Check for updates

Vitamin D regulates transepithelial acid secretion in zebrafish (*Danio rerio*) larvae

Chia-Hao Lin[1,2]*, Sian-Tai Liu[1,3], Yu-Chun Wang[4], Yi-Ling Tsou[5] and Huei-Jyun Hu[5]

[1]Department of Marine Biotechnology, National Kaohsiung University of Science and Technology, Kaohsiung, Taiwan, [2]The Integrative Evolutionary Galliforms Genomics Research (iEGG) and Animal Biotechnology Center, National Chung Hsing University, Taichung, Taiwan, [3]Department of Life Science, National Taiwan University, Taipei, Taiwan, [4]Planning and Information Division, Fisheries Research Institute, Keelung City, Taiwan, [5]Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan

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 ($1\alpha,25(OH)_2D_3$), the bioactive vitamin D, were significantly increased in 3 days post-fertilization zebrafish larvae after exposure to acidic freshwater (AFW, pH 4.0). Exogenous $1\alpha,25(OH)_2D_3$ (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 $1\alpha,25(OH)_2D_3$ treatment. This study is the first to indicate that vitamin D is involved in acid secretion in fish.

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 ($1\alpha,25$ $(OH)_2D_3$), 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ (Reschly et al., 2007; Howarth et al., 2008; Krasowski et al., 2011). Although the two isoforms of medaka VDRs show different responses to $1\alpha,25(OH)_2D_3$, 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

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 $1\alpha,25(OH)_2D_3$ levels have been found in NHE3 knockout mice (Pan et al., 2012). Treatment with $1\alpha,25(OH)_2D_3$ 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, $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ synthesis and $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$, 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ (CAS No. 32222-06-3, Sigma, USA) were used for an incubation experiment. Zebrafish eggs were incubated in $1\alpha,25(OH)_2D_3$ media immediately after fertilization and were sampled at 3 dpf for subsequent analysis. The incubation media were changed with new $1\alpha,25(OH)_2D_3$ 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 $12,000 \times g$ 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 $12,000 \times g$ 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$_2$HPO$_4$, and 0.002 mM KH$_2$PO$_4$; 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.
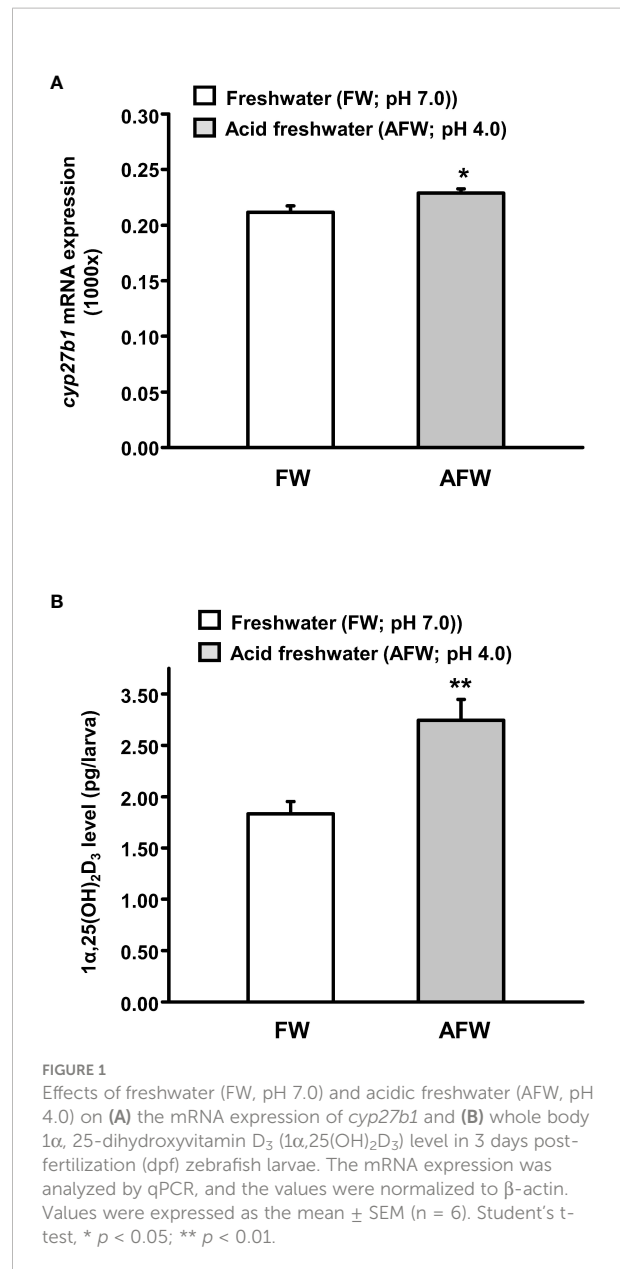
Statistical analysis

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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$, and whole-body $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 ($1\alpha,25(OH)_2D_3$) 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ levels were significantly upregulated in zebrafish larvae in AFW (Figure 1). Therefore, we further investigated whether exogenous $1\alpha,25(OH)_2D_3$ (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 $1\alpha,25(OH)_2D_3$ 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

FIGURE 2
Effects of exogenous $1\alpha,25(OH)_2D_3$ 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 $\pm$ SEM (n = 6−12). Student's t-test, $* p < 0.05$; $** p < 0.01$.

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).

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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ (20 μg/L) treatment on the expression of *gcm2*, a key transcription factor controlling the differentiation of HRCs (Chang et al., 2009). Exogenous $1\alpha,25(OH)_2D_3$ treatment significantly stimulated *gcm2* transcripts, while the stimulation was prevented in 3 dpf zebrafish larvae with *vdra/b* knockdown (Figure 5).

Effects of exogenous $1\alpha,25(OH)_2D_3$ 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
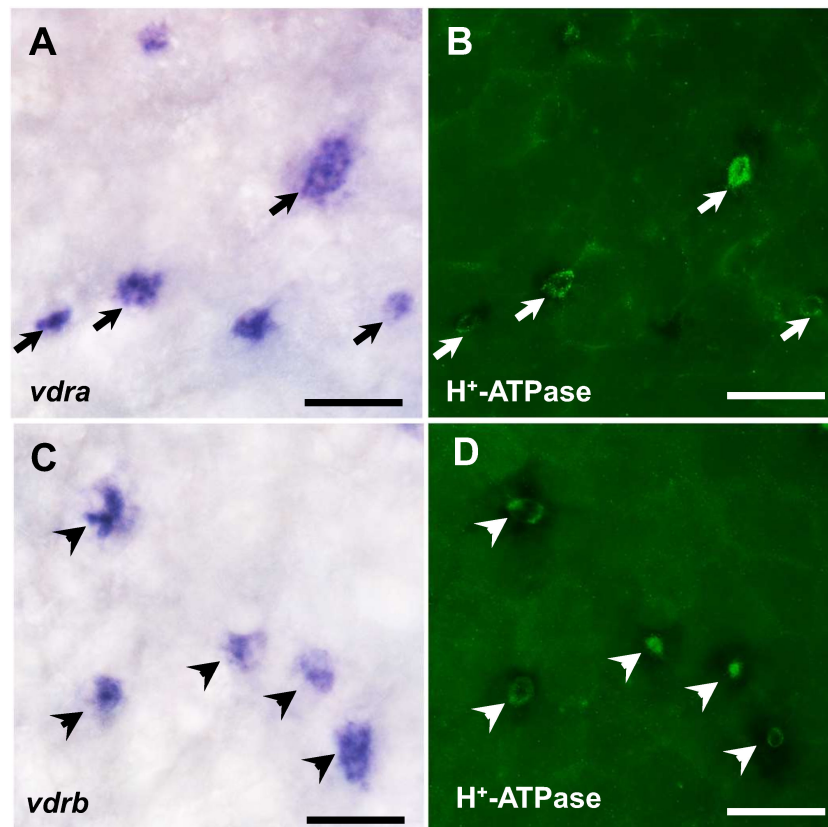
FIGURE 3
In situ hybridization staining of *vdra* and *vdrb* **(A, C)** and immunocytochemical staining of H$^+$-ATPase **(B, D)** in 3 dpf zebrafish larvae. Co-localization of *vdra* mRNA and H$^+$-ATPase protein signals in the same cell is indicated by arrows. Scale bar: 20 µm.

$1\alpha,25(OH)_2D_3$ (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*
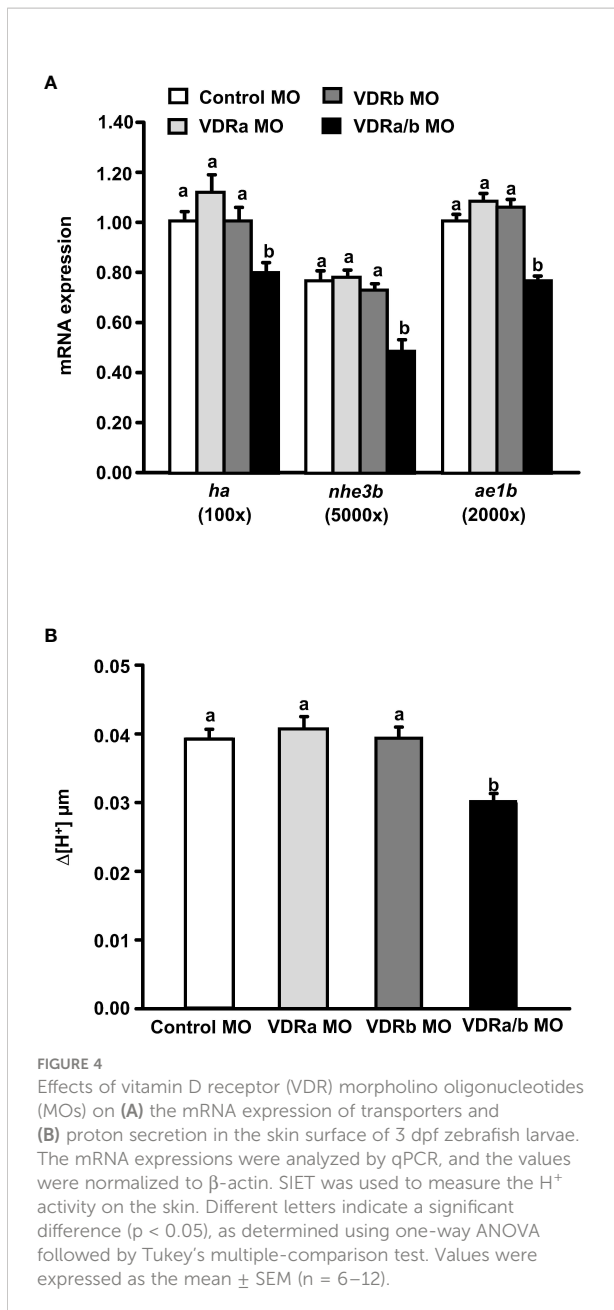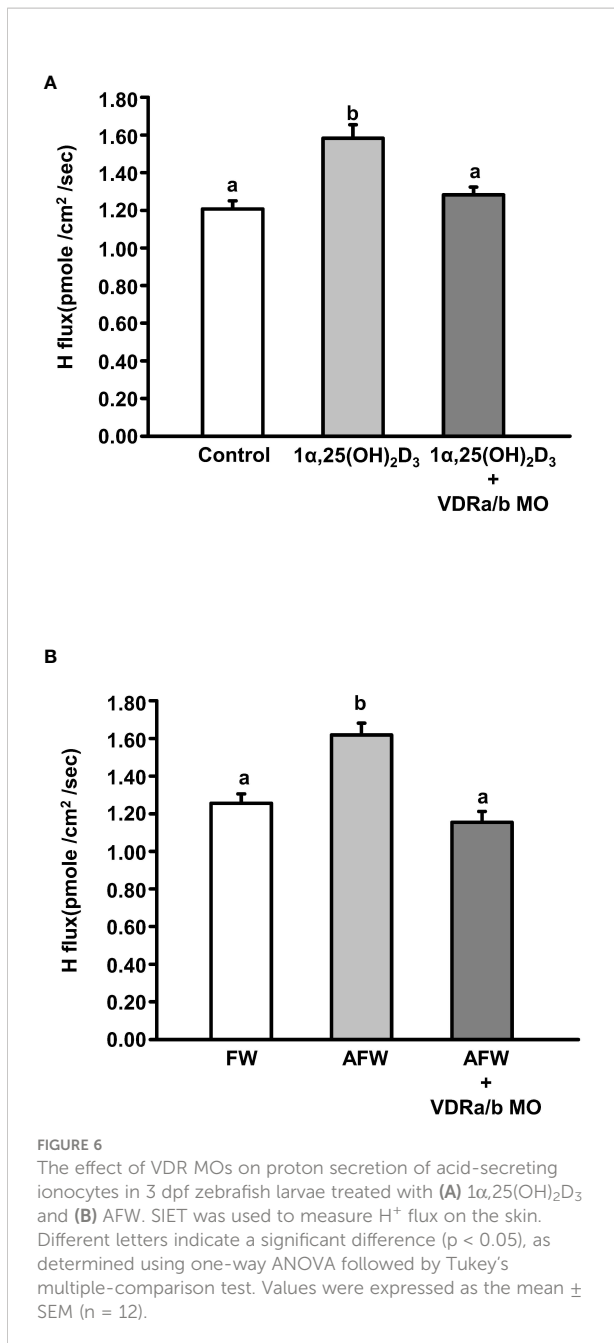
expression. However, Lin et al. (2015) reported that the mRNA expression level of *ha* was far greater than *nhe3b*—approximately 50-fold—in 3 dpf zebrafish larvae, and the present study also found a similar result. Thus, the increased *ha* expression by 1α,25(OH)₂D₃ treatment predominantly contributed to the increased acid secretion in zebrafish larvae. The mRNA expression of *ae1b* also increased in zebrafish larvae treated with 1α,25(OH)₂D₃. *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 HCO₃⁻ out of cells to fulfill the function of epithelial
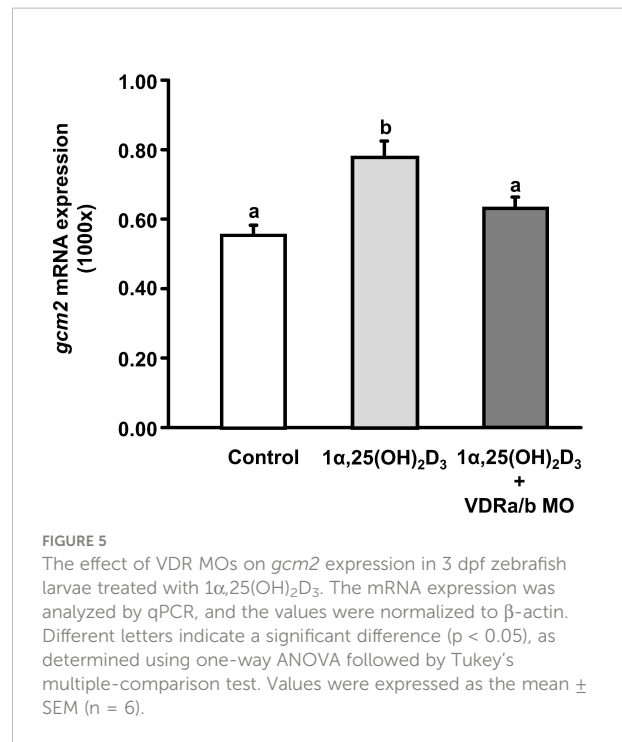
acid secretion (Lee et al., 2011). Therefore, 1α,25(OH)₂D₃ treatment may increase the capacity for acid secretion at each HRC by enhancing the expression of apical and basolateral acid-secreting transporters in zebrafish larvae.

Acid secretion at skin surface of the entire body and at each HRC of zebrafish was elevated by 1α,25(OH)₂D₃ treatment. Moreover, 1α,25(OH)₂D₃ 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)** $1\alpha,25(OH)_2D_3$ 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 $\pm$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$- 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $1\alpha,25(OH)_2D_3$ 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.

Funding

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.990502/full#supplementary-material

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