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Effects of carbonate alkalinity on branchial gene expression in the large-scale loach (*Paramisgurnus dabryanus*)

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Elucidating the mechanisms of alkaline tolerance in freshwater teleosts will help in the development of commercial saline-alkaline aquaculture. The large-scale loach (*Paramisgurnus dabryanus*) is a viable species for such aquaculture, but the mechanisms of its tolerance of alkaline water are unclear. Large-scale loach was exposed to 40, 50, and 60 mmol L^{-1} $NaHCO_3$ for 12, 48, and 96 h to evaluate the transcriptional changes of branchial Rhesus (Rh) glycoproteins, and aquaporins (Aqp)1 and Aqp3. *Rhag* transcript levels increased with longer exposure times. *Rhag* expression also rose considerably at higher carbonate alkalinities. *Rhbg* mRNA levels declined significantly under carbonate alkalinity exposure. A marked up-regulation of *Rhcg* was observed in the gills of the loach. Exposure to 60 mmol L^{-1} $NaHCO_3$ also induced a significant up-regulation of *aqp1*. By contrast, *aqp3* expression was significantly lower after 48 h exposure. The current findings reveal that the large-scale loach up-regulates *Rhag* and *Rhcg* to enhance ammonia efflux from the gills when exposed to high alkalinity. It is proposed that this species maintains appropriate osmolality when adapting to an alkaline environment by down-regulating *aqp3* (to impede urea removal) and up-regulating *aqp1* in the gills (to excrete excessive internal water).

KEYWORDS

alkaline water, Rh glycoproteins, Aquaporins, air-breathing fish, osmoregulation, amonia excretion

Introduction

Saline-alkaline water is widely distributed across the world, with an estimated 45.87 million hectares in China alone (Lin et al., 2013; Song et al., 2021). It was characterized by high salinity, high carbonate alkalinities, and high pH, that pose homeostatic challenges to aquatic species (Geng et al., 2016; KoKou et al., 2019; Pellegrin et al., 2020; Song et al., 2021). Only a few native species, such as *Gymnocypris przewalskii* (Li et al., 2020) and *Leuciscus waleckii* (Xu et al., 2013), can inhabit such environments. Selecting or breeding saline-alkaline-resistant fish strains and developing commercial saline-alkaline

aquaculture production have become efficient means of sustaining fisheries as the worldwide shortage of freshwater resources worsens. Consequently, there is an urgent need to improve our understanding of the mechanisms by which fish adapt to saline-alkaline waters.

The high pH of saline-alkaline water reduces H^+ levels in the body, which limits NH_3 efflux from the gills (Bolner and Baldisserotto, 2007). High pH also restricts Na^+ influx, resulting in osmolar imbalance in fish (Cui et al., 2020). Thus, effective osmoregulation and NH_3 excretion are essential for fish inhabiting saline-alkaline waters. The gills are the dominant site of osmoregulation and excretion of nitrogen waste in teleosts (Evans et al., 2005). Several channel transporters play important roles in small molecule exchange across the gills into the surrounding water. For example, Rhesus (Rh) glycoproteins, including Rh blood group-associated glycoproteins (Rhag), Rh family B glycoprotein (Rhbg), and Rh family C glycoprotein (Rhcg) have been shown to enhance NH_3 outflow (Wright and Wood, 2009; Eom et al., 2020). Aquaporins (Aqps) 1 and 3 are modulated by ambient salinity and alkalinity in *Oreochromis mossambicus* female × *O. urolepis hornorum* male (Su et al., 2020), suggesting that Aqps contribute to osmoregulation in fish. Consequently, elucidating the alkaline adaptation strategies of freshwater teleosts requires an understanding of the regulatory mechanisms of channel transporters localized in their gills.

The large-scale loach (*Paramisgurnus dabryanus*) is one of the most economically significant farmed species in East Asia (Liu et al., 2018), with worldwide production reaching 368,406 metric tons in 2020 (valued at 865.527 million US dollars). The large-scale loach is a typical air-breathing species (Zhang et al., 2016) that exhibits excellent resistance to environmental stresses, including ammonia tolerance (Zhang et al., 2017; Zhang et al., 2019; Zhang et al., 2021; Shang et al., 2021), and may be a viable species for saline-alkaline aquaculture. However, the mechanisms of its tolerance of alkaline water remain unclear. The present study aims to examine the regulation of Rh glycoproteins and Aqps-related genes in response to high carbonate alkalinity in order to reveal their significance in the alkaline tolerance of large-scale loach. The findings will inform our understanding of the mechanisms by which freshwater fish become acclimatized to saline-alkaline environments and provide information for the breeding and selection of alkali-tolerant strains.

Materials and methods

Ethics statement

This study was carried out in accordance with the principles of the Basel Declaration and the recommendations of the Guide for the Care and Use of Laboratory Animals published by the Animal Care Committee of Anhui Agricultural University (Hefei, China). The protocol was approved by the Animal Care Committee of Anhui Agricultural University.

Experimental fish

Experimental large-scale loach (20.3 ± 2.8 g, unsexed) were obtained from a fish market in Hefei (China) and acclimated in laboratory cycling tanks containing aerated freshwater (water temperature 23.0 ± 1.0 °C, dissolved oxygen ≥ 5.0 mg L^{-1}) for 7 days. During acclimatization, the fish were fed a commercial meal twice daily (crude protein 35%, crude lipid 7%).

Experimental design

After 24 h fasting, fish were exposed to various concentrations (nominally 40, 50, and 60 mmol L^{-1}; measured values: 39.6 mmol L^{-1} pH 9.40, 48.0 mmol L^{-1} pH 9.50, and 59.6 mmol L^{-1} pH 9.60, respectively) of carbonate alkalinity (as $NaHCO_3$) for 12, 48, and 96 h in a volume of 10 L at 25.0 ± 1.0 °C using a plastic box (diameter 35.5 cm, height 15.5 cm). The carbonate alkalinity levels were designed according to an acute $NaHCO_3$ toxicityin this species reported by Wu et al., 2017. The $NaHCO_3$ solution was completely exchanged every 24 h to maintain the alkaline level. Control fish were collected at the beginning of the experiment (0 h). Three replicate tanks containing four fish were sampled for each exposure period. Fish were not fed during the experimental period.

Sample collection

At the end of each exposure period all the fish were anaesthetized with tricaine methane sulfonate (MS-222, 200 mg L^{-1}), executed by a blow to the head, and the gills removed by dissection. Until RNA isolation, all specimens were stored at -80 °C.

Real-time qPCR

Total RNA was isolated from samples of large-scale loach gills using an Ultrapure RNA Kit (CoWin Biotech, Beijing) following manufacturer's instructions. The quality and integrity of the isolated RNA were verified by 2% agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20 °C. RNA (1 μg) from each sample was reverse transcribed to cDNA using MonScript RTIII Super Mix with dsDNase (Monad Biotech, Wuhan) and a thermal cycler (T100, BIO-RAD, USA) following the manufacturer's protocol.

Gene-specific primers were used to measure relative gene expression. (Table 1). Quantitative real-time PCR (qPCR) was carried out using AceQ qPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing) and a quantitative thermal cycler (CFX Connect 96, BIO-RAD, USA) in a 20 μl reaction volume. Amplification conditions were as follows: pre-denaturation for 5 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Relative mRNA levels were calculated using the $2^{-\triangle\triangle Ct}$

TABLE 1 Primer sequences of detected genes for qPCR.

Gene	Primer	Sequence (5'-3')	Tm °C	Product size (bp)
β-actin	β-actin-F	TCTTGGGTATGGAGTCTTGCGGT	58	113
	β-actin-R	TCTTGATTTTCATTGTGCTGGGG		
Rhag	Rhag-F	ATTGCTTTGGTGGGTGGACTCATC	58	132
	Rhag-R	CTCTTCGTGCTCGTGTTCTTCCTC		
Rhbg	Rhbg-F	TGACTGGAGCGTTGGACAACAAG	60	133
	Rhbg-R	TGTATCTGGAGGAGCACCGTAGAC		
Rhcg	Rhcg-F	TCTCCCCAAAATGGCAAT	60	124
	Rhcg-R	CTGGTATTTGTGTAGCCCTC		
aqp1	aqp1-F	GCTGGTTGGCATGACCCTCTTC	58	121
	aqp1-R	AGTGGCGATGGACAAACCGAAAG		
aqp3	aqp3-F	TCCATCATTGGCGTGATTGTGTACC	58	134
	aqp3-R	AGGCTAGATCCATCCTTACTGGTGAC		

All the primers were designed based on transcriptome assemblies (Shang et al., 2021) and synthesized by Sangon Biotech (Shanghai, China).

method as described by Livak and Schmittgen (2001), with the housekeeping gene *β-actin* being used as an internal standard to normalize the results.

Data analysis

Values are expressed as mean ± standard error. The variance homogeneity of the data was examined using Levene's test. One-way (exposure time, carbonate alkalinity concentration) and two-way (exposure time × carbonate alkalinity concentration) analysis of variance (ANOVA) were used to compare mRNA expression levels. Tukey's multiple tests were performed when significant differences were found at the 0.05 level. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Changes in Rh glycoproteins-related gene expression in response to carbonate alkalinity

Significant interactions were observed between carbonate alkalinity concentration and exposure time on *Rhag*, *Rhbg*, and *Rhcg* expression in the gills of large-scale loach after various periods of acute exposure (two-way ANOVA, $P < 0.05$, Table 2). *Rhag* transcript levels increased markedly as exposure time increased ($P < 0.05$, Figure 1A). High carbonate alkalinity also induced a significant increase in *Rhag* expression during the various periods of $NaHCO_3$ exposure ($P < 0.05$, Figure 1A). Up-regulation of the *Rhag* gene was previously reported in the gills of *Anabas testudineus* exposed to high levels of ammonia (Chen et al., 2017). Rhag in erythrocytes have been shown to enhance NH_3 efflux from red cells into plasma (Wright and Wood, 2009). Rhag may be present in pillar cells between the lamellar blood space and the gill epithelium, allowing NH_3 to permeate the branchial epithelium (Wright and Wood, 2009). Ammonia excretion was clearly reduced by exposure to high alkalinity (Li et al., 2020). Thus, the observed high level of Rhag transcripts accompanying the blocking of ammonia excretion might be attributed to the development of ammonia tolerance. The various periods of carbonate alkalinity exposure induced significant declines in *Rhbg* mRNA level ($P < 0.05$), while *Rhbg* expression was unaffected by $NaHCO_3$ concentrations ($P > 0.05$, Figure 1B). Similarly, changes in *Rhbg* transcription in response to ammonia were not observed in *Micropterus salmoides* (Egnew et al., 2019), *Oncorhynchus mykiss*, or *Cyprinus carpio* (Sinha et al., 2013). The present results suggest that the function of Rhbg in large-scale loach is not

TABLE 2 Two-way ANOVA *P* value in a carbonate alkalinity exposure trial.

Significance		*Rhag*	*Rhbg*	*Rhcg*	*aqp1*	*aqp3*
Two-way ANOVA	CA	< 0.001	0.945	0.312	< 0.001	0.065
	ET	< 0.001	< 0.001	< 0.001	0.029	< 0.001
	CA × ET	0.001	< 0.001	< 0.001	0.010	< 0.001

CA, carbonate alkalinity concentration; ET, exposure time.

10.3389/fmars.2022.983615

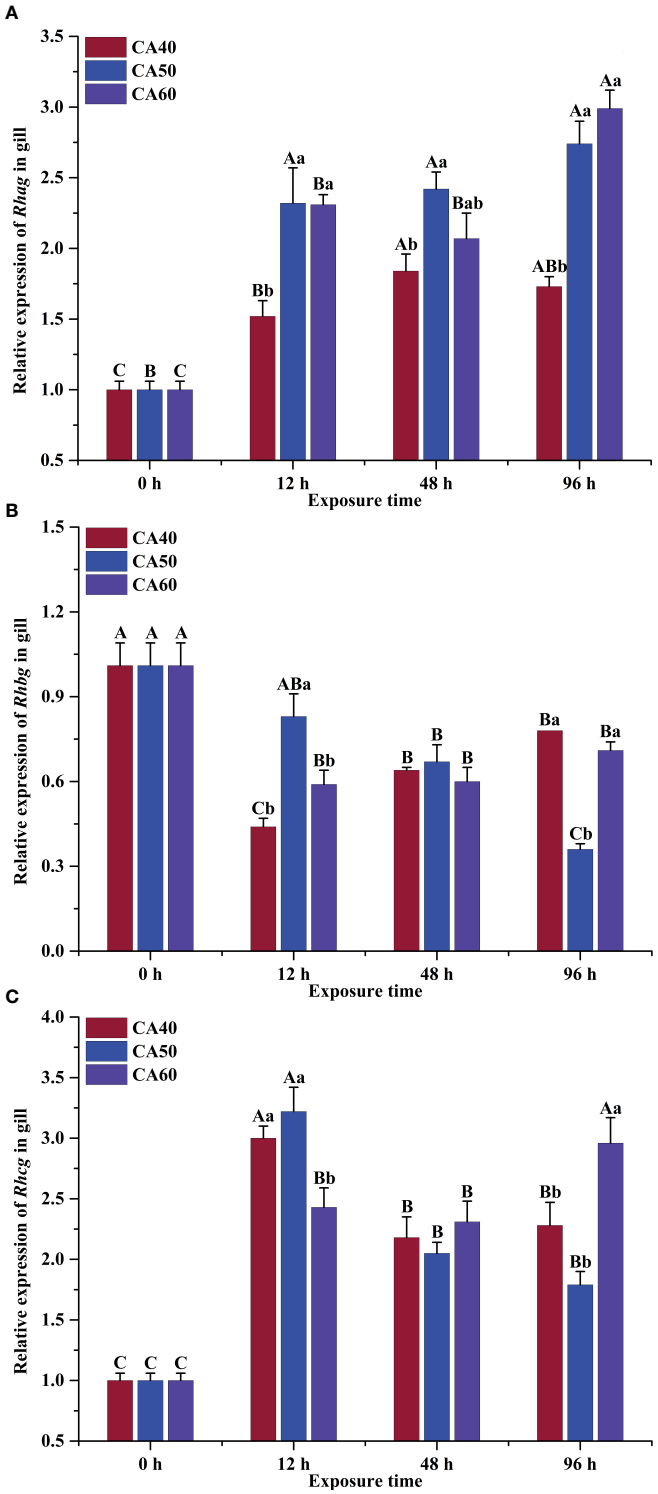

FIGURE 1
Effects of carbonate alkalinity (CA) on the relative expression of *Rhag* **(A)**, *Rhbg* **(B)** and *Rhcg* **(C)** in the gills of large-scale loach. The different capital letters are significant differences among the different exposure times in the same CA concentration. The different lowercase letters are significant differences among the different CA concentrations at the same exposure time. The bars represent the mean ± S.E. (n=3).

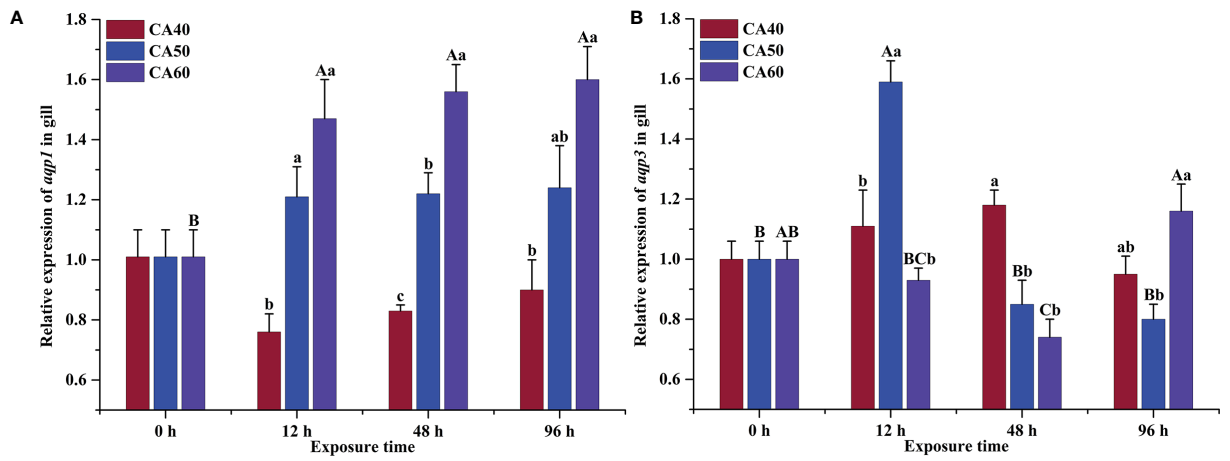

FIGURE 2
Effects of carbonate alkalinity (CA) on the relative expression of *aqp1* **(A)** and *aqp3* **(B)** in the gills of large-scale loach. The different capital letters are significant differences among the different exposure times in the same CA concentration. The different lowercase letters are significant differences among the different CA concentrations at the same exposure time. The bars represent the mean ± S.E. (n = 3).

primarily relevant to ammonia elimination during alkalinity stress. A marked up-regulation of *Rhcg* was observed in the fish after extended alkalinity exposure ($P < 0.05$, Figure 1C), but it was unaffected by alkalinity concentrations ($P > 0.05$, Table 2). Rhcg facilitates the movement of NH_3 across the apical gill membrane and its excretion into surrounding water (Wright and Wood, 2009). Our results suggest that the Rhcg presented on the apical side of the branchial epithelium facilitate NH_3 excretion from the gills, in accordance with observations in *M. salmoides* (Egnew et al., 2019), *Dicentrarchus labrax* (Shrivastava et al., 2019), and *O. mykiss* (Eom et al., 2020).

Changes in Aqps-related genes expression in response to carbonate alkalinity loading

There was a significant interaction between carbonate alkalinity concentration and exposure time on *aqp1* and *aqp3* expression in the gills of large-scale loach (two-way ANOVA, $P < 0.05$, Table 2). Exposure to 60 mmol L^{-1} $NaHCO_3$ induced a significant up-regulation of *aqp1* ($P < 0.05$), while no effect was observed after the various periods of 40 and 50 mmol L^{-1} $NaHCO_3$ exposure ($P > 0.05$, Figure 2A). Moreover, *aqp1* mRNA levels increased obviously as carbonate alkalinity concentration increased (Figure 2A). The physiological function of Aqp1 in teleosts is primarily related to passive water exchange (Deane et al., 2011). Elevated ambient alkalinity resulted in the high transcription of *aqp1* in the gills of large-scale loach to facilitate excretion of excess internal water and to maintain osmotic balance. Similar results were observed in the gills of hybrid tilapia after 24 h of alkaline treatment (Su et al., 2020). By contrast, *aqp3* expression was found to be significantly lower in the gills of large-scale loach after 48 h of 60 mmol L^{-1} $NaHCO_3$ exposure ($P < 0.05$), while expression in 40 and 50 mmol L^{-1} groups were not statistically different from the control ($P > 0.05$, Figure 2B). In addition to affecting water permeabilization, Aqp3 has been shown to transport urea (Cutler et al., 2007). Decreased mRNA transcription of *aqp3* was also reported in *O. mossambicus* during seawater acclimation (Breves et al., 2016). Therefore, the absence of Aqp3 in the gills of large-scale loach may result in the blocking of urea elimination to maintain high internal osmolality.

In summary, branchial ammonia transport-related and osmoregulation-related gene expression in large-scale loach are significantly affected by carbonate alkalinity. The large-scale loach up-regulate *Rhag* and *Rhcg* to facilitate NH_3 efflux from the gills during exposure to high alkalinity. Elevated transcription of *aqp1* in the gills in order to excrete excess internal water, and down-regulation of *aqp3* in order to block urea elimination together maintain appropriate osmolality as an adaptation to alkaline environments.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal Care Committee of Anhui Agricultural University (Hefei, China).

Author contributions

MH, wrote the paper. M-XW, L-JZ, and DM performed the experiments and analyzed the data. Y-LZ, reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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