

## RESEARCH ARTICLE

# Reassessing the contribution of the Na<sup>+</sup>/H<sup>+</sup> exchanger Nhe3b to Na<sup>+</sup> uptake in zebrafish (*Danio rerio*) using CRISPR/Cas9 gene editing

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## ABSTRACT

Freshwater fishes absorb Na<sup>+</sup> from their dilute environment using ion-transporting cells. In larval zebrafish (*Danio rerio*), Na<sup>+</sup> uptake is coordinated by (1) Na<sup>+</sup>/H<sup>+</sup> exchanger 3b (Nhe3b) and (2) H<sup>+</sup>-ATPase-powered electrogenic uptake in H<sup>+</sup>-ATPase-rich (HR) cells and by (3) Na<sup>+</sup>-Cl<sup>-</sup>-cotransporter (Ncc) expressed in NCC cells. The present study aimed to better understand the roles of these three proteins in Na<sup>+</sup> uptake by larval zebrafish under 'normal' (800 μmol l<sup>-1</sup>) and 'low' (10 μmol l<sup>-1</sup>) Na<sup>+</sup> conditions. We hypothesized that Na<sup>+</sup> uptake would be reduced by CRISPR/Cas9 knockout (KO) of *slc9a3.2* (encoding Nhe3b), particularly in low Na<sup>+</sup> where Nhe3b is believed to play a dominant role. Contrary to this hypothesis, Na<sup>+</sup> uptake was sustained in *nhe3b* KO larvae under both Na<sup>+</sup> conditions, which led to the exploration of whether compensatory regulation of H<sup>+</sup>-ATPase or Ncc was responsible for maintaining Na<sup>+</sup> uptake in *nhe3b* KO larvae. mRNA expression of the genes encoding H<sup>+</sup>-ATPase and Ncc was not altered in *nhe3b* KO larvae. Moreover, morpholino knockdown of H<sup>+</sup>-ATPase, which significantly reduced H<sup>+</sup> flux by HR cells, did not reduce Na<sup>+</sup> uptake in *nhe3b* KO larvae, nor did rearing larvae in chloride-free conditions, thereby eliminating any driving force for Na<sup>+</sup>-Cl<sup>-</sup>-cotransport via Ncc. Finally, simultaneously treating *nhe3b* KO larvae with H<sup>+</sup>-ATPase morpholino and chloride-free conditions did not reduce Na<sup>+</sup> uptake under normal or low Na<sup>+</sup>. These findings highlight the flexibility of the Na<sup>+</sup> uptake system and demonstrate that Nhe3b is expendable to Na<sup>+</sup> uptake in zebrafish and that our understanding of Na<sup>+</sup> uptake mechanisms in this species is incomplete.

**KEY WORDS:** Compensatory regulation, H<sup>+</sup>-ATPase, Ionocyte, Ion regulation, Na<sup>+</sup>-Cl<sup>-</sup>-cotransporter, Ncc, Rhesus glycoprotein, Rhcgb, Rhcg1

## INTRODUCTION

For nearly a century, comparative physiologists have sought to determine the mechanisms by which freshwater (FW) fishes absorb ions from their dilute hypoionic environment to counteract passive salt loss. Zebrafish (*Danio rerio*) has emerged as a powerful model for understanding ionoregulatory mechanisms by FW fishes (Hwang et al., 2011; Dymowska et al., 2012; Kumai and Perry, 2012; Guh et al., 2015), owing primarily to its ease of husbandry, short generation time and amenability to genetic manipulations.

A model for ion uptake in zebrafish has been established whereby multiple subtypes of ion-transporting cells (ionocytes) coordinate the absorption of specific ions. Na<sup>+</sup> uptake, in particular, is coordinated by three distinct pathways: (i) Na<sup>+</sup>/H<sup>+</sup> exchange via Slc9a3.2 (Na<sup>+</sup>/H<sup>+</sup> exchanger 3b; Nhe3b) expressed in H<sup>+</sup>-ATPase-rich (HR) cells, (ii) electrogenic Na<sup>+</sup> uptake via a putative Na<sup>+</sup> channel powered by Atp6v1aa (H<sup>+</sup>-ATPase) expressed in HR cells and (iii) Na<sup>+</sup>-Cl<sup>-</sup>-cotransport by Slc12a10.2 (Na<sup>+</sup>-Cl<sup>-</sup>-cotransporter-like 2; hereafter referred to as Ncc) expressed in NCC cells (Hwang et al., 2011; Dymowska et al., 2012; Hwang and Chou, 2013; Hwang and Lin, 2014; Guh et al., 2015).

Nhe3b was first localized to HR cells of zebrafish using immunohistochemical labelling and *in situ* hybridization (Yan et al., 2007) and its role in Na<sup>+</sup> uptake was supported by a reduction in whole-body Na<sup>+</sup> uptake and HR cell-specific Na<sup>+</sup> accumulation in 4 days post-fertilization (dpf) larvae by treatment with 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), a specific inhibitor of Na<sup>+</sup>/H<sup>+</sup> exchangers (NHE) (Esaki et al., 2007). Moreover, epithelial Na<sup>+</sup> influx, measured by the scanning ion-selective electrode technique (SIET), was reduced in response to EIPA treatment and in response to antisense morpholino oligonucleotide knockdown (KD) of Nhe3b (Shih et al., 2012). It is important to note, however, that EIPA was also shown to have no inhibitory effect on Na<sup>+</sup> uptake in zebrafish larvae and adults in other studies (Boisen et al., 2003; Kumai and Perry, 2011; Kwong and Perry, 2013; Dymowska et al., 2015). The reductions in epithelial Na<sup>+</sup> gradients that were observed in response to EIPA and Nhe3b KD (Shih et al., 2012) were demonstrated only in larvae reared in a low ambient Na<sup>+</sup> concentration (5 μmol l<sup>-1</sup>). In fact, several studies have demonstrated that *nhe3b* gene expression increases in response to low Na<sup>+</sup> acclimation in zebrafish (Yan et al., 2007; Shih et al., 2012; Lin et al., 2016). An increased role for Nhe3b in low Na<sup>+</sup> is surprising given that NHE function even in 'typical' FW (~1 mmol l<sup>-1</sup> Na<sup>+</sup>) has been debated on thermodynamic grounds (Parks et al., 2008). However, the thermodynamic constraints of NHE function in FW are believed to be overcome by close association with apical ammonia-transporting Rhesus (Rh) glycoproteins. In zebrafish, Rhcgb (formerly Rhcg1) is expressed on the apical membrane of HR cells (Nakada et al., 2007; Ito et al., 2013) and is believed to facilitate Na<sup>+</sup>/H<sup>+</sup> exchange by binding cytosolic NH<sub>4</sub><sup>+</sup>, stripping off H<sup>+</sup>, and conducting NH<sub>3</sub>, after which it is titrated by H<sup>+</sup> in the apical boundary layer (reviewed by Hwang and Lin, 2014; Wright and Wood, 2009, 2012; Zimmer et al., 2017a). This arrangement of Nhe3b and Rhcgb, in addition to the presence of cytosolic carbonic anhydrase (Wright and Wood, 2009; Ito et al., 2013), is believed to create an acidic microenvironment in the cytosol and an alkaline microenvironment in the apical boundary layer that provides a sufficient H<sup>+</sup> gradient to drive Na<sup>+</sup>/H<sup>+</sup> exchange against a Na<sup>+</sup> concentration gradient. The role of this

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Rhcgbl/Nhe3b metabolon in  $\text{Na}^+$  uptake is also supported by the observation that gill *rhcgbl* mRNA expression increased in response to low  $\text{Na}^+$  conditions (Nakada et al., 2007; Shih et al., 2012) and morpholino KD of Rhcgbl reduced epithelial  $\text{Na}^+$  influx in 4 dpf larvae reared in low  $\text{Na}^+$  (Shih et al., 2012).

The  $\text{H}^+$ -ATPase model of  $\text{Na}^+$  uptake was originally proposed as a more thermodynamically feasible mechanism of  $\text{Na}^+$  uptake in FW fishes (Avella and Bornancin, 1989). In larval zebrafish, treatment with the  $\text{H}^+$ -ATPase inhibitor bafilomycin A1 reduced  $\text{H}^+$  flux at HR cells by nearly 80% (Lin et al., 2006) and  $\text{H}^+$ -ATPase KD resulted in reductions in both epithelial  $\text{H}^+$  gradients and whole-body  $\text{Na}^+$  content (Hornig et al., 2007). Interestingly, this reduction in whole-body  $\text{Na}^+$  content in  $\text{H}^+$ -ATPase morphants (individuals treated with a morpholino) was observed only in larvae reared under low  $\text{Na}^+$  ( $10 \mu\text{mol l}^{-1}$ ) conditions, contrary to previous reports suggesting that the role of  $\text{H}^+$ -ATPase in  $\text{Na}^+$  uptake is diminished under low  $\text{Na}^+$  conditions and that Nhe3b is the dominant pathway in low  $\text{Na}^+$  (Yan et al., 2007; Shih et al., 2012). Treatment with bafilomycin also reduced the accumulation of  $\text{Na}^+$  in HR cells (Esaki et al., 2007) and inhibited whole-body  $\text{Na}^+$  uptake in zebrafish larvae acclimated to  $\sim 800 \mu\text{mol l}^{-1}$   $\text{Na}^+$  (Esaki et al., 2007; Kumai and Perry, 2011; Kwong and Perry, 2013), providing further support for  $\text{H}^+$ -ATPase-mediated  $\text{Na}^+$  uptake in zebrafish. In adult zebrafish, however, the effect of bafilomycin on  $\text{Na}^+$  uptake is more equivocal, being dependent on water chemistry and exposure duration (Boisen et al., 2003). The electrogenic apical entry of  $\text{Na}^+$ , driven by  $\text{H}^+$ -ATPase, has long been controversial because a homologous epithelial  $\text{Na}^+$  channel (ENaC) gene has not been found in the genome of any fish species examined to date (Hwang et al., 2011; Dymowska et al., 2012) and the ENaC-specific blocker phenamil has been demonstrated to reduce  $\text{Na}^+$  uptake in FW fishes in some instances (Grosell and Wood, 2002; Reid et al., 2003; Preest et al., 2005; Brix and Grosell, 2012), but not others (Preest et al., 2005; Dymowska et al., 2014). More recently, the acid-sensing ion channel (ASIC) *Asic4b* has been implicated as the channel involved in  $\text{H}^+$ -ATPase-mediated electrogenic  $\text{Na}^+$  uptake in rainbow trout (Dymowska et al., 2014) and zebrafish (Dymowska et al., 2015; Zimmer et al., 2018).

In addition to HR cell-mediated  $\text{Na}^+$  uptake by Nhe3b and  $\text{H}^+$ -ATPase, Ncc also is implicated in  $\text{Na}^+$  uptake by FW fishes (Hiroi et al., 2008; Wang et al., 2009; Hwang et al., 2011; Dymowska et al., 2012; Hwang and Chou, 2013; Hwang and Lin, 2014; Guh et al., 2015), despite the fact that the function of this electroneutral transporter in FW suffers from some of the same thermodynamic limitations as Nhe3b (Parks et al., 2008). In zebrafish, Ncc is expressed in a subset of cells on the yolk sac epithelium that do not express  $\text{H}^+$ -ATPase (NCC cells; Kwong et al., 2016; Wang et al., 2009) and treatment with the NCC-specific pharmacological blocker metolazone resulted in a significant reduction in whole-body  $\text{Na}^+$  uptake in larvae (Wang et al., 2009), although this blocker was also shown to have no effect on  $\text{Na}^+$  uptake in other studies (Esaki et al., 2007; Kwong and Perry, 2013). In response to NCC KD,  $\text{Na}^+$  uptake and whole-body  $\text{Na}^+$  levels increased in larval zebrafish (Wang et al., 2009; Chang et al., 2013; Kwong and Perry, 2016), probably as a function of increased flux via Nhe3b (Wang et al., 2009; Chang et al., 2013). It is likely, therefore, that Ncc is an important pathway for  $\text{Na}^+$  uptake, requiring compensatory changes in  $\text{Na}^+$  uptake mechanisms when eliminated via morpholino KD. Moreover, in at least one study, *slc12a10.2* mRNA expression (encoding Ncc) was shown to be upregulated in larval zebrafish in response to low  $\text{Na}^+$  acclimation (Lin et al., 2016).

The goal of the present study was to further elucidate the roles of the three putative pathways of  $\text{Na}^+$  uptake (Nhe3b,  $\text{H}^+$ -ATPase mediated, Ncc) under typical ('normal';  $800 \mu\text{mol l}^{-1}$ ) and 'low' ( $10 \mu\text{mol l}^{-1}$ )  $\text{Na}^+$  conditions using a combination of clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9), morpholino KD and water chemistry manipulation. In particular, we hypothesized that CRISPR/Cas9 knockout (KO) of *slc9a3.2* (encoding Nhe3b) would result in reduced  $\text{Na}^+$  uptake rates specifically under low  $\text{Na}^+$  conditions. Alternatively, compensatory regulation might occur in *nhe3b* KO mutants, such that  $\text{Na}^+$  uptake would be maintained by the other two  $\text{Na}^+$  uptake pathways ( $\text{H}^+$ -ATPase-mediated and/or Ncc pathways), as previously described in response to morpholino KD of Nhe3b, Ncc (Wang et al., 2009; Chang et al., 2013) and the cation channel *Asic4b* (Zimmer et al., 2018) that is thought to be involved in  $\text{H}^+$ -ATPase-mediated  $\text{Na}^+$  uptake in fishes (Dymowska et al., 2014, 2015). Therefore, we predicted that if compensatory upregulation of  $\text{H}^+$ -ATPase and/or Ncc were to occur,  $\text{H}^+$ -ATPase KD and/or rearing larvae under chloride-free conditions (to limit  $\text{Na}^+$ - $\text{Cl}^-$ -cotransport) would result in a reduction in  $\text{Na}^+$  uptake in *nhe3b* KO larvae. The effect of CRISPR/Cas9 KO of *rhcgbl* on  $\text{Na}^+$  uptake was also assessed and we predicted that if compensatory regulation of  $\text{Na}^+$  uptake occurred in *nhe3b* KO larvae, the same compensatory mechanisms would be upregulated in *rhcgbl* KO larvae as this protein is presumed to be a requisite for Nhe3b-mediated  $\text{Na}^+$  uptake in zebrafish. Overall, we found that  $\text{Na}^+$  uptake was not reduced in *nhe3b* or *rhcgbl* KO larvae in normal or low  $\text{Na}^+$  conditions and that the maintenance of  $\text{Na}^+$  uptake in *nhe3b* KO larvae did not appear to be a function of compensatory regulation by the  $\text{H}^+$ -ATPase-mediated or Ncc pathways.

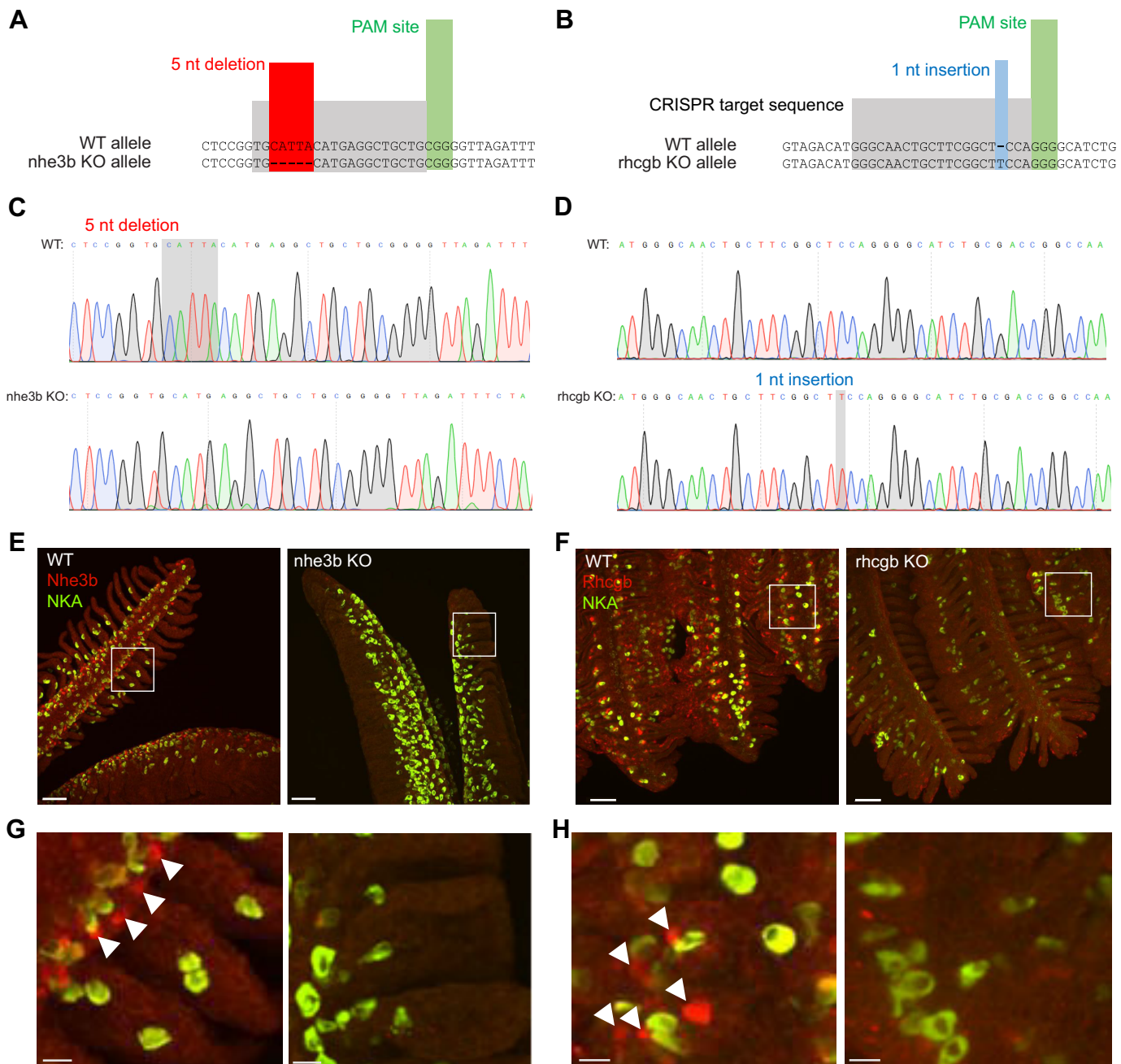
## MATERIALS AND METHODS

### Zebrafish

Mutant zebrafish (*nhe3b* KO and *rhcgbl* KO) were generated using CRISPR/Cas9 gene editing (see below) and were treated and handled in the same way as wild-type fish. Adult zebrafish, *Danio rerio* (F. Hamilton 1822), originally purchased from the pet trade, were bred and raised in the University of Ottawa animal holding facilities. Fish were kept in plastic aquaria receiving re-circulating dechloraminated City of Ottawa tap water (in  $\text{mmol l}^{-1}$ :  $0.8 \text{ Na}^+$ ,  $0.4 \text{ Cl}^-$ ,  $0.25 \text{ Ca}^{2+}$ ; pH 7.6) and were maintained at  $28^\circ\text{C}$  with a 14 h:10 h light:dark photoperiod. Fish were bred by placing two female and one male zebrafish overnight in a plastic container with a perforated bottom insert. The following morning, embryos were collected and were reared in Petri dishes containing 50 ml of reconstituted water that differed in composition depending on the experiment (see below). Embryos were reared at a density of 40 individuals per 50 ml in an incubator set to  $28^\circ\text{C}$ ; water was replaced daily. All fish handling and experimental procedures were in accordance with the guidelines of the Canadian Council of Animal Care and were approved by the University of Ottawa Animal Care Committee (protocol BL-1700).

### Generation of *nhe3b* KO and *rhcgbl* KO zebrafish

CRISPR/Cas9 gene editing was used to knock out the expression of *slc9a3.2* (*nhe3b*; NCBI accession number: NM\_00113479.1) and *rhcgbl* (formerly *rhcg1*; NCBI accession number: NM\_001320382.1). CRISPR/Cas9 single guide RNAs (sgRNAs) were designed using CHOPCHOP (Montague et al., 2014; Labun et al., 2016) and were specific to exon 1 of *slc9a3.2* (TGCATTACATGAGGCTGCTG), upstream of a protospacer-adjacent motif (PAM) (CGG), and to exon 1 of *rhcgbl* (GGGCAACTGCTTCGGCTCCA), upstream of a PAM



**Fig. 1. CRISPR single guide RNA (sgRNA) targets, chromatogram sequences and whole-mount immunohistochemical knockout confirmation for *nhe3b* and *rhcgb* knockout (KO) mutants.** (A,B) Schematic diagram showing CRISPR sgRNA target sequences (grey shaded areas), indel mutation sites (red or blue shaded areas) and protospacer-adjacent motif (PAM) recognition sites (green shaded areas) for *nhe3b* KO (A) and *rhcgb* KO (B) mutants. WT, wild-type. (C,D) Chromatogram confirmation of indel mutations in *nhe3b* KO (C) and *rhcgb* KO (D) mutants. (E–H) Whole-mount immunohistochemical staining of adult gills. Gills of *nhe3b* KO mutants (E,G) were incubated with anti-Nhe3b (red) and anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA; green) antibodies. Gills of *rhcgb* KO mutants (F,H) were incubated with anti-Rhcgb (red) and anti-NKA (green) antibodies. G and H are higher magnification views of the boxed regions in E and F, respectively. Scale bars in E and F: 50  $\mu$ m; scale bars in G and H: 10  $\mu$ m

(GGG) (Fig. 1). sgRNAs were synthesized using a cloning-free method described previously (Talbot and Amacher, 2014) but with slight modifications. First, a set of primers was employed to amplify DNA constructs (Table S1) that acted as templates for *in vitro* sgRNA transcription. The constructs were amplified in 50  $\mu$ l PCR reactions consisting of 250 nmol l<sup>-1</sup> *slc9a3.2* or *rhcgb* template oligo, 250 nmol l<sup>-1</sup> universal template oligo 2, 20 nmol l<sup>-1</sup> forward amplification oligo, 20 nmol l<sup>-1</sup> reverse amplification oligo (Table S1), 200  $\mu$ mol l<sup>-1</sup> dNTPs, 5  $\mu$ l 10 $\times$  ThermoPol buffer

[New England Biolabs (NEB), Ipswich, MA, USA] and 0.25  $\mu$ l Taq (NEB). Reactions started with initial denaturation at 95°C for 30 s followed by 35 cycles of 95°C for 30 s (denaturation), 55°C for 1 min (annealing) and 72°C for 45 s (extension), and a final extension at 72°C for 10 min. The PCR products were column-purified (Qiagen, Hilden, Germany) and the size and quality of the resulting PCR constructs were verified by gel electrophoresis. DNA concentration was determined spectrophotometrically (NanoDrop 2000, ThermoFisher, Waltham, MA, USA). The sgRNAs were

transcribed from PCR constructs using a HiScribe T7 high yield RNA synthesis kit (NEB) with 200 ng DNA template, following the manufacturer's protocol. sgRNA size and quality were verified by gel electrophoresis, and RNA concentration was determined spectrophotometrically following purification (RNeasy mini kit, Qiagen).

Zebrafish codon-optimized Cas9 (pCS2-nls-zCas9-nls) was obtained from Jao et al. (2013) and was used as a template to synthesize Cas9 mRNA. The pCS2-nls-zCas9-nls plasmid was linearized by NotI digestion and purified using a QIAquick purification kit (Qiagen). Capped nls-zCas9-nls RNA was synthesized using the mMESSAGE mMACHINE SP6 kit (Invitrogen, Carlsbad, CA, USA). Following purification (RNeasy Mini kit, Qiagen), the size and quality of the Cas9 mRNA was checked by gel electrophoresis and Cas9 mRNA concentration was determined spectrophotometrically.

Wild-type zebrafish embryos at the 1-cell stage were injected with 1 nl of injection solution containing 150 pg Cas9 mRNA, 50 pg of *slc9a3.2* or *rhcgb* sgRNA, and 0.01% Phenol Red (for visualization) suspended in Danieau buffer [in mmol l<sup>-1</sup>: 58 NaCl, 0.7 KCl, 0.4 MgSO<sub>4</sub>, 0.6 Ca(NO<sub>3</sub>)<sub>2</sub> and 5.0 Hepes; pH 7.6]. At 1 dpf, a subset of embryos was genotyped to determine whether the injections resulted in the generation of mutant embryos. Embryos were killed with an overdose of Tris-neutralized tricaine methanesulfonate (MS-222) and DNA was extracted by digesting embryos individually in 20 µl of 50 mmol l<sup>-1</sup> NaOH at 95°C for 10 min and neutralizing with 2 µl of 1 mol l<sup>-1</sup> Tris HCl (pH 8). PCR amplification of the CRISPR target regions of *slc9a3.2* and *rhcgb* was performed in 25 µl reactions containing 200 nmol l<sup>-1</sup> forward and reverse sequencing primers (Table S1), 200 µmol l<sup>-1</sup> dNTPs, 2.5 µl 10× DreamTaq buffer (ThermoFisher), 0.125 µl DreamTaq Hot Start DNA polymerase (ThermoFisher) and 5 µl of DNA digest, with a temperature cycle of 94°C for 3 min, and 35 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 45 s, and a final 72°C step for 10 min. Amplified PCR products were sequenced by Sanger Sequencing (Genome Quebec, McGill University, Montreal, QC, Canada), which confirmed that a portion (~80%) of the embryos had a mutated *slc9a3.2* or *rhcgb* gene. The remaining embryos were reared to sexual maturity (60–90 dpf) and mutants in this adult F0 population were identified by clipping a small portion of the caudal fin, and genotyping the fin clip following the same protocol described above for embryos. Identified mutants were outcrossed with wild-type fish to establish an F1 heterozygous line. F1 fish were reared to sexual maturity, genotyped, and heterozygous mutants carrying a 5 bp deletion mutation (*nhe3b* KO mutants) or 1 bp insertion mutation (*rhcgb* KO mutants) were used as founders for the KO lines. F1 females and males were intercrossed and F2 homozygous *slc9a3.2* mutants (*nhe3b* KO) and *rhcgb* mutants (*rhcgb* KO) were identified by fin clipping and genotyping of adult individuals. A breeding population of approximately 20–30 adult F2 *nhe3b* KO or *rhcgb* KO mutants was established and these fish were intercrossed to provide F3 mutants on which all experiments were performed. Note that larvae used in this study were obtained from multiple breeding events using several different wild-type or F2 mutant parents. Null mutations in *slc9a3.2* and *rhcgb* were confirmed via Sanger sequencing and whole-mount immunohistochemistry.

### Morpholino KD

Antisense morpholino oligonucleotides (Gene-Tools, LLC, Philomath, OR, USA) were used to knock down the expression of *Slc9a3.2* (*Nhe3b*) or *Atp6v1aa* (H<sup>+</sup>-ATPase) using techniques

described in previous studies (Shih et al., 2012; Chang et al., 2013). Briefly, 1-cell stage embryos were injected with 2 or 4 ng of a sham (standard control) morpholino (5'-CCTCTTACCTCAGTTACAA-TTTATA-3') that has no biological target in zebrafish, 2 ng of a translation-blocking morpholino that targeted the translation start site of *slc9a3.2* (5'-CAGTTGCCCATGTCTACAGCTTGAG-3') and has been validated in a previous study (Chang et al., 2013), or 4 ng of a translation-blocking morpholino that targets the translation start site of *atp6v1aa* (5'-ATCCATCTTGTGTGTAGAAACTG-3'; Horng et al., 2007). *Atp6v1aa* (H<sup>+</sup>-ATPase) KD was confirmed using whole-mount immunohistochemistry. Morpholinos were suspended in Danieau buffer with 0.01% Phenol Red and heated to 65°C for 10 min prior to injection at a volume of 1 nl per embryo.

### Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was used to confirm KO of *Nhe3b* and *Rhcgb* in adult gills and KD of H<sup>+</sup>-ATPase in 4 dpf larvae. Adult wild-type, *nhe3b* KO and *rhcgb* KO zebrafish were killed with an overdose of neutralized MS-222 and gills were removed and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C. Following fixation, individual gill filaments were removed from the arch to obtain more even and consistent antibody accessibility. Sham and *Atp6v1aa* morphant larvae were killed with neutralized MS-222 overdose and were fixed overnight in 4% PFA in PBS. In both gill filaments and larvae, antigen retrieval was performed by incubating the samples in 150 mmol l<sup>-1</sup> Tris HCl (pH 9) for 10 min at room temperature, followed by 15 min at 65°C. Samples were rinsed in PBS containing 0.1% Tween-20 (PBST) 5 times and blocked and permeabilized in PBST containing 3% BSA and 0.8% Triton X-100 for 2 h at room temperature. Gill filaments from wild-type and *nhe3b* KO fish were probed overnight at 4°C with a custom rabbit polyclonal anti-*Nhe3b* antibody raised against a synthetic peptide corresponding to the C-terminal region of *Nhe3b* (CEPAADEETPEEKPA; 1:100 dilution; GenScript, Piscataway, NJ, USA) and a commercial mouse monoclonal anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase antibody (a5; Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA, USA; 1:250 dilution) diluted in PBST containing 3% BSA and 0.8% Triton X-100. Filaments from wild-type and *rhcgb* KO fish were probed overnight at 4°C with a rabbit polyclonal anti-*Rhcgb* antibody raised against amino acid residues 425–488 of *Rhcgb* (1:100 dilution; Nakada et al., 2007) and the anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase antibody (1:250 dilution). Larvae were probed overnight at 4°C with a rabbit polyclonal anti-H<sup>+</sup>-ATPase antibody (AEMPADSGYPAYLGAR; 1:2000 dilution; courtesy of Dr M. Uchiyama, University of Toyama, Toyama, Japan). Samples were subsequently rinsed 5 times in PBST and incubated with secondary antibodies (1:500 dilution; Alexa 568-conjugated anti-rabbit IgG and/or Alexa 488-conjugated anti-mouse IgG; Invitrogen). Gill filaments and larvae were finally washed 5 times in PBST and mounted in depression slides in SlowFade Gold antifade mounting medium (ThermoFisher). Images were acquired using a scanning confocal laser microscope (A1R+, Nikon Instruments, Melville, NY, USA).

### Manipulation of water ion content

Wild-type, mutant and/or morphant larvae were reared in water containing 500 µmol l<sup>-1</sup> Cl<sup>-</sup>, 250 µmol l<sup>-1</sup> Ca<sup>2+</sup>, 40 µmol l<sup>-1</sup> K<sup>+</sup> and either 800 µmol l<sup>-1</sup> Na<sup>+</sup> ('normal' Na<sup>+</sup>) or 10 µmol l<sup>-1</sup> Na<sup>+</sup> ('low' Na<sup>+</sup>) (pH 7.6) that was prepared by adding CaCl<sub>2</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O, KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub> and Na<sub>2</sub>SO<sub>4</sub> salts to ultrapure water (Table 1). 'Chloride-free' water was achieved by replacing CaCl<sub>2</sub> with CaSO<sub>4</sub> (Table 1). Water changes were performed daily

**Table 1. Nominal ion concentrations in reconstituted water used in experimental treatments**

Experimental treatment	[Na <sup>+</sup> ] (μmol l <sup>-1</sup> )	[Cl <sup>-</sup> ] (μmol l <sup>-1</sup> )	[Ca <sup>2+</sup> ] (μmol l <sup>-1</sup> )	[SO <sub>4</sub> <sup>2-</sup> ] (μmol l <sup>-1</sup> )	[K <sup>+</sup> ] (μmol l <sup>-1</sup> )	[Mg <sup>2+</sup> ] (μmol l <sup>-1</sup> )
Normal Na <sup>+</sup> /control Cl <sup>-</sup>	800	500	250	550	40	150
Low Na <sup>+</sup> /control Cl <sup>-</sup>	10	500	250	155	40	150
Normal Na <sup>+</sup> /Cl <sup>-</sup> -free	800	0	250	800	40	150
Low Na <sup>+</sup> /Cl <sup>-</sup> -free	10	0	250	405	40	150

and pH was adjusted daily as needed by the addition of 0.1 mol l<sup>-1</sup> KOH or H<sub>2</sub>SO<sub>4</sub>.

### Real-time PCR

Real-time PCR was used to assess the expression of genes involved in Na<sup>+</sup> uptake in 4 dpf wild-type, *nhe3b* KO and *rhcg* KO larvae. Larvae were killed via an overdose of neutralized MS-222 and 20 larvae were homogenized via sonication in 0.5 ml Trizol (ThermoFisher). RNA was extracted from these homogenates following the manufacturer's protocol. RNA concentration was determined via spectrophotometry and RNA quality was verified via gel electrophoresis. RNA was treated with DNase (ThermoFisher) prior to cDNA synthesis from 1 μg total RNA using a commercial kit (iScript, Bio-Rad, Hercules, CA, USA). Real-time PCR was performed in 10 μl reactions containing 5 μl SsoFast EvaGreen supermix (Bio-Rad), 500 nmol l<sup>-1</sup> forward and reverse primers (Table S2) and 4 μl cDNA template. No-template controls were run in every assay and non-reverse transcribed samples were run for every primer pair. Reaction efficiency for all primer pairs was between 90% and 110%.

### Measurement of Na<sup>+</sup> and Cl<sup>-</sup> uptake

Na<sup>+</sup> uptake was measured in 4 dpf wild-type, mutant or morphant larvae reared under various water chemistry conditions; measurements were made under the same conditions under which the larvae were reared. Experiments on mutant or morphant larvae were always run in parallel with wild-type or sham-treated fish. Larvae were transferred to 2 ml centrifuge tubes (12 larvae per tube for 800 μmol l<sup>-1</sup> Na<sup>+</sup> treatments; 2 larvae per tube for 10 μmol l<sup>-1</sup> Na<sup>+</sup> treatments) containing 1.6 ml of the respective acclimation water, incubated in a 28°C water bath. For larvae reared under 800 μmol l<sup>-1</sup> Na<sup>+</sup>, a density of 12 larvae per tube was used in order to minimize radioisotope cost and waste. Larvae were allowed to settle for 30 min, after which <sup>22</sup>Na was added to each tube [0.4 μCi for 800 μmol l<sup>-1</sup> Na<sup>+</sup>, mean specific activity 0.34±0.04 cpm pmol<sup>-1</sup> (*n*=12); 0.05 μCi for 10 μmol l<sup>-1</sup> Na<sup>+</sup>, mean specific activity 3.41±0.42 cpm pmol<sup>-1</sup> (*n*=12)], marking the beginning of the 3 h Na<sup>+</sup> flux period. Initial and final 0.7 ml water samples were collected for gamma radioactivity and [Na<sup>+</sup>] analyses and at the end of the flux period, larvae were killed, rinsed 4 times in 10 mmol l<sup>-1</sup> NaCl to displace loosely bound <sup>22</sup>Na<sup>+</sup>, and then collected as separate samples (2 larvae per sample; *n*=1). Gamma radioactivity of larvae and water samples was determined by gamma counting (2470 Wizard2, Perkin Elmer) and [Na<sup>+</sup>] of water samples was determined by flame atomic absorption spectrophotometry (Spectra AA 220FS, Varian, Palo Alto, CA, USA).

Cl<sup>-</sup> uptake was measured using the same experimental approach described for Na<sup>+</sup> uptake except that <sup>36</sup>Cl<sup>-</sup> was added to each tube (1 ml per tube; 12 fish per tube for all water chemistry conditions; 0.4 μCi per tube). Samples (0.1 ml) were collected at the beginning and end of a 3 h flux period for scintillation counting and larvae were killed and rinsed 4 times in 10 mmol l<sup>-1</sup> NaCl. Larvae were collected in pairs (*n*=1) and digested overnight at 65°C in 50 μl 2 mol l<sup>-1</sup> HNO<sub>3</sub>. Digests and water samples were added to 10 ml

EcoLume scintillation cocktail (MP Biomedicals, Solon, OH, USA) and incubated for 3 h in the dark to reduce chemiluminescence prior to scintillation counting (LS-6500 scintillation counter, Beckman Coulter, Brea, CA, USA). [Cl<sup>-</sup>] of water used in these experiments was measured using a colorimetric assay (Zall et al., 1956). Na<sup>+</sup> and Cl<sup>-</sup> uptake rates (pmol larva<sup>-1</sup> h<sup>-1</sup>) were calculated using the following equation:

$$\text{Ion uptake rate} = R_{\text{larvae}}/SA/t/n, \quad (1)$$

where  $R_{\text{larvae}}$  is the total radioactivity (cpm) of the pooled larvae in a sample,  $SA$  is the average specific activity of the water (cpm pmol<sup>-1</sup>),  $t$  is flux duration (h) and  $n$  is the number of larvae.

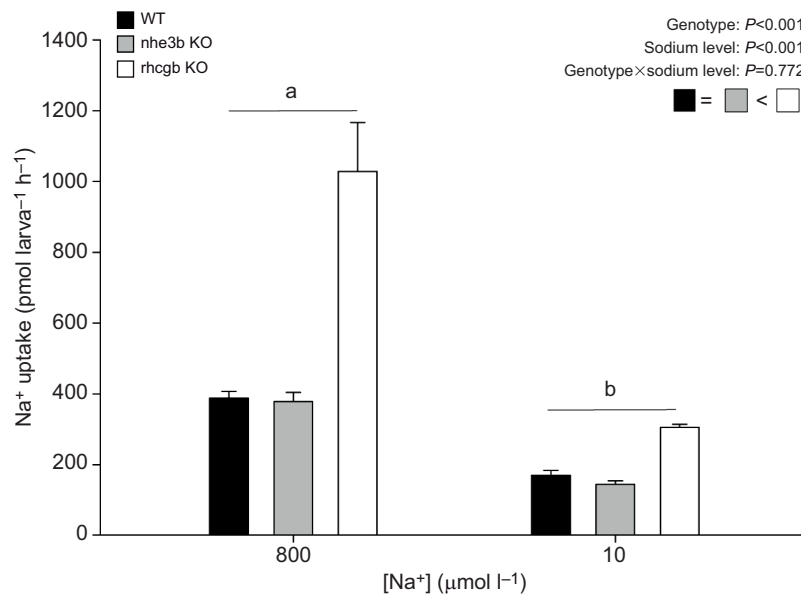
### SIET

SIET was used to measure H<sup>+</sup> flux at HR cells on the yolk sac epithelium of wild-type and *nhe3b* KO larvae treated with sham or H<sup>+</sup>-ATPase morpholino. To visualize HR cells under a fluorescence microscope, live larvae were stained with 0.05 mg ml<sup>-1</sup> concanavalin A (conA) conjugated to Alexa 488 (Invitrogen) for 30 min at room temperature. SIET was performed based on methods described previously (Donini and O'Donnell, 2005; Lin et al., 2006). H<sup>+</sup>-selective probes were constructed by pulling borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) to a tip diameter of ~5 μm with a micropipette puller (P-2000, Sutter Instrument, Novato, CA, USA) and silanizing the glass pipettes with *N,N*-dimethyltrimethylsilylamine (Sigma-Aldrich, St Louis, MO, USA) on a hot plate covered by a glass Petri dish for 1 h. Pipettes were then back-filled with a solution of 100 mmol l<sup>-1</sup> NaCl and 100 mmol l<sup>-1</sup> sodium citrate (pH 6) and front-filled with a ~100 μm column of H<sup>+</sup> ionophore I cocktail B (Sigma-Aldrich). H<sup>+</sup>-selective probes were then calibrated in 'normal' Na<sup>+</sup> water containing 1 mmol l<sup>-1</sup> Hepes buffer and 0.5 mg l<sup>-1</sup> MS-222 (needed to immobilize larvae during measurements) adjusted to pH 6, 7 or 8. The average Nernstian slope obtained from this calibration was 54.1±0.8 mV (*n*=4).

Larvae were secured in place in modified Petri dishes described previously (Hughes et al., 2019) containing 'normal' Na<sup>+</sup> water with 1 mmol l<sup>-1</sup> Hepes buffer and 0.5 mg l<sup>-1</sup> MS-222 (pH 7.6). The Petri dish was positioned under a fluorescence dissecting microscope (SMZ 1500, Nikon) and HR cells were identified by Alexa 488-conjugated conA staining. The H<sup>+</sup>-selective probe was positioned ~5 μm above the surface of HR cells on the lateral side of the yolk sac epithelium and 5 replicate scans with an excursion distance of 20 μm in the *z* direction, a wait time of 5 s and measure time of 1 s were performed at each HR cell; 3–5 HR cells were scanned for each fish (*n*=1). H<sup>+</sup> flux (pmol cm<sup>-2</sup> s<sup>-1</sup>) was calculated using the following equations (Donini and O'Donnell, 2005):

$$\Delta C = C_B \times 10^{(\Delta V/S)} - C_B, \quad (2)$$

where  $\Delta C$  is the concentration gradient (pmol cm<sup>-3</sup>) between the origin and excursion point,  $C_B$  is the average ion concentration (pmol cm<sup>-3</sup>) calculated from voltage measured at the origin and excursion points,  $\Delta V$  is the voltage gradient (μV) and  $S$  is the slope



**Fig. 2. Na<sup>+</sup> uptake in wild-type, *nhe3b* KO and *rhcgb* KO larvae.** Na<sup>+</sup> uptake rate in 4 dpf wild-type, *nhe3b* KO and *rhcgb* KO larvae reared and assayed in 800 μmol l<sup>-1</sup> (normal) or 10 μmol l<sup>-1</sup> (low) Na<sup>+</sup>. Data are means±s.e.m. Different letters indicate a statistically significant effect of sodium level within a genotype and boxes in the upper right corner depict the overall statistical effect of genotype as determined by a two-way ANOVA followed by a Holm–Šidák *post hoc* test on rank-transformed data. *n*=16 for wild-type; *n*=8 for *nhe3b* KO and *rhcgb* KO.

of the electrode (μV). This concentration gradient was then converted into a flux using Fick's law:

$$H^+ \text{ flux} = D \times \Delta C / \Delta x, \quad (3)$$

where *D* is the diffusion coefficient of H<sup>+</sup> (2.09×10<sup>-5</sup> cm<sup>2</sup> s<sup>-1</sup>), Δ*C* is concentration gradient (pmol cm<sup>-3</sup>) and Δ*x* is the excursion distance (cm).

### Statistical analyses

All data are presented as means±s.e.m. and statistical significance was accepted at the *P*<0.05 level. Two-factor comparisons among multiple means were conducted using a two-way ANOVA followed by a Holm–Šidák *post hoc* test. In cases where normality or equal variance was not achieved, data were normalized by square or log transformations. Specific details on the statistical analyses and transformations used for each dataset are included in the corresponding figure captions. All statistical analyses were conducted with SigmaStat version 3.5 (Systat Software Inc., San Jose, CA, USA).

## RESULTS

### Na<sup>+</sup> uptake in *nhe3b* KO and *rhcgb* KO larvae and the effects of *Nhe3b* KD

CRISPR/Cas9 sgRNAs were targeted to exon 1 of *slc9a3.2* and *rhcgb* and resulted in 5 bp deletion and 1 bp insertion mutations, respectively (Fig. 1A,B), confirmed by Sanger sequencing (Fig. 1C, D). Both indel mutations were nonsense mutations, resulting in predicted premature stop codons and truncated proteins (Table S3). KO was confirmed at the protein level by whole-mount immunohistochemistry on gill filaments from adult fish. In wild-type filaments, staining with anti-Nhe3b (Fig. 1E,G) and anti-Rhcgb (Fig. 1F,H) antibodies confirmed cell-specific staining that was not co-localized with anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) staining using the α5 antibody (see Materials and Methods), a marker for Ca<sup>2+</sup>-transporting NKA-rich (NaR) cells (Liao et al., 2007). In the gills of *nhe3b* KO and *rhcgb* KO adults, staining of anti-Nhe3b and anti-Rhcgb antibodies, respectively, was absent, though anti-NKA staining was still present (Fig. 1E,F). Using the same anti-Nhe3b antibody that was able to detect Nhe3b expression in the gills of WT adults (Fig. 1E), we were unsuccessful in visualizing Nhe3b

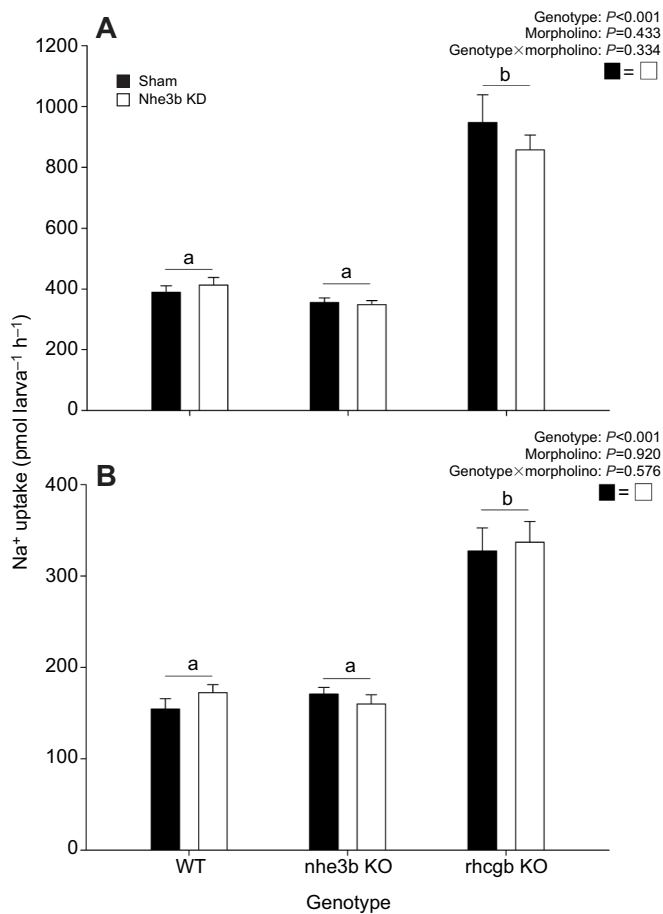
staining in larvae or a band of the appropriate size in western blots; numerous western blots using an array of conditions repeatedly yielded a single non-specific band of approximately 45 kDa that was retained in the *nhe3b* KOs (data not shown).

An overall effect of Na<sup>+</sup> level on Na<sup>+</sup> uptake by 4 dpf larvae of all three genotypes was observed whereby Na<sup>+</sup> uptake was lower in larvae that were reared and assayed in 10 μmol l<sup>-1</sup> Na<sup>+</sup> relative to the 800 μmol l<sup>-1</sup> Na<sup>+</sup> treatment (Fig. 2). Na<sup>+</sup> uptake between wild-type and *nhe3b* KO 4 dpf larvae was not significantly different; however, Na<sup>+</sup> uptake in *rhcgb* KO larvae was significantly greater than in both wild-type and *nhe3b* KO larvae, as indicated by an overall effect of genotype across Na<sup>+</sup> levels (Fig. 2).

Similar to the effect of *nhe3b* KO, *Nhe3b* KD had no effect on Na<sup>+</sup> uptake in wild-type larvae at either Na<sup>+</sup> level (Fig. 3). Importantly, as a control, the *Nhe3b* morpholino was injected into *nhe3b* KO larvae (Fig. 3) in order to ensure that any effect of the morpholino was not recapitulated in the mutant line lacking *Nhe3b* protein. We found this to be an essential control in the use of a different *Nhe3b* morpholino (Fig. S1; see Discussion). The elevation in Na<sup>+</sup> uptake observed in *rhcgb* KO larvae (Fig. 2) was not affected by *Nhe3b* KD (Fig. 3). Overall, *Nhe3b* loss of function via CRISPR/Cas9 KO or morpholino KD did not result in alterations in Na<sup>+</sup> uptake, nor did it alter the pattern of response in the *rhcgb* KO larvae.

### Expression of genes involved in Na<sup>+</sup> uptake

In order to determine whether compensatory regulation occurred in *nhe3b* KO and *rhcgb* KO larvae, whole-body gene expression of genes involved in Na<sup>+</sup> uptake was measured. Expression of *slc9a3.2* (encoding *Nhe3b*) was significantly greater in 4 dpf larvae reared in 10 μmol l<sup>-1</sup> Na<sup>+</sup>, relative to those reared in 800 μmol l<sup>-1</sup> Na<sup>+</sup>, across all genotypes; there were no significant differences in *slc9a3.2* gene expression among the genotypes (Fig. 4A). The expression of *atp6v1aa* (encoding H<sup>+</sup>-ATPase) was not significantly influenced by genotype or Na<sup>+</sup> level (Fig. 4B). Larvae experiencing *rhcgb* KO displayed significantly lower *asic4b* expression compared with wild-type and *nhe3b* KO larvae, which were not significantly different from one another; *asic4b* expression was not affected by Na<sup>+</sup> level (Fig. 4C). Expression of *slc12a10.2* (encoding *Ncc*) was not significantly different across genotypes or Na<sup>+</sup> levels (Fig. 4D).



**Fig. 3.  $\text{Na}^+$  uptake in response to Nhe3b knockdown (KD) in wild-type, nhe3b KO and rhcgb KO larvae.**  $\text{Na}^+$  uptake rate in 4 dpf wild-type, nhe3b KO and rhcgb KO larvae treated with sham or Nhe3b (Nhe3b KD) morpholino and reared and assayed in  $800 \mu\text{mol l}^{-1}$  (A) or  $10 \mu\text{mol l}^{-1}$  (B)  $\text{Na}^+$ . Data are means  $\pm$  s.e.m. Different letters indicate a statistically significant effect of genotype within a morpholino treatment and boxes in the upper right corner depict the overall statistical effect of morpholino treatment as determined by a two-way ANOVA followed by a Holm–Šidák *post hoc* test.  $n=14$ – $20$ .

### Effects of *nhe3b* KO on $\text{H}^+$ secretion and $\text{Cl}^-$ uptake

Compensatory regulation of  $\text{Na}^+$  uptake by  $\text{H}^+$ -ATPase and Ncc was also assessed at the physiological level by measuring  $\text{H}^+$  flux and  $\text{Cl}^-$  uptake.  $\text{H}^+$  flux at the surface of HR cells (Fig. 5A) of larvae reared and assayed in  $800 \mu\text{mol l}^{-1}$   $\text{Na}^+$  was not significantly different between wild-type and nhe3b KO larvae (Fig. 5B); however, there was an overall inhibitory effect of  $\text{H}^+$ -ATPase KD on  $\text{H}^+$  flux by HR cells (Fig. 5B); successful KD was confirmed by immunohistochemistry (Fig. 5C,D).

$\text{Cl}^-$  uptake was significantly increased in the nhe3b KO larvae in comparison to that in wild-type larvae;  $\text{Na}^+$  level had no effect on  $\text{Cl}^-$  uptake (Fig. 6).

### $\text{Na}^+$ uptake in response to $\text{H}^+$ -ATPase KD and chloride-free conditions

To further probe the mechanisms promoting compensatory regulation of  $\text{Na}^+$  uptake in nhe3b KO larvae, larvae were subjected to  $\text{H}^+$ -ATPase KD and chloride-free rearing conditions. Interestingly, while in prior experiments there were no differences in  $\text{Na}^+$  uptake rate between wild-type and nhe3b KO larvae (Figs 1 and 2), the following experiments revealed a stimulatory effect of nhe3b KO on  $\text{Na}^+$  uptake, which was present only in some instances and

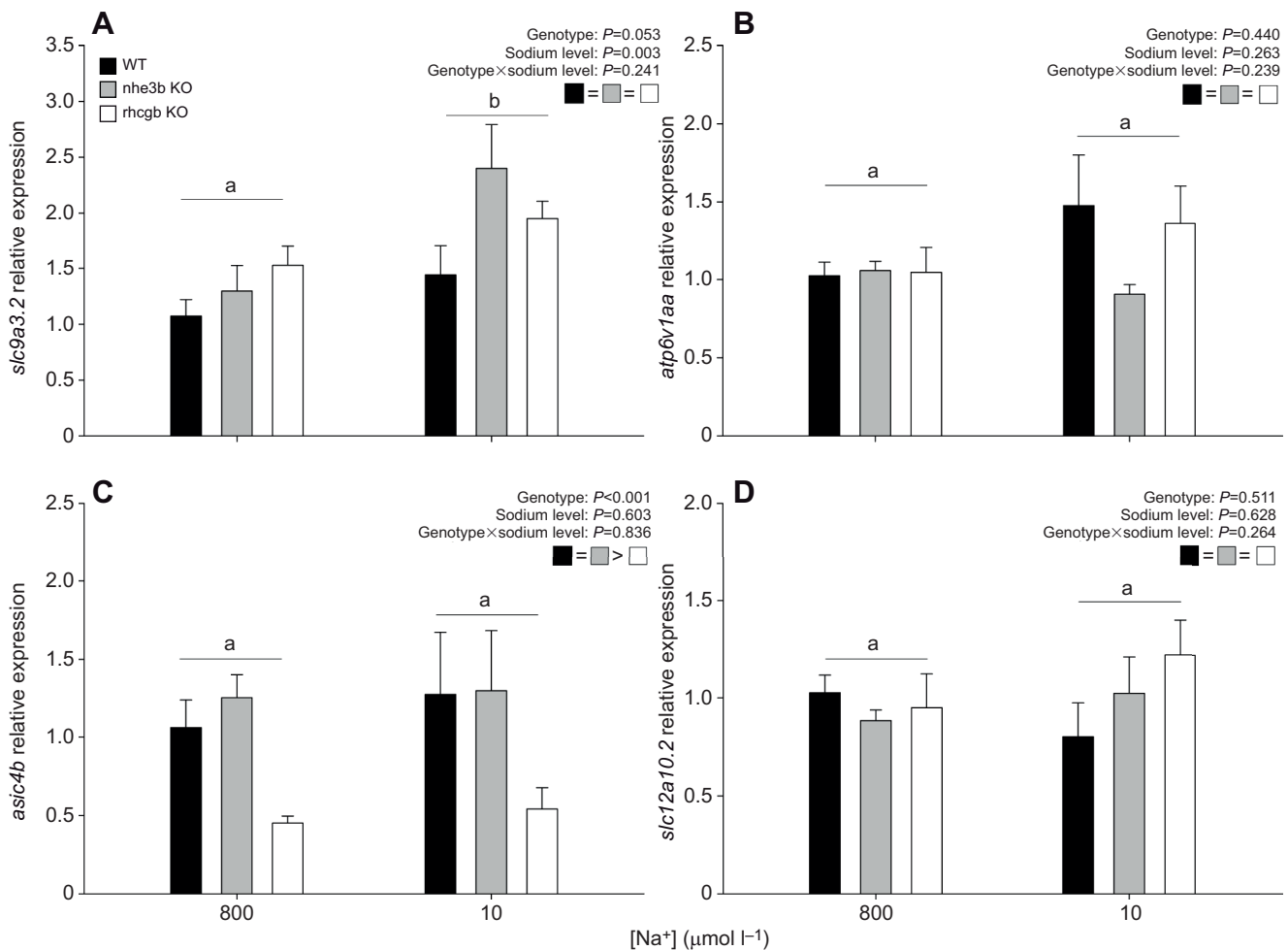
did not appear to depend on  $\text{Na}^+$  level (Figs 7, 8A and 9B). In  $800 \mu\text{mol l}^{-1}$   $\text{Na}^+$ ,  $\text{H}^+$ -ATPase KD resulted in a stimulation of  $\text{Na}^+$  uptake in 4 dpf wild-type larvae, while this effect was absent in nhe3b KO larvae (Fig. 7A). This stimulatory effect of  $\text{H}^+$ -ATPase KD also was absent in  $10 \mu\text{mol l}^{-1}$   $\text{Na}^+$  (Fig. 7B). Rearing and assaying larvae in chloride-free conditions had no effect on  $\text{Na}^+$  uptake regardless of genotype or  $\text{Na}^+$  level (Fig. 8). Most surprisingly,  $\text{Na}^+$  uptake in nhe3b KO larvae experiencing  $\text{H}^+$ -ATPase KD that were reared (and assayed) in chloride-free conditions was not reduced relative to  $\text{Na}^+$  uptake by wild-type larvae treated with sham morpholino and reared under control chloride conditions at either  $\text{Na}^+$  level (Fig. 9). In fact, under low  $\text{Na}^+$  conditions,  $\text{Na}^+$  uptake was highest in this group experiencing a functional ablation of all three putative  $\text{Na}^+$  uptake pathways (nhe3b KO,  $\text{H}^+$ -ATPase KD, chloride-free conditions).

### DISCUSSION

In the present study, we aimed to further elucidate the mechanisms of  $\text{Na}^+$  uptake utilized by larval zebrafish, particularly under low  $\text{Na}^+$  conditions. Using CRISPR/Cas9 gene editing, we tested the hypothesis that loss of Nhe3b or Rhcgb, an ammonia-transporting protein thought to be integral to the thermodynamic operation of Nhe3b in FW (Wright and Wood, 2009), would result in a reduced capacity to absorb  $\text{Na}^+$  from a dilute environment. Contrary to our predictions, nhe3b and rhcgb KO larvae maintained  $\text{Na}^+$  uptake at the same or greater rate as wild-type larvae, even under low  $\text{Na}^+$  conditions (Fig. 2). This led us to explore the possibility of compensatory regulation by alternative  $\text{Na}^+$  uptake pathways in nhe3b KO larvae, previously shown to occur after morpholino KD of Nhe3b, Ncc (Chang et al., 2013) and Asic4b (Zimmer et al., 2018). Such compensatory regulation is believed to be a fairly widespread consequence of gene KO in zebrafish (El-Brolosy and Stainier, 2017). Through several experiments, we concluded that neither the  $\text{H}^+$ -ATPase-mediated nor Ncc pathways of  $\text{Na}^+$  uptake compensated for the loss of Nhe3b, and that  $\text{Na}^+$  uptake persisted when all three putative pathways for  $\text{Na}^+$  uptake were targeted simultaneously.

### The role of Nhe3b in $\text{Na}^+$ uptake by zebrafish

In zebrafish and other fishes, Nhe3b and Rhcgb are believed to associate as part of a functional metabolon that alleviates the presumed thermodynamic constraints on electroneutral  $\text{Na}^+/\text{H}^+$  exchange in FW (Wright and Wood, 2009; Wu et al., 2010; Dymowska et al., 2012; Ito et al., 2013; Guh et al., 2015). Surprisingly, in zebrafish, the contribution of Nhe3b to  $\text{Na}^+$  uptake increased in response to acclimation to low  $\text{Na}^+$  (Lin et al., 2016; Shih et al., 2012; Yan et al., 2007; Fig. 4A), a condition which would theoretically exacerbate the thermodynamic limitations of Nhe3b function. However, the expression of *rhcgb* also appears to be increased in response to low  $\text{Na}^+$  acclimation (Nakada et al., 2007; Shih et al., 2012), further indicating the potential importance of Rhcgb in maintaining  $\text{Na}^+$  uptake via Nhe3b. Based on these findings, we hypothesized that  $\text{Na}^+$  uptake would be limited, particularly under low  $\text{Na}^+$  conditions, in nhe3b and rhcgb KO larvae. However, neither KO resulted in a reduction in  $\text{Na}^+$  uptake under normal ( $800 \mu\text{mol l}^{-1}$ ) or low ( $10 \mu\text{mol l}^{-1}$ )  $\text{Na}^+$  (Fig. 2). Notably, the mean specific activity (SA; see Materials and Methods) in our study was an order of magnitude lower in the  $800 \mu\text{mol l}^{-1}$   $\text{Na}^+$  experiments than in the  $10 \mu\text{mol l}^{-1}$   $\text{Na}^+$  experiments. To our knowledge, the influence of SA on ion uptake rates in fish has never been tested directly. Regardless, the most important comparisons made in the present study were those within a  $\text{Na}^+$  level treatment,



**Fig. 4. Whole-body mRNA expression of Na<sup>+</sup> uptake genes in wild-type, *nhe3b* KO and *rhcgb* KO larvae.** Relative gene expression (normalized to the expression of *actb2* and expressed relative to that of wild-type larvae reared in 800 μmol l<sup>-1</sup> Na<sup>+</sup>) of *slc9a3.2* (A; encoding Nhe3b), *atp6v1aa* (B; encoding H<sup>+</sup>-ATPase), *asic4b* (C) and *slc12a10.2* (D; encoding Ncc) in 4 dpf wild-type, *nhe3b* KO and *rhcgb* KO larvae reared in 800 or 10 μmol l<sup>-1</sup> Na<sup>+</sup>. Data are means±s.e.m. Different letters indicate a statistically significant effect of sodium level within a genotype and boxes in the upper right corner depict the overall statistical effect of genotype as determined by a two-way ANOVA followed by a Holm–Sidak *post hoc* test. *n*=5–7.

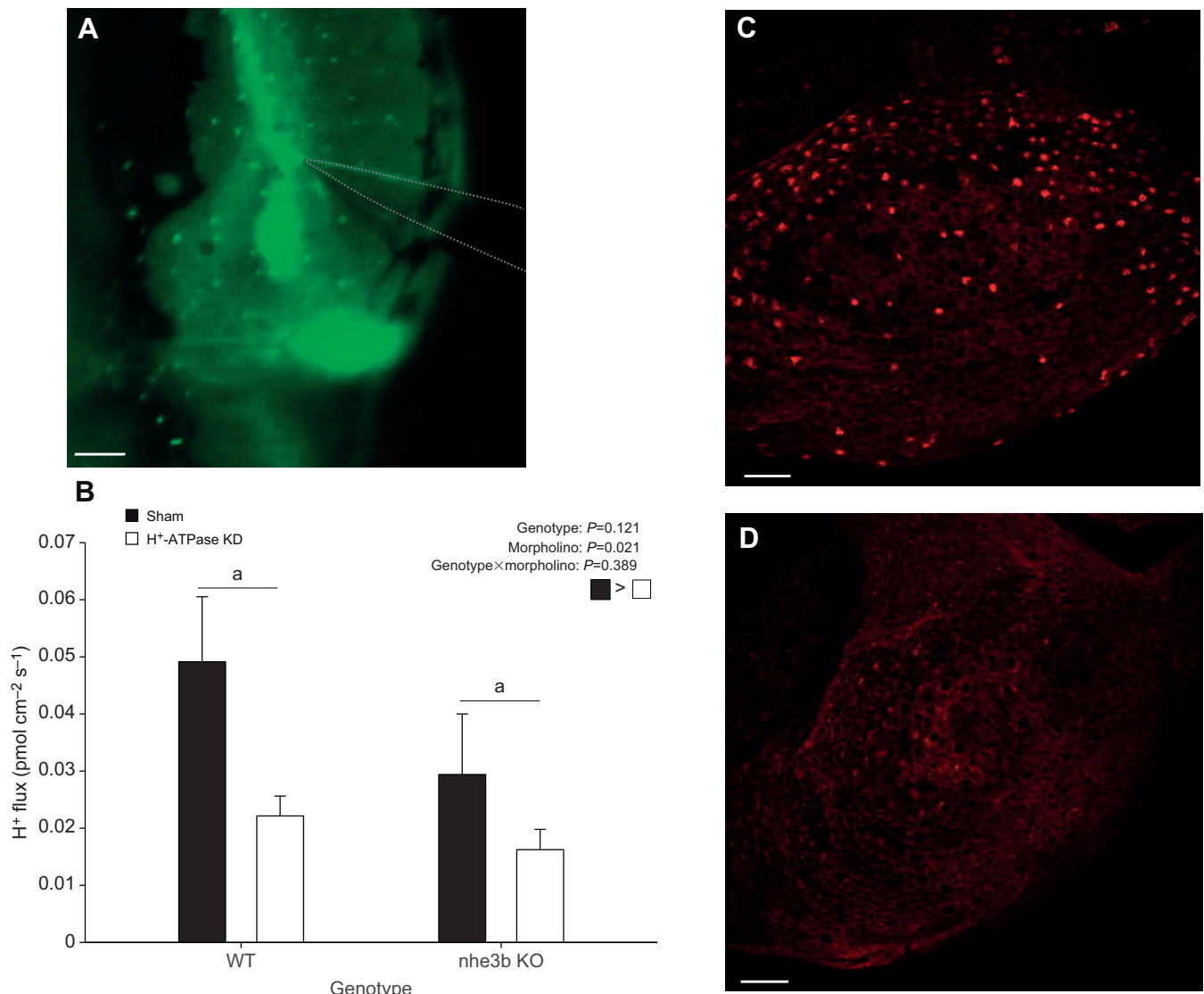
and therefore the potentially confounding influence of SA on Na<sup>+</sup> uptake would not affect the interpretation of our results. In previous work, morpholino KD of Nhe3b and Rhcgb reduced Na<sup>+</sup> uptake by zebrafish larvae under low Na<sup>+</sup> (Shih et al., 2012) and low pH (Kumai and Perry, 2011) acclimation conditions.

Using the same Nhe3b morpholino and dose that were used in the present study, Shih et al. (2012) demonstrated that epithelial Na<sup>+</sup> influx (measured by SIET) was reduced in larvae reared in 5 μmol l<sup>-1</sup> Na<sup>+</sup>. While we were unable to replicate this effect (Fig. 3), it is possible that the differences between these studies reflects the method with which Na<sup>+</sup> uptake was assessed. It is conceivable that localized reductions in epithelial Na<sup>+</sup> influx in Nhe3b morphants measured by SIET (Shih et al., 2012) cannot be detected when Na<sup>+</sup> uptake is measured at the whole-body level. Regardless, the results obtained with Nhe3b KD in the current study were consistent with the phenotype of *nhe3b* KO mutants. Moreover, there were no off-target effects of the morpholino when injected into *nhe3b* KO embryos, an important control for morpholino experiments (Zimmer et al., 2019). A caveat to our results is that we were unable to confirm Nhe3b KD because the Nhe3b antibody failed to detect Nhe3b expression in whole-mount immunostaining or western blotting of larvae, despite working well in adult gills (Fig. 1E). However, given

that the same dose of this Nhe3b morpholino (2 ng per embryo) was shown to reduce Nhe3b expression in a previous study (Chang et al., 2013), it seems likely that successful KD was also achieved in the current study. Nevertheless, it is clear that zebrafish larvae do not require Nhe3b to sustain whole-body Na<sup>+</sup> uptake under normal or low Na<sup>+</sup> conditions.

Nhe3b was also proposed to contribute to Na<sup>+</sup> uptake under acidic conditions. Kumai and Perry (2011) found that KD of Nhe3b using a different morpholino from that used in the present study and by Shih et al. (2012) resulted in a significant reduction in Na<sup>+</sup> uptake when larvae were reared at pH 4 and acutely transferred to circumneutral pH at 4 dpf. In initial experiments, we employed the morpholino used by Kumai and Perry (2011) to knock down Nhe3b and found a nearly 50% reduction in Na<sup>+</sup> uptake in wild-type and *rhcgb* KO larvae reared and assayed in low Na<sup>+</sup> (Fig. S1). However, this same effect was recapitulated in *nhe3b* KO larvae lacking Nhe3b (Fig. S1), indicating that this morpholino exhibits off-target effects on Na<sup>+</sup> uptake that cannot be attributed to Nhe3b KD. Moreover, we discovered that the Rhcgb morpholino employed by Kumai and Perry (2011) was mistakenly designed to target *rhcgl1* (*rhcgl1*, formerly *rhcg2*), a different *rhcg* paralogue for which we currently have little information in terms of localization and





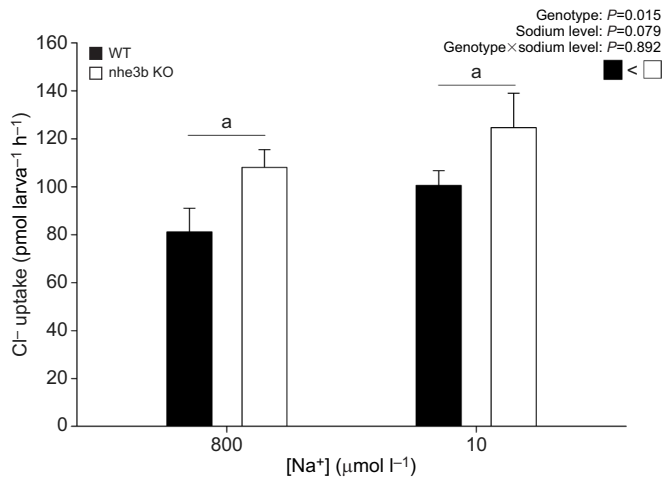
**Fig. 5. H<sup>+</sup> flux at the surface of H<sup>+</sup>-ATPase-rich (HR) cells in response to H<sup>+</sup>-ATPase KD in wild-type and *nhe3b* KO larvae.** (A) Representative image of a H<sup>+</sup>-selective probe positioned above the surface of a HR cell labelled with conA conjugated to Alexa 488. Scale bar: 50  $\mu$ m. (B) H<sup>+</sup> flux at the surface of HR cells of 4 dpf wild-type and *nhe3b* KO larvae treated with sham or H<sup>+</sup>-ATPase (H<sup>+</sup>-ATPase KD) morpholino and reared and assayed in 800  $\mu$ mol l<sup>-1</sup> Na<sup>+</sup>. Data are means  $\pm$  s.e.m. Different letters indicate a statistically significant effect of genotype within a morpholino treatment and boxes in the upper right corner depict the overall statistical effect of morpholino treatment as determined by a two-way ANOVA followed by a Holm–Šidák *post hoc* test.  $n=4-5$ . (C, D) Immunohistochemical staining of H<sup>+</sup>-ATPase in the yolk sac of 4 dpf wild-type larvae treated with sham morpholino (C) or H<sup>+</sup>-ATPase morpholino (D). Scale bars: 50  $\mu$ m.

function. Therefore, it appears that the contribution of the *Rhcg*b/*Nhe3b* metabolon to Na<sup>+</sup> uptake under acidic conditions remains equivocal. Indeed, in other studies, mRNA expression of *slc9a3.2* was found to be decreased in the gills of adult zebrafish acclimated to acidic conditions (Yan et al., 2007; Chang et al., 2013).

#### Compensatory regulation in gene loss of function approaches

In response to gene KD or KO, compensatory regulation of paralogous or functionally related genes has been documented in a number of studies on zebrafish (see El-Brolosy and Stainier, 2017), such that null mutations may not always result in any obvious phenotype (Balciunas, 2018). The mechanism(s) by which this compensatory regulation occurs is not yet clear, but may involve signalling from mutant mRNA (El-Brolosy et al., 2019), at least in the case of gene KO. In zebrafish, compensatory regulation has been demonstrated in response to morpholino KD of several genes

involved in Na<sup>+</sup> uptake. For example, KD of *Nhe3b* in zebrafish reduced whole-body Na<sup>+</sup> content in 2–4 dpf larvae, but a concomitant increase in the density of NCC cells reversed this effect by 5 dpf (Chang et al., 2013). While NCC cell density was not assessed in *nhe3b* KO larvae, it is possible that Na<sup>+</sup> flux via *Ncc* was elevated given that Cl<sup>-</sup> uptake rate was significantly greater in *nhe3b* KO larvae compared with that of wild-type larvae (Fig. 6), although *slc12a10.2* mRNA expression was unchanged (Fig. 4). Notably, however, Cl<sup>-</sup> uptake was unaffected by Na<sup>+</sup> levels in wild-type or *nhe3b* KO larvae, suggesting that Cl<sup>-</sup> uptake is mediated by non-*Ncc* pathways, such as *Slc26a* anion exchangers (Perry et al., 2009; Bayaa et al., 2009), given that the driving force for Na<sup>+</sup>-Cl<sup>-</sup> cotransport would be minimal under low Na<sup>+</sup> conditions. Compensatory regulation also occurred in response to *Ncc* KD in zebrafish. *Ncc* morphants displayed a significant reduction in Cl<sup>-</sup> uptake, but a stimulation of Na<sup>+</sup> uptake (Wang et al., 2009; Kwong and Perry, 2016), indicating compensation from alternative

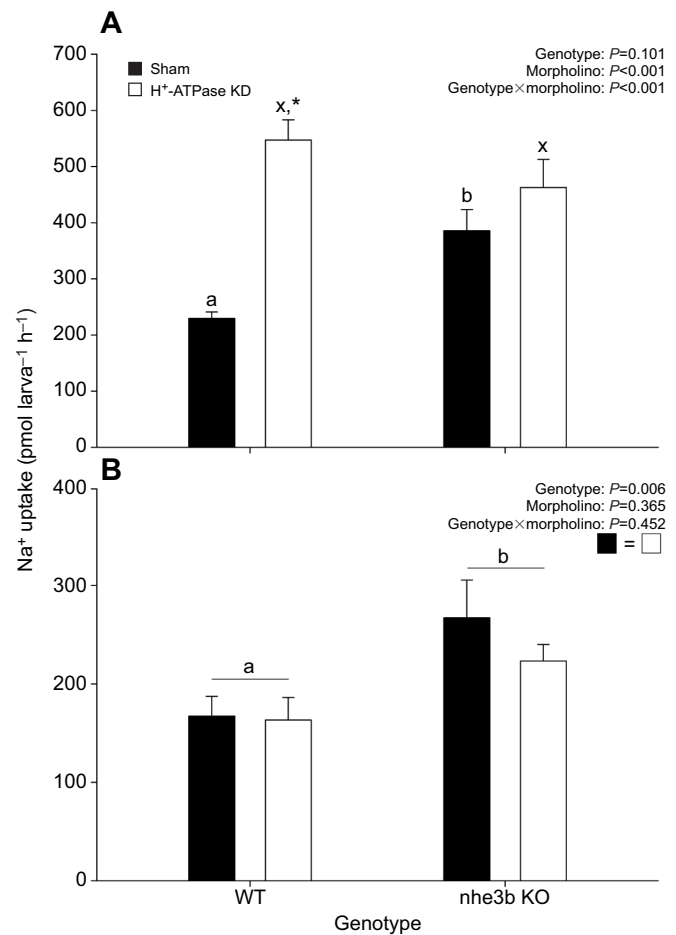


**Fig. 6. Cl<sup>-</sup> uptake in wild-type and nhe3b KO larvae.** Cl<sup>-</sup> uptake rate in 4 dpf wild-type and nhe3b KO larvae reared and assayed in 800 or 10 μmol l<sup>-1</sup> Na<sup>+</sup>. Data are means±s.e.m. Different letters indicate a statistically significant effect of sodium level within genotype and boxes in the upper right corner depict the overall statistical effect of genotype as determined by a two-way ANOVA followed by a Holm–Šidák *post hoc* test. *n*=12.

Na<sup>+</sup> uptake pathways. Indeed, *nhe3b* mRNA expression (Wang et al., 2009) and HR cell density (Chang et al., 2013) were increased in Ncc morphant zebrafish by 4 dpf. In addition, KD of *Asic4b*, an acid-sensing cation channel that may be involved in H<sup>+</sup>-ATPase-mediated Na<sup>+</sup> uptake in zebrafish (Dymowska et al., 2015), resulted in an increase in the density of HR cells and increased mRNA expression of *nhe3b* and *slc12a10.2*, which may have served to maintain normal rates of Na<sup>+</sup> uptake (Zimmer et al., 2018). Clearly, the phenomenon of compensatory regulation is widespread in the Na<sup>+</sup> uptake system of zebrafish, which led to the hypothesis in the current study that Na<sup>+</sup> uptake by nhe3b KO larvae may be sustained by the H<sup>+</sup>-ATPase and/or Ncc-mediated pathways.

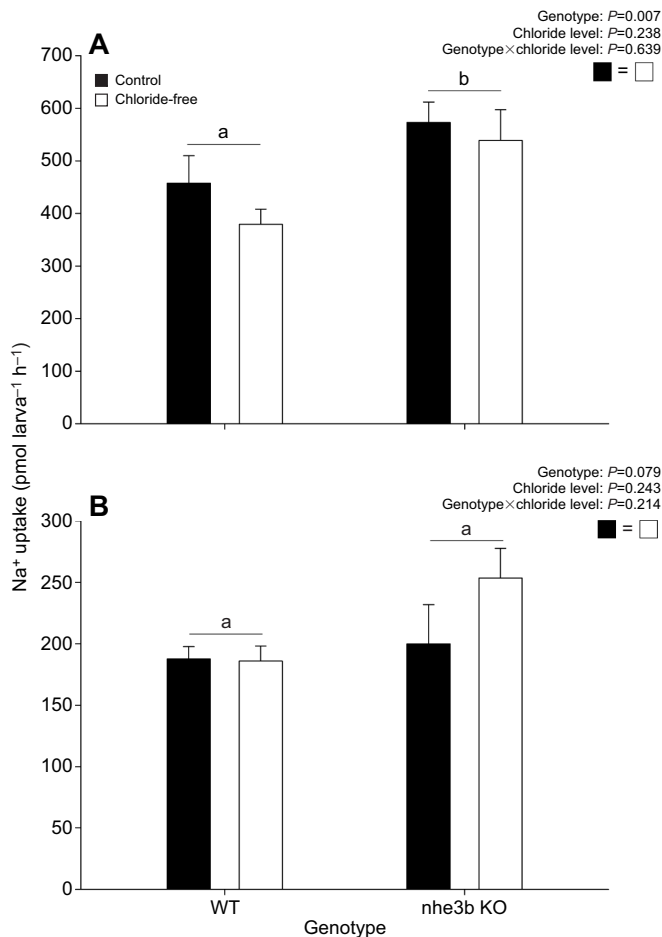
#### Compensatory regulation does not account for maintenance of Na<sup>+</sup> uptake by nhe3b KO larvae

None of the approaches used to assess the contribution of H<sup>+</sup>-ATPase to Na<sup>+</sup> uptake in nhe3b KO larvae revealed compensatory regulation from this pathway. mRNA expression of *atp6v1aa* was not elevated in nhe3b KO larvae or rhcgb KO larvae (Fig. 4B), and H<sup>+</sup> flux at HR cells was not significantly elevated in nhe3b KO larvae (Fig. 5B), which would have indicated an increase in H<sup>+</sup>-ATPase activity. Furthermore, KD of H<sup>+</sup>-ATPase did not significantly reduce Na<sup>+</sup> uptake in nhe3b KO larvae under normal or low Na<sup>+</sup> conditions (Fig. 7), despite a clear reduction in H<sup>+</sup> flux by HR cells, at least under normal Na<sup>+</sup> conditions (Fig. 4B). In fact, Na<sup>+</sup> uptake was stimulated by H<sup>+</sup>-ATPase KD in wild-type larvae, but not in nhe3b KO larvae, under normal conditions (Fig. 7A). To our knowledge, this is the first demonstration of a stimulatory effect of H<sup>+</sup>-ATPase KD on Na<sup>+</sup> uptake. The mechanisms responsible for the stimulation of Na<sup>+</sup> uptake in *Atp6v1aa* morphants are unclear but possibilities include increased expression of Ncc, similar to the results of KD of *gcm2*, a transcription factor that regulates the proliferation of HR cells (Chang et al., 2013; Kwong and Perry, 2016), or an increase in Nhe3b expression given that Na<sup>+</sup> uptake was not stimulated by H<sup>+</sup>-ATPase KD in nhe3b KO larvae. However, H<sup>+</sup> flux did not differ in wild-type and nhe3b KO larvae treated with H<sup>+</sup>-ATPase morpholino (Fig. 5B), which might indicate that Nhe3b does not supplant the function of H<sup>+</sup>-ATPase in wild-type H<sup>+</sup>-ATPase morphants.

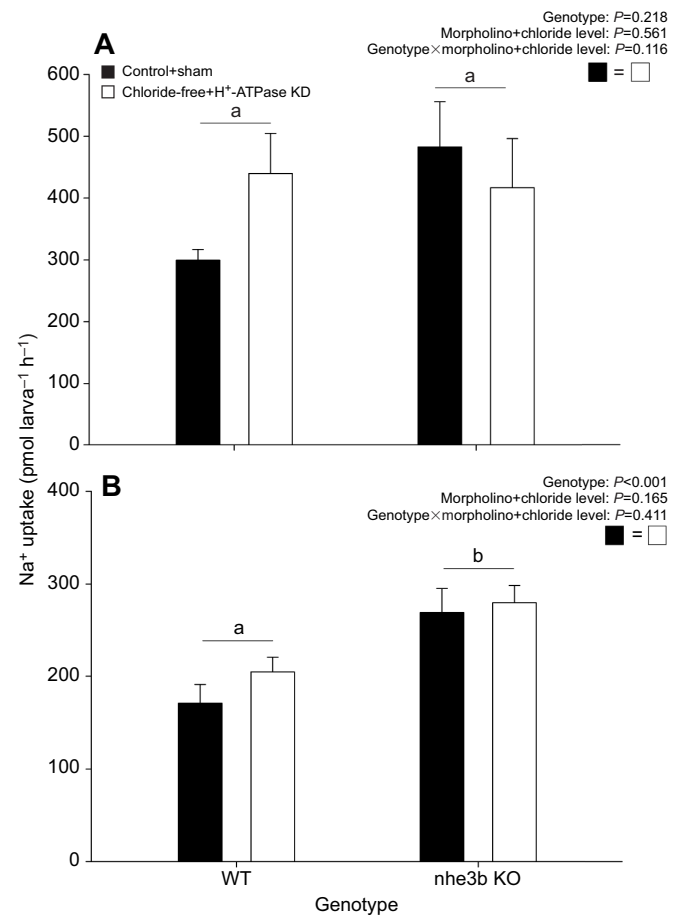


**Fig. 7. Na<sup>+</sup> uptake in response to H<sup>+</sup>-ATPase KD in wild-type and nhe3b KO larvae.** Na<sup>+</sup> uptake rate in 4 dpf wild-type and nhe3b KO larvae treated with sham or H<sup>+</sup>-ATPase (H<sup>+</sup>-ATPase KD) morpholino and reared and assayed in 800 μmol l<sup>-1</sup> (A) or 10 μmol l<sup>-1</sup> (B) Na<sup>+</sup>. Data are means±s.e.m. Different letters indicate a statistically significant effect of genotype within a morpholino treatment, asterisks represent a significant effect of morpholino treatment when the interaction term was significant, and boxes in the upper right corner depict the overall statistical effect of morpholino treatment when the interaction term was not significant as determined by a two-way ANOVA followed by a Holm–Šidák *post hoc* test. *n*=10–14 in A; *n*=6 in B.

Cl<sup>-</sup> uptake increased significantly in response to nhe3b KO (Fig. 6), potentially indicating compensatory regulation by Ncc, as was demonstrated for Nhe3b KD (Chang et al., 2013). However, rearing larvae under chloride-free conditions, achieved by replacing Cl<sup>-</sup> with SO<sub>4</sub><sup>2-</sup>, had no effect on Na<sup>+</sup> uptake by wild-type or nhe3b KO larvae, regardless of Na<sup>+</sup> level. In a previous study, chloride removal was demonstrated to have no effect on Na<sup>+</sup> uptake under ‘normal’ Na<sup>+</sup> conditions in 4 dpf larvae (Kwong and Perry, 2016), although a nearly 50% reduction in Na<sup>+</sup> uptake was observed in 3 dpf larvae in an earlier study (Kwong and Perry, 2013). Chloride-free conditions have also been shown to attenuate increases in Na<sup>+</sup> uptake observed during recovery from acute acid exposure (Kwong and Perry, 2016) and in response to morpholino KD of Claudin-b, a tight junction protein (Kwong and Perry, 2013). Importantly, the increase in Na<sup>+</sup> uptake observed in both acute acid treatment and Claudin-b KD were attributed to Ncc (Kwong and Perry, 2013, 2016), suggesting that chloride-free treatment is an effective way to assess the contribution of Ncc to Na<sup>+</sup> uptake. Therefore, despite an increase in Cl<sup>-</sup> uptake, it does



**Fig. 8. Na<sup>+</sup> uptake in response to chloride-free conditions in wild-type and nhe3b KO larvae.** Na<sup>+</sup> uptake rate in 4 dpf wild-type and nhe3b KO larvae reared and assayed in 800 μmol l<sup>-1</sup> (A) or 10 μmol l<sup>-1</sup> (B) Na<sup>+</sup> containing 500 μmol l<sup>-1</sup> Cl<sup>-</sup> (control) or no Cl<sup>-</sup> (chloride-free). Data are means+s.e.m. Different letters indicate a statistically significant effect of genotype within chloride treatment and boxes in the upper right corner depict the overall statistical effect of chloride treatment as determined by a two-way ANOVA followed by a Holm–Šidák *post hoc* test. *n*=6.



**Fig. 9. Na<sup>+</sup> uptake in response to H<sup>+</sup>-ATPase KD and chloride-free conditions in wild-type and nhe3b KO larvae.** Na<sup>+</sup> uptake rate in 4 dpf wild-type and nhe3b KO larvae treated with either sham morpholino and control Cl<sup>-</sup> conditions (500 μmol l<sup>-1</sup> Cl<sup>-</sup>) or H<sup>+</sup>-ATPase morpholino (H<sup>+</sup>-ATPase KD) and chloride-free conditions (no Cl<sup>-</sup>), reared and assayed in 800 μmol l<sup>-1</sup> (A) or 10 μmol l<sup>-1</sup> (B) Na<sup>+</sup>. Data are means+s.e.m. Different letters indicate a statistically significant effect of genotype within a morpholino plus chloride treatment and boxes in the upper right corner depict the overall statistical effect of morpholino plus chloride treatment as determined by a two-way ANOVA followed by a Holm–Šidák *post hoc* test. *n*=7–8.

not appear that Ncc plays a significant role in sustaining Na<sup>+</sup> uptake in nhe3b KO larvae.

Given the apparent flexibility of the Na<sup>+</sup> uptake system of zebrafish, it is conceivable that even after Na<sup>+</sup> uptake is reduced to only one of the three putative pathways (e.g. nhe3b KO larvae experiencing H<sup>+</sup>-ATPase KD), Na<sup>+</sup> uptake can continue at normal rates. Therefore, an experiment whereby all three pathways were targeted simultaneously was conducted. Surprisingly, in nhe3b KO larvae treated with H<sup>+</sup>-ATPase morpholino and reared and assayed under chloride-free conditions, Na<sup>+</sup> uptake was not reduced relative to that of wild-type larvae treated with sham morpholino and reared and assayed under control chloride conditions (Fig. 9). How can Na<sup>+</sup> uptake be sustained in the absence of Nhe3b and H<sup>+</sup>-ATPase and in the absence of a driving force (Cl<sup>-</sup>-free) for Na<sup>+</sup>-Cl<sup>-</sup>-cotransport? First, it is possible that compensatory regulation of another *nhe* paralogue could supplant the function of *nhe3b*. Indeed, *nhe2* is expressed in the gills of adult zebrafish (Yan et al., 2007) and this gene is thought to be an important component of Na<sup>+</sup> uptake in rainbow trout (Zimmer et al., 2010, 2017b). However, no study to our knowledge has localized its expression to ionocytes of larval

zebrafish. Second, it is possible that a Na<sup>+</sup> channel might function in the absence of a H<sup>+</sup> electromotive force generated by H<sup>+</sup>-ATPase. Recently, Esbaugh et al. (2019) suggested that NKA isoform switching might allow NKA to function at exceedingly low levels of intracellular Na<sup>+</sup> (~10 μmol l<sup>-1</sup>) by changing the stoichiometry of the pump, thereby lowering its activation energy. Indeed, the expression of one particular paralog, *atp1a1a.5*, that is predicted to have a 1:1 Na<sup>+</sup>:K<sup>+</sup> stoichiometry and therefore lower activation energy, was increased in response to low Na<sup>+</sup> (<10 μmol l<sup>-1</sup>) acclimation in the gills of adult zebrafish (Esbaugh et al., 2019). Thus, it is possible that a concentration gradient between the surrounding environment and cytosol could be maintained such that a Na<sup>+</sup> channel alone could transport Na<sup>+</sup> in the absence of H<sup>+</sup>-ATPase. Third, if SO<sub>4</sub><sup>2-</sup> can substitute for Cl<sup>-</sup> via Ncc, perhaps Ncc can still function to absorb Na<sup>+</sup> under the chloride-free conditions used in the present study. However, as discussed earlier, this method of chloride removal has been effective in reducing Na<sup>+</sup> uptake in previous work under conditions in which Ncc function is upregulated (Kwong and Perry, 2013, 2016) and the hydrated ionic radius of SO<sub>4</sub><sup>2-</sup> (0.242 nm) is substantially larger than that of Cl<sup>-</sup>

(0.180 nm) (Marcus, 1988), further suggesting that it is unlikely that  $\text{Na}^+$  is absorbed through  $\text{Na}^+\text{-SO}_4^{2-}$ -cotransport via Ncc. Finally, there may be an alternative pathway for  $\text{Na}^+$  uptake that has not yet been discovered in FW fishes.

### Conclusions and perspectives

Despite a nearly century-long effort to elucidate the mechanisms of  $\text{Na}^+$  uptake by FW fishes, there are many aspects of this vital physiological function that remain unclear. Even with powerful techniques such as CRISPR/Cas9 gene editing, the apparent flexibility and plasticity of the  $\text{Na}^+$  uptake system make uncovering its mechanisms challenging. The present study unequivocally demonstrated that Nhe3b is expendable to  $\text{Na}^+$  uptake in larval zebrafish, but the pathways supplanting the function of this protein, especially in low  $\text{Na}^+$  where its role is increased, remain unclear. We hypothesized that ablating the function of the other two pathways of  $\text{Na}^+$  uptake ( $\text{H}^+$ -ATPase mediated and Ncc) in nhe3b KO larvae would reveal mechanisms of compensatory regulation in response to nhe3b KO. However, these experiments instead pointed towards the possibility that an unknown pathway for  $\text{Na}^+$  uptake may exist in zebrafish and that this may be an important avenue to explore in future research.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: A.M.Z., R.W.K., S.F.P.; Methodology: A.M.Z., K.S.-M., R.W.K.; Software: A.M.Z.; Validation: A.M.Z., K.S.-M., R.W.K.; Formal analysis: A.M.Z.; Resources: S.F.P.; Writing - original draft: A.M.Z.; Writing - review & editing: A.M.Z., R.W.K., S.F.P.; Supervision: S.F.P.; Project administration: S.F.P.; Funding acquisition: S.F.P.

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### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.215111.supplemental>

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