Characterization, functional analysis, and expression levels of three carbonic anhydrases in response to pH and saline–alkaline stresses in the ridgetail white prawn *Exopalaemon carinicauda*

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Abstract

Carbonate alkalinity, salinity, and pH are three important stress factors for aquatic animals in saline–alkaline water. Carbonic anhydrases (CAs) catalyze the reversible reaction of CO₂ reported to play an important role in the acid–base regulation in vertebrates. To explore the molecular mechanism of CAs efficacy in shrimp after their transfer into saline–alkaline water, the cDNAs of three CAs (*EcCAc*, *EcCag*, and *EcCab*) were cloned from *Exopalaemon carinicauda*. Sequence analysis showed that *EcCAc* and *EcCag* both possessed a conserved α -CA domain and a proton acceptor site, and *EcCab* contained a Pro-CA domain. Tissue expression analysis demonstrated that *EcCAc* and *EcCag* were most abundantly in gills, and *EcCab* was highly expressed in muscle. The cumulative mortalities remained below 25% under exposure to pH (pH 6 and pH 9), low salinity (5 ppt), or high carbonate alkalinity (5 and 10 mmol/L) after 72 h of exposure. However, mortalities increased up to 70% under extreme saline–alkaline stress (salinity 5 ppt, carbonate alkalinity 10 mmol/L, and pH 9) after 14 days of exposure. The *EcCAc* and *EcCag* expressions in gills were significantly upregulated during the early period of pH and saline–alkaline stresses, while the *EcCab* expressions showed no regular or large changes. The two-way ANOVA found significant interactions between salinity and carbonate alkalinity observed in *EcCAc*, *EcCag*, and *EcCab* expressions ($p < 0.05$). Furthermore, an RNA interference experiment resulted in increased mortality of *EcCAc*- and *EcCag*-silenced prawns under saline–alkaline stress. *EcCAc* knockdown reduced expressions of Na⁺/H⁺ exchanger (*EcNHE*) and sodium bicarbonate cotransporter (*EcNBC*), and *EcCag* knockdown reduced *EcCAc*, *EcNHE*, *EcNBC*, and V-type H⁺-ATPase (*EcVTP*) expressions. These results suggest *EcCAc* and *EcCag* as important modulators in response to pH and saline–alkaline stresses in *E. carinicauda*.

Keywords Carbonic anhydrase · *Exopalaemon carinicauda* · pH stress · Saline–alkaline stress · Expression analysis · RNA interference

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Introduction

Large areas of saline–alkaline water are a widespread environmental problem throughout the world. Carbonate alkalinity, salinity, and pH are considered as the three of the major stressors that affect survival, growth, and reproduction of aquatic animals in saline–alkaline water (Lin et al. 2013). CO₃²⁻ and HCO₃⁻ are the major ions in saline–alkaline water, and highly alkaline lake water poses as a challenge where water carbon dioxide tension is very low, which decreases blood carbon dioxide tension levels and creates persistent alkalosis in aquatic animals (Yao et al. 2016). Therefore, understanding the mechanism of animal tolerance to saline–alkaline stress will be beneficial for the exploitation of saline–alkaline regions. The ridgetail

white prawn (*Exopalaemon carinicauda*) is one of the most commercially pond-reared shrimp species and accounts for 30% of the gross production of the polyculture ponds in eastern China (Xu et al. 2010). Apart from the merits of high reproductive performance and fast growth, *E. carinicauda* can also tolerate a wide range of temperatures (2–38 °C with an optimum of 22–26 °C), salinity (4–35 ppt with an optimum of 22–28 ppt), and pH (4.8–10.5 with an optimum of 7.9–8.6) (Liang 2013). Recently, *E. carinicauda* has been successfully cultured and bred in the saline–alkaline ponds at Dongying City, Shandong province (approximate salinity 5–8 ppt, pH 8.5–9.5, carbonate alkalinity 1.4–8.0 mmol/L) and Cangzhou City, Hebei province (approximate salinity 10–25 ppt, pH 8.3–9.2, carbonate alkalinity 3.5–13 mmol/L), China, suggesting that it has a high tolerance to saline–alkaline stress. In summary, *E. carinicauda* may be a valuable species to study the shrimp saline–alkaline adaptation mechanism.

Acid–base and ionic regulation in an aquatic animal are tightly coupled. The ionic regulation system of aquatic animal depends on several osmoregulatory proteins, such as Na⁺/K⁺-ATPase (NKA), carbonic anhydrase (CA), V-type H⁺-ATPase (VTP), sodium bicarbonate cotransporter (NBC), and Na⁺/H⁺ exchanger (NHE) (Pham et al. 2016; Gilmour and Perry 2009; Perry et al. 2003; Cai et al. 2017). Among these, CA is a Zn-metalloenzyme that catalyzes the inter-conversion of CO₂ and HCO₃[−] through the reversible reaction, CO₂ + H₂O $\rightleftharpoons$ H⁺ + HCO₃[−], where H⁺ and HCO₃[−] serve as anti-ports for Na⁺/H⁺ (NH₄⁺) exchangers and Cl[−]/HCO₃[−] cotransporter (Gilmour 2012). Thus, CA plays an important role in pH balance, ion transport, and gas exchange. So far, three forms of CA have been identified in arthropods: cytoplasmic CA (CAc), glycosylphosphatidylinositol-linked CA (CAG), and beta CA (CAB) (Syrjänen et al. 2010). CAc and CAG are both members of the alpha carbonic anhydrase gene family and have an independent origin compared to β-CA, which is a member of the beta carbonic anhydrase gene family (Ali 2016). Previous research suggested that CAc exists in the cytoplasm and provides counter ions (H⁺ and HCO₃[−]) for the Na⁺/H⁺ and Cl[−]/HCO₃[−] exchanger, driving ion uptake in gills. Furthermore, CAG is mainly located on the outer membrane of cells and has been suggested to facilitate the mobilization of hemolymph HCO₃[−] to molecular CO₂ and assists with CO₂ excretion via the gills. However, the available information about the location or function of CAB is very limited (Ali 2016). CAc and CAG have been reported to play a role in the response to pH stress in several crustacean species including *Litopenaeus vannamei*, *Portunus trituberculatus*, and *Cherax quadricarinatus* (Liu et al. 2015; Pan et al. 2016; Ali et al. 2015b). In general, the main CA enzyme that has been shown to respond to pH changes

and that plays a significant role in systematic acid–balance in crustaceans is CAc, while the role of CAG and CAB remains unknown.

Various researchers have studied the functions of CA under either salinity or pH stress in crustaceans, while studies that investigate a combination of saline–alkaline stress have not been published to date (Roy et al. 2007; Pongsomboon et al. 2009; Henry et al. 2002; Pan et al. 2016; Ali et al. 2015c). It is worth noting that the CA was always selected as candidate saline–alkaline tolerant gene in fish in relevant research. Yao et al. (2012) examined genome-wide gene expression profiles of the Japanese medaka in response to carbonate alkalinity stress and found that CA was significantly upregulated. Xu et al. (2013a) generated high-throughput RNA-Seq data from *Leuciscus waleckii* living in a soda lake and a freshwater lake and identified CAs as significantly differentially expressed genes (DEGs). To date, no report has been published on the role of CAs in shrimp exposed to saline–alkaline stress. To investigate the potential contributions of CAs in response to saline–alkaline stress, three full-length cDNA sequences of CAs in *E. carinicauda* (*EcCAc*, *EcCAG*, and *EcCAB*) were cloned; their tissue distribution and expression levels under pH and saline–alkaline stresses were examined, and then their function was determined by RNA interference (RNAi). In addition, the effects of pH and saline–alkaline exposure on the mortality were assessed. The obtained results further enhance our understanding of the response mechanism of *E. carinicauda* to pH stress and saline–alkaline stimulation.

Materials and methods

Animal and experimental design

E. carinicauda healthy adults (body length 3.7 ± 0.5 cm, body weight 1.32 ± 0.30 g) were obtained from a commercial farm in Qingdao, China, and acclimated in 200 L polyvinyl chloride polymer (PVC) tanks containing aerated and sand-filtrated seawater (salinity 30 ppt, pH 8.0 ± 0.2, carbonate alkalinity 1 mmol/L) at 23 ± 2 °C for 2 weeks before the start of the experiment. During the acclimation period, all prawns were fed regularly with formulated feed pellets and two-thirds of water each tank was renewed daily.

Prawns for the pH stress experiment A total of 360 prawns—were placed in four pH treatments (pH of 6, 7, 8, and 9). Each treatment consisted of two branches; one branch included three replicates with 10 individuals per replicate and was used to monitor mortality, while the other branches included three replicates with 20 individuals per replicate, which were used to sample. pH 8 was the control group, and pH 6, pH 7, and pH 9 were prepared by adjusting natural seawater with either 1 M HCl or 1 M NaOH. The pH of each treatment was

adjusted every 2 h to maintain stability and was monitored using a pH electrode (METTLER TOLEDO SG2, Schwerzenbach, Switzerland). The cumulative mortality was calculated at 0, 6, 12, 24, 48, and 72 h by the following formula: $M(\%) = [D_1 + D_2 + \dots + D_t] / Nt \times 100$, where M , D , t , and N indicates the cumulative mortality, the number of deaths, the sampling time, and the total number of prawns in each parallel, respectively (Ge et al. 2015). Gills from three prawns (one was taken randomly per replicate tank) in each corresponding treatment were sampled at 0, 6, 12, 24, 48, and 72 h.

Prawns for the saline–alkaline stress experiment A total of 540 prawns were placed in six saline–alkaline treatments including two salinity levels (5 ppt and 30 ppt) and three carbonate alkalinity levels (1, 5, and 10 mmol/L), named S5C1, S5C5, S5C10, S30C1, S30C5, and S30C10, respectively. S30C1 was used as the control group. The design of salinities and carbonate alkalinities was based on the actual farming process of *E. carinicauda* in saline–alkaline ponds. Each treatment contained two branches; one branch included three replicates with ten individuals per replicate and was used to monitor mortality, while the other branches included three replicates with 20 individuals per replicate and were used to sample. Salinity of 5 ppt was gradually achieved by adding fresh water to seawater and was continually measured by salinity meter (YSI model 30, Yellow Springs, OH, USA). Carbonate alkalinities of 5 and 10 mmol/L were adjusted using NaHCO_3 and Na_2CO_3 and measured by an acid-based titration method. During the whole experiment, the water pH in S5C5, S5C10, S30C5, and S30C10 groups was monitored using a pH electrode (METTLER TOLEDO SG2, Schwerzenbach, Switzerland) and maintained at about 8.8, 9.0, 8.5, and 8.6, respectively. The cumulative mortality was calculated by the same method above. Gills from three prawns (one was taken randomly per replicate tank) in each treatment were sampled at 12 h, 1 d, 3 d, 7 d, and 14 d.

Cloning the full-length cDNA of *EcCAc*, *EcCAg*, and *EcCAb*

The RACE cDNA was synthesized from gills RNA isolation referring to the method of the SMARTTM RACE cDNA amplification kit (Clontech, USA) and stored at -20°C . The RACE primers (Table 1) were designed to extend the partial cDNA sequences of *EcCAc*, *EcCAg*, and *EcCAb* based on comparative transcriptomic sequencing of *E. carinicauda* (SRA accession number: SRP096646). The 5'/3' RACE PCR reaction was performed with the RACE primers and the anchor primer UPM according to manufacturer instruction. For 3' RACE of *EcCAc* and *EcCAb*, PCR was performed initially with primers *EcCAc*-3'RACE, *EcCAb*-3'RACE, and UPM (Table 1), followed by nested PCR with *EcCAc*-n3' RACE, *EcCAb*-n3'RACE, and nested universal primer

(NUP). The PCR products were separated on 1% agarose gel, and then purified with a PCR purification kit (Tiangen, Beijing, China). The purified PCR products were ligated into pEASY-T1 vectors (TransGen Biotech, Beijing, China), and transformed into Trans1-T1 Phage Resistant Chemically Competent cells. Randomly selected positive clones were sequenced (Sangon Biotech, Shanghai, China).

Sequence analysis

The ExPASy Translate tool (www.expasy.ch/tools/dna.html) was used to predict the amino acid sequences, protein molecular weight, and an isoelectric point of *EcCAc*, *EcCAg*, and *EcCAb*. To identify sequences and similarities, the predicted amino acid sequences were checked against the NCBI database in the GenBank using the blastP programs. The SMART (smart.embl-heidelberg.de) platforms were used to identify the conserved domains and active sites. The signal peptide and motif were predicted by SignalP 3.0 program (<http://www.cbs.dtu.dk/services/SignalP/>). The N-glycosylation sites were predicted by the NetNGlyc server (<http://www.cbs.dtu.dk/services/NetNGlyc/>). A multiple sequence alignment of amino acid sequences was performed using the programs of DNAMAN, and phylogenetic analyses of CAs were made by MEGA 6.0 software using the neighbor-joining method (Arizona State University, USA).

Tissue expressions of the three CA genes

Various tissues including gill, antennary gland, hemocytes, hepatopancreas, muscle, intestine, stomach, and heart from *E. carinicauda* were used to detect the distribution of three CAs. The methods of RNA isolation, cDNA synthesis, and quantitative real-time RT-PCR (qRT-PCR) assay were based on our previous studies (Ge et al. 2015; Ge et al. 2016). The expression levels of *EcCAc*, *EcCAg*, and *EcCAb* in these tissues were detected by qRT-PCR (Livak and Schmittgen 2001; Ge et al. 2016).

Survival rate assay and expression levels of osmoregulatory genes in dsRNA injected prawn

To investigate the function of *EcCAc*, *EcCAg*, and *EcCAb* under saline–alkaline stress, RNAi was used to perform gene silencing. Sequence-specific primers linked to the T7 promoter at the 5' terminus were designed using Snap Dragon tools (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl) (Table 1) and were used to amplify the partial cDNA as templates for dsRNA synthesis. DsRNAs were synthesized in vitro with a Transcription T7 Kit (TaKaRa, Japan) following the manufacturer's protocols. The dsRNA was purified by phenol/chloroform extraction and then ethanol precipitation, dried, and dissolved in RNase-free water. DsRNA of GFP synthesized in the same way was used as the control.

Table 1 Primers used in this study

Primer	Primer sequence (5'-3')	Purpose	Amplicon size (bp)
<i>EcCAc</i>	ATACGCCAAAGGAAACGGAC AGCATTCGCTTGGGTGGTAG		691
<i>EcCAg</i>	GACTTACACTGGACAACACGGC TTTCGCCTGAGACCAAGACC	For partial cDNA sequences	763
<i>EcCAb</i>	CAAAGGCGGTATTTTTCACA ATACTCGCCCTCGCACTAAT		520
<i>EcCAc</i> -5'	TAGCATTCGGCTTGGGTGGTAGGACTT	For RACE	788
<i>EcCAc</i> -3'	ATCAAATGCCGAGGATGCTGTGAGGACT		925
<i>EcCAc</i> -n3'	GACAGGAGCACGATGAAATGAACAAGAT		576
<i>EcCAg</i> -5'	TTTCTCAGACACTGGGATGGCGTTAGCG		816
<i>EcCAg</i> -3'	GGGCTCTTATGGAACCTTGGGAAATGCT		1944
<i>EcCAb</i> -5'	GCCCTCGCACTAATCCATCTCGCAT		754
<i>EcCAb</i> -3'	CAGCCGTATGTTACCCACAAGATTAC		1056
<i>EcCAb</i> -n3'	TAGAAATGCTGGTAACTTGGTCCCTCA		999
UPM	CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT		
NUP	AAGCAGTGGTATCAACGCAGAGT		
<i>qEcCAc</i>	GAAGCTGAACCGTCTCCAAG GGACCTCCAGTTAGGCATGA	For qRT-PCR	116
<i>qEcCAg</i>	AACGAAGTCGTCACCTGGAC GTGGACGGAAGTTGTTGACC		120
<i>qEcCAb</i>	TGGTCACTCAGATTGCAAGG TAGATGAATGCCCATGACGA		124
<i>qEcNHE</i>	GCGGTCTTTGAAGAAATCCA CTATTTTCGCTGAACCCCTTCG		120
<i>qEcNBC</i>	GAGGACAGATCCCGAAACAA GCCGCCATTCATTTCTACAT		119
<i>qEcVTP</i>	TCGAACACGAAGTCAAGCTG ATACACTCGGCGATCCAGAC		123
<i>18 s</i>	TATACGCTAGTGGAGCTGGAA GGGGAGGTAGTGACGAAAAAT		147
GFP-T7	GATCACTAATACGACTCACTATAGGGATGGT GAGCAAGGGGGAGGA GATCACTAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA	For RNAi	720
<i>EcCAc</i> -T7	GATCACTAATACGACTCACTATAGGGGAAGCTGAACCGTCTCCA AG GATCACTAATACGACTCACTATAGGGATTTCATCGTGCTCCTGTCC		338
<i>EcCAg</i> -T7	GATCACTAATACGACTCACTATAGGGTTTGGCTGTGTTGGGTGTTA GATCACTAATACGACTCACTATAGGGCATGTTTCCTCCTTGGTCTGT		300
<i>EcCAb</i> -T7	GATCACTAATACGACTCACTATAGGGTCGTCATGGGCATTCATCTA GATCACTAATACGACTCACTATAGGGGAAGTGGACAGCCATGGAAT		521

In dsRNA-mediated RNAi experiments, 36 healthy prawns (1.08 ± 0.15 g) were intramuscularly injected with each target dsRNA dissolved in PBS (2 µg/g prawn) and the control prawns were injected with the same volume dsGFP. The total RNA from gills of three prawns in each group was extracted at 24 h and 48 h after dsRNA injection. The qRT-PCR was performed to analyze the RNAi efficiency of each *EcCA*. The expression levels of osmoregulatory genes including *EcCAs*, *EcNHE*, *EcNBC*, and *EcVTPd* were detected in each group. Meanwhile, to determine the influence of each dsRNA

on survival for *E. carinicauda* under saline–alkaline stress, the survival rates in each treatment group with three replicates after saline–alkaline (salinity 5 ppt and carbonate alkalinity 10 mmol/L) stress were recorded at 0, 6, 12, 18, 24, and 48 h.

Quantitative analysis of gene expression

qRT-PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) to investigate the *EcCAc*, *EcCAg*, and *EcCAb* mRNA expression patterns. The

primers of the target genes were shown in Table 1. The 18S rRNA gene of *E. carinicauda* (GenBank accession number: HQ172894.1) was used as an internal control to verify the qRT-PCR reaction. The efficiency of qRT-PCR was in the optimal range of 90–110% (slope of standard curves 3.1–3.6) for all of the primer pairs used. The qRT-PCR amplifications were carried out in triplicate in a total volume of 20 μ L containing 10 μ L SYBR® Premix Ex Taq™ II (TaKaRa), 2 μ L of the cDNA template, 0.8 μ L each of forward and reverse primer (10 mmol L⁻¹), 0.4 μ L ROX Reference Dye II (50 $\times$), and 6.0 μ L DEPC-treated water. The PCR program was 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 34 s, followed by 1 cycles of 95 °C 15 s, 60 °C for 1 min and 95 °C for 15 s. The relative expression levels of CA genes were calculated according to the $2^{-\Delta\Delta CT}$ method. All samples for qRT-PCR analysis were replicated in triplicate.

Data analysis

All data were presented as mean $\pm$ SE. Statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL, USA). The two-way analysis of variance (ANOVA) was used to determine the main effects of salinity, carbonate alkalinity, and their interactions on the expressions of *EcCAc*, *EcCag*, and *EcCab*. If significant interaction between salinity and carbonate alkalinity levels was observed, data were analyzed by the one-way ANOVA followed by the Student–Newman–Keuls test to find the significant difference. The expression data in different tissues and pH treatments were analyzed with one-way ANOVA. The Student–Newman–Keuls test was used to determine significant difference among the treatments. In the RNAi experiments (RNAi efficiency and expression data of osmoregulatory genes), there were only two treatments (PBS and dsRNA injection), therefore the independent samples *t* test was performed instead of post hoc test. All tests used a significance level of $p < 0.05$.

Results

Effects of pH and saline–alkaline stresses on mortality

The prawns placed in pH 7 and pH 8 water for 72 h showed no mortality. Deaths occurred at 48 h and 12 h under pH 6 and pH 9 stress, respectively. In total, 3.33 and 23.33% of prawns died in the pH 6 and pH 9 treatments at 72 h, respectively (Fig. 1a).

In the normal salinity treatments, no mortality was found in the S30C5 group and only a mortality of 16.7% was found in the S30C10 group. Under salinity 5 ppt stress, the cumulative mortality was increased with the carbonate alkalinity and the cumulative mortalities of S5C1, S5C5, and S5C10 groups on

the 14th day were 13.3%, 33.3%, and 66.7%, respectively (Fig. 1b).

cDNA cloning and sequence characterization of *EcCAc*, *EcCag*, and *EcCab*

The full-length cDNA sequences of *EcCAc*, *EcCag*, and *EcCab* were obtained and submitted to GenBank (Accession number: MF403002, MF403003, MF403004). The open reading frames (ORF) of *EcCAc*, *EcCag*, and *EcCab* consisted of 810, 926, 774 nucleotides, encoding 269, 308, 257 amino acids, respectively. A signal peptide and a putative N-linked glycosylation site are present in the *EcCag* protein sequence. The deduced *EcCAc* and *EcCag* proteins both contained an alpha carbonic anhydrase domain, an alpha carbonic anhydrases signature, a proton acceptor site, three zinc 1 binding sites (histidine amino acid residues), and three zinc 2 binding sites (Fig. S1A and Fig. S1B). The predicted *EcCab* protein contained a Pro-CA domain and four amino acid residues involved in zinc binding (Fig. S1C). Multiple alignment of the *EcCAc* with the orthologue genes in other crustaceans, arthropods, and human showed that three histidine amino acid residues for the zinc cofactor binding were conserved in all the aligned sequences. The proton acceptor site in most species was histidine residues, but replaced by Ser in *EcCAc* (Fig. S2A). Multiple alignment of the *EcCag* with the homologous genes in other crustaceans, amphipod, bivalvia, and arthropod showed that the proton acceptor site and three histidine residues for the zinc cofactor binding were conserved in all the aligned sequences (Fig. S2B). Multiple alignment of the *EcCab* with the homologous genes in other crustaceans and arthropods showed that the four amino acid residues involved in zinc binding were conserved as amino acid residues associated with the hydrogen bond around the active site (Fig. S2C).

Phylogenetic analysis of carbonic anhydrases

By phylogenetic analysis (as shown in Fig. 2), the CAs could be classified into three branches. The deduced amino acid of *EcCAc* was in the same sub-group as the CAc and CAI from decapod crustaceans and clustered with the arthropod CAs and the human CA1 and CA2. The deduced amino acid of *EcCag* was resolved in well-supported clade that contained GPI-anchored CA from decapod crustaceans and CAs from arthropods. The *EcCab* was sister to the beta CA clade from decapod crustaceans and aligned with other CAs from arthropods. The branch of *EcCAc* shared a phylogenetically close relationship with the branch of *EcCag*, while *EcCab* revealed a long evolutionary distance from *EcCAc* and *EcCag*.

Fig. 1 Cumulative mortality rates of prawns under pH stress (a) and saline-alkaline stress (b). Each bar represents the mean value from three repetitions with standard error (SE)

Tissue distribution of *EcCac*, *EcCag*, and *EcCab*

EcCac, *EcCag*, and *EcCab* were expressed in all tested tissues of *E. carinicauda*. It was detected that *EcCac* mRNA expression was mostly found in gill, intestine, and heart (Fig. 3a). *EcCag* mRNA was extremely abundant in gill compared with the other tissues ($p < 0.05$) (Fig. 3b). *EcCag* mRNA expression was much higher in muscle, hemocytes, and heart (Fig. 3c).

EcCac, *EcCag*, and *EcCab* expressions under pH and saline-alkaline stresses

The expression profiles of the three *EcCAs* in gills were detected in response to different pH stress. The *EcCac* expression was upregulated at all sampled time points in pH 6 and pH 9 treatments, and at 12 h in the pH 7 treatment (Fig. 4a). The *EcCag* expression was upregulated throughout the experiment time in the pH 6 treatment and only at 6 h in the pH 7 treatment. However, the expression was upregulated at 6–24 h and then recovered to the normal level after 48 h in the pH 9 treatment (Fig. 4b). Compared with the pH 8 treatment, the *EcCab* expression only showed slight fluctuation at 6 h and 24 h in the pH 6 treatment and at 12 h in the pH 9 treatment, while it showed no significant difference in the pH 7 treatment (Fig. 4c).

The two-way ANOVA showed that increasing salinity and carbonate alkalinity levels significantly influenced *EcCac*, *EcCag*, and *EcCab* expressions ($p < 0.05$) and the significant interactions between salinity and carbonate alkalinity were observed in the expressions of *EcCac*, *EcCag*, and *EcCab* at each sampling time point ($p < 0.05$) (Table 2). In the salinity 5 ppt treatments, the *EcCac* expression was significantly upregulated under different carbonate alkalinity stress on days 1, 3, 7, and 14 ($p < 0.05$) and reached to a maximum at 3 d. In the normal salinity treatments, the *EcCac* expression was

upregulated during the first 3 days and then gradually recovered to the normal level under different carbonate alkalinity stresses (Fig. 5a). The low salinity and high carbonate alkalinity could both induce the upregulated expression of *EcCac*. In the 5 ppt salinity treatments, the *EcCag* expression was significantly upregulated after 3 days under different carbonate alkalinity stress compared with the control group ($p < 0.05$). In the normal salinity treatments, the *EcCag* expression was upregulated during the first 3 days and then recovered to the normal level in the S30C5 group. In the S30C10 group, the *EcCag* expression was upregulated during the first day and was then significantly downregulated ($p < 0.05$) (Fig. 5b). During the whole experiment period, there were no regular or large changes of *EcCab* expressions in the six groups (Fig. 5c).

Survival rates and expression levels of osmoregulatory genes in dsRNA injected prawn

Compared with the GFP group, prawns receiving *EcCac*, *EcCag*, and *EcCab* dsRNAs efficiently suppressed the transcript levels of each target gene at both 24 and 48 h after injection (Fig. S3). It indicated that *EcCac*, *EcCag*, and *EcCab* were knocked down successfully in *E. carinicauda*.

Furthermore, to learn the function of *EcCac*, *EcCag*, and *EcCab* during saline-alkaline stress, the effect of each gene silencing on the survival rate of prawns was also determined. After 48 h of saline-alkaline stress, the survival rate of control prawns injected with GFP dsRNA was 63.3%, prawns injected with dsRNA of *EcCab* showed no significant difference ($p > 0.05$), whereas prawns injected with dsRNA of *EcCac* and *EcCag* showed survival rate of 20% and 30%, respectively, which was statistically significant compared with the control group ($p < 0.05$) (Fig. S4).

The expression levels of other osmoregulatory genes in dsRNA injected prawns were investigated by qRT-PCR.

Fig. 2 Phylogenetic analysis of CAs in various species based on neighbor-joining method. The species with the GenBank accession numbers are *Aedes albopictus* CAc (XP_019541540.1), *Anopheles aquasalis* CAc (JAA98551.1), *Anopheles gambiae* CAc (ABF66618.1), *Bemisia tabaci* CA13 (XP_018906696.1), *Callinectes sapidus* CAc (ABN51213.1), *Cherax cainii* CA (AJO70180.1), *Cherax destructor* CA (AJO69996.1), *Cherax quadricarinatus* CAc (AIW68600.1), *Drosophila rhopaloea* CA (XP_016989668.1), *Homo sapiens* CA1 (NP_001729.1), *Homo sapiens* CA2 (NP_000058.1), *Limulus polyphemus* CA2 (XP_013785730.1), *Litopenaeus vannamei* CAI (ADM16544.2), *Panopeus monodon* CAI (ABV65904.1), *Portunus trituberculatus* CAc (AFV46144.1), *Tribolium castaneum* CA1 (XP_974322.1); *Anolis carolinensis* CA15 (XP_008115548.1), *Branchiostoma belcheri* CA (XP_019637467.1), *Callinectes sapidus* CAg (ABN51214.1), *Carcinus maenas* CAg (ABX71209.1), *Cherax cainii* CAg (AJO70181.1), *Cherax destructor* CAg (AJO69997.1), *Crassostrea gigas* CA (XP_

019930779.1), *Daphnia magna* α -CA (KZS12866.1), *Hadrurus spadix* CA1 (JAV48261.1), *Halocaridina rubra* CAg (AIM43573.1), *Hyalomma azteca* CA2 (XP_018027123.1), *Limulus polyphemus* CA1 (XP_013777310.1), *Litopenaeus vannamei* CAg (AGC70493.1), *Monopterus albus* CA4 (XP_020456274.1), *Patella vulgata* CA (CCJ09593.1), *Portunus trituberculatus* CAg (AFV46145.1); *Camponotus floridanus* CAb1 (XP_011261355.1), *Cherax cainii* CAb (AJO70182.1), *Cherax destructor* CAb (AJO69999.1), *Cherax quadricarinatus* CAb (AIW68602.1), *Culex quinquefasciatus* CA (XP_001849341.1), *Daphnia magna* CAb (JAN40855.1), *Drosophila rhopaloea* CAb1 (XP_016978326.1), *Fopius arisanus* CAb1 (XP_011300350.1), *Hyalomma azteca* CAb1 (XP_018006429.1), *Nasonia vitripennis* CAb1 (XP_001606972.1), *Zootermopsis nevadensis* CAb1 (KDR11644.1). The numbers at the forks indicate the bootstrap proportions

After injection of *EcCac* dsRNA, the expression of *EcNHE* and *EcNBC* decreased significantly ($p < 0.05$) compared with the GFP group, while other genes showed no significant change ($p < 0.05$). After injection of *EcCag* dsRNA, the expression of all four osmoregulatory genes including *EcCac*, *EcNHE*, *EcNBC*, and *EcVTP* decreased significantly ($p < 0.05$) (Fig. 6).

Discussion

To date, studies on the function of CAs in crustaceans, especially on CAb, have been limited. In order to preliminarily elucidate what roles different CAs play in acid–base regulation of *E. carinicauda*, the full-length cDNA sequences of three CAs were cloned in the present study, and their sequence

structures, phylogenetic status, tissue distribution, and expression profiles under pH and saline–alkaline stresses were comparatively analyzed.

Sequence analysis showed that *EcCag* contains a signal peptide with 19 amino acid residues in the N-terminal, while neither *EcCac* nor *EcCab* does contain a signal peptide. This may imply that CAg is secreted to the periplasm and extracellular medium or combined with the cell membrane, while CAC and CAb are not a secreted form of CA and may distribute in the cytoplasm (Serrano et al. 2007; Muesch et al. 1990). The N-linked glycosylation site indicates *E. carinicauda EcCag* as a glycoprotein. With regard to the functional domains, *EcCac* and *EcCag* both have the representative structure of an alpha carbonic anhydrase domain and an alpha carbonic anhydrases signature, indicating that both *EcCac* and *EcCag* are the alpha CA type, and not identical to *EcCab*. The result of multiple

Fig. 3 Relative expression levels of *EcCac* (a), *EcCag* (b), and *EcCab* (c) in different tissues of *E. carinicauda*. Vertical bars represent the mean $\pm$ SD from three prawns ($n = 3$). Different lowercase letters meant significant differences ($p < 0.05$)

sequence alignment indicates that three histidine amino acid residues were highly conserved in *EcCac* and *EcCag*. The same patterns of *EcCac* and *EcCag* have been previously reported in various species such as *P. monodon* (Pongsomboon et al. 2009), *L. vannamei* (Liu et al. 2015),

P. trituberculatus (Pan et al. 2016), and in humans (Frost and McKenna 2013). These His amino acid residues have been suggested supposedly to constitute zinc binding site 1, which directly binds to the zinc ions (Christianson and Fierke 1996). Although the position of His shows some differences, such as

Fig. 4 Relative expression levels of *EcCac* (a), *EcCag* (b), and *EcCab* (c) in response to pH stress in *E. carinicauda*. At every time point of pH stress, different lowercase letters indicated significant differences ($p < 0.05$) among different pH treatments

Table 2 The two-way ANOVA of *EcCAc*, *EcCag*, and *EcCab* expression under saline–alkaline stress with two different levels salinity and three different levels of carbonate alkalinity

		<i>EcCAc</i> expression			<i>EcCag</i> expression			<i>EcCab</i> expression		
		S	C	S × C	S	C	S × C	S	C	S × C
12 h	F	764.284	419.848	606.642	797.416	128.881	105.297	32.586	1.056	9.988
	P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.378	0.003
1d	F	0.016	59.277	77.142	69.045	25.363	185.789	20.689	23.792	5.002
	P	0.903	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.026
3d	F	871.123	85.804	54.355	445.828	36.287	48.216	270.227	21.750	65.658
	P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7d	F	117.461	64.553	12.603	341.904	15.395	39.381	66.061	16.485	7.230
	P	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.009
14d	F	817.639	12.056	15.546	5665	568.564	266.461	66.357	4.403	42.473
	P	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.037	0.000

S indicated salinity, C indicated carbonate alkalinity, S × C indicated interactive effect of salinity and carbonate alkalinity

EcCAc and *PtCAc*, the distance between them is identical, which is possibly a necessity for space fold (Liu et al. 2015). In addition, the *EcCAc* and *EcCag* both contain a proton acceptor site, which was also found to be conserved with His64 in human CA-II and acts as a proton shuttle in CO₂ hydration in high activity CAs (Supuran et al. 2003), while the proton acceptor site His residues of CAs in most species including *EcCag* replaced by Ser in *EcCAc* may indicate the different CA activity of *EcCAc*.

To evaluate the molecular evolutionary relationships between *EcCAc*, *EcCag*, and *EcCab*, and similar proteins of other species, a phylogenetic tree was constructed. As it can be seen, *Cac* and *Cag* form two different sub-groups rather than forming two genes via gene variability of shear. The *EcCab*-containing sub-group is far away from the sub-groups *EcCAc* and *EcCag*. This matches previous research indicating that *Cac* and *Cag* are both members of the alpha carbonic anhydrase gene family while *Cab* is a member of the beta carbonic anhydrase gene family in *C. quadricarinatus* (Ali 2016). The phylogenetic tree shows that strictly evolutionary relationships exist in CA, which matches findings reported for other arthropods.

Gill is an important osmoregulatory and acid/base regulatory organ in crustaceans (Supuran et al. 2003; Martinez et al. 2005; Santos et al. 2007), and many pH and salinity regulatory genes were found to express in gill at high levels (Cai et al. 2017; Henry et al. 2012; Pan et al. 2016). Thus, the high expression of *EcCAc* and *EcCag* in gills indicates that *EcCAc* and *EcCag* were probably related to the osmoregulatory and acid-base regulation. Low expression level of *EcCab* in the gills indicates that the β isoform of CA identified here may not play an important role in acid–base balance in *E. carinicauda*. In fact, recent studies have shown that the homologous β -CA protein from *Drosophila* is a highly active mitochondrial enzyme (Syrjanen et al. 2010).

pH, salinity, and carbonate alkalinity have significantly influence crustaceans. In particular, many researchers reported the effects of pH stress on shrimp survival. Wang et al. (2002) observed that when placed in pH 6.0 and pH 8.5 environments, *Penaeus chinensis* showed 34.5% and 28.5% mortality after 14 days, respectively. Zhou et al. (2009) indicated that the mortalities of *L. vannamei* placed in pH 5.6 and pH 9.3 environments were 35% and 65% after 24 h. This

Fig. 5 Relative expression levels of *EcCAc* (a), *EcCag* (b), and *EcCab* (c) in response to saline–alkaline stress in *E. carinicauda*. Different lowercase letters indicate significant differences ($p < 0.05$) among different saline–alkaline treatments at the same time point

Fig. 6 Effect of prawns receiving *EcCac* and *EcCag* dsRNA on the expression levels of osmoregulatory genes. Prawns injected with GFP dsRNA was set as control group. Significant difference relative to GFP control was indicated with asterisk symbol ($p < 0.05$) at the same time point

present study shows that the mortality of *E. carinicauda* was relatively low under pH 6 and pH 9 stresses, indicating that this species may have good tolerance to acidic and alkaline conditions. In addition, for *L. vannamei*, the survival rate was significantly affected when the water pH exceeded 9.0 and the water carbonate alkalinity was 3 mmol/L. With increasing water carbonate alkalinity, the survival rate decreased and the tolerance ability decreased significantly with increasing water pH (Yao et al. 2010). The mortality of *E. carinicauda* increased under high carbonate alkalinity combined with low salinity, while most prawns survived under high carbonate alkalinity combined with normal salinity. This indicates that *E. carinicauda* can tolerate high carbonate alkalinity when in its suitable salinity range. These results provide evidence that *E. carinicauda* is a potential species for farming in low saline–alkaline ponds.

In this study, both the *EcCac* and *EcCag* expressions in gills were significantly upregulated throughout the experiment under pH 6 and pH 9 stresses. Expressions showed little to no change under pH 7 or pH 8 conditions, while the *EcCab* expressions showed smaller variations in the four tested pH groups. This suggests that *EcCac* and *EcCag* may play a more important role in response to sharp pH stress than *EcCab*. This difference in the expression of *Cac*, *Cag*, and *Cab* transcripts is likely attributed to phylogenetic differentiation with distinct osmoregulatory domains and binding sites in both *Cac* and *Cag*. Previous studies reported increased levels of *EcCac* and *EcCag* expression in crustaceans under pH stress. For example, Liu et al. (2015) reported that *Cac* and *Cag* expressions in *L. vannamei* increased at 48 h and 12 h respectively after pH 7.4 and pH 9.0 challenge. Pan et al. (2016) found that *Cag* expression in *P. trituberculatus* increased significantly at 12–48 h after pH 7.4 and pH 9.0 challenge. Among *Cac*, *Cag*, *Cab*, *VTP*,

and arginine kinase, Ali et al. (2015a) found that only the *Cac* gene in *C. quadricarinatus* showed significant differences in expression across pH 6, pH 7, and pH 8 treatments, while other *CA* genes likely only play a limited role.

In euryhaline crustaceans, the expression of osmoregulatory genes typically increases dramatically in the gills during comparable salinity transfers (Havird et al. 2013). For example, *Cag* and *Cac* have been reported to show high expression levels in *Callinectes sapidus* and *Carcinus maenas* after exposure to salinity stress (Serrano et al. 2007; Serrano and Henry 2008). In this study, *EcCac* and *EcCag* expressions increased significantly under 5 ppt salinity stress, suggesting that *EcCac* and *EcCag* may play important roles in the response to low salinity stress in *E. carinicauda*. Following this gene expression change, the synthesis of the *CA* enzyme increased to adapt to the salinity fluctuation induced by the involvement in osmoregulation (Henry et al. 2003). During the early stage of carbonate alkalinity stress, the expressions of *EcCac* and *EcCag* were upregulated, which demonstrated that both *EcCac* and *EcCag* may be involved in mediating alkaline stress in *E. carinicauda*. Furthermore, Xu et al. (2013a) and Xu et al. (2013b) identified five carbonic anhydrase genes that showed strong positive selection under severe alkaline-saline stress, which were also expressed differentially under alkaline water and fresh water conditions. At a later stress stage, *EcCac* mRNA expressions recovered to the normal level, while *EcCag* mRNA expressions were suppressed in the prawns, suggesting that *E. carinicauda* may decrease its *Cag* expression to balance for a respiratory alkalosis and to aid in osmoregulation during the transition from normal seawater to a saline–alkaline pond (Yao et al. 2016). In addition, significant interactions between salinity and carbonate alkalinity were observed in *EcCac* and *EcCag* expressions using the two-way ANOVA ($p < 0.05$). The highest significant

increases of *EcCAc* and *EcCag* expressions were observed in response to the increase of carbonate alkalinity in the salinity 5 ppt treatment. This indicated that salinity and carbonate alkalinity seem to have a common induction to *EcCAc* and *EcCag* expression.

To confirm the biological function of *EcCAc*, *EcCag*, and *EcCab* involved in saline–alkaline regulation, an RNAi experiment was conducted. The survival rates of *EcCAc*- and *EcCag*-silenced prawns were significantly higher than that of both control prawns and *EcCab*-silenced prawns, suggesting that *EcCAc* and *EcCag* can contribute to the adaption to a saline–alkaline environment in *E. carinicauda*. This result is highly consistent with the expression profiles of *EcCAc*, *EcCag*, and *EcCab* under saline–alkaline stress. To further investigate the effects of *EcCAc* or *EcCag* knockdown in the shrimp acid/base homeostasis, the expression levels of several major osmoregulatory genes were observed. Knockdown of *EcCAc* resulted in a reduction of the expressions of *EcNHE* and *EcNBC*, while knockdown of *EcCag* resulted in a reduction of the expression of *EcCAc*, *EcNHE*, *EcNBC*, and *EcVTP*. This indicates that *EcCAc* and *EcCag* regulate the expression of different osmoregulatory genes. The results of sequence analysis indicate that *CAc* exists in the cytoplasm and *Cag* is distributed in the periplasm and extracellular medium or combined with the cell membrane. Previous research has shown that cytoplasmic *CAII* in mammals could interact with numerous transport proteins of the membrane such as anion exchanger, *NBC*, or *NHE* to regulate intracellular homeostasis (Esbaugh and Tufts 2006; Vince and Reithmeier 2000). This may be similar to *CAc* in shrimp. *Cag* is mainly located on the outer membrane of cells and has been suggested to facilitate mobilization of hemolymph HCO_3^- to molecular CO_2 and to assist with CO_2 excretion via the gills of the blue crab (Henry 1987). Similarly, Wang et al. (2017) reported that *CAII-1* in *Crassostrea gigas* was detected on the membrane of hemocytes after CO_2 exposure in an immunohistochemical assay. This indicates that *CgCAII-1* might interact with membrane proteins, which further indicates that the *CA* located in the membrane may perform different functions with *CA* exists in the cytoplasm by regulating different osmoregulatory genes. All of these studies indicate a relationship between *EcCAc/EcCag* and other osmoregulatory genes; however, the mechanism of *EcCAc* or *EcCag* regulation in osmoregulation remains unknown and requires further research.

Conclusions

In conclusion, the full-length cDNAs of three *CAs* were cloned in *E. carinicauda*. Three *CAs* were found to have different sequence structures, and tissue expression analyses suggested different functions of the three *CAs*. The

low mortality under pH, salinity, or carbonate alkalinity stress suggests that this species may achieve good adaption in saline–alkaline water after domestication. Expression levels of the *EcCAc* and *EcCag* were upregulated under pH and saline–alkaline stresses, while *EcCab* did not show strong changes. The two-way ANOVA found significant interactions between salinity and carbonate alkalinity in *EcCAc*, *EcCag*, and *EcCab* expressions. Moreover, an RNA interference experiment resulted in increased mortality of *EcCAc*- and *EcCag*-silenced prawns under saline–alkaline stress and reduced in the expression levels of different osmoregulatory genes. Therefore, it can be concluded that *EcCAc* and *EcCag* were involved in the response to pH and saline–alkaline stresses in *E. carinicauda*.

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Authors' contributions Qianqian Ge and Jian Li conceived and designed the experiments; Qianqian Ge, Jiajia Wang and Zhengdao Li performed the experiments; Qianqian Ge and Jiajia Wang analyzed the data; Jitao Li contributed materials; Qianqian Ge drafted the manuscript; and Jian Li made a critical revision of the manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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