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mRNA EXPRESSION AND ACTIVITY OF ION-TRANSPORTING PROTEINS IN GILLS OF THE BLUE CRAB *CALLINECTES SAPIDUS*: EFFECTS OF WATERBORNE COPPER

CAMILA M. G. MARTINS, DANIELA VOLCAN ALMEIDA, LUIS FERNANDO FERNANDES MARINS, and ADALTO BIANCHINI*
Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (FURG), 96201-900 Rio Grande, Rio Grande do Sul, Brazil

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Abstract—Waterborne Cu effects on the transcription of genes encoding ion-transporting proteins and the activities of these proteins were evaluated in gills of the blue crab *Callinectes sapidus* acclimated to diluted (2‰) and full (30‰) seawater. Crabs were exposed (96 h) to an environmentally relevant concentration of dissolved Cu (0.78 μM) and had their posterior (osmoregulating) gills dissected for enzymatic and molecular analysis. Endpoints analyzed were the activity of key enzymes involved in crab osmoregulation (sodium-potassium adenosine triphosphatase [Na^+/K^+-ATPase], hydrogen adenosine triphosphatase [H^+-ATPase], and carbonic anhydrase [CA]) and the mRNA expression of genes encoding these enzymes and the sodium-potassium-chloride ($Na^+/K^+/2Cl^-$) cotransporter. Copper effects were observed only in crabs acclimated to diluted seawater (hyperosmoregulating crabs) and were associated with an inhibition of the expression of mRNA of genes encoding the Na^+/K^+-ATPase and the $Na^+/K^+/2Cl^-$ cotransporter. However, Cu did not affect Na^+/K^+-ATPase activity, indicating that the gene transcription is downregulated before a significant inhibition of the enzyme activity can be observed. This also suggests the existence of a compensatory response of this enzyme to prevent osmoregulatory disturbances after short-term exposure to environmentally relevant Cu concentrations. These findings suggest that Cu is a potential ionoregulatory toxicant in blue crabs *C. sapidus* acclimated to low salinity. The lack of Cu effect on blue crabs acclimated to full seawater would be due to the reduced ion uptake needed for the regulation of the hemolymph osmotic concentration in full seawater (30‰). Also, this could be explained considering the lower bioavailability of toxic Cu (free ion) associated with the higher ionic content and dissolved organic matter concentration in high salinity (30‰) than in diluted seawater (2‰). Environ. Toxicol. Chem. 2011;30:206–211. © 2010 SETAC

Keywords—Crab Copper mRNA expression Ion transporters Salinity

INTRODUCTION

Marine environmental contamination by Cu is most likely to occur in coastal and estuarine ecosystems as a result of human activities around these areas. In fact, Cu is one of the most relevant inorganic contaminants reaching coastal waters in sandy beaches in Southern Brazil. Studies on trace metal concentrations in the waters from the Patos Lagoon estuary (Rio Grande, Rio Grande do Sul, Southern Brazil) have shown significant increases (from 2 μg/L in early 1980s to 34 μg/L in 1998) in environmental Cu concentration as a consequence of industrial and harbor activities [1,2].

Although Cu plays an essential role in a number of biological processes in crustaceans [3–5], this metal has the potential to exert toxic effects. Therefore, a homeostatic regulation for Cu based on mechanisms involved in metal absorption and excretion ensures sufficient amounts of Cu for essential processes while preventing the toxicity of Cu excess [6].

Aquatic organisms can take up Cu directly from ambient water, and elevated Cu concentrations lead to metal accumulation in several tissues [7–12]. Although oxidative stress is accepted as one important cause of Cu toxicity, other effects have been described in organs involved in osmoregulation of aquatic organisms [11]. Physiological studies have demonstrated that the key mechanism of waterborne Cu toxicity is associated with an impairment of Na^+ and Cl^- regulation in both freshwater and marine osmoregulating fish and invertebrates. Generally, the gill is the main organ involved in osmotic and ionic regulation and is consequently the main target for Cu toxicity in these animals [10,11,13].

Toxicological studies clearly indicate that gills serve as a major route of Cu uptake in aquatic animals. This metal can compete with other ions such as Na^+ [10,13] and Ca^{2+} [14,15] for binding physiologically active sites at the gill, consequently being incorporated by the animal. In fact, components of Na^+ transport, including the enzymes sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase), hydrogen adenosine triphosphatase (H^+-ATPase), and carbonic anhydrase (CA), as well as the membrane sodium-hydrogen exchanger (Na^+/H^+) and sodium-potassium-chloride cotransporter ($Na^+/K^+/2Cl^-$), are involved in Cu transport across the gill epithelium [11,13,16]. Therefore, Na^+ transport across the gills can be affected by the presence of waterborne Cu, resulting in osmoregulatory disturbances. In fact, the inhibition of Na^+ transport across the gills of the freshwater rainbow trout, *Salmo gairdneri,* was paralleled by inhibition of the gill Na^+/K^+-ATPase activity [7]. Also, inhibition of the activity of gill CA was observed in the estuarine crab *Chasmagnathus granulata* in the presence of high levels of waterborne Cu [17]. Despite these studies, only few reports describe the biochemical and physiological effects of Cu on ion-transporting proteins in euryhaline invertebrates, especially at different environmental salinities. Also, information is lacking at the molecular level.

Thus, the objective of the present study was to evaluate the effect of Cu on the activity of key enzymes (Na^+/K^+-ATPase, H^+-ATPase, and CA) involved in gill Na^+ transport in the blue crab *Callinectes sapidus* as well as on the transcription of genes encoding these enzymes and the $Na^+/K^+/2Cl^-$

* To whom correspondence may be addressed (adaltobianchini@furg.br).
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cotransporter. Given that *C. sapidus* is an euryhaline species living in estuaries and coastal areas, Cu effects were evaluated at two ambient salinities (2 and 30‰), corresponding to two different physiological conditions (hyperosmoregulating and osmoconforming blue crabs, respectively) [18,19].

MATERIALS AND METHODS

Experimental animals and Cu exposure

Experiments were performed on adult male blue crabs (*Callinectes sapidus*) in stage C or early D of the intermolt cycle [20] collected from nonmetal-contaminated sites at the Patos Lagon estuary (Rio Grande, Rio Grande do Sul, Southern Brazil) [2,21]. Crabs were collected, transferred to the laboratory, and acclimated to diluted (2‰) or full (30‰) seawater for at least two weeks, as previously described [22]. Low salinity (2‰) was obtained by diluting full seawater with distilled water. Acclimation media were continuously filtered (chemical and biological filters) and aerated. Temperature and photoperiod were fixed at 20°C and 12:12-h light:dark, respectively. Three times a week, crabs were fed until satiation with chopped fish.

Acclimated crabs were acutely exposed (96 h) to Cu (nominal = 1 μM Cu as $CuCl_2$) using filtered seawater (5-μm-mesh filter) at salinity 2 or 30‰. Copper was added to the experimental media from a stock solution (5 mM). Copper concentration was checked by atomic absorption spectrophotometry (AAS; Avanta 932 Plus; GBC) using standard solutions prepared in saltwater at the experimental salinity [12,13,23]. Copper was added to the water 3 h prior to crab introduction in the test chamber to allow Cu equilibration with the experimental medium [23]. Control exposures (no Cu addition to the exposure medium) were also tested in water at salinities 2 and 30‰.

Experimental media were completely renewed every 24 h. At the beginning and after 24 h of exposure, nonfiltered and filtered (0.45-μm-mesh filter) water samples were collected from the experimental media for further analyses of total (nonfiltered samples) and dissolved (filtered samples) Cu concentrations by AAS (Avanta 932 Plus; GBC), as previously described [12,13,23]. Six blue crabs were tested under each experimental condition.

After Cu exposure, crabs were cryoanesthetized and killed and had their posterior gills (gills 6–8) dissected. These gills were analyzed because they are the main organs involved in ion uptake from water in blue crabs, whereas anterior gills (gills 1–4) are considered as mainly respiratory. In *C. sapidus*, the fifth pair of gills is a transitional one, showing respiratory and ion-transporting functions [19]. Dissected gills were dried on filter paper. The sixth pair of gills (G6) was immediately used for RNA extraction, and the seventh pair of gills (G7) was stored (−80°C) for further enzymatic assays.

RNA extraction and semiquantitative polymerase chain reaction

Total RNA was extracted from G6 using Trizol reagent (Invitrogen) and used to synthesize the complementary DNA (cDNA), using the reverse transcriptase (Superscript III RT) and the corresponding primer AP (5′-GGCCACGCGTCGAC-TAGTAC$(T)_{17}$-3′), following the manufacturer's instructions (Invitrogen). cDNA produced was employed as template for gene-specific polymerase chain reaction (PCR). All reactions were performed in 12.5 μl of a solution containing 1.25 μl of 10 × PCR buffer, 0.375 μl $MgCl_2$ (50 mM), 0.25 μl dNTP (10 mM), 0.25 μl primer forward (0.01 mM), 0.25 μl primer reverse (0.01 mM), 0.5 μl cDNA (dilution 1:10), 0.1 μl Platinum Taq polymerase (5 U/L), and 9.525 μl pure water. All reagents were purchased from Invitrogen. The PCR protocol consisted of 2 min at 94°C, 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, with a final extension step of 5 min at 72°C. For genes with a sequence size lower than 500 base pair (bp), the last step of the PCR cycle was 30 s at 72°C. The number of cycles in the PCRs for each gene was established in order to avoid the reaction's plateau phase. Analyzed genes, number of cycles, and primer sequences used are described in Table 1. Note that the number of cycles is different between salinity 2 and 30‰.

The β-actin gene transcription was used to normalize data on mRNA expression. It is important to note that expression of the gill β-actin mRNA was not affected either by salinity (2 and 30‰) or by Cu exposure (1 μM; see *Results*). Products of PCR were revealed in 2% agarose gel with ethidium bromide (500 ng/ml) and photographed. Images were analyzed by densitometry employing the 1 Dscan Ex software version 3.1 (Scanalytics).

Na^+/K^+-ATPase activity assay

The seventh pair of gills (G7) was thawed on ice, homogenized in 1 ml cold buffer (150 mM sucrose, 10 mM ethylenediaminetetraacetic acid, 50 mM imidazole, pH 7.3) containing 0.1% sodium deoxycholate, and centrifuged (1 min, 4°C, 10,000 × *g*; Mikro22R; Hettich). Supernatant was split in two aliquots. One aliquot was immediately frozen (−80°C) for further analysis of the H^+-ATPase activity; the

Table 1. Gene-specific primers used for polymerase chain reaction assays with posterior gills of the blue crab *Callinectes sapidus*[a]

Gene (No. GB)[b]	AS[c] (bp)	NC[d] salinity 2‰	NC[d] 30‰	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-Actin (DQ084066)	321	25	30	AAGATCTGGCACCACACTTTCTA	GTGACCTTACCGACTACCTGATG
NaK-ATPase (AF327439)	538	25	28	GATCAGTCACTTCATCCACATCA	GTGTGGAACTTGCTATTGGAGAC
H-ATPase (AF189780)	254	27	30	TCAAGCAGGACTTTGAAGAGAAC	GTGGTTTCCCAGGTTACATGTAC
CasCag (EF375491)	927	28	30	TGGTGGACTTAAGGGAGAATACA	TCTACTTCATGACCTCTATGGCA
CasCac (EF375490)	816	26	30	GGTACACTGGAACAAGAGCAAGT	GTCAGGAAGTACAGTGACAAGCAA
NKCC (AF190129)	600	34	28	CTATTGGCCTTATGTTCACCTTG	TAGCTTAACAGGCAGTGTTGACA

[a] NaK-ATPase = gene of the sodium-potassium adenosine triphosphatase; H-ATPase = gene of the hydrogen adenosine triphosphatase; CasCag = gene of the carbonic anhydrase associated to gill membrane; CasCac = gene of the cytoplasmic carbonic anhydrase; NaKCC = gene of the sodium-potassium-chloride cotransporter.
[b] GenBank accession number.
[c] PCR amplicon size expressed in number of base pairs (bp).
[d] NC = number of cycles used for each pair of primes for experiments performed at water salinity of 2 and 30‰.

other was immediately used for the Na^+/K^+-ATPase activity measurement. Na^+/K^+-ATPase activity was measured by coupling ouabain-sensitive ATP hydrolysis to piruvate kinase- and lactate dehydrogenase-mediated oxidation of reduced nicotinamide adenine dinucleotide (NADH), as previously described [12,24]. Data were normalized to protein content in the supernatant.

H^+-ATPase activity assay

H^+-ATPase activity measurements were performed in the second aliquot of the G7 supernatant obtained as described above. They were carried out following the protocol used to measure the Na^+/K^+-ATPase activity, but using *N*-ethylmaleimide (100 mM; Sigma-Aldrich) as a specific inhibitor of the H^+-ATPase [25]. Data were normalized to protein content in the supernatant.

Carbonic anhydrase activity assay

The seventh pair of gills (G7) was thawed on ice, homogenized (10% weight:volume) in cold phosphate buffer (225 mM mannitol, 75 mM sucrose, 10 mM Trizma base, 10 mM NaH_2PO_4, pH adjusted to 7.4), sonicated on ice for 30 s (Sonozap 4180; Sonaer Ultrasonics), and centrifuged ($10{,}000 \times g$) for 20 min at 4°C (Mikro22R; Hettich). An aliquot (200 μl) of the supernatant was used as enzyme source to determine the total CA activity. The remaining supernatant was centrifuged ($100{,}000 \times g$) for 90 min at 4°C to obtain the cytoplasmic CA fraction. Gill CA activity was determined in the supernatant using the ΔpH method, as previously described [17]. Data were normalized to protein content in the supernatant.

Data analyses

Data were expressed as mean ± standard error ($n = 6$). Mean values from mRNA expression and enzyme activity data were subjected to analysis of variance, followed by the Tukey test. The significance level adopted was 95% ($\alpha = 0.05$). Analysis of variance assumptions (data normality and homogeneity of variances) were previously verified.

RESULTS

Mean Cu concentrations in diluted (2‰) and full (30‰) seawater were not significantly different. Therefore, only one mean was calculated (nominal = 1 μM; total Cu = 0.99 ± 0.13 μM; dissolved Cu = 0.78 ± 0.10 μM). No mortality was observed in either control or Cu-exposed crabs.

In control crabs, salinity did not affect transcription of the β-actin gene (2‰ salinity = 801.1 ± 173.0 densitometry units; 30‰ salinity = 914.8 ± 166.3 densitometry units). Also, waterborne Cu exposure did not affect β-actin mRNA expression (2‰ salinity = 619.3 ± 34.5; 30‰ salinity = 854.4 ± 69.5 densitometry units). Therefore, the mRNA expression of this gene was used to normalize the data obtained for the other genes analyzed in the present study.

In control crabs, salinity did not affect the transcription of genes encoding the ion-transporting proteins analyzed, except for that encoding the $Na^+/K^+/2Cl^-$ cotransporter. Transcription of this gene was higher in diluted seawater (2‰) than in full seawater (30‰; Table 2).

In crabs acclimated to diluted seawater (2‰), waterborne Cu exposure markedly inhibited the expression of mRNA encoding the Na^+/K^+-ATPase enzyme (88% inhibition) and for the $Na^+/K^+/2Cl^-$ cotransporter (87% inhibition). Although the Cu effect on the transcription of gene encoding the H^+-ATPase was not significant, an important reduction (60% inhibition) was also observed. On the other hand, Cu exposure did not significantly affect the expression of the mRNA encoding for the ion-transporting proteins analyzed in crabs acclimated to full seawater (30‰; Table 2). Waterborne Cu exposure did not significantly change the Na^+/K^+-ATPase (Fig. 1), H^+-ATPase (Fig. 2), total CA (Fig. 3A), or cytoplasmic CA (Fig. 3B) activity in gill of blue crabs acclimated to diluted or full seawater.

Table 2. Gene transcription (target mRNA/β-actin mRNA) of the sodium-potassium adenosine triphosphatase (NaK-ATPase gene), hydrogen adenosine triphosphatase (H-ATPase gene), membrane carbonic anhydrase (CasCag gene), cytoplasmic carbonic anhydrase (CasCac gene), and sodium-potassium-chloride cotransporter (NaKCC gene) in posterior gills (sixth pair of gills) of the blue crab *Callinectes sapidus* kept under control condition (no copper addition to the water) or acutely exposed (96 h) to waterborne Cu (1 μM) at water salinity of 2 and 30‰[a]

	Salinity 2‰		Salinity 30‰	
Gene	Control	Copper	Control	Copper
NaK-ATPase	1.32 ± 0.72A	0.16 ± 0.10B	1.26 ± 0.15A	1.03 ± 0.24AB
H-ATPase	0.84 ± 0.34A	0.34 ± 0.03A	0.50 ± 0.13A	0.51 ± 0.12A
CasCAg	1.59 ± 0.63A	1.62 ± 0.52A	1.57 ± 0.26A	1.23 ± 0.32A
CasCac	1.72 ± 0.16A	2.06 ± 0.48A	1.73 ± 0.62A	1.72 ± 0.14A
NaKCC	0.85 ± 0.30A	0.11 ± 0.07B	0.40 ± 0.04B	0.31 ± 0.08B

[a] Data are expressed as mean ± standard error ($n = 3$–6). Different uppercase letters indicate significantly different mean values ($p < 0.05$) between treatments and salinities tested for each gene.

DISCUSSION

In crustaceans inhabiting diluted seawater or freshwater, such as the blue crab *Callinectes sapidus*, active transbranchial NaCl absorption constitutes one element of the hyperosmoregulatory process. The other element is represented by water excretion. Both processes have been intensively studied in many species. The diffusive loss of ions from the hemolymph to the diluted media is compensated by actively pumping Na^+ and Cl^- across the gills, especially the posterior ones. These

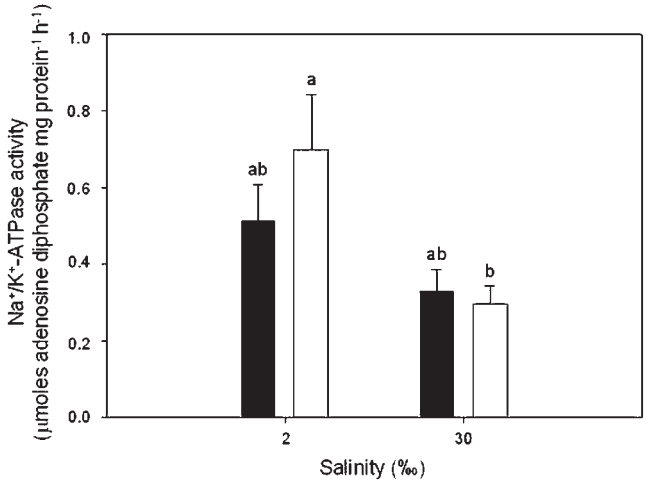

Fig. 1. Sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity in the seventh pair of gills (G7) of the blue crab *Callinectes sapidus* kept under control conditions (solid bars) or acutely exposed (96 h) to waterborne copper (1 μM; open bars) at water salinity of 2 and 30‰. Data are expressed as mean ± standard error ($n = 6$). Different lowercase letters indicate significantly different mean values ($p < 0.05$) between treatments and salinities tested.

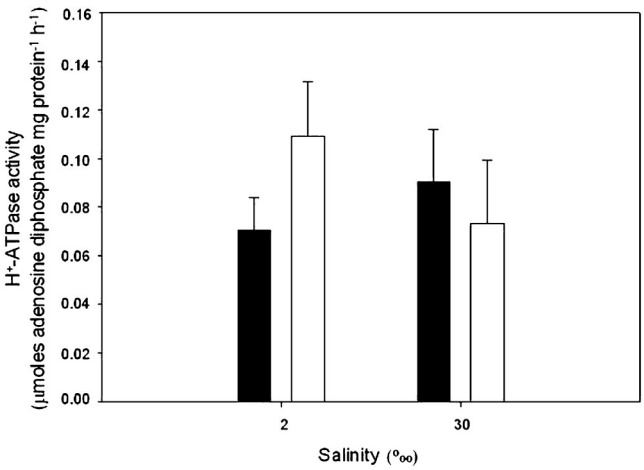

Fig. 2. Hydrogen adenosine triphosphatase (H^+-ATPase) activity in the seventh pair of gills (G7) of the blue crab *Callinectes sapidus* kept under control conditions (solid bars) or acutely exposed (96 h) to waterborne copper (1 μM; open bars) at water salinity of 2 and 30‰. Data are expressed as mean ± standard error ($n = 6$). No significant difference ($p > 0.05$) was observed between treatments (Cu exposure and salinities).

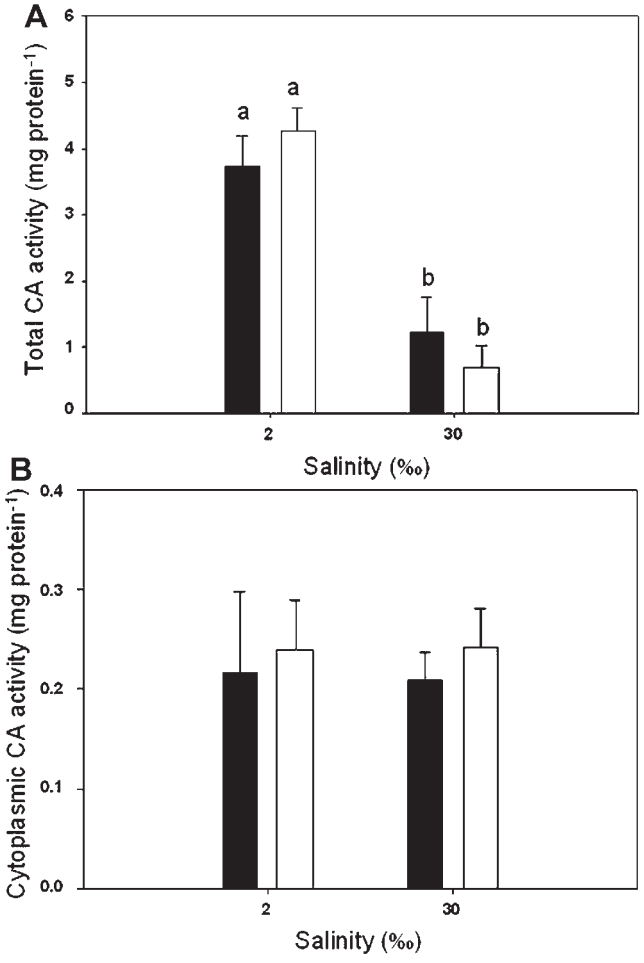

Fig. 3. Total (**A**) and cytoplasmic (**B**) carbonic anhydrase (CA) activity in the pair of gills 7 (G7) of the blue crab *Callinectes sapidus* kept under control conditions (solid bars) or acutely exposed (96 h) to waterborne copper (1 μM; open bars) at water salinity of 2 and 30‰. Data are expressed as mean ± standard error ($n = 6$). For total CA activity, different letters indicate significantly different mean values ($p < 0.05$) between treatments and salinities tested. For cytoplasmic CA, no significant difference ($p > 0.05$) was observed between treatments (Cu exposure and salinities).

gills show higher activities of enzymes related to ion transport, especially the Na^+/K^+-ATPase and have more mitochondria-rich cells than the anterior ones. Otherwise, anterior gills are considered as largely respiratory, showing passive Na^+ movements. On the other hand, most crustacean species living in seawater are osmoconformers. In this case, the total osmotic pressure of their hemolymph is nearly identical to that of seawater. Therefore, the osmotic stress is minimal and activity of the mechanisms involved in the regulation of the hemolymph osmotic concentration is reduced [26–28]. For example, the blue crab *C. sapidus* hyperosmoregulates its hemolymph osmotic concentration in low-salinity water and acts as an osmoconformer species in water of salinities above 27‰ [19,29]. In the present study, the response of the blue crabs to Cu exposure was analyzed at these two different osmotic conditions (2 and 30‰ salinities).

In the present study, Cu was tested at 1 μM (nominal concentration) in both experimental salinities (2 and 30‰). This concentration was selected based on the fact that at 1 μM virtually all Cu is dissolved in saltwater, whereas significant metal precipitation is observed at higher Cu concentrations in the experimental salinities tested in the present study. Also, Cu was tested at 1 μM because a significant metal accumulation in the gills (anterior and posterior gills) of the blue crab *C. sapidus* was observed at that concentration in both experimental salinities (2 and 30‰). However, very low crab mortality rates were observed [30]. As expected, no mortality was observed in the present study when crabs were exposed to 0.78 μM dissolved Cu in both experimental salinities (2 and 30‰). In fact, the dissolved Cu concentration tested in the present study (0.78 μM) corresponded to 14.4 and 1.47% of the 96-h LC50 (5.40 and 52.99 μM) for blue crabs *C. sapidus* exposed to Cu at 2 and 30‰ salinity, respectively [30]. Therefore, the dissolved Cu concentration tested in the present study (0.78 μM) can be considered as sublethal and environmentally relevant. This statement is based on the fact that dissolved Cu concentrations close to that tested in the present study have been observed in estuarine waters where the blue crab *C. sapidus* is abundant [2].

In blue crabs acclimated to seawater (30‰ salinity), Cu did not affect gene transcription (Table 2) or activity of the ion-transporting proteins analyzed (Figs. 1–3). Little or no variation in Na^+/K^+-ATPase activity was observed in seawater fish [11] and crustaceans [12] exposed to Cu as well. The lack of a Cu effect in seawater crustaceans, including the blue crab *C. sapidus*, would be due to the reduced ion uptake needed for regulation of the hemolymph osmotic concentration in seawater (30‰). Also, reduced Cu toxicity resulting from the lower free metal bioavailability in seawater [12] cannot be ruled out.

Waterborne Cu exposure, however, inhibited transcription of the gene encoding the gill Na^+/K^+-ATPase of blue crabs acclimated to low salinity (2‰; Table 2). However, no significant effect was observed on the enzyme activity (Fig. 1). These findings indicate, for the first time, that the gene transcription is down-regulated at an environmentally relevant Cu concentration, before a significant inhibition of the enzyme activity can be observed. In fact, Cu is reported as an osmoregulatory toxicant to both freshwater fish [31] and crustaceans [32]. Furthermore, this toxicity is clearly related to an inhibition of the gill Na^+/K^+-ATPase activity. Free Cu has shown to bind covalent SH groups of Na^+/K^+-ATPase, thus interfering with conformational changes of the protein. Also, Cu is able to interact specifically with Na^+/K^+-ATPase magnesium binding sites, readily inhibiting the activity of this pump [33,34].

Na^+/K^+-ATPase activity in blue crabs can be modulated by insertion of pre-existing enzyme units into the cell membrane or synthesis of new enzyme units [29]. Considering that Cu exposure induced an approximately 90% inhibition of mRNA expression, the lack of Cu effect on gill Na^+/K^+-ATPase activity can be explained considering an insertion of pre-existing enzyme units at the basolateral membrane of gill cells. The amount of enzyme units inserted would be enough to maintain the maximum enzyme activity, compensating for the possible inhibitory effect induced by the Cu exposure. It is important to note that the exposure time used in the present study was 96 h, i.e., a classical condition used in toxicological tests with fish and macroinvertebrates [35]. Perhaps a significant inhibition of the gill Na^+/K^+-ATPase activity would be observed with a longer time of exposure to Cu.

As reported for the Na^+/K^+-ATPase, an approximately 90% inhibition of the NaKCC mRNA expression induced by Cu was also observed in gills of blue crabs acclimated to low salinity (2‰; Table 2). This gene encodes the $Na^+/K^+/2Cl^-$ cotransporter, which is a key transporter for Na^+ and Cl^- uptake in weak hyperosmoregulators such as the crabs *Carcinus maenas* and *C. sapidus* [36–38]. Preliminary evidence suggests that this cotransporter is expressed preferentially in apical membranes of posterior gills of *C. sapidus*, suggesting its involvement in the osmotic control [37]. The significant decrease (~50%) in NaKCC gene transcription observed in control blue crabs acclimated to seawater (30‰) compared with those acclimated to diluted seawater (2‰; Table 2) supports the idea that the $Na^+/K^+/2Cl^-$ cotransporter is involved in the hemolymph ion regulation in *C. sapidus*.

Considering the important roles of Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter in the osmoregulatory processes in *C. sapidus* and the marked Cu effects on the transcription of the genes encoding these proteins in gills of blue crabs acclimated to low salinity (2‰; Table 2), we suggest that this metal is also a potential osmoregulatory toxicant to *C. sapidus* under this osmotic condition. Unfortunately, it was not possible in the present study to evaluate the influence of the acclimation salinity and the Cu effect on the activity of the gill $Na^+/K^+/2Cl^-$ cotransporter.

Conversely, no significant effect of Cu on transcription of the gene encoding the H^+-ATPase (Table 2) or the enzyme activity (Fig. 2) was observed in posterior gills of blue crabs acclimated to salinity 2‰. Also, no significant difference between control blue crabs acclimated to seawater and diluted seawater was observed (Table 2 and Fig. 2). These results were expected, given that this enzyme is involved mainly in osmoregulatory mechanisms of strong hyperosmoregulators such as freshwater fish and crustaceans such as the rainbow trout and the Chinese crab *Eriocheir sinensis*, respectively [27,38]. An apical H^+-ATPase activity is apparently required to complement the Na^+/K^+-ATPase in driving osmoregulatory ion uptake from the dilute medium [39]. It generally occurs together with apical Na^+ channels (ENaC). This pump is electrically linked to the ENaC, macroscopically affecting the equimolar Na^+/H^+ exchange. The cellular substrate for the proton pump is supplied via hydration of CO_2 catalyzed by CA [27].

Data from several molecular and physiological studies have indicated that the H^+-ATPase found in gills of weak hyperosmoregulator crabs is not involved in osmoregulatory processes but probably serves in the acidification of intracellular vesicles. This evidence was reported for the shore crab *C. maenas*, which cannot survive in salinities lower than 8‰ [39]. Despite the fact that the blue crab is considered as a moderate osmoregulator, as with the shore crab, it can tolerate salinities as low as 2‰ as tested in the present study. Therefore, the H^+-ATPase present in gills of the blue crab *C. sapidus* could also be implicated in ion uptake in this crab species as described for the freshwater *E. sinensis*. However, additional studies should be performed to clarify the possible role of the H^+-ATPase present in the gill apical membrane in the osmoregulation of the blue crab *C. sapidus*. Although the Cu effect on the transcription of the gene encoding the H^+-ATPase was not significant in blue crabs acclimated to diluted seawater (2‰ salinity), a noticeable reduction (~60%) in mRNA expression was observed (Table 2). This finding reinforces the idea that Cu is toxic to blue crab *C. sapidus* by disturbing the osmoregulatory processes.

Carbonic anydrase is probably the first nonmetabolic enzyme to be associated with epithelial ion transport. It does not generate transmembrane transport per se but accelerates the CO_2 dissociation, providing H^+ and HCO_3^- to their respective transporters. An increased branchial CA activity was observed in blue crabs *C. sapidus* acclimated to low salinity, the magnitude of CA induction being inversely proportional to the acclimation salinity [40]. In the present study, the CA activity was also higher in posterior gills of control crabs acclimated to diluted seawater than in those acclimated to seawater. However, this increased activity was detected only for total CA (Fig. 3A), not for the cytoplasmic CA (Fig. 3B). Furthermore, no significant change in CA gene transcription as a function of acclimation salinity was observed (Table 2). Physiological studies reveal more than one isoform of CA with different functions. The membrane-associated CA is the isoform related to crab respiration, facilitating CO_2 excretion, whereas the cytoplasmic CA isoform would be directly involved in osmotic and ionic regulation [41]. Therefore, the increased total CA activity observed in control blue crabs acclimated to diluted seawater could result from a higher activity and/or expression of the CA membrane-associated isoform.

In summary, data reported here suggest that the mechanism of Cu toxicity in seawater acclimated crabs (osmoconforming crabs) is clearly not related to metal interaction with the ion-transporting proteins analyzed. However, they also indicate that Cu is a potential osmoregulatory toxicant to *C. sapidus* in diluted seawater, as observed for other osmoregulating crustaceans. Data clearly show that dissolved Cu at an environmentally relevant concentration (0.78 μM) can be toxic to euryhaline crustaceans living in estuaries (diluted seawater). This statement is based on the fact that mRNA expression of some key proteins involved in osmoregulation in these animals was markedly reduced after exposure to an environmentally relevant concentration of Cu. Therefore, it is fundamental to assess the amount of Cu that is environmentally realistic in order to prevent further damage to those organisms and the whole ecosystem.

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