oxygen measurements were conducted using fiber-optic cables secured to the outside walls of glass half-chambers to illuminate a fiber-optic sensor spot glued to the inside wall of each respective half-chamber. Each cable was connected to a separate single-channel oxygen meter (Fibox 3) used in conjunction with Oxy-View software (PST3-V6.02; PreSens, Regensburg, Germany). A standard curve and/or two-point calibrations were used to convert the signal (i.e., phase angle Φ) to oxygen content using salines pre-gassed with air for 100% air saturation and supersaturated with sodium sulfite for 0% oxygen calibration. O_2 measurements were recorded every second at 22°C ± 1 °C with automatic temperature compensation. Intermittent-flow respirometry was performed to determine oxygen consumption rates of isolated tissue by flushing and replacing salines using a peristaltic pump (WPI Peri-Star). Flush cycles (~3 min) were optimized for complete saline replacement between closed cycles, and time intervals (~15 min) during closed measurements were monitored to ensure that O_2 concentrations did not drop below 80% air saturation. Tissue MO_2 was calculated from the sum of mucosal and serosal O_2 consumption rates and normalized to the mass of the exposed tissue. Background respiration rates (blanks) were measured using parafilm to separate the two half-chamber respirometers and were found to be negligible.

2.4 Statistical analyses

Data are presented as means ± standard error of the mean (SEM). ANOVAs, followed by Tukey *post hoc* tests, were used to analyze isolated esophageal and intestinal respirometry data, whereas repeated measures ANOVAs, followed by Tukey *post hoc* tests, were used to analyze SMR and resting metabolic rate (RMR; unfed and fully acclimated fish displaying only routine activity) data. All statistical tests were performed in R using the jamovi platform (version 0.7.5.4; jamovi project 2017).

3 Results

3.1 Whole-animal respirometry

SMR was 1,682 ± 180 μmol O_2 kg^{-1} h^{-1} in 34 ppt seawater (n = 13). A higher SMR was observed in fish acclimated to 9 ppt ($p < 0.05$) while the SMR of fish acclimated to 60 ppt was not significantly different from that of 34 ppt acclimated fish (Figure 1). A similar pattern was observed for RMR with 1825 ± 208 μmol O_2 kg^{-1} h^{-1} in 34 ppt seawater (n = 13) ($p < 0.05$).

3.2 Theoretical calculations for MO_2 in esophageal and intestinal epithelia

We estimated ATP consumption related to Cl^- absorption, from Cl^- absorption rates and various assumptions of transporter stoichiometries, to be 94.8 and 117.5 μmol ATP kg^{-1} h^{-1} for the esophagus and the intestine, respectively (Table 1). For the intestine, where at least four parallel Cl^- absorption pathways operate, ~95% of Cl^- absorption occurs *via* the least metabolically costly NKCC2 pathway with SLC26a6 pathways accounting for the remaining ~5% (Figure 2).

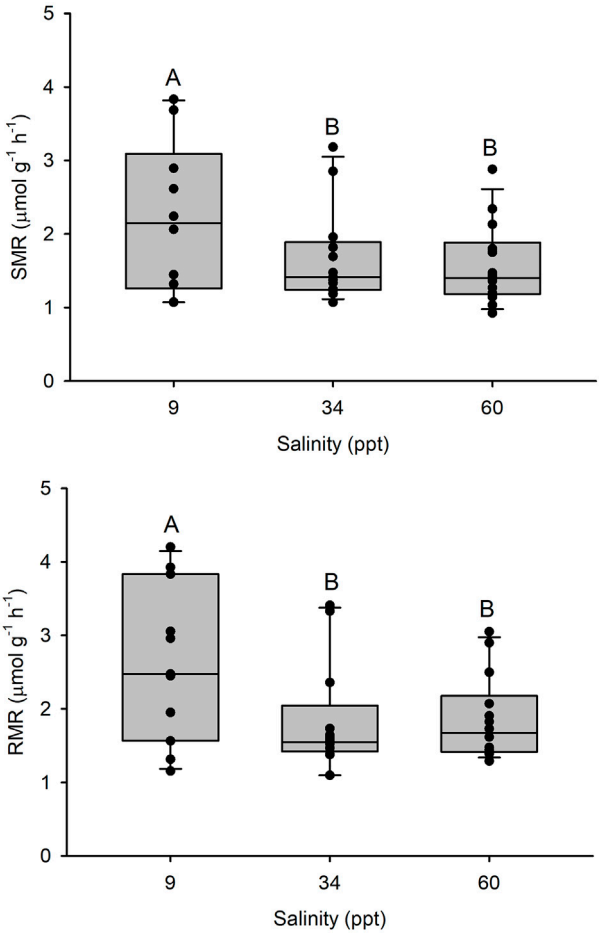

FIGURE 1
Top panel: Standard metabolic rate (SMR) and bottom panel: Resting metabolic rate (RMR) in Gulf toadfish (*Opsanus beta*) acclimated to 9, 34 or 60 ppt. Median, range, upper and lower quartile as well as individual observations (n = 10, 13, 14, respectively). Groups labelled with different letters are statistically significant different ($p < 0.05$).

The SLC26a6 mediated pathways are generally more metabolically demanding (0.22–1 mol ATP/mol Cl^-) than the NKCC2 pathway (0.17 mol ATP/mol Cl^-) with the CAc mediated pathways being most costly (Table 1; Figure 2). The calculated ATP consumption rates allowed us to estimate O_2 consumption rates associated with Cl^- absorption to be 19 and 23.5 μmol O_2 kg^{-1} h^{-1}, for the esophagus and the intestine, respectively (Table 1; Figure 2). Whole animal SMR was 1.68 ± 180 μmol O_2 g^{-1} h^{-1} and the esophageal and intestinal contribution to SMR is thus estimated to be 1.22% and 1.4%, respectively, for an overall contribution of osmoregulatory costs in the gastro-intestinal tract of 2.62% of SMR.

Empirical measures of MO_2 in isolated esophageal and intestinal tissue preparations

There was a significant effect of salinity acclimation on mean intestinal MO_2 (df = 15; F = 6.99; $p = 0.007$), where individuals

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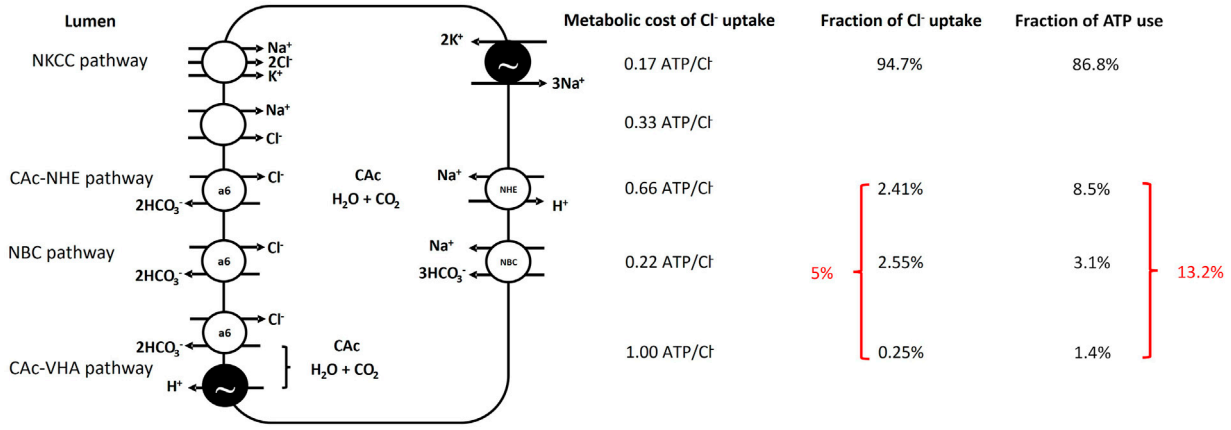

FIGURE 2
Schematic representation of Cl^- absorption pathways in the Gulf toadfish (and likely most marine teleosts) intestine along the theoretical ATP consumption required for Cl^- absorption by each pathway, the fraction of overall Cl^- absorption conducted by each pathway and finally the resulting fraction of overall ATP consumption related to Cl^- absorption by individual pathways. The NKCC2 pathway is responsible for the majority of Cl^- absorption but there is no evidence for involvement of the Na^+, Cl^-—cotransporter. The anion transport pathways are generally more metabolically costly than the NKCC2 pathway and specifically, the pathways involving hydration of endogenous CO_2 via carbonic anhydrase are the most metabolically costly. Note that the modest contribution by anion exchange pathways to Cl^- absorption of 5% corresponds to 13.2% of the ATP consumption required for Cl^- uptake. See text and Table 1 for further information.

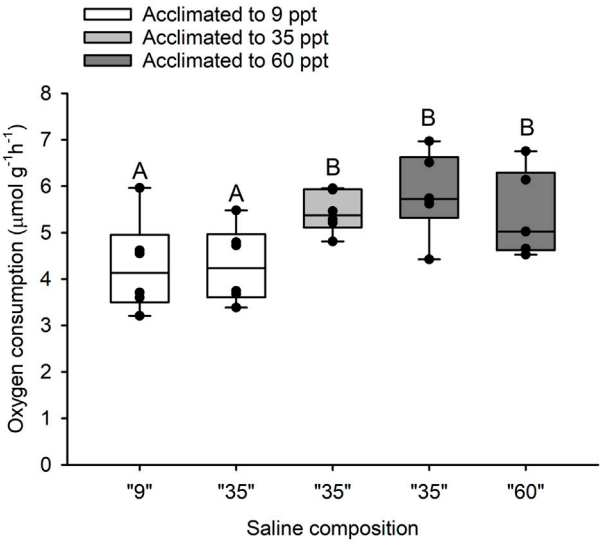

FIGURE 3
Oxygen consumption rate by isolated anterior intestinal epithelia from toadfish acclimated to 9 (open boxes), 35 (light grey) and 60 (dark grey) with different luminal salines representative of *in vivo* intestinal fluid composition at 9, 35 and 60 ppt (*X*-axis). Median, range, upper and lower quartile as well as individual observations. N = 6 for 9, 35 and 60 ppt. Groups labelled with different letters are statistically significant different ($p < 0.05$).

acclimated to 34 ppt and 60 ppt had significantly higher mean intestinal MO_2 (5.44 μmol $g^{-1}h^{-1}$ and 5.83 μmol $g^{-1}h^{-1}$, respectively) than those acclimated to 9 ppt (4.28 μmol $g^{-1}h^{-1}$; df = 15; t = 2.685; p = 0.042 and df = 15; t = −3.595; p = 0.007, respectively) (Figure 3). For fish acclimated to 9 ppt, luminal saline representing intestinal fluids of fish acclimated to 9 and 35 ppt caused no differences in MO_2 of isolated intestinal tissue (Figure 3). Similarly, for fish acclimated to 60 ppt, luminal saline representing intestinal fluids of fish acclimated to 35 and 60 ppt resulted in no difference in MO_2 of isolated intestinal tissue (Figure 3). Despite the small increase in mean MO_2, there was no significant difference between fish acclimated to 34 ppt and 60 ppt (df = 15; t = −0.911; p = 0.642). There was no significant effect of salinity acclimation on mean esophageal MO_2 between 9 ppt (4.31 μmol $g^{-1}h^{-1}$), 34 ppt (3.24 μmol $g^{-1}h^{-1}$) and 60 ppt (3.80 μmol $g^{-1}h^{-1}$; df = 17; F = 0.523; p = 0.602) (Figure 4).

3.3 Metabolic costs of osmoregulation in esophageal and intestinal tissues

The metabolic costs of osmoregulation in the intestine and esophagus were calculated from empirical data relative to mean SMR in seawater (1,682 ± 180 μmol O_2 kg^{-1} h^{-1}). For the intestine, mean intestinal MO_2 for fish acclimated to 9 ppt was subtracted from the mean intestinal MO_2 for fish acclimated to 34 ppt (5.44 μmol g^{-1} h^{-1}—4.28 μmol g^{-1} h^{-1} = 1.17 μmol g^{-1} h^{-1}), multiplied by mean relative (%) intestinal mass relative to whole body mass (1.17 μmol g^{-1} h^{-1} x 1.90% = 22.17 μmol kg^{-1} h^{-1}), and divided by mean SMR (22.17 μmol kg^{-1} h^{-1}/1,682 μmol kg^{-1} h^{-1} = 1.32% SMR). There was no difference in esophageal MO_2 with salinity acclimation (see above). However, the primary function of the esophagus in marine teleost fish is ion transport. Thus, mean esophageal MO_2 was multiplied by mean relative (%) esophageal mass (3.56 μmol g^{-1} h^{-1} x 0.56% = 19.89 μmol kg^{-1} h^{-1}), and divided by mean SMR (19.89 μmol kg^{-1} h^{-1}/1,682 μmol kg^{-1} h^{-1} = 1.18% SMR) to calculate absolute esophageal osmoregulatory costs.

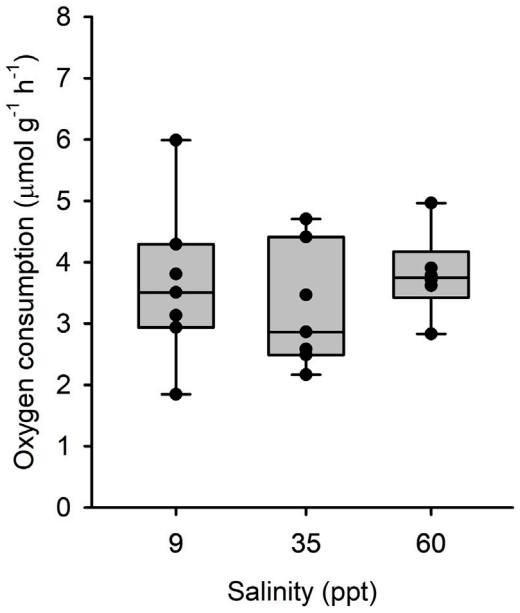

FIGURE 4
Oxygen consumption rate by isolated esophageal epithelia from toadfish acclimated to 9 (open bars), 35 (light grey) and 60 (dark grey) with different luminal salines representative of *in vivo* conditions. Median, range, upper and lower quartile as well as individual observations. N = 7, 7 and 6 for 9, 35 and 60 ppt, respectively. No statistical differences were observed.

Thus, esophageal and intestinal osmoregulatory processes, based on isolated tissue respirometry, account for approximately 2.5% SMR.

4 Discussion

4.1 Cost of esophageal and intestinal transport

The first empirical data on metabolic cost of osmoregulation by the esophagus and intestinal epithelium of a marine teleost fish presented here suggests that 2.5% of SMR is devoted to this essential physiological function. Corresponding theoretical estimates of metabolic cost of osmoregulatory processes were possible for the gulf toadfish for which detailed information is available for the relevant ion transport pathways. These estimates predicted a cost of intestinal osmoregulatory processes of 2.6% of SMR and are thus in close agreement with our empirical data. Although isolated epithelia under Ussing chamber style conditions are generally accepted to be healthy and viable, any deviation from in vivo-like conditions would likely result in underestimates of true metabolic rates because the isolated tissue lacks primary and secondary circulation, neuroendocrine stimulation, and may not have optimal mixtures of metabolic fuel. Similarly, theoretical estimates of metabolic cost of ion transport could underestimate true metabolic cost as these calculations assume 100% efficiency of transport processes and no significant back flux of ions. However, the most parsimonious explanation for the close agreement between the two approaches seems to be that neither process is associated with significant error. Errors of the same magnitude, caused by different potential limitations, constraints on isolated tissues *versus* erroneous assumptions of transport efficiencies, on two widely distinct approaches seem less likely. We did not include the rectal epithelium in our measurements or estimates. The rectal epithelium does contribute to osmoregulation in toadfish (Ruhr et al., 2016) but represents a small fraction of length and mass compared to the intestine and its contribution to overall metabolic cost of osmoregulation is therefore modest.

The metabolic cost of intestinal osmoregulatory processes of 2.5%–2.6% of SMR is modest. However, the metabolic rate of the intestinal tissue (5.44 μmol g^{-1} h^{-1} at 22°C) is similar to previously reported values from this species (8.9 μmol g^{-1} h^{-1} at 25°C) (Taylor and Grosell, 2009) and 3.2 times higher than the corresponding mass specific whole animal metabolic rate illustrating the overall high metabolic demand of this tissue. Similar results were found for the esophageal tissue, with its mass specific metabolic rate 2.1-fold higher than the corresponding whole animal metabolic rate. Further, 21% (1.17/5.44 μmol g^{-1} h^{-1}) of this high tissue specific metabolic demand is allocated to ion transport processes in the intestine, while most of the metabolic demand of the esophageal tissue appears to be associated with ion absorption. Our observations add perspective to previous work showing elevated cardiac output devoted mainly to increased gut blood flow in the euryhaline rainbow trout (*Oncorhynchus mykiss*) following acclimation to seawater (Brijs et al., 2015; Brijs et al., 2016). This increase in gut blood flow is necessary to sustain the higher metabolic demand of intestinal tissue associated with osmoregulation in seawater and allowed for normal specific dynamic action and postprandial increases in gut blood flow in seawater acclimated rainbow trout despite the increased metabolic cost associated with osmoregulation (Brijs et al., 2016).

Kirschner (Kirschner, 1993) used a similar theoretical approach to the one we employed to determine cost of osmoregulation by the intestine and the gill in seawater acclimated rainbow trout (*Oncorhyncus mykiss*) and European flounder (*Platichthys flesus*) and found that 1.4% and 3.2% of SMR could be ascribed to osmoregulatory costs by the intestine for rainbow trout and European flounder, respectively. Note that Kirschner's original data suggests that intestinal costs are 7.4% of BMR for flounder. However, as Kirschner points out, the values for European flounder are overestimates since ion flux rates were measured at 17°C–22°C, while the BMR measurements were obtained at 10°C. Assuming a Q_{10} of 2.3 to adjust the BMR to 20°C provides an estimate of the 3.2% of BMR associated with intestinal osmoregulatory functions, as mentioned above. Kirschner assumed that all Na^+ and Cl^- absorption was performed by the intestine and did not account for the component of Na^+ and Cl^- absorption that occurs across the esophagus via passive transport, a factor that would tend to overestimate metabolic cost. Further, Kirschner assumed that all intestinal Na^+ and Cl^- absorption occurred via NKCC. However, both European flounder (Grosell and Jensen, 1999; Grosell et al., 2005) and rainbow trout (Wilson et al., 1996; Grosell et al., 2009a) rely on anion exchange pathways in addition to the NKCC and although these pathways contribute little to overall Cl^- uptake, they are less energy efficient from a Cl^- uptake perspective. The assumption of exclusive NKCC-driven ion absorption likely represents a modest underestimation of cost of intestinal ion transport, as anion exchange pathways combined represent only ~13% of total metabolic costs, at least in toadfish (Figure 2).

Despite these relatively minor differences between Kirschner's original approach and ours, there is reasonable agreement among estimates of 1.4% and 3.2% of SMR for rainbow trout and European flounder, respectively (Kirschner, 1993), and our estimate of 2.6% of SMR for toadfish esophageal and intestinal osmoregulatory process. Importantly, the average of these estimates (~2.4% of SMR) are validated by the empirical data (2.5%) in the present study.

The three intestinal ion transport pathways involving anion exchange are all energetically more costly than the NKCC pathway and although they only contribute around 5% of overall Cl^- uptake, they are responsible for >13% of the overall metabolic cost (Figure 2). Since the NKCC pathway is more efficient, the adaptive significance of these transport pathways does not appear to be Cl^- absorption. Rather, the significance of these transport pathways is likely to raise luminal HCO_3^- and CO_3^{2-} concentrations to facilitate formation of $CaCO_3$ ("ichthyocarbonates"), which act to reduce luminal osmotic pressure by up to 100 mOsm (Wilson et al., 2002; Grosell et al., 2009b) thereby facilitating water absorption. Our previous work has demonstrated that intestinal water absorption, which is coupled to Na^+ and Cl^- absorption, can proceed against osmotic gradients of up to 35 mOsm (Genz et al., 2011) and intestinal fluids are near isosmotic to the blood plasma. Thus, without this precipitation reaction and assuming constant plasma osmotic pressure, the osmotic pressure of the intestinal lumen would exceed that of the blood by 100 mOsm and not only prevent intestinal water absorption but also result in fluid loss into the intestine.

4.2 Cost of branchial and renal transport

Few studies have directly addressed the metabolic cost of osmoregulatory process in the gill despite the important role this organ plays in teleost fish osmoregulation in both freshwater and seawater. In Kirschner's pioneering work from 1993 (Kirschner, 1993), metabolic cost of branchial ion regulatory processes was estimated using the theoretical approach to be 5.7% and 4.1% for seawater acclimated rainbow trout and European flounder, respectively, with the value for flounder derived using a temperature adjusted SMR as described in the preceding paragraph. These values are comparable but slightly higher than the only tissue specific metabolic rate measurement performed on gill tissue from seawater acclimated fish (Morgan and Iwama, 1999) indicating that 2.4% of BMR was required for branchial osmoregulatory processes but overall suggests that average branchial cost of osmoregulation in seawater teleost is, on average ~4% of BMR.

Metabolic costs of renal osmoregulatory processes have yet to be estimated or measured as it is challenging, if not impossible, to work on the kidney in isolation. However, the agreement between empirical and theoretical estimates of osmoregulatory costs observed for the intestinal tissue in the present study is encouraging for future work aiming to determine the cost of osmoregulatory processes of the renal system. With known renal urine flow rates and composition, urinary bladder transport functions and assumptions about the secretory and absorptive processes in renal tubules, theoretical estimates of renal osmoregulatory costs should be possible although beyond the scope of this study.

4.3 Whole animal cost of osmoregulation

To estimate whole animal metabolic cost of osmoregulation in marine or seawater acclimated euryhaline teleost fish, it seems safe to assume that renal contributions are minor, possibly in the order of ~1%, which when combined with an average esophageal/intestinal cost of 2.4% and an average branchial cost of 4% adds up to 7%–8% of BMR. This estimate is in close agreement with Kirschner's cost of osmoregulation estimates, not including renal contributions, of 7.5% and 7.3% of SMR for rainbow trout and European flounder, respectively (applying the temperature correction of SMR for the European flounder as discussed above). Dozens of studies (reviewed by (Ern et al., 2014)) have attempted to determine the cost of osmoregulation by comparing metabolic rates in fish acclimated to different salinities with the expectation that the differences between metabolic rates at low or high salinities would exceed the corresponding metabolic rates at isosmotic salinities where cost of osmoregulation would approach zero and that these differences would reflect metabolic cost of osmoregulation. However, there is no consensus among the whole animal metabolic rate studies and estimates range from undetectable to >30% of BMR (Ern et al., 2014; Christensen et al., 2017; Christensen et al., 2018; Christensen et al., 2019). Similar difficulty was observed in the present study where the highest SMR and RMR was observed in fish acclimated to isosmotic 9 ppt, suggesting a negative cost of osmoregulation in seawater acclimated fish which is obviously meaningless. In our view, at least two factors contribute to difficulties in assessing metabolic costs of osmoregulation, and other vital processes, by comparing whole animal metabolic rates. First, whole animal metabolic rates vary considerably among individuals, even when care is taken to select animals of similar size and history, to standardize digestive status, and to allow for animals to settle in respirometers before measurements are initiated (Clark et al., 2013). Using the present dataset as an example, toadfish SMR in seawater of 1,682 μmol O_2 kg^{-1} h^{-1} was associated with an SEM of ±180 (=STDEV of ± 649) μmol O_2 kg^{-1} h^{-1}, n = 13, which is equivalent to 39% variation. Assuming a whole animal metabolic cost of osmoregulation of 7.5%, α = 0.05, ß = 0.2 and a power of 0.8, a power analysis revealed that an n-number of 416 individuals per experimental group would be required to detect a statistically significant differences of ~7.5% of BMR associated with osmoregulation in the present study and that of Kirschner (Kirschner, 1993). None of the studies aiming to detect cost of osmoregulation from whole animal metabolic rates, including ours, have applied replication anywhere near this requirement. Second, by nature, experiments comparing metabolic rates across salinities use euryhaline and estuarine species. For many of these species, salinity fluctuations, tidal or seasonal, may represent differences in prey abundance, predatory pressures, and reproductive status and, by association, elicit salinity induced changes in metabolic rates that are evident in estimates of SMR, but are not strictly related to the cost of osmoregulation. This second factor may explain how investigators, including us in the present study, have documented significant effects of salinity on metabolic rates even if the power of experimental design was insufficient to detect the true cost of osmoregulation.

Considering the high degree of replication required and the possible salinity-associated confounding factors that may influence whole animal metabolic rates, it seems that whole animal respirometry is

impractical for studying cost of osmoregulation. Rather, isolated tissue respirometry or estimates based on ion transport rates, which were found in close agreement in the present study, are better suited to answer future questions about metabolic cost of osmoregulation. Important questions to be addressed in future studies include how osmoregulatory costs may change with climate change, size/age of the organism, and active *versus* sedentary lifestyles.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by University of Miami has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is #A-3224-01, approved through November 30, 2023. We are registered with USDA APHIS, registration # 58-R-007, approved through December 3, 2023. We have full accreditation with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International), site 001069, latest effective date, June 22, 2022.

Author contributions

AL contributed to study design, acclimated fish to appropriate salinities, performed the isolated tissue measurements and associated analyses, and drafted part of the manuscript. CP and JS performed the whole animal respirometry experiments. MG outlined study design, secured funding, performed the estimates of metabolic cost from ion transport rates, and drafted part of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Anandakrishnan, R., and Zuckerman, D. M. (2017). Biophysical comparison of ATP-driven proton pumping mechanisms suggests a kinetic advantage for the rotary process depending on coupling ratio. *PloS one* 12, e0173500. doi:10.1371/journal.pone.0173500

Brijs, J., Axelsson, M., Grans, A., Pichaud, N., Olsson, C., and Sandblom, E. (2015). Increased gastrointestinal blood flow: An essential circulatory modification for euryhaline rainbow trout (*Oncorhynchus mykiss*) migrating to sea. *Sci. Rep-Uk* 5, 10430. doi:10.1038/srep10430

Brijs, J., Grans, A., Ekstrom, A., Olsson, C., Axelsson, M., and Sandblom, E. (2016). Cardiorespiratory upregulation during seawater acclimation in rainbow trout: Effects on gastrointestinal perfusion and postprandial responses. *Am. J. Physiology-Regulatory Integr. Comp. Physiology* 310, R858–R865. doi:10.1152/ajpregu.00536.2015

Chabot, D., Steffensen, J. F., and Farrell, A. P. (2016). The determination of standard metabolic rate in fishes. *J. fish Biol.* 88, 81–121. doi:10.1111/jfb.12845

Chang, M. H., Plata, C., Kurita, Y., Kato, A., Hirose, S., and Romero, M. F. (2012). Euryhaline pufferfish NBCe1 differs from nonmarine species NBCe1 physiology. *Am.J.Physiol Cell Physiol.* 302, C1083–C1095. doi:10.1152/ajpcell.00233.2011

Christensen, E. A. F., Illing, B., Iversen, N. S., Johansen, J. L., Domenici, P., and Steffensen, J. F. (2018). Effects of salinity on swimming performance and oxygen consumption rate of shiner perch *Cymatogaster aggregata*. *J. Exp. Mar. Biol. Ecol.* 504, 32–37. doi:10.1016/j.jembe.2018.04.002

Christensen, E. A. F., Stieglitz, J. D., Grosell, M., and Steffensen, J. F. (2019). Intra-specific difference in the effect of salinity on physiological performance in European perch (*Perca fluviatilis*) and its ecological importance for fish in estuaries. *Biology-Basel* 8, 89. doi:10.3390/biology8040089

Christensen, E. A. F., Svendsen, M. B. S., and Steffensen, J. F. (2017). Plasma osmolality and oxygen consumption of perch *Perca fluviatilis* in response to different salinities and temperatures. *J. fish Biol.* 90, 819–833. doi:10.1111/jfb.13200

Clark, T. D., Sandblom, E., and Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: Respirometry, relevance and recommendations. *J. Exp. Biol.* 216, 2771–2782. doi:10.1242/jeb.084251

Colin, D. A., Nonnotte, G., Leray, C., and Nonnotte, L. (1985). Na-transport and enzyme-activities in the intestine of the fresh-water and sea-water adapted trout (Salmo-Gairdnerii R). *Comp. Biochem. Physiology A-Physiology* 81, 695–698. doi:10.1016/0300-9629(85)91049-7

Ern, R., Huong, D. T. T., Cong, N. V., Bayley, M., and Wang, T. (2014). Effect of salinity on oxygen consumption in fishes: A review. *J. fish Biol.* 84, 1210–1220. doi:10.1111/jfb.12330

Esbaugh, A. J., and Grosell, M. (2014). Esophageal desalination is mediated by Na^+, H^+ exchanger-2 in the gulf toadfish (*Opsanus beta*). *Comp. Biochem. Physiology a-Molecular Integr. Physiology* 171, 57–63. doi:10.1016/j.cbpa.2014.02.012

Fuentes, J., Rey, P., and Rebolledo, E. (1997). Progressive transfer to seawater enhances intestinal and branchial Na^+-K^+ ATPase activity in non-anadromous rainbow trout. *Aquac. Int.* 5, 217–227. doi:10.1023/a:1018387317893

Genz, J., McDonald, D. M., and Grosell, M. (2011). Concentration of $MgSO_4$ in the intestinal lumen of *Opsanus beta* limits osmoregulation in response to acute hypersalinity stress. *Am. J. Physiol.* 300, R895–R909. doi:10.1152/ajpregu.00299.2010

Genz, J., Taylor, J. R., and Grosell, M. (2008). Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*. *J. Exp. Biol.* 211, 2327–2335. doi:10.1242/jeb.016832

Grosell, M., and Genz, J. (2006). Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. *Am. J. Physiol.* 291, R1145–R1156. doi:10.1152/ajpregu.00818.2005

Grosell, M., Genz, J., Taylor, J. R., Perry, S. F., and Gilmour, K. M. (2009a). The involvement of H^+-ATPase and carbonic anhydrase in intestinal HCO_3^- secretion on seawater-acclimated rainbow trout. *J. Exp. Biol.* 212, 1940–1948. doi:10.1242/jeb.026856

Grosell, M. (2006). Intestinal anion exchange in marine fish osmoregulation. *J. Exp. Biol.* 209, 2813–2827. doi:10.1242/jeb.02345

Grosell, M. (2014). "Intestinal transport," in *Physiology of fishes* David Evans Claiborne, (Claiborne and Suzie Currie), 175–205.

Grosell, M., and Jensen, F. B. (1999). NO2- uptake and HCO3- excretion in the intestine of the European flounder (Platichthys flesus). *J. Exp. Biol.* 202, 2103–2110. doi:10.1242/jeb.202.15.2103

Grosell, M., Mager, E. M., Williams, C., and Taylor, J. R. (2009b). High rates of HCO_3^- secretion and Cl^- absorption against adverse gradients in the marine teleost intestine: The involvement of an electrogenic anion exchanger and H^+-pump metabolon? *J. Exp. Biol.* 212, 1684–1696. doi:10.1242/jeb.027730

Grosell, M., Wood, C. M., Wilson, R. W., Bury, N. R., Hogstrand, C., Rankin, C., et al. (2005). Bicarbonate secretion plays a role in chloride and water absorption of the European flounder intestine. *Am. J. Physiology-Regulatory Integr. Comp. Physiology* 288, R936–R946. doi:10.1152/ajpregu.00684.2003

Guffey, S., Esbaugh, A., and Grosell, M. (2011). Regulation of apical H^+-ATPase activity and intestinal HCO_3^- secretion in marine fish osmoregulation. *Am. J. Physiology-Regulatory Integr. Comp. Physiology* 301, R1682–R1691. doi:10.1152/ajpregu.00059.2011

Herskin, J. (1999). Effects of social and visual contact on the oxygen consumption of juvenile sea bass measured by computerized intermittent respirometry. *J. fish Biol.* 55, 1075–1085. doi:10.1111/j.1095-8649.1999.tb00742.x

Hinkle, P. C. (2005). P/O ratios of mitochondrial oxidative phosphorylation. *Biochimica Biophysica Acta-Bioenergetics* 1706, 1–11. doi:10.1016/j.bbabio.2004.09.004

Hirano, T., and Mayer-Gostan, N. (1976). Eel esophagus as an osmoregulatory organ. *Proc. Natl. Acad. Sci. U. S. A.* 73, 1348–1350. doi:10.1073/pnas.73.4.1348

Jampol, L. M., and Epstein, F. H. (1970). Sodium-potassium-activated adenosine triphosphatase and osmotic regulation by fishes. *Am. J. Physiol.* 218, 607–611. doi:10.1152/ajplegacy.1970.218.2.607

Jordan, A. D., and Steffensen, J. F. (2007). Effects of ration size and hypoxia on specific dynamic action in the cod. *Physiological Biochem. Zoology* 80, 178–185. doi:10.1086/510565

Kelly, S. P., Chow, I. N. K., and Woo, N. Y. S. (1999). Effects of prolactin and growth hormone on strategies of hypoosmotic adaptation in a marine teleost, Sparus sarba. *General Comp. Endocrinol.* 113, 9–22. doi:10.1006/gcen.1998.7159

Kirschner, L. B. (1993). The energetics of osmotic regulation in ureotelic and hypoosmotic fishes. *J. Exp. Zoology* 267, 19–26. doi:10.1002/jez.1402670104

Kurita, Y., Nakada, T., Kato, A., Doi, H., Mistry, A. C., Chang, M. H., et al. (2008). Identification of intestinal bicarbonate transporters involved in formation of carbonate precipitates to stimulate water absorption in marine teleost fish. *Am. J. Physiology-Regulatory Integr. Comp. Physiology* 294, R1402–R1412. doi:10.1152/ajpregu.00759.2007

Larsen, E. H., Deaton, L. E., Onken, H., O'Donnell, M., Grosell, M., Dantzler, W. H., et al. (2014). Osmoregulation and excretion. *Compr. Physiol.* 4, 405–573. doi:10.1002/cphy.c130004

Madsen, S. S., Mccormick, S. D., Young, G., Endersen, J. S., Nishioka, R. S., and Bern, H. S. (1994). Physiology of seawater acclimation in the striped bass, *Morone saxatilis* (Walbaum). *Fish. Physiol.Biochem.* 13, 1–11. doi:10.1007/BF00004114

Mager, E. M., Esbaugh, A. J., Stieglitz, J. D., Hoenig, R., Bodinier, C., Incardona, J. P., et al. (2014). Acute embryonic or juvenile exposure to deepwater horizon crude oil impairs the swimming performance of mahi-mahi (*Coryphaena hippurus*). *Environ. Sci. Technol.* 48, 7053–7061. doi:10.1021/es501628k

Maxson, M. E., and Grinstein, S. (2014). The vacuolar-type H^+-ATPase at a glance - more than a proton pump. *J. Cell Sci.* 127, 4987–4993. doi:10.1242/jcs.158550

McDonald, M. D., and Grosell, M. (2006). Maintaining osmotic balance with an aglomerular kidney. *Comp. Biochem. Physiology A-Molecular Integr. Physiology* 143, 447–458. doi:10.1016/j.cbpa.2005.12.029

Morgan, J. D., and Iwama, G. K. (1999). Energy cost of NaCl transport in isolated gills of cutthroat trout. *Am. J. Physiology-Regulatory Integr. Comp. Physiology* 277, R631–R639. doi:10.1152/ajpregu.1999.277.3.R631

Parmelee, J. T., and Renfro, J. L. (1983). Esophageal desalination of seawater in flounder: Role of active sodium transport. *Am. J. Physiol.* 245, R888–R893. doi:10.1152/ajpregu.1983.245.6.R888

Ruhr, I. M., Takei, Y., and Grosell, M. (2016). The role of the rectum in osmoregulation and the potential effect of renoguanylin on SLC26a6 transport activity in the Gulf toadfish (*Opsanus beta*). *Am. J. Physiology-Regulatory Integr. Comp. Physiology* 311, R179–R191. doi:10.1152/ajpregu.00033.2016

Sattin, G., Mager, E. M., and Grosell, M. (2010). Cytosolic carbonic anhydrase in the gulf toadfish is important for tolerance to hypersalinity. *Comp. Biochem.Physiol A Mol.Integr.Physiol* 156, 169–175. doi:10.1016/j.cbpa.2010.01.018

Secor, S. M., Taylor, J. R., and Grosell, M. (2012). Selected regulation of gastrointestinal acid-base secretion and tissue metabolism for the diamondback water snake and Burmese python. *J. Exp. Biol.* 215, 185–196. doi:10.1242/jeb.056218

Seidelin, M., Madsen, S. S., Blenstrup, H., and Tipsmark, C. K. (2000). Time-course changes in the expression of the Na^+, K^+-ATPase in gills and pyloric caeca of Brown trout (*Salmo trutta*) during acclimation to seawater. *Physiological Biochem. Zoology* 73, 446–453. doi:10.1086/317737

Shehadeh, Z. H., and Gordon, M. S. (1969). The role of the intestine in salinity adaptation of the rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol.* 30, 397–418. doi:10.1016/0010-406x(69)92011-8

Smith, H. W. (1930). The absorption and excretion of water and salts by marine teleosts. *Am. J. Physiology* 93, 480–505. doi:10.1152/ajplegacy.1930.93.2.480

Steffensen, J. F., Schurmann, H., and Bushnell, P. G. (1994). Oxygen-consumption in 4 species of teleosts from Greenland - No evidence of metabolic cold adaptation. *Polar Biol.* 14, 49–54. doi:10.1007/BF00240272

Stieglitz, J. D., Mager, E. M., Hoenig, R. H., Benetti, D. D., and Grosell, M. (2016). Impacts of Deepwater Horizon crude oil exposure on adult mahi-mahi (*Coryphaena hippurus*) swim performance. *Environ. Toxicol. Chem.* 35, 2613–2622. doi:10.1002/etc.3436

Svendsen, J. C., Steffensen, J. F., Aarestrup, K., Frisk, M., Etzerodt, A., and Jyde, M. (2012). Excess posthypoxic oxygen consumption in rainbow trout (*Oncorhynchus mykiss*): Recovery in normoxia and hypoxia. *Can. J. Zoology* 90, 1–11. doi:10.1139/z11-095

Taylor, J. R., and Grosell, M. (2009). The intestinal response to feeding in seawater gulf toadfish, *Opsanus beta*, includes elevated base secretion and increased epithelial oxygen consumption. *J. Exp. Biol.* 212, 3873–3881. doi:10.1242/jeb.034579

Tomashek, J. J., and Brusilow, W. S. A. (2000). Stoichiometry of energy coupling by proton-translocating ATPases: A history of variability. *J. Bioenergetics Biomembr.* 32, 493–500. doi:10.1023/a:1005617024904

Wilson, R. W., Gilmour, K. M., Henry, R. P., and Wood, C. M. (1996). Intestinal base excretion in the seawater-adapted rainbow trout: A role in acid-base balance? *J. Exp. Biol.* 199, 2331–2343. doi:10.1242/jeb.199.10.2331

Wilson, R. W., Wilson, J. M., and Grosell, M. (2002). Intestinal bicarbonate secretion by marine teleost fish-why and how? *Biochim. Biophys. Acta*. 1566, 182–193. doi:10.1016/s0005-2736(02)00600-4

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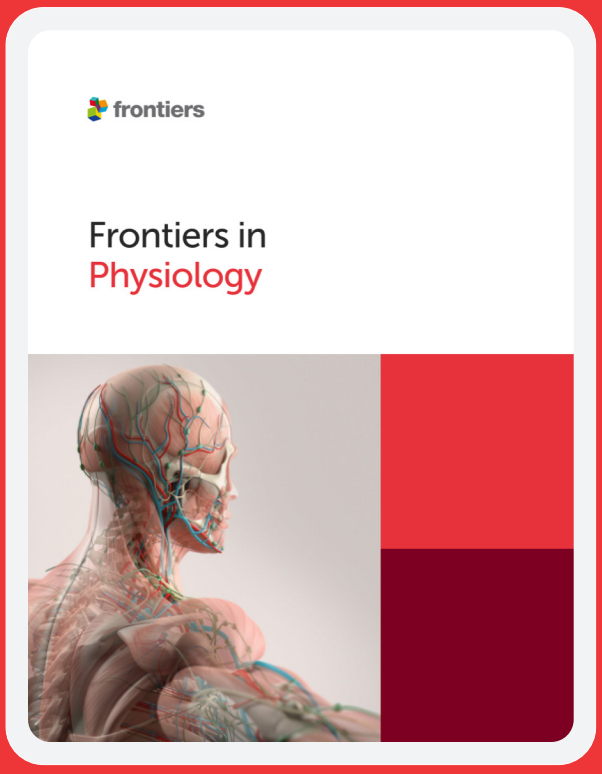

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