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Muhammad Yousuf Ali, Ana Pavasovic, Peter B Mather, Peter J Prentis

Osmoregulation and systemic acid-base balance in decapod crustaceans are largely controlled by a set of transport-related enzymes including carbonic anhydrase (CA), Na^+/K^+-ATPase (NKA) and V-type- H^+-ATPase (HAT). Variable pH levels and changes in osmotic pressure can have a significant impact on the physiology and behaviour of crustaceans. Therefore, it is crucial to understand the mechanisms via which an animal can maintain its internal pH balance and regulate the movement of ions into and out of its cells. Here, we examined expression patterns of the cytoplasmic (CAc) and membrane-associated form (CAg) of CA, NKA α subunit and HAT subunit a in gills of the freshwater crayfish *Cherax quadricarinatus*. Expression levels of the genes were measured at three pH levels, pH 6.2, 7.2 (control) and 8.2 over a 24 hour period. All genes showed significant differences in expression levels, either among pH treatments or over time. Expression levels of CAc were significantly increased at low pH and decreased at high pH conditions 24 h after transfer to these treatments. Expression increased in low pH after 12 h, and reached their maximum level by 24 h. The membrane-associated form CAg showed changes in expression levels more quickly than CAc. Expression increased for CAg at 6 h post transfer at both low and high pH conditions, but expression remained elevated only at low pH (6.2) at the end of the experiment. Expression of CqNKA significantly increased at 6 h after transfer to pH 6.2 and remained elevated up to 24 h. Expression for HAT and NKA showed similar patterns, where expression significantly increased 6 h post transfer to the low pH conditions and remained significantly elevated throughout the experiment. The only difference in expression between the two genes was that HAT expression decreased significantly 24 h post transfer to high pH conditions. Overall, our data suggest that CAc, CAg, NKA and HAT gene expression is induced at low pH conditions in freshwater crayfish. Further research should examine the physiological underpinnings of these changes in expression to better understand systemic acid/base balance in freshwater crayfish.

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Muhammad Yousuf Ali[1], Ana Pavasovic[2], Peter B Mather[1] and Peter J Prentis[1*]

[1]School of Earth, Environmental and Biological Sciences, Queensland University of Technology, Brisbane, QLD 4001, Australia
[2]School of Biomedical Sciences, Queensland University of Technology, Brisbane, QLD 4001, Australia

[*] Corresponding author:

Peter J Prentis

2 George Street, Brisbane, 4001, Australia

email: p.prentis@qut.edu.au

Email addresses:

MYA: my.ali@qut.edu.au
AP: a.pavasovic@qut.edu.au
PBM: p.mather@qut.edu.au
PJP: p.prentis@qut.edu.au

ABSTRACT

Osmoregulation and systemic acid-base balance in decapod crustaceans are largely controlled by a set of transport-related enzymes including carbonic anhydrase (CA), Na^+/K^+-ATPase (NKA) and V-type-H^+-ATPase (HAT). Variable pH levels and changes in osmotic pressure can have a significant impact on the physiology and behaviour of crustaceans. Therefore, it is crucial to understand the mechanisms via which an animal can maintain its internal pH balance and regulate the movement of ions into and out of its cells. Here, we examined expression patterns of the cytoplasmic (CAc) and membrane-associated form (CAg) of CA, NKA α subunit and HAT subunit a in gills of the freshwater crayfish *Cherax quadricarinatus*. Expression levels of the genes were measured at three pH levels, pH 6.2, 7.2 (control) and 8.2 over a 24 hour period. All genes showed significant differences in expression levels, either among pH treatments or over time. Expression levels of CAc were significantly increased at low pH and decreased at high pH conditions 24 h after transfer to these treatments. Expression increased in low pH after 12 h, and reached their maximum level by 24 h. The membrane-associated form CAg showed changes in expression levels more quickly than CAc. Expression increased for CAg at 6 h post transfer at both low and high pH conditions, but expression remained elevated only at low pH (6.2) at the end of the experiment. Expression of CqNKA significantly increased at 6 h after transfer to pH 6.2 and remained elevated up to 24 h. Expression for HAT and NKA showed similar patterns, where expression significantly increased 6 h post transfer to the low pH conditions and remained significantly elevated throughout the experiment. The only difference in expression between the two genes was that HAT expression decreased significantly 24 h post

transfer to high pH conditions. Overall, our data suggest that CAc, CAg, NKA and HAT gene expression is induced at low pH conditions in freshwater crayfish. Further research should examine the physiological underpinnings of these changes in expression to better understand systemic acid/base balance in freshwater crayfish.

KEY WORDS: osmoregulation, acid-base, pH balance, gills, expression, crayfish, Redclaw

INTRODUCTION

Of all currently available farmed freshwater crayfish, Redclaw (*Cherax quadricarinatus*) is the most important commercial species developed for aquaculture production in Australia. It is also an important commercial species in other areas of the world, most notably Mexico, Ecuador, Uruguay, Argentina and China (FAO 2010; Saoud et al. 2012). Redclaw occurs naturally across Northern Australia as well as Southern Papua New Guinea. Wild Australian population of Redclaw are distributed over a distinct pH gradient; one area has low pH (≈6.2) in north Queensland and the other area higher pH (≈8.2) on the western side of Northern Territory (Baker et al. 2008; Bryant & Papas 2007; Macaranas 1995). Previous research on this species reported a fixed allozyme difference at a carbonic anhydrase (CA) allozyme locus between *C. quadricarinatus* populations collected from either side of this pH gradient (Macaranas 1995). The authors suggested that this CA isoform may play an important role in maintaining systemic acid–base balance and ion regulation under different water chemistry (Macaranas 1995). This indicates that CA genes and potentially other genes involved in systemic acid-base balance or ion transport probably play an important role in the response to changes in water chemistry.

In the areas *Cherax* species occur and are cultured, pH Levels fluctuate not only among natural water bodies, but vary widely within water-bodies over time (Boyd 1990). For example, many aquaculture ponds are built in areas with acid sulphate soils or areas with acid precipitation which can lead to decreased pH levels within water-bodies (Haines 1981). Regardless of the causes, fluctuations in pH have been demonstrated to have a great impact on the distribution, growth, behaviour and physiology in many crustaceans including *Cherax* crayfish (Chen & Chen

2003; Haddaway et al. 2013; Kawamura et al. 2015; Kim et al. 2015; Pan et al. 2007; Pavasovic et al. 2004; Yue et al. 2009). It is also evident that environmental pH has a great impact on acid-base balance and electrolyte concentrations in the haemolymph of freshwater crayfish (Morgan & McMahon 1982; Wheatly et al. 1996; Wood & Rogano 1986; Zanotto & Wheatly 1993). For example, water with a low pH level has been shown to cause acid–base imbalance (decreased pH in haemolymph) and disturbed ion regulation (decreased Na^+ and Cl^- concentrations and increased K^+) in freshwater crayfish (Morgan & McMahon 1982; Wheatly et al. 1996; Wood & Rogano 1986). Therefore, a better understanding of the effect of external pH on gene expression in pH induced and ion transport-related genes in gills of freshwater crayfish is needed to elucidate what role these important genes are playing in response to changes in water pH.

Systemic acid-base balance and ion-regulation in crustaceans are largely controlled by a set of transport-related enzymes including, carbonic anhydrase (CA), Na+/K+-ATPase (NKA) and V-type-H^+-ATPase (HAT); and gills are the main organs where these functions take place (Freire et al. 2008). CA produce H^+ and HCO_3^- ions through a reversible reaction, $CO_2+H_2O \leftarrow\rightarrow H^++HCO_3^-$, thereafter the H^+ and HCO_3^- serve as anti-porters for $Na^+/H^+(NH_4^+)$ exchangers and Cl^-/HCO_3^- cotransporter (Freire et al. 2008; Henry et al. 2012; Romano & Zeng 2012). NKA pumps Na^+ ions out of the cell and draws K^+ ions in, and thus establishes an electrochemical gradient that acts as a driving force for transport of Na^+ and K^+ ions by other transporters including $Na^+/K^+/2Cl^-$ cotransporter (Jayasundara et al. 2007; Leone et al. 2015; Li et al. 2015; Lucu & Towle 2003). H^+-ATPase pumps protons (H^+) and acidifies intracellular organelles that help to maintain pH

balance in crustacean taxa (Boudour-Boucheker et al. 2014; Faleiros et al. 2010; Lee et al. 2011; Lucena et al. 2015; Towle et al. 2011).

Recently, two distinct forms of CA as well as key systemic acid-base balance genes Na+/K+-ATPase (NKA) and V-type-H^+-ATPase (HAT) were identified through transcriptome sequencing in multiple species of the genus *Cherax*; *Cherax quadricarinatus, C. destructor* and *C. cainii* (Ali et al. 2015a; Ali et al. 2015b; Ali et al. 2015c). More recently, comparative molecular analysis between these species showed that there were very few non-synonymous mutations in CA or other key osmoregulatory genes that are likely to lead to large differences in protein function within and between these species (Ali et al., unpublished data). Therefore, we hypothesize that differential expression of CA genes and other important ion transport and systemic acid-base balance genes may enable Redclaw crayfish to survive in both acidic and alkaline water conditions. However, this hypothesis has to be thoroughly investigated through a gene expression study at varied pH conditions.

Despite the fact that expression and activity levels of the ion-transport enzymes are probably regulated by extracellular pH, studies on the effects of pH on the expression of these genes in crustaceans is limited (Liu et al. 2015; Lucena et al. 2015; Pan et al. 2007; Wang et al. 2012). Most of the previous work has investigated the effect of salinity on expression of ion-transport genes in decapod crustaceans (for example; CA (Pongsomboon et al. 2009; Serrano et al. 2007; Serrano & Henry 2008); NKA (Chaudhari et al. 2015; Han et al. 2015; Leone et al. 2015; Li et al. 2015; Mitchell & Henry 2014); HAT (Havird et al. 2014; Luquet et al. 2005). However, to the best of our knowledge, all the studies have been undertaken in euryhaline species, and none

have investigated freshwater crayfish. Recently, we have reported gene expression of CA and HAT in *C. quadricarinatus*, but the study was limited to one sampling point and mainly comprised the description of a transcriptome dataset (Ali et al. 2015b). Thus, in this new study we have undertaken a time course of expression patterns for the key genes involved in pH-balance and osmoregulation under varied pH conditions in *C. quadricarinatus*. The study reports the expression patterns of two forms of alpha carbonic anhydrase (a cytoplasmic form, referred to as CqCAc and a membrane-associated form, referred to as CqCAg); sodium-potassium pump Na+/K+-ATPase α subunit (CqNKA); and proton (H^+) pump V-type-H^+-ATPase subunit a (CqHAT) at three different pH levels, pH 6.2, 7.2 (control) and 8.2, in the gills of *C. quadricarinatus* over a time course of 24 hours.

MATERIALS AND METHODS

Sample Preparation

Live inter-moult *C. quadricarinatus* were obtained from Theebine, Queensland, Australia. Animals were housed in rectangular glass tanks (size: 25×18×15 cm, capacity: 27 L each) at QUT (Queensland University of Technology) Aquaculture facility and acclimated at pH 7.2 ± 0.14 for three weeks before the experiment. Other water quality parameters were as follows: temperature 20.9±0.9^{0}C, conductivity 405±42 µS/cm. Water quality was maintained with a computer-controlled filtration system (Technoplant). During the acclimation period all animals were fed regularly with formulated feed pellets.

Feeding was stopped 24 h before the pH treatments were undertaken. A total of 45 animals (weight 41±4 g and length 11.8±0.6) were distributed into separate tanks. The animals were

stressed by two pH treatments, pH 6.2 and pH 8.2, a treatment within the tolerance range of this species (Bryant & Papas 2007; Macaranas 1995); and pH 7.2 was used as a no change control. Gills were extracted from three individuals as biological replicates at 0 h, 3 h, 6 h, 12 h and 24 h post-exposure for each treatment.

RNA extraction and cDNA synthesis

Prior to tissue extraction, animals were euthanized in crushed ice for 5-10 minutes. Gill tissue were dissected and immediately frozen in liquid nitrogen. Total RNA was extracted from individual gill tissue, from 0.1 g of tissue from each animal, using a TRIZOL/Chloroform extraction (Chomczynski & Mackey 1995) and then purified using a RNeasy Midi Kit (cat # 75144, QIAGEN) using an existing protocol (Prentis & Pavasovic 2014). Genomic DNA was digested with Turbo DNA-free kit (REF-AM1907, Ambion RNA, Life Technologies, USA) and RNA quality and concentration were checked using a Bioanalyzer 2100 RNA nanochip (Agilent Technologies).

Complementary DNA (cDNA) was synthesized by reverse transcription from 1 µg of total RNA using SensiFAST cDNA synthesis protocol (Bioline, Australia, Cat # BIO-65054). The reaction was made in a final volume of 20 µl with 1 µg RNA template, 1x TransAmp Buffer, 1 µl Reverse Transcriptase and of DNase/RNase free water as required. The resulting cDNA samples were stored at −20°C until used as templates for real-time quantitative PCR.

Quantification of mRNA by quantitative Real-Time-PCR (qRT-PCR)

The relative abundance of mRNA levels was measured using the quantitative real-time PCR machine LightCycler 96 (Roche, Version 04) using FastStart Essential DNA Green Master (Roche, Germany, Cat. No.06924204001). Three replicate animals were used for all sampling points in all experimental treatments and all qRT-PCR amplifications were carried out in triplicate. Gene-specific quantitative real-time PCR primers were designed in primer3 using the settings from Amin et al. (2014) for transcripts that were identified as: two forms from the alpha CA gene family (CqCAc and CqCAg), CqNKA, CqHAT and 18s rRNA (Cq18s) (Ali et al. (2015b) (Supplementary Table 1). The RT-PCR reaction contained 1 µl cDNA template, 1 µl Green Master, 2 µl primers (1 µl forward and 1 µl reverse, concentration 10 µmole) and DNase free water required to make the final volume up to 20 µl. Real-time PCR conditions included a pre-incubation of 95^0C for 5 minutes, followed by a total 45 cycles of three-step amplification of 95^0C for 10 seconds; 60^0C for 10 seconds and 72^0C for 10 seconds. Ribosomal 18S was used as an internal control gene (whose expression levels did not change under different treatments) to normalize sample-to-sample variation. Negative controls (without cDNA template) were also used. The relative expression of the target genes were measured as a ratio (concentration of target gene/concentration of 18S gene) according to Pfaffl (2001).

Data analysis

The relative expression values for the genes were obtained using the Relative Quant analysis tool described in the Light Cycler 96 system operator's guide, version 2.0. Statistical analyses were performed using Minitab software (version 17). A one-way ANOVA was undertaken with

Fisher's tests to determine if any of the genes showed significant expression differences across the three pH treatments or time points at $p<0.05$.

RESULTS

Carbonic anhydrase (CA)

We analysed expression patterns of two forms of carbonic anhydrase, CqCAc and CqCAg. The results showed that CqCAc was only differentially expressed at 24 hour time point, across all three treatments, low pH (6.2), high pH (8.2) and the control pH treatment (7.2) (Fig. 1). At 24 h, the expression levels of CqCAc were significantly upregulated with an approximately 3 fold increase at pH 6.2 ($p=0.007$, F-value 22.14) whereas, at pH 8.2, the expression levels were significantly down-regulated ($p=0.023$, F-value 18.60). CqCAc expression levels were consistent in control group (pH 7.2) over the experiment. For pH 6.2 treatment, CqCAc showed consistent expression up to 6 h mark; after this expression of CqCAc started increasing dramatically with the initial increase of ≈2 fold at 12 h, and 3 fold at 24 h ($p=0.007$, F-value 22.14). At pH 8.2, CqCAc expression did not change significantly up to the 12 h mark, but there was a significant decrease in expression 24 h post transfer ($p=0.023$, F-value 18.60).

The pattern of CqCAg expression was similar to CqCAc, but CqCAg was induced more quickly than the CqCAc (Fig. 2). The expression of CqCAg initially increased at 6 h post transfer at both pH 6.2 and pH 8.2; although these increases were not statistically significant ($p>0.05$, F value 1.79). The expression levels remained elevated at pH 6.2, but non-significantly, for any time

points post-transfer. Expression levels of CqCAg in the pH 8.2 treatment gradually increased until 6 h but started to decrease 12 h post transfer and expression decreased significantly by ≈2.5 fold at 24 h as compared to the initial expression level (p=0.01, F-value 33.82).

Na^+/K^+-ATPase (NKA)

Expression of CqNKA was significantly different between treatments (p= 0.021, F-value 9.19) from 6 h onwards. At pH 6.2 expression of CqNKA increased ≈4.5 fold at 6 h and remained differentially expressed up to 24 h post transfer (≈3 fold expression increase compared to time 0)(Fig. 3, p= 0.016 and F-value 16.37). In contrast, the magnitude of increase in expression of CqNKA at pH 8.2 was less than that at pH 6.2. At pH 8.2, the highest level of expression was reached at 12 h post-transfer with an increase of ≈2.5 fold, but the change was not statistically significant (p=0.36, F-value 1.02). At 24 h post-exposure to high pH (pH 8.2) the expression levels sharply decreased almost to its initial level of expression (0 h) (Fig. 3).

V-type H^+-ATPase (HAT)

The expression of CqHAT was similar to that of CqNKA, and demonstrated significantly higher levels of expression 6 h after transfer to pH 6.2 (p=0.023 and F-value 18.74 at 6h; p=0.005 and F-value 53.30 at 24 h). This pattern of increased expression remained fairly constant approximately 2-3 fold higher than initial expression until the experiment ceased (Fig. 4). For pH 8.2 the expression of CqHAT was not significantly different from that of the control group until 24 h post-transfer. At 24 h post-exposure the expression level dropped sharply to less than the initial level of expression (≈35% of the control level, p=0.012, F-value 29.62).

DISCUSSION

The expression of all candidate genes was induced by transfer to low pH. The timing of induction varied between the candidate genes as did the fold increase of expression. The role that these candidate genes may play in systemic acid-base balance is discussed in the following subsections.

Carbonic anhydrase (CA)

In our study, expression levels of CqCAc increased upon exposure to low pH conditions (≈3 fold at 24 hour post-exposure) (Fig. 1). Other studies have reported increased levels of CA expression in crustaceans after being exposed to low pH conditions (Liu et al. 2015) or low salinity levels (Jayasundara et al. 2007; Mitchell & Henry 2014; Pongsomboon et al. 2009; Serrano et al. 2007; Serrano & Henry 2008). For instance, Liu et al. (Liu et al. 2015) reported a 5 fold increase in expression of CAc in the euryhaline shore crab *Pachygrapsus marmoratus* 12h after transfer to a pH 7.4 treatment (control pH 8.2). A large induction of CAc was also reported by Serrano et al. (Serrano et al. 2007) (100 fold increase in blue crab *Callinectus sapidus*), Serrano and Henry (Serrano & Henry 2008) (100 fold increase in green crab *Carcinus maenas*), Mitchell and Henry (Mitchell & Henry 2014) (90 fold increase in *C. sapidus*), and Henry et al. (2006) (10 fold increase in *C. maenas*) following transfer to low salinity water. The pattern of CAc expression we found in our study was similar to that seen in other studies where CAc expression was more pronounced at low salinity or low pH conditions (Jayasundara et al. 2007; Serrano et al. 2007; Serrano & Henry 2008). It is suggested that this change in expression occurs due to the fact that cytoplasmic CA provides the counter ions H^+ and HCO_3^- ions to the

242 Na^+/H^+ and Cl^-/HCO_3^- exchanger to drive ion uptake in the gills of crustaceans when exposed to
243 low pH or low salinity environments (Henry 1988; Henry 2001; Henry & Cameron 1983).

244 Previous studies have shown decreased level of Cl^- concentrations in freshwater crayfish
245 including *Procambarus clarkia*, *Orconectes propinguus* and *Orconectes rusticus* after exposure
246 to low pH conditions (Wood & Rogano 1986; Zanotto & Wheatly 1993). Therefore, it is possible
247 that in *C. quadricarinatus*, also a freshwater crayfish species, that internal Cl- concentrations
248 may decrease in haemolymph under low pH conditions; increasing the activity of Cl^-/HCO_3^-
249 exchanger to compensate for the loss of Cl^-. The activity of CAc should increase under low-pH
250 stress because HCO_3^- are supplied by CAc through hydration of CO_2. From these findings, we
251 can infer that the cytoplasmic CA form from *C. quadricarinatus* may play an important role in
252 maintaining internal pH conditions in low streams across northern Australia where they occur
253 naturally.

254 Our study showed that the timing for initial induction of CqCAg was faster than that of CqCAc in
255 both pH conditions. For CqCAc, the initial increase in expression was observed only after 12 h
256 post-exposure, and only at the lower pH (pH 6.2); while for CqCAg, the initial increase started at
257 6 h post-transfer, in both low pH and high pH conditions (Fig. 2). This finding suggests that
258 CqCAg is more sensitive to pH changes, and responds quicker. Liu et al. (Liu et al. 2015) also
259 reported similar pattern of expression for the same two forms of CA in *Litopenaeus vannamei*,
260 where CAg was induced more quickly than the CAc. This indicates that CAg might be more
261 sensitive to changes in pH than CAc at both low and high pH levels.

We also observed that the cytoplasmic CA (CqCAc) was induced more at low pH than high pH (Fig. 1); and had a greater magnitude of 'inductive scope' (degree of differences in expression between the maximal level of expression and baseline expression). In contrast, CAg showed increased induction at higher pH levels. Liu et al. (Liu et al. 2015) also observed similar patterns of CA gene expression, where they found that CAc was induced more under low pH conditions, and that CqCAc was induced at a higher level compared to CAg. Very interestingly, the patterns of sensitivity and induction at different pH conditions are very similar to expression patterns of Cag and CAc under different salinity conditions in other decapod crustaceans (Serrano et al. 2007; Serrano & Henry 2008).

Na^+/K^+-ATPase (NKA)

In our study, expression of CqNKA was significantly upregulated at low pH conditions but non-significantly increased at high pH (pH 8.2) (Fig. 3). Previous studies have reported similar patterns of NKA expression, where they found increased levels of either NKA transcription or NKA enzyme activity under both low and/or high pH conditions (Pan et al. 2007; Wang et al. 2012). In an experiment with *L. vannamei*, Pan et al. (2007) reported 3-4 fold increases in NKA activity 24 h after exposure to low pH (pH 7.1) and high pH conditions (pH 9.1). In the present study, we report a maximum increase of 4.5 fold in expression at low pH conditions and 2.5 fold increase at high pH conditions. This indicates that Na^+/K^+-ATPase from *C. quadricarinatus* is induced more strongly to low pH, rather than the high-pH conditions. Similarly, Wang et al. (Wang et al. 2012) in *L. vannamei* documented a 17 fold and 4 fold increase in NKA expression after exposure to low pH (at 6 h post-exposure to pH 5.6) and high pH conditions (at 3h post-exposure to pH 9.3). The main reason for the differences reported in the level of NKA expression among our study and the two *L. vannamei* studies is probably related to differences in the pH treatments (transfer from pH 7.4 to pH 5.6 and 9.3).

In crustacean species, a large number of previous studies have examined the effect of salinity on expression of transport-related genes, but few studies have focused on the effects of external pH (Li et al. 2015; Pan et al. 2007; Wang et al. 2012; Wang et al. 2002). Changes in expression patterns of CqNKA induced by pH in our study are quite similar with that induced by salinity in other crustacean species, i.e. higher induction levels at low salinity exposure (Han et al. 2015; Havird et al. 2013; Jayasundara et al. 2007; Leone et al. 2015; Li et al. 2015; Luquet et al. 2005; Pan et al. 2007; Serrano et al. 2007; Wang et al. 2012). As published reports show that

294 Na^+ concentration decreases and Na^+ concentration increases in the haemolymph of the
295 freshwater crayfish *Procambarus clarkia* and *Orconectes rusticus* upon exposure to low-pH
296 conditions (Morgan & McMahon 1982; Wheatly et al. 1996; Wood & Rogano 1986), it is logical
297 that the NKA expression level should also increase in *C. quadricarinatus* under similar
298 conditions. The main reason of increased expression levels for CqNKA in *C. quadricarinatus* may
299 be attributed to increased level of NKA activity in the gills. Because, Na+/K+-ATPase is a key
300 enzyme that pumps Na^+ into haemolymph and draws K^+ in the cell, it establishes
301 electrochemical gradients that act as a driving force for trans-epithelial movement of many
302 monovalent ions including Na^+, K^+, H^+, Cl^- across the gills in crustacean taxa (Chaudhari et al.
303 2015; Han et al. 2015; Havird et al. 2013; Leone et al. 2015; Li et al. 2015). Overall, these
304 findings suggest that NKA plays a role in maintaining systemic acid-base balance in *Cherax*
305 crayfish.

306 **Vacuolar-type H^+-ATPase**

307 In the present study, expression of Vacuolar-type H^+-ATPase in *C. quadricarinatus* was
308 upregulated at low-pH and down-regulated at high-pH conditions (Fig. 4). Previous studies in
309 crustacean species have also reported that expression levels of Vacuolar-type H^+-ATPase are
310 changed under low and high pH conditions in a similar pattern, but this pattern can vary among
311 species (Havird et al. 2013; Pan et al. 2007; Wang et al. 2012). For example, in a study of HAT
312 enzyme activity in *L. vannamei*, Pan et al. (2007) reported a negative correlation between
313 activity of HAT and pH levels. HAT expression is probably affected by changes in external pH,
314 because it is one of the key enzymes that acidifies intracellular organelles which in turn

315 influence the activity of other ion-transport enzymes including $Na^+/H^+(NH_4^+)$ and Cl^-/HCO_3^-
316 exchanger (Faleiros et al. 2010; Havird et al. 2013; Luquet et al. 2005; Martin et al. 2011;
317 Martin Tresguerres et al. 2006; Pan et al. 2007; Wang et al. 2012; Weihrauch et al. 2012;
318 Weihrauch et al. 2004; Weihrauch et al. 2009; Weihrauch et al. 2002).

319 In the present study, we report a maximum increase of 2-3 fold in mRNA expression of CqHAT
320 between 6-12 h after exposure to low pH (6.2) and a 3 fold decrease at high pH (pH 8.2)
321 conditions (Fig. 4). Our results are similar to a recent study that reported a significant down-
322 regulation of 2.5 fold in gill $V(H^+)$-ATPase activity of river shrimp *M. amazonicum* at pH 8.5, as
323 compared to that at pH 7.5 (Lucena et al. 2015). Wang et al. (2012), however, reported
324 increased levels of HAT expression in *L. vannamei* at both low- and high-pH conditions, but
325 expression levels were higher at low pH. This variation in the expression of HAT transcripts or
326 HAT activity are probably attributed to species-specific physiological differences in species with
327 distinct osmoregulatory capabilities (Anger & Hayd 2010; Charmantier & Anger 2011; Faleiros
328 et al. 2010; Lucena et al. 2015). These findings suggest that $V(H^+)$-ATPase likely plays an
329 important role in systemic acid-base balance in freshwater crayfish.

330

331 **CONCLUSIONS**

332 From the gene expression data, we suggest that cytoplasmic carbonic anhydrase, Na^+/K^+-
333 ATPase and V-type-H^+-ATPase may play important role in the maintenance of pH balance in
334 freshwater crayfish, while the membrane-associated CA probably plays a more limited or
335 indirect role in this process. We can also infer that cytoplasmic CA, Na^+/K^+-ATPase and V-type-

336 H^+-ATPase are induced more strongly at low pH conditions compared with high pH conditions.
337 These data can help to provide a better understanding of which genes are involved in systemic
338 pH balance in freshwater crayfish.

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343

344 **ADDITIONAL INFORMATION AND DECLARATIONS**

345 **Competing interest**

346 The authors declare that they have no competing interests.

347 **Authors' contributions**

348 Designed experiment: MYA, PP, AP, PM. Conducted research and laboratory work: MYA. Research
349 supervision: PP, AP, PM. Undertook analysis: MYA, PP, AP. Wrote manuscript: MYA. All authors read,
350 corrected and approved the final manuscript.

351 **Abbreviations**

352 CA: Carbonic anhydrase; CAc: cytoplasmic carbonic anhydrase; CAg: membrane-associated
353 Glycosyl-phosphatidylinositol-linked carbonic anhydrase; Cq: *Cherax quadricarinatus*; NKA:

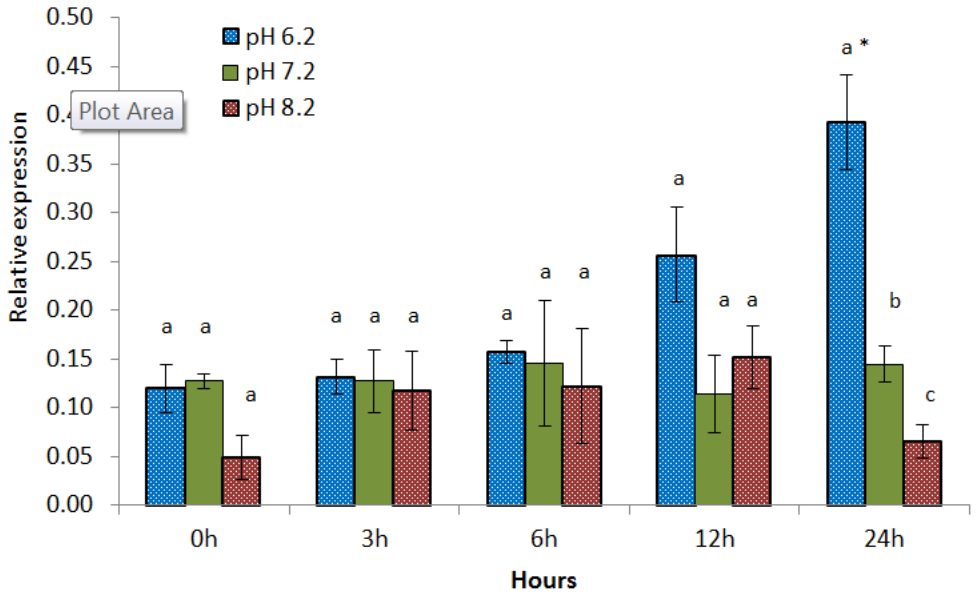
354 Na^+/K^+-ATPase; V-type: Vacuolar-type; HAT: *H^+-ATPase*; qRT-PCR: quantitative real-time
355 polymerase chain reaction.

356

357

358 **FIGURES**

359



360

361 **Fig 1. Relative expression of cytoplasmic carbonic anhydrase**

362 Relative mRNA expression of CqCAc (cytoplasmic carbonic anhydrase) in the gills of Redclaw crayfish (*Cherax*
363 *quadricarinatus*) acclimated to pH 7.2 (control) and after being transferred to pH 6.2 and pH 8.2 at various times
364 for up to 24 hours. Vertical bars represent the mean±s.e.m (n=3). Different letters above the bars denote
365 significant differences from the control group in the same time of sampling at the 0.05 level (one-way ANOVA,
366 Tukey's and Fisher post-hoc tests). Asterisk (*) indicate the significant differences (at 0.05 level) in expression
367 levels over the course of exposure time compared with the initial level (0 h) of mRNA-expression within the same
368 treatment group. Expression levels were normalized with respect to reference gene 18S (internal control gene) for
369 the same sample, and calculated as a ratio (conc. of target gene/conc. of 18S gene) according to Pfaffl (2001).

370

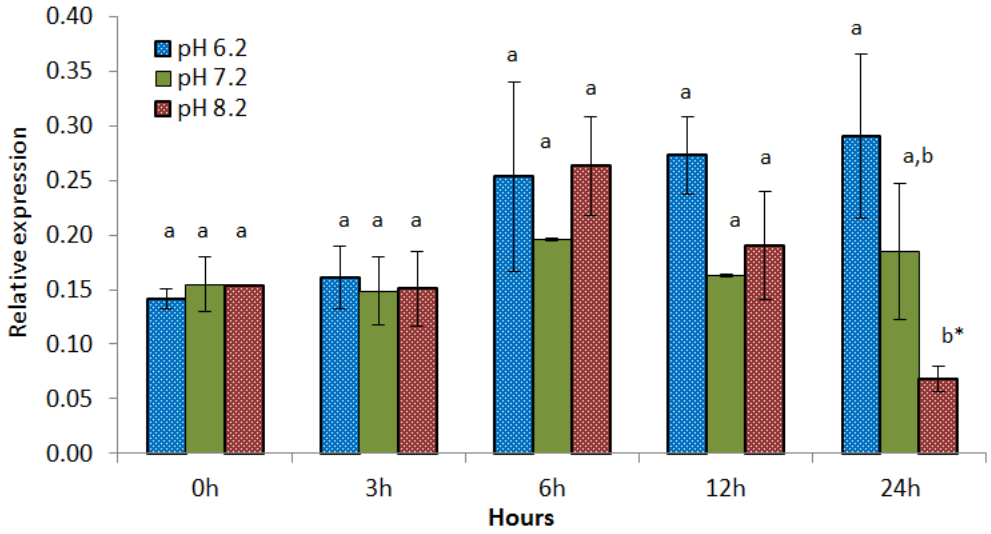


Fig. 2. Relative expression of membrane-associated carbonic anhydrase

Relative mRNA expression of CqCAg (membrane-associated carbonic anhydrase) in the gills of Redclaw crayfish (*C. quadricarinatus*) acclimated to pH 7.2 (control) and after being transferred to pH 6.2 and pH 8.2 at various times for up to 24 hours. Vertical bars represent the mean±s.e.m (n=3). Different letters above the bars denote significant differences from the control group in the same time of sampling at the 0.05 level (one-way ANOVA, Tukey's and Fisher post-hoc tests). Asterisk (*) indicate the significant differences (at 0.05 level) in expression levels over the course of exposure time compared with the initial level (0 h) of mRNA-expression within the same treatment group.

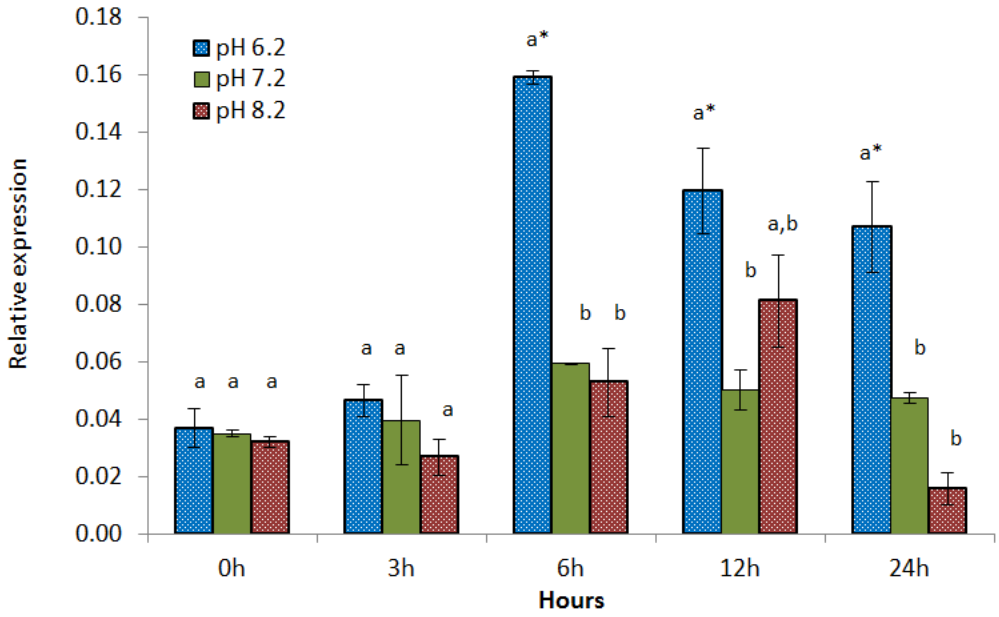


Fig. 3. Relative expression of Na^+/K^+-ATPase

Relative mRNA expression of CqNKA (Na^+/K^+-ATPase) in the gills of Redclaw crayfish (*C. quadricarinatus*) acclimated to pH 7.2 (control) and after being transferred to pH 6.2 and pH 8.2 at various times for up to 24 hours. Vertical bars represent the mean±s.e.m (n=3). Different letters above the bars denote significant differences from the control group in the same time of sampling at the 0.05 level (one-way ANOVA, Tukey's and Fisher post-hoc tests). Asterisk (*) indicate the significant differences (at 0.05 level) in expression levels over the course of exposure time compared with the initial level (0 h) of mRNA-expression within the same treatment group.

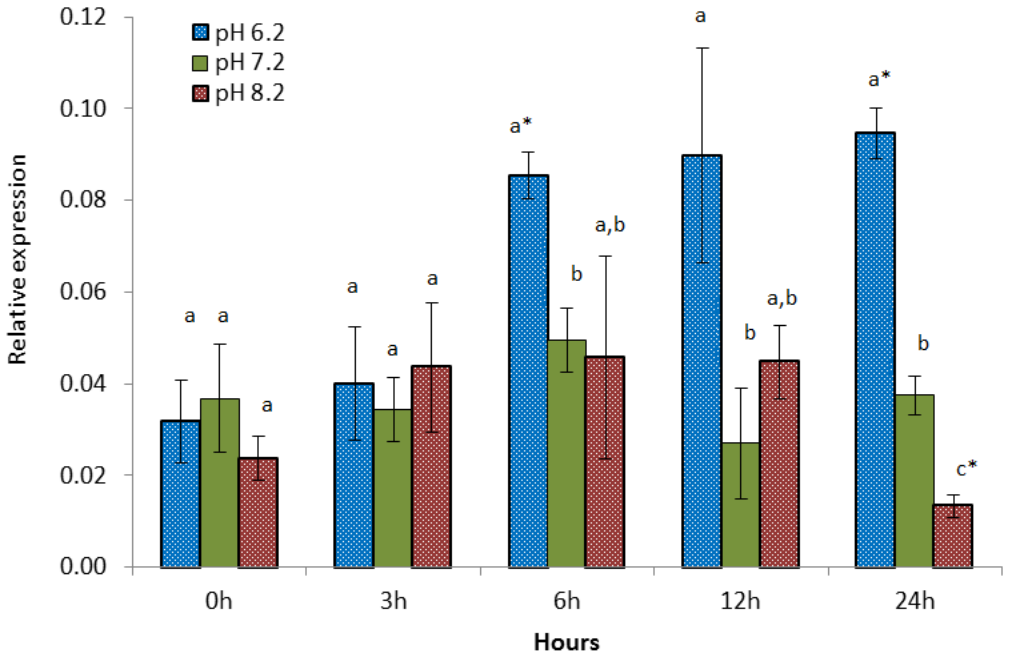


Fig. 4. Relative expression of V-type-H$^+$-ATPase

Relative mRNA expression of CqHAT (V-type-H$^+$-ATPase) in the gills of Redclaw crayfish (*C. quadricarinatus*) acclimated to pH 7.2 (control) and after being transferred to pH 6.2 and pH 8.2 at various times for up to 24 hours. Vertical bars represent the mean±s.e.m (n=3). Different letters above the bars denote significant differences from the control group in the same time of sampling at the 0.05 level (one-way ANOVA, Tukey's and Fisher post-hoc tests). Asterisk (*) indicate the significant differences (at 0.05 level) in expression levels over the course of exposure time compared with the initial level (0 h) of mRNA-expression within the same treatment group.

403 **Supplementary Table 1.**

404 Oligonucleotide primers used in the experiment for amplification of the target genes at RT-PCR

Target genes	Primers	Primer Sequence (5′→3′)	T_m (^{0}C)	Product Size (bp)
18s (internal control)	Cq18s-F	ACCTTGAGACCAAACTGCGT	56.9	103
	Cq18s-R	GTGCTGACCTCTCACACGAA	57.1	
Cytoplasmic CA	CqCAc-F	CTTGCTGTCCTGGGAATGTT	64.0	196
	CqCAc-R	CATAGCATGGTGGAGTGGTG	64.1	
GPI-linked CA	CqCAg-F	GGCACTAGGCTCTGAACACA	57.0	149
	CqCAg-R	CTGACACCTCCAGCATCACT	56.8	
Na^+K^+ATPase alpha	CqNKA-F	TGGTGTTGAGGAGGGAAGAC	64.2	130
	CqNKA-R	ACCCAATGGTAGAGGCACAG	63.9	
V-typeH^+ATPase subunit a	CqHAT-F	ATCGAGTATTGGCTGCTGCT	63.8	142
	CqHAT-R	ACTGGGATCCAACATTCAGC	63.9	

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