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Genome-wide identification of the NHE gene family in Coilia nasus and its response to salinity challenge and ammonia stress

Jun Gao1,2, Zhijuan Nie2, Gangchun Xu1,2* and Pao Xu1,2*

Abstract

Background: In aquatic environments, pH, salinity, and ammonia concentration are extremely important for aquatic animals. NHE is a two-way ion exchange carrier protein, which can transport Na\(^+\) into cells and exchange out H\(^+\), and also plays key roles in regulating intracellular pH, osmotic pressure, and ammonia concentration.

Results: In the present study, ten NHEs, the entire NHE gene family, were identified from Coilia nasus genome and systematically analyzed via phylogenetic, structural, and synteny analysis. Different expression patterns of C. nasus NHEs in multiple tissues indicated that expression profiles of NHE genes displayed tissue-specific. Expression patterns of C. nasus NHEs were related to ammonia excretion during multiple embryonic development stages. To explore the potential functions on salinity challenge and ammonia stress, expression levels of ten NHEs were detected in C. nasus gills under hypotonic stress, hypertonic stress, and ammonia stress. Expression levels of all NHEs were upregulated during hypotonic stress, while they were downregulated during hypertonic stress. NHE2 and NHE3 displayed higher expression levels in C. nasus larvae and juvenile gills under ammonia stress.

Conclusions: Our study revealed that NHE genes played distinct roles in embryonic development, salinity stress, and ammonia exposure. Syntenic analysis showed significant difference between stenohaline fish and euryhaline fishes. Our findings will provide insight into effects of C. nasus NHE gene family on ion transport and ammonia tolerance and be beneficial for healthy aquaculture of C. nasus.

Keywords: Na\(^+\)-H\(^+\) exchangers, Chinese tapertail anchovy, Hypotonic stress, Hypertonic stress, High environmental ammonia (HEA), Gene expression

Background

Na\(^+\)/H\(^+\) exchanger (NHE) is a transmembrane protein that exists in all eukaryotic cells. Nine NHEs have been identified since human NHE1 cDNA was successfully cloned [1]. Based on subcellular localization and phylogenetic analysis, NHEs can be classified into plasmalemmal subgroup (NHE1–5, SLC9A1–5) and intracellular subgroup (NHE6–9, SLC9A6–9) in fish [2]. Plasmalemmal NHEs usually cooperate with bicarbonate transporter to regulate cytoplasmic pH, cell volume, and intracellular fluid secretion, thereby maintaining the balance of acid-base, electrolyte, and cell volume in the entire life system [3, 4]. Intracellular NHEs can not only transport Na\(^+\), Li\(^+\), and K\(^+\), but can also limit the excess acidification of organelles caused by vacuolar H\(^+\)-ATPase (HA) [4, 5]. NHE1 has been demonstrated to be involved in cardiac remodeling and myocardial fibrosis [6]. NHEs, such as NHE6 and NHE9, can inhibit proliferation and migration in a variety of tumors [7].

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In aquatic environments, pH, salinity, and ammonia concentration are extremely important for aquatic animals. NHE is a two-way ion exchange carrier protein, which can transport Na\(^+\) into cells and exchange out H\(^+\), and also plays key roles in regulating intracellular pH, osmotic pressure, and ammonia concentration [8–11]. In fish, the plasma pH cannot be regulated via excreting CO\(_2\). Thus, H\(^+\) and HCO\(_3^-\) transport in fish gills plays a critical role in acid-base regulation [10, 12]. In hypertonic environments, fish excrete metabolic acids through the apical NHE, which is generally believed to play a critical role in ionocytes [13]. NHE can also coordinate with carbonic anhydrase and bicarbonate transporter in Tribolodon hakonensis and medaka (Oryzias latipes) at acidic environment [14, 15]. Salinity in aquatic environment is an important environmental factor for survival of aquatic animals. The expression level of NHE3 was higher in brackish water than seawater in Atlantic stingray (Dasyatis Sabina) and bull shark (Carcharhinus leucas) [16, 17]. Besides gills in elasmobranch species, in banded hound shark (Triakis scyllium), the expression changes of NHE3 were also detected in the kidney and intestine at different salinity environments [9]. At present, mechanisms of the ammonia nitrogen tolerance have been studied in fish, including inhibiting protein and amino acid catabolism, reducing environmental pH, NH\(_4^+\) and NH\(_3\) continuous excretion, synthesis of non-toxic glutamine, and synthesis of urea, etc. [18–20]. Ammonia is mainly excreted as NH\(_3\) in zebrafish (Danio rerio) and medaka embryos [21, 22]. It is essential for NH\(_3\) excretion to form NH\(_4^+\) via combine H\(^+\) [21]. Based on the acid-trapping hypothesis of ammonia excretion [23], NH\(_3\) excretion was promoted in acidic environment which would increase the conversion of NH\(_3\) to NH\(_4^+\). NHE proteins are essential in the process of ammonia excretion [22] and has a combined effect with carbonic anhydrase (CA) and Rhesus-type ammonia transporter (Rh) [2, 24].

The Chinese tapertail anchovy (Coilia nasus) is an economically valuable fish widely distributed in China, Japan, and Korea. The sexually mature fish run thousands of kilometers from marine to river [25]. Ion uptake is activated in C. nasus during hypotonic environment, and ion excretion and water conservation are promoted in C. nasus during hypertonic environment [26]. Moreover, excessive ammonia nitrogen could lead to mass death of C. nasus larvae and juvenile during artificial breeding [27]. Based on previous studies, NHEs are involved in osmoregulation [26] and ammonia stress [27] in gills of C. nasus. To localize NHE genes in the C. nasus genome and their functions on salinity challenge and ammonia stress, we identified NHE gene family, and detected their expression levels under salinity and ammonia stress. Our findings will provide insight into their effects on ion transport and ammonia tolerance and be beneficial for healthy aquaculture of C. nasus.

**Results**

**Identification of NHE genes**

The entire NHE gene family, 10 NHE genes, were identified in C. nasus genome, including NHE1, NHEβ, NHE2, NHE2-like, NHE3, NHE5, NHE6a, NHE6b, NHE7, and NHE8. The detail information of C. nasus NHE genes were displayed in Table 1.

**Chromosomal distribution of NHE genes**

NHE1, NHE2, NHE2-like, NHE5, NHE6a, NHE6b, NHE7, and NHE8 were located on chromosome 19 (LG 19), LG 12, LG 17, LG 18, LG 20, LG 1, LG 14, and LG 21, respectively (Fig. 1). NHEβ and NHE3 were located on LG 6 (Fig. 1).

**Phylogenetic analysis**

NHEs can be classified into plasmalemmal subgroup (NHE1–5) and intracellular subgroup (NHE6–9) based on subcellular localization and phylogenic analysis [4]. Our phylogenic analysis showed that C. nasus NHEs were categorized into plasmalemmal subgroup (NHE1, NHEβ, NHE2, NHE2-like, NHE3, NHE5) and intracellular subgroup (NHE6a, NHE6b, NHE7, and NHE8) (Fig. 2). Moreover, phylogenic analysis cannot distinguish C. nasus NHE1 and NHEβ.

**Structural analysis of the NHE genes**

To further explore the characteristics of C. nasus NHE genes, analysis of gene structure, conserved domains and motif was performed. The exon numbers of NHE1 were the minimum (6 exons), and the exon numbers of NHE7 owned maximum exons (19 exons) (Table 1, Fig. 3A). All of C. nasus NHEs contained the Na_H_Exchanger domain, except NHE8 which contained the Na_H_Exchanger superfamily domain (Fig. 3B). NHEβ, NHE3, NHE6a, NHE6b, and NHE8 contained only one domain, while others contained two or more domains. Motif analysis showed that motif 3, 4, and 6 existed in all of C. nasus NHEs. Noticeably, motif 5 only existed in plasmalemmal subgroup (NHE1, NHEβ, NHE2, NHE2-like, NHE3, NHE5), and motif 10 only existed in intracellular subgroup (NHE6a, NHE6b, and NHE7) (Fig. 3C). The sequences of each motif were shown in Supplementary material: Fig. S1.

**Syntenic analysis**

The syntenic analysis was performed to further explore the evolutionary relationship of NHE genes between C. nasus and other fish species. The number of homolog pairs between C. nasus NHEs and other fish species,
including channel catfish, Nile tilapia, common carp, Atlantic salmon, and Atlantic herring, were 7, 8, 2, 7, and 10, respectively (Fig. 4A-E).

**Expression profiles NHEs of multiple tissues and embryonic development stages**

Expression profiles of NHEs was detected via qRT-PCR in *C. nasus* brain, eye, gill, heart, head kidney, kidney, intestine, liver, muscle, and spleen (Fig. 5A). NHE2 displayed higher expression in brain, gill, and heart. **NHE3** displayed higher expression in brain, gill, and kidney. High expression level of NHE2-like was displayed in brain, eye, gill, heart, head kidney, kidney, liver, and muscle. High expression level of NHE5 was displayed in eye, heart, head kidney, kidney, intestine, and muscle. High expression level of NHE6a was displayed in eye, heart,

| Gene name | Chromosome location | CDS length (bp) | Exon number | Protein length (aa) | Protein molecular weight (Da) | Isoelectric point (pI) | ID in *C. nasus* genome |
|-----------|---------------------|-----------------|-------------|---------------------|-----------------------------|-----------------------|-------------------------|
| NHE1      | LG19                | 2220            | 6           | 1076                | 82,508                      | 5.89                  | augustus-scaffold8-processed-gene-12.25-mRNA-1 |
| NHEβ      | LG6                 | 1746            | 8           | 581                 | 64,374                      | 8.04                  | maker-scaffold214-augustus-gene-3.54-mRNA-1 |
| NHE2      | LG12                | 2610            | 15          | 869                 | 98,001                      | 5.27                  | maker-scaffold134-augustus-gene-2.29-mRNA-1 |
| NHE2-like | LG17                | 2322            | 11          | 773                 | 87,191                      | 8.53                  | augustus-scaffold63-processed-gene-6.3-mRNA-1 |
| NHE3      | LG6                 | 3231            | 18          | 1076                | 120,098                     | 6.42                  | maker-scaffold60-augustus-gene-4.23-mRNA-1 |
| NHE5      | LG18                | 3549            | 12          | 1182                | 130,950                     | 9.18                  | maker-scaffold18-augustus-gene-8.24-mRNA-1 |
| NHE6a     | LG20                | 2142            | 14          | 713                 | 78,416                      | 6.14                  | maker-scaffold89-augustus-gene-4.19-mRNA-1 |
| NHE6b     | LG14                | 1584            | 6           | 527                 | 57,652                      | 9.33                  | maker-scaffold127-augustus-gene-4.25-mRNA-1 |
| NHE7      | LG1                 | 3297            | 19          | 1098                | 121,677                     | 6.05                  | maker-scaffold241-augustus-gene-2.55-mRNA-1 |
| NHE8      | LG21                | 2373            | 12          | 790                 | 87,695                      | 6.95                  | augustus-scaffold304-processed-gene-2.2-mRNA-1 |

**Fig. 1** Chromosomal location of NHE gene family. The chromosomes were characterized by yellow bars. Chromosome numbers are shown on the left of the chromosomes. NHE genes are marked in red on the right of the chromosomes. LG: the name of chromosomes in *C. nasus* genome.
and heart. **NHE6b** displayed higher expression in gill, liver, and spleen. **NHE7** displayed higher expression in head kidney, intestine, liver, muscle, and spleen. **NHEβ** displayed higher expression in brain, eye, intestine, and liver. The expression of **NHE1** and **NHE8** were almost undetectable in all tissues used in our study.

Expression profiles of **NHEs** was detected via qRT-PCR at fertilized stage, 2-cell stage, multi-cell stage, midgastrula stage, neural stage, muscle burl stage, prehatching, post-hatching, before first feeding, and after first feeding (Fig. 5B). **NHEβ** displayed higher expression at fertilized stage and before first feeding. High expression level of **NHE7** was displayed in fertilized stage and midgastrula stage. High expression level of **NHE3** was displayed in 2-cell stage. High expression level of **NHE5** was displayed in 2-cell stage, multi-cell stage, midgastrula stage, and neural stage. High expression level of **NHE2-like** was displayed in midgastrula stage, neural stage, and muscle burl stage. **NHE2** displayed highest expression at neural stage, and prehatching stage. **NHE1** displayed higher expression at midgastrula stage, neural stage, and prehatching stage. **NHE6b** displayed higher expression at 2-cell stage, neural stage, muscle burl stage, prehatching.
Fig. 3 Structural analysis of *C. nasus* NHE genes. (A) gene structure, (B) conversed domains and (C) motifs. The plasmalemmal subgroup (*NHEβ*, *NHE1–5*), and the intracellular subgroup (*NHE6–8*) were differentiated by pink and green.
Expression of NHEs in response to salinity challenge and ammonia stress

To explore *C. nasus* NHEs in response to hypotonic stress and hypertonic stress, their expression profiles were detected under hypotonic stress (F vs C) and hypertonic stress (S vs C) in the gill via qRT-PCR (Fig. 6). Overall, expression profiles of all NHEs were significantly upregulated at first, and then significantly downregulated under hypotonic stress. The converse trend was displayed in hypertonic stress. NHEs were significantly downregulated at first, and then significantly upregulated. During hypotonic stress, *NHE1*, *NHE2*, *NHE7* showed highest expression at 6 h. *NHE2-like*, *NHE3*, *NHE6a*, and *NHE8* showed highest expression at 12 h. *NHEβ*, *NHE5*, and *NHE6b* showed lowest expression at 12 h. During hypertonic stress, *NHE1*, *NHE2*, *NHE7* showed lowest expression at 6 h. *NHE2-like*, *NHE3*, *NHE6a*, and *NHE8* showed lowest expression at 12 h. *NHEβ*, *NHE5*, and *NHE6b* showed lowest expression at 24 h.

To explore the potential functions of NHEs of *C. nasus* larvae and juveniles in response to ammonia stress, their expression patterns were detected in the gill via qRT-PCR (Fig. 7). In *C. nasus* larvae, the expression of *NHE2*, *NHE2-like*, *NHE3*, and *NHE6a* were significantly enhanced under ammonia stress, while other NHEs displayed no significant difference. In juveniles, the expression of *NHE2* and *NHE3* were significantly enhanced under ammonia stress, while other NHEs displayed no significant difference.
Discussion

Ten NHE genes were identified in _C. nasus_. Based on analysis of other vertebrates NHE gene family, NHEβ was only identified in teleost species, and NHE4 is generally missing in teleost fishes [28–30], which was generally identified in mammals [2]. In the present study, NHEβ was identified, and NHE4 missed in _C. nasus_ NHE gene family. Furthermore, _C. nasus_ NHE9 was not identified. As the oldest NHE gene, NHE9 lost in some fish [31]. Moreover, the synteny analysis showed that the number of homolog pairs between _C. nasus_ NHEs and other fish species, including channel catfish, Nile tilapia, Atlantic salmon, and Atlantic herring, were 7, 8, 7, and 10, respectively, which indicated that the genetic relationship between _C. nasus_ and these fish species was close. The genetic relationship between _C. nasus_ and common carp is closer than channel catfish and Nile tilapia based on traditional fish taxonomy, while homolog pairs of NHEs between _C. nasus_ and common carp is only 2. Common carp is stenohaline fish, while other fish species are euryhaline fishes. These results suggested that most of NHEs between stenohaline fish and euryhaline fish are not conserved, or most of NHEs has been lost in stenohaline fish genome.

Based on tissue-specific expression patterns, NHE2 and NHE3 displayed higher expression levels in _C. nasus_ gills. NHE2 and NHE3 are the primary isoforms expressed in gills of multiple fishes, including zebrafish [32], Pacific dogfish (_Squalus suckleyi_) [33], rainbow trout (_Oncorhynchus mykiss_) [34, 35], and _Fundulus heteroclitus_ [36], which was similar to our results. In winter flounder (_Pseudopleuronectes americanus_) and zebrafish, NHE1 displayed high expression levels in red blood cells [32, 37]. Therefore, the expression level of NHE1 was almost undetectable in all tissues used in our study. However, few research focused on tissue distribution other NHEs.

In the present study, NHE2-like and NHE6b showed higher expression levels in the spleen, and NHE8 higher expression levels in the liver of European sea bass (_Lateolabrax maculatus_) [2]. These results were different from our results, which implied that the tissue distributions of NHEs were different from different fish species considering the habitats and lifestyle. _C. nasus_ NHEs displayed different expression patterns during multiple embryonic development stages. Yolk proteins and amino acids are the main energy source in most teleost fish during embryonic development [38–40]. Their metabolism can continuously produce a waste product, ammonia. NHEs involved in ammonia excretion and Na⁺ uptake [41, 42]. NHE3 can induce Rhesus glycoprotein (Rh) proteins to involve
in ammonia excretion during embryonic development stage in *Coryphaena hippurus* [43]. Similar results have been reported in medaka, zebrafish, and rainbow trout [42, 44]. Moreover, as a consequence, embryos of oviparous organisms are exposed to high respiratory CO₂ within the egg capsule due to their increasing metabolic rate and the egg capsule wall acting as a diffusion barrier [45, 46]. High respiratory CO₂ can decrease pH in the embryo and larvae. In acid-secreting ionocytes, NHEs are believed to be specialized in the secretion of acid equivalents [47]. It is proposed that ammonia transporters from the Rh family in combination with NHE3, expressed in HR cells are key players in mediating the active secretion of ammonia and protons in seawater teleost [22, 48]. A previous study demonstrated that NHE3 expressing epidermal ionocytes of cephalopod embryos are also involved in active secretion of acid equivalents [49]. Together, different expression patterns of *C. nasus* NHEs during multiple embryonic development stages seem to involve in ammonia excretion, Na⁺ uptake, and maintaining cellular pH homeostasis, or other physiological processes.

Gill is an indispensable tissue in fish, playing critical roles in osmoregulation and ammonia excretion. NHE is a two-way ion exchange carrier protein, which can transport Na⁺ into cells and exchange out H⁺ (or NH4⁺) [50, 51]. The expression of NHE3 was increased in the apical membrane of mitochondria-rich cells of *Dasyatis sabina* under low-salinity stress, thereby promoting the absorption of Na⁺ [16]. The mRNA expression level of NHE3 in Mozambique tilapia (*Oreochromis mossambicus*) gills in freshwater environment is higher than that in seawater environment [52]. Similar results have been reported in other fish, including *D. sabina* [16], zebrafish [32], banded hound shark [9], and Pacific dogfish [33]. In the present study, NHE2, NHE2-like and NHE3 with higher expression in the gills were displayed in *C. nasus* under hypotonic stress, which was consistent with other fish species. However, they were downregulated in the gills of *C. nasus* under hypertonic stress. This finding was also occurred in European sea bass [2]. Besides NHE2 and NHE3, expression levels of other NHE genes were also detected, and they showed higher expression levels in the gills of *C. nasus* under hypertonic stress and lower expression levels in hypotonic stress, which was similar to European sea bass [2]. These results indicated that *C. nasus* NHEs appear to have differing functions in hypotonic and hypertonic regulation via exchanging extracellular Na⁺ for intracellular H⁺.

Besides osmoregulation, NHEs are also essential for ammonia excretion. In the present study, the expression levels of NHE2, NHE2-like, NHE3, and NHE6a were significantly upregulated in *C. nasus* larvae gills under ammonia stress for 24 h. And the mRNA expression of NHE2 and NHE3 were significantly upregulated in *C. nasus* juvenile gills. Similarly, the mRNA expression of NHE2 and NHE3 were upregulated in *Boleophthalmus pectinirostris* under ammonia stress [41]. The mRNA expression level of NHE was upregulated more significantly in *B. pectinirostris* than *Periophthalmus magnuspinatus* subjected to treatment with high environmental ammonia for 72 h [53]. Based on transport physiology, it is generally believed that NH₃ and CO₂ move across biological membranes to a much higher degree via membrane channels than simple, passive diffusion [54]. Ammonia excretion in fishes occurs via a “Na⁺/NH₄⁺-exchange metabolon” which involves NHEs [22, 41, 55]. NH₃ can diffuse from cells into water via Rh glycoproteins. As soon as it enters the water, NH₃ combines with H⁺ which is pumped from the gill cell by H⁺-ATPase and/or by one or more NHE proteins, to form NH₄⁺ [41]. There is indirect coupling of NH₄⁺ efflux to Na⁺ uptake by either of these H⁺ efflux mechanisms. These results implied that significant up-regulation of NHE in *C. nasus* larvae and juveniles promoted NH₃ to form NH₄⁺ via pumping H⁺ into water to reduce ammonia toxicity. Moreover, the expression levels of Rhcg1, H⁺-ATPase, NHE, Na⁺/Ka⁺-ATPase (NKA), and Na⁺/Ka⁺/Cl⁻ cotransporter (NKCC) were upregulated in *Takifugu rubripes* exposed to high external ammonia, and they showed combined effects on ammonia excretion [48]. The combined effects between NHEs and other proteins are required to further study in *C. nasus* under ammonia stress.

**Conclusions**

In the present study, 10 NHE genes were systematically identified from *C. nasus* genome. Phylogenetic analysis showed that NHE4 and NHE9 were lost in *C. nasus* genome. Syntenic analysis showed significant difference between stenohaline fish and euryhaline fishes. Different expression patterns of *C. nasus* NHE genes
Fig. 6 (See legend on previous page.)
were displayed in multiple tissues. Different expression patterns of *C. nasus* NHE genes during multiple embryonic development stages were related to ammonia excretion. During hypotonic stress, *C. nasus* NHE genes were significantly upregulated. During hypertonic stress, they were significantly downregulated. During ammonia stress, NHE2 and NHE3 were significantly upregulated in *C. nasus* larvae and juveniles. These studies will provide insights into molecular mechanism of osmoregulation and ammonia tolerance in teleost.

**Methods and materials**

**Identification of NHE gene family in *C. nasus***

To identify NHE gene family in *C. nasus*, the whole genome databases (GenBank GCA_007927625.1) were searched using BLAST GUI Wrapper on TBtools (v1.0692) according to amino sequences of human (*Homo sapiens*), zebrafish, and Atlantic herring (*Clupea harengus*) downloaded from Ensembl (http://www.ensembl.org) and NCBI (http://www.ncbi.nlm.nih.gov/) databases (cutoff value <1e-5). After removing repeated sequences, the unique sequences were validated via BLASTN against
NCBI non-redundant protein database. Moreover, the Na\(_{\text{H}}\)Exchanger domain (PF00999.21) was downloaded on Pfam database (http://pfam.xfam.org/). The Simple HMM search on TBtools (v1.0692) was used for seeking \(C.\ nasus\) NHE proteins with E-value < 0.01. The generated NHE proteins were verified on the Pfam databases. After removing the repeated sequences sought from the two methods above, the unique sequences were retained for further analysis.

The chromosomal location was performed based on their locations on \(C.\ nasus\) genome via Gene Location Visualize (Advanced) on the TBtools (v1.0692). The molecular weight and pI of NHE family were detected on ExPASy-Compute pl/Mw tool (https://web.expasy.org/compute_pi/).

**Phylogenetic analysis of NHEs**

The phylogenetic analysis of NHE sequences between \(C.\ nasus\) and several vertebrates were performed by MEGA X software. The accession numbers used for phylogenetic analysis were listed on Supplementary Table S1. The phylogenetic tree beautification was performed beautified on Interactive Tree of Life (iTOL, http://itol.embl.de/).

**Structure analysis of NHEs**

The gene structure of NHE genes were analyzed based on \(C.\ nasus\) genome database (gff3 file). Conserved domains of NHEs were analyzed via Conserved Domain Search Service (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Motifs of NHEs were analyzed via Multiple Em for Motif Elicitation (MEME) (https://meme-suite.org/meme/tools/meme). Gene Structure View (Advanced) on TBtools was used for the visualization of gene structure, conversed domains, and motifs.

**Synteny analysis of NHEs**

One Step MCScanX on TBtools was used to examine gene duplication [56, 57]. Homology of NHE genes was analyzed between \(C.\ nasus\) and other five fishes, including channel catfish (\(Ictalurus\) Punetaus), Nile tilapia (\(Oreochromis\) niloticus), common carp (\(Cyprinus\) carpio), Atlantic salmon (\(Salmo\) salar), and Atlantic herring. Dual Systeny Plot for MCScanX on TBtools was used for the visualization of synteny analysis.

**Salinity challenge, ammonia stress, and samples collection**

Healthy \(C.\ nasus\) (5.54 ± 0.63 cm, 2.25 ± 0.83 g) were randomly allocated into three groups (in triplicates): control group (C, salinity 10 ppt), hypotonicity group (F, salinity ~ 1 ppt), and hypertonicity group (S, salinity 30 ppt). After exposure for 0 hour (h), 3 h, 6 h, 12 h, 24 h, 24 h, 48 h, and 72 h, the treated fish were anesthetized via 70 mg/L buffered tricaine methanesulfonate (MS-222) (Greenhengxing, Beijing, China), and their gills were immediately collected and then stored at −80°C for further molecular assays.

Healthy \(C.\ nasus\) larvae (2.33 ± 0.25 cm, 1.11 ± 0.21 g) and juveniles (5.12 ± 0.45 cm, 2.35 ± 0.47 g) were reared for 10 days (27 ± 1.5 °C, pH 8.0 ± 0.4, salinity < 1.7, ammonia nitrogen < 0.005 mg/L). After acclimation, larvae and juveniles were randomly allocated into control group (ammonia nitrogen < 0.005 mg/L) and ammonia stress group (concentration of ammonia ~ 280 umole/L) (in triplicates), respectively. Stock solution (1.0 mol/L) of high purity NH\(_4\)Cl was used as the source of the total ammonia. After exposed for 24 hours (h) (28 ± 2.1 °C, pH 7.5 ± 0.3, salinity < 2.3), gills were immediately collected and then stored at −80°C for further molecular assays.

For analysis of tissue distribution, three \(C.\ nasus\) (24.7 ± 1.68 cm, 11.9 ± 0.62 g) were anesthetized via 70 mg/L buffered MS-222, and then eye, gill, brain, liver, spleen, intestines, heart, head-kidney, kidney, and muscle were immediately collected and then stored at −80°C for further molecular assays. Tissue samples from all 3 fish were pooled together to make one sample. For ontogenetic expression profiles, embryos and larvae during multiple developmental stages were collected following natural spawning of the brood stock. Every developmental stage was examined by microscope observation. 30 embryos at fertilized egg (0 hour post fertilization (hpf)), 2-cell (1 hpf), muti-cell (3 hpf), midgastrula (4 hpf), neural (11 hpf), muscle burl (21 hpf), prehatching (28 hpf), and post-hatching (30 hpf); 20 larvae before first feeding (96 hpf) and after first feeding (144 hpf) were immediately pooled and frozen in liquid nitrogen.

**Quantification of the NHEs expression by quantitative real-time-PCR (qRT-PCR)**

cDNA was synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa, Tokyo, Japan). Primer Premier 5 software was used to design the primers used for qRT-PCR (Supplementary material: Table S2). The reactions were carried out on the Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The reaction system (20.0 µL) included 10.0 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 2.0 µL of cDNA, 1.0 µL of each primer (10 µmol/L), and 6.0 of PCR-grade DEPC water. Reactions were performed in
triplicate per sample, and cycling parameter was set as following: 94 °C for 2 min, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 45 s at 72 °C. The geometric means of β-actin, 18S rRNA, and GAPDH, housekeeper genes were used to normalize expression levels of NHE genes [58]. All samples were detected in triplicate and the relative expression levels of NHE genes were calculated using the 2−ΔΔCT method [59].

Statistical analysis
The data of C. nasus NHEs expression during salinity and ammonia stress were analyzed by two-way ANOVA. Kolmogorov-Smirnov and Shapiro-Wilk methods were used to test the normal distribution. Interactive effect was tested using conversed non-normally distributed data. When P < 0.05, two-way ANOVA was performed. Data analysis was performed on SPSS 20.0. All data were displayed as mean ± SE. P < 0.05 indicates a significant difference. Histograms were drawn via GraphPad 8.0.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12864-022-08761-9.

Additional file 1.

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Not applicable.

Authors’ contributions
PX, GCX, and JG designed the experiment, JG collected the samples, JG and GCX analyzed the data, and prepared the manuscript under supervision of PX and GCX. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analysed during the current study are available in the NCBI database (https://www.ncbi.nlm.nih.gov/assembly/GCA_007927625.1/).

Declarations

Ethics approval and consent to participate
All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) (No. YZB201134) and approved by the Institutional Animal Care and Use Committee of Nanjing Agriculture University (Nanjing, China). The Collia nasus were anesthetized with 70 mg/L MS-222 (Greenhengxing, Beijing, China). The muscles, eyes, brains, gills, and livers were extracted based on the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) (No. YZB201134). The study is also reported in accordance with ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest.

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