Arch Environ Contam Toxicol (2017) 72:461–470
DOI 10.1007/s00244-017-0379-8

The Effects of Acute Copper and Ammonia Challenges on Ammonia and Urea Excretion by the Blue Crab *Callinectes sapidus*

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Received: 9 August 2016 / Accepted: 15 February 2017 / Published online: 4 March 2017

Abstract Copper (Cu) is a persistent environmental contaminant that elicits several physiological disturbances in aquatic organisms, including a disruption in ammonia regulation. We hypothesized that exposure to Cu in a model crustacean (blue crab, *Callinectes sapidus*) acclimated to brackish water (2 ppt) would lead to hyperammonemia by stimulating an increase in ammonia production and/or by inhibiting ammonia excretion. We further hypothesized that urea production would represent an ammonia detoxification strategy in response to Cu. In a pilot experiment, exposure to 0, 100, and 200 μg/L Cu for 6 h caused significant concentration-dependent increases in ammonia excretion (J_{amm}). Based on these results, an acute 24-h 100 μg/L Cu exposure was conducted and this similarly caused an overall stimulation of J_{amm} during the 24-h period, indicative of an increase in ammonia production. Terminal haemolymph total ammonia content (T_{amm}) was unchanged, suggesting that while ammonia production was increased, there was no inhibition of the excretion mechanism. In support of our second hypothesis, urea excretion (J_{urea}) increased in response to Cu exposure; haemolymph [urea] was unaffected. This suggested that urea production also was increased. To further test the hypothesis that J_{urea} increased to prevent hyperammonemia during Cu exposure, crabs were exposed to high environmental ammonia (HEA; 2.5 mmol/L NH_4HCO_3) for 12 h in a separate experiment. This led to a fourfold increase in haemolymph T_{amm}, whereas J_{urea} increased only transiently and haemolymph [urea] was unchanged, indicating that urea production likely does not contribute to the attenuation of hyperammonemia in blue crabs. Overall, Cu exposure in blue crabs led to increased ammonia and urea production, which were both eliminated by excretion. These results may have important implications in aquaculture systems where crabs may be exposed to elevated Cu and/or ammonia.

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Copper (Cu) is found naturally at relatively low levels in many aquatic systems, ranging from >1 to 30 μg/L, depending on geochemical properties of the environment. In areas affected by anthropogenic activities, however, Cu may be found at much greater levels, reaching 100 μg/L and up to 200 mg/L in some heavily impacted areas (Bidone et al. 2001; USEPA 2007). While Cu is an essential element, acting as a cofactor in many enzymes and other proteins, it may be toxic when present at elevated levels. In crustaceans, Cu has been well-documented to act as an ionoregulatory and osmoregulatory toxicant (Bambang et al. 1995; Bamber and Depledge 1997; Bianchini et al. 2004; Bjerregaard and Vislie 1986; Boitel and Truchot 1989, 1990; Brooks and Mills 2003; Hansen et al. 1992; Pinho et al. 2007; Thurberg et al. 1973; Weeks et al. 1993). In addition, a number of studies have suggested that exposure to waterborne Cu can disrupt nitrogen metabolism, leading to changes in the excretion of ammonia (Correa 1987; Garnacho et al. 2001; Harris and Santos 2000; Vosloo et al. 2002), the primary nitrogenous waste

excreted by decapod crustaceans (Regnault 1987). The effects of Cu on nitrogen metabolism by fish have been much more widely studied in the past, and findings have suggested that Cu causes hyperammonemia (elevated blood ammonia levels) by inhibiting the excretion of ammonia by the gill via the inhibition of specific transport proteins, and/or by increasing endogenous ammonia production by stimulating an increase in circulating cortisol levels (see Grosell 2012 for review). This may be potentially problematic in aquaculture settings where levels of environmental ammonia can be high due to the accumulation of metabolic waste and where Cu contamination from antifouling or algicidal agents can occur (Flemming and Trevors 1989; Randall and Tsui 2002).

Ammonia production in crustaceans occurs primarily as a function of amino acid catabolism and ammonia is ultimately excreted via the gills (Cameron and Batterton 1978). Ammonia excretion (J_{amm}) accounts for the majority (80%) of N excretion in crustaceans, whereas the remaining proportion is comprised of urea, free amino acid, nitrate, and nitrite excretions (Chen and Cheng 1993a, b; Chen et al. 1994; Regnault 1987). The current model for ammonia excretion by the gills of crustaceans includes the combined actions of a basolateral Na^+/NH_4^+-ATPase, the apical release of NH_4^+-containing acidified vesicles, and the diffusion of NH_3 down a partial pressure gradient, potentially facilitated by Rhesus (Rh) proteins (Kormanik and Cameron 1981; see Weihrauch et al. 2009 for review). Maintaining constant ammonia excretion is particularly important in most aquatic animals, because this N waste is toxic at elevated levels (Chen and Kou 1993). The excretion of ammonia and other N compounds by crustaceans is influenced by a number of environmental stressors (in addition to Cu), including alterations in salinity and high environmental ammonia (HEA) exposure. In general, acclimation to lower salinities appears to increase ammonia excretion (J_{amm}) in crustaceans (Chen and Chia 1996; Chen et al. 1994; Mangum et al. 1976; Spaargaren 1982), potentially due to the higher metabolic cost of hyper iono- and osmoregulation in dilute water or to the catabolism of intracellular amino acids when intracellular osmolality equilibrates with decreasing haemolymph osmolality. HEA exposure also dramatically alters nitrogen metabolism, primarily by inhibiting or even reversing ammonia excretion. Also notable are the increases in urea excretion (J_{urea}) and haemolymph urea content observed in many crustacean species exposed to HEA (Chen and Cheng 1993a, b; Chen and Chen 1997; Chen et al. 1994; Cheng et al. 2004; Hong et al. 2007; Liu et al. 2014; Ren et al. 2015), suggesting that ammonia detoxification via urea production may be a general feature of crustaceans.

The overall purpose of this study was to characterize the effects of acute waterborne Cu exposure on nitrogen metabolism in a model crustacean, the blue crab (*Callinectes sapidus*). While Cu is known to cause systemic hyperammonemia in fish (Grosell 2012), the effects of waterborne Cu exposure on nitrogen metabolism in crustaceans are relatively unknown. In our study, blue crabs were acclimated to 2 ppt—salinity within the range (freshwater to full strength seawater) experienced naturally in the wild (Millikin and Williams 1984). We chose this salinity because we predicted that it would be more physiologically challenging in the face of Cu exposure, based on the fact that ammonia production tends to be greater in hyper-regulating crabs (Chen and Chia 1996; Chen et al. 1994; Mangum et al. 1976; Spaargaren 1982). In addition, previous studies have demonstrated that crustaceans acclimated to lower salinities are generally more sensitive to Cu exposure, at least in terms of ionoregulatory disruption (Boitel and Truchot 1990; Martins et al. 2011a, b; Thurberg et al. 1973).

We hypothesized that exposure to Cu (nominally 100 μg/l) would lead to an increase in haemolymph ammonia content (hyperammonemia) of *C. sapidus* acclimated to 2 ppt via an inhibition of ammonia excretion and/or an increase in endogenous ammonia production. We further hypothesized that Cu-induced hyperammonemia would be accompanied by an increase in urea production (J_{urea} and/or haemolymph urea content), because many crustaceans have demonstrated the capacity to increase urea production in response to ammonia loading. To our knowledge, this was the first study to assess this potential detoxification pathway in response to waterborne Cu in a crustacean species. We additionally assessed the effects of Cu (100 μg/L) on haemolymph [Na^+], because this parameter is commonly affected by Cu exposure in hyper-regulating crustaceans (Boitel and Truchot 1990; Thurberg et al. 1973), likely by the inhibition of Na^+ uptake pathways (see Grosell 2012 for review). To understand the overall capacity of blue crabs to detoxify ammonia to urea, we induced hyperammonemia exogenously via HEA exposure and measured ammonia and urea excretion as well as haemolymph ammonia and urea content. We hypothesized that blue crabs would increase urea production to limit increases in haemolymph ammonia content during HEA exposure, demonstrating a capacity for ammonia detoxification via urea production. This additional experiment was critical to understanding whether the effects of Cu on J_{urea} and haemolymph urea content were related specifically to ammonia detoxification.

Materials and Methods

Crab Collection and Acclimation

Juvenile blue crabs (*C. sapidus*; mean wet mass = 38.5 ± 1.2 g; approximate carapace width = 4–6 cm) were

collected from clean reference sites at the Patos Lagoon estuary (Rio Grande, Rio Grande do Sul, Southern Brazil) and held in natural sea water diluted with dechlorinated Rio Grande tap water to a salinity of 2 ppt (water chemistry reported by Martins et al. 2011b) at 20 °C for at least 2 weeks before any experimentation. Crabs were fed to satiation three times per week with a mix of chopped fish and were fasted for at least 48 h prior to experimentation. Water was recirculated through a biological filter and was continuously aerated. An artificial 12-h light:12-h dark photoperiod was maintained throughout the entire housing period. All experiments were performed at the same salinity, temperature, and photoperiod as the holding conditions described above for the acclimation period.

Pilot Experiments

An initial, 6-h pilot experiment was performed to determine an appropriate concentration to elucidate the effects of waterborne Cu exposure on ammonia excretion by blue crabs. Crabs ($n = 3$–4 per treatment) were randomly selected and placed individually into 1-L beakers containing 400 mL of aerated brackish water (2 ppt) to which $CuSO_4$ had been added to nominal concentrations of 0, 100, and 200 μg/L Cu. Crabs were allowed to settle in the beakers for 10 min and thereafter initial 1-mL water samples were collected from each beaker. Following a 6-h flux period, final water samples were collected and crabs were weighed. Samples were stored at −20 °C for subsequent ammonia analysis. Based on the stimulatory effects of Cu on ammonia excretion in this pilot experiment (see "Results"), a concentration of 100 μg/L Cu, which was closer to measured levels of Cu in the local environment (~30 μg/L; Baumgarten and Niencheski 1998), was chosen for an acute 24-h exposure.

An additional 3-h pilot experiment was conducted to determine an appropriate concentration for the subsequent 12-h HEA experiment designed to cause internal ammonia loading in the absence of Cu. Crabs ($n = 3$–4) were selected randomly from holding tanks and placed individually into beakers containing 400 mL of aerated control brackish water (2 ppt) to which NH_4HCO_3 had been added to concentrations of 0, 0.5, 1, 2, 2.5, or 3.5 mmol/L. Crabs were allowed to settle in the beakers for 10 min, and thereafter initial 1-mL water samples were collected from each beaker. Following a 3-h flux period, final 1-mL water samples were collected, haemolymph samples (200 μL) were then collected from each crab via puncture at the base of the third or fourth pair of pereiopods, and crabs were weighed. Water samples were stored at −20 °C, and haemolymph samples were snap frozen in liquid nitrogen and stored at −80 °C for subsequent ammonia analyses. From this pilot experiment, a concentration of 2.5 mmol/L of NH_4HCO_3 was chosen as the lowest concentration resulting in a reversal of ammonia excretion, indicating ammonia uptake from the water, and a substantial increase in haemolymph T_{amm}. Note that these experiments, in addition to the 12-h HEA experiment described below, were conducted in brackish water to which no Cu was added.

Acute 24-h Copper Exposure

For the 24-h Cu exposure, crabs ($n = 6$ per treatment) were randomly selected from the holding tank and were placed individually into 1-L glass beakers containing 800 mL of aerated brackish water (2 ppt) to which nominally 0 or 100 μg/L Cu had been added. After an initial 10-min adjustment period to allow crabs to settle in the beakers, initial 1-mL water samples were collected from each beaker, marking the beginning of the experimental period. Thereafter, water samples were collected from each beaker at 3, 6, 9, and 12 h. At 12 h, to minimize the buildup of ammonia in the experimental beakers, crabs were transferred to new beakers containing fresh brackish water (2 ppt) to which the respective Cu concentration had been added, and water samples were collected from each beaker. A final water sample was collected at 24 h (12 h after water change). Haemolymph samples (200 μL) were then collected from each crab via puncture at the base of the third or fourth pair of pereiopods, and individual crab weights were recorded. Water samples were stored at −20 °C until subsequent total ammonia (T_{amm}) and [urea] analyses. Haemolymph samples were snap frozen in liquid nitrogen and stored at −80 °C until subsequent T_{amm}, [Na^+], and [urea] analyses. At the end of the experiment, 10-mL water samples were collected from a subset of beakers, filtered through a 0.45-μm filter, and acidified to 1% HNO_3 for the later determination of dissolved Cu concentrations in the exposure water.

12-h High External Ammonia Exposure

Crabs ($n = 6$) were randomly selected from holding tanks and placed individually into beakers containing 400 mL of aerated control brackish water (2 ppt), with no ammonia added. Initial 1-mL water samples were collected from each beaker, marking the beginning of the 3-h pre-HEA control period. After 1.5 h, haemolymph samples (200 μL) were collected from each crab following the protocol described above. At the 3-h time point, final water samples were collected from each beaker and crabs were transferred individually into beakers containing 400 mL of brackish water (2 ppt) to which 2.5 mmol/L NH_4HCO_3 had been added (HEA). Following transfer to HEA, 1-mL water samples were collected from each beaker at 0, 3, 6, 9, and 12 h, whereas haemolymph samples (200 μL) were

collected from each crab at 1, 3, 6, and 12 h. Following this 12-h HEA period, crabs were then transferred into beakers containing 800 mL of control water (2 ppt, no ammonia-added) for a 12-h recovery. During this period, water samples (1 mL) were collected at 0, 3, 6, and 12 h while haemolymph samples (200 µL) were collected at 3 and 12 h. All water and haemolymph samples were handled and stored as described above for the Cu exposure.

Analytical Techniques and Calculations

T_{amm} of water samples was determined spectrophotometrically using the indophenol-salicylate method described by Verdouw et al. (1978). J_{amm} (µmol/g/h) was then calculated using the following equation:

$$J_{amm} = [(T_{amm}i - T_{amm}f) * V]/\text{wt}/t \quad (1)$$

where $T_{amm}i$ and $T_{amm}f$ are initial and final water T_{amm} (µmol/L), respectively; V is water volume (L); wt is the weight of the crab (g); and t is time (h). Note that negative J_{amm} is representative of excretion of ammonia into the surrounding environment while positive J_{amm} represents uptake of ammonia from the surrounding environment.

Urea concentration (expressed as µmol N, accounting for two moles N per mole urea) was measured using an assay modified to measure low levels of urea based on the methods described by Rahmatullah and Boyde (1980). J_{urea} (µmol N/g/h) was calculated using the following equation:

$$J_{urea} = [([\text{urea}]_i - [\text{urea}]_f) * V]/\text{wt}/t \quad (2)$$

where $[\text{urea}]_i$ and $[\text{urea}]_f$ are initial and final water urea concentration (µmol N/L), respectively; V is water volume (L); wt is the weight of the crab (g); and t is time (h). Note that negative J_{urea} is representative of urea excretion into the surrounding water.

Haemolymph samples were removed from the −80 °C freezer and thawed on ice. Haemolymph T_{amm} was measured using a commercial assay kit (Raichem Ammonia Kit, Cliniqa, San Marcos, CA, USA), which is based on the glutamate dehydrogenase method and haemolymph [urea] was measured using the same method described for water samples above. $[Na^+]$ in haemolymph samples was measured by flame atomic absorption spectroscopy (AAS 932 Plus, GBC, Hampshire, IL, USA). Dissolved Cu concentrations in acidified water samples were also measured using flame atomic absorption spectroscopy (AAS 932 Plus, GBC, Hampshire, IL, USA) as per methods described previously (Zimmer et al. 2012).

Statistical Analyses

All data are represented as mean ± SEM (n = number of crabs) and significance was accepted at $P < 0.05$. The linear relationship between external [Cu] and % J_{amm} stimulation in the 6-h pilot experiment was determined using linear regression. In the 24-h Cu exposure, significant differences in mean J_{amm} or J_{urea} between crabs exposed to control (nominal 0 µg/L) and 100 µg/L over 24 h were determined using a two-way ANOVA, with copper and time as variables. Significant differences in terminal haemolymph T_{amm}, $[Na^+]$, and [urea] between the two groups were determined using a Student's two-tailed t test. Significant differences in J_{amm}, haemolymph T_{amm}, J_{urea}, and haemolymph [urea] over 12 h of HEA and 12 h of recovery were determined using a repeated measures one way-ANOVA followed by a Holm–Sidak post hoc test or a repeated measures ANOVA on Ranks followed by a Student–Newman–Keuls Method post hoc test, in the case of failed normality tests. All statistical analyses were performed with Sigma Stat v3.5 software (Systat Software, Inc.), and specific details for statistical approaches in each experiment are included in corresponding figure captions.

Results

Pilot Experiments

In the Cu pilot experiment, exposure to waterborne Cu for 6 h caused a stimulation of J_{amm} in a concentration-dependent manner ($R = 0.99$). There were 42 and 77% increases in J_{amm} at 100 and 200 µg/L Cu respectively (Fig. 1).

In the HEA pilot experiment, exposure to 2.5 mmol/l NH_4HCO_3 for 3 h resulted in a threefold increase in haemolymph T_{amm} and a reversal of J_{amm}, indicating an uptake of ammonia from the environment (data not shown). This was the lowest of the five concentrations tested to result in a marked reversal of J_{amm}, which was necessary to ensure adequate ammonia loading in the subsequent 12-h experiment.

Acute 24-h Cu Exposure

Mean dissolved Cu concentration in the 24-h Cu exposure for 100 µg/L (nominal value) was 147.6 ± 9.4 µg/L ($n = 5$). Cu concentration in control exposures was below the limit of detection (5 µg/L) of our spectrometer. Crabs exposed to nominally 100 µg/L Cu for 24 h exhibited an increase in J_{amm}, which was significant as an overall Cu effect in the two-way ANOVA (Fig. 2; denoted by a single asterisk, $P = 0.025$), although rates at specific times were not significantly elevated. Terminal haemolymph T_{amm} (Fig. 3a) and $[Na^+]$ values (Fig. 3b) were not significantly altered. J_{urea}, for which 12-h increments were needed for reliable detection of differences in water [urea], increased

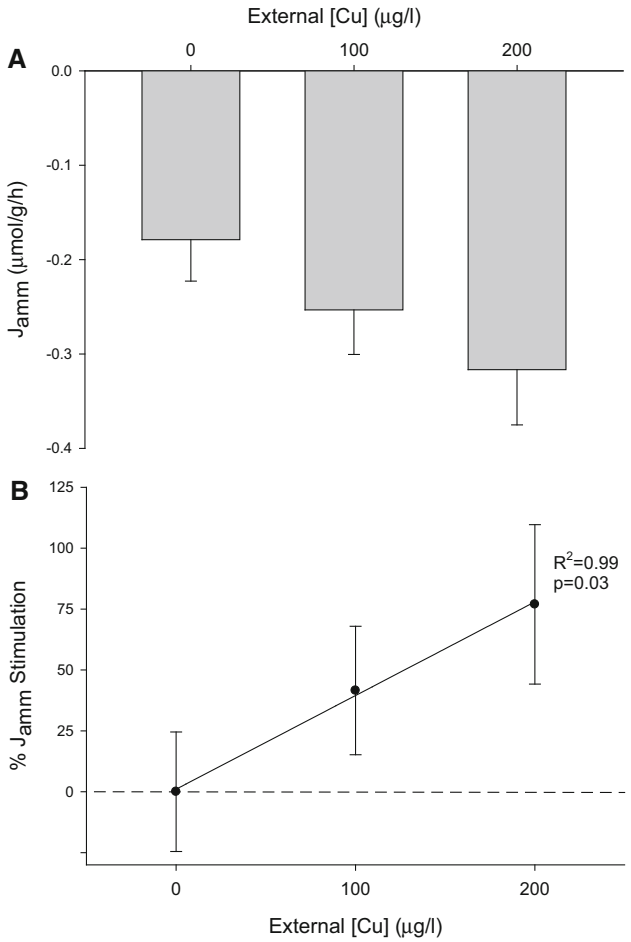

Fig. 1 **a** Ammonia excretion (J_{amm}) in blue crabs exposed to nominal 0, 100, and 200 µg/L Cu for 6 h. **b** Data from panel A expressed as % change in J_{amm} relative to the rates from crabs exposed to nominal 0 µg/L Cu. A significant concentration-dependent effect of Cu on J_{amm} ($R = 0.99$; $P = 0.03$) was determined by linear regression. Data are mean ± SE ($n = 3$–4)

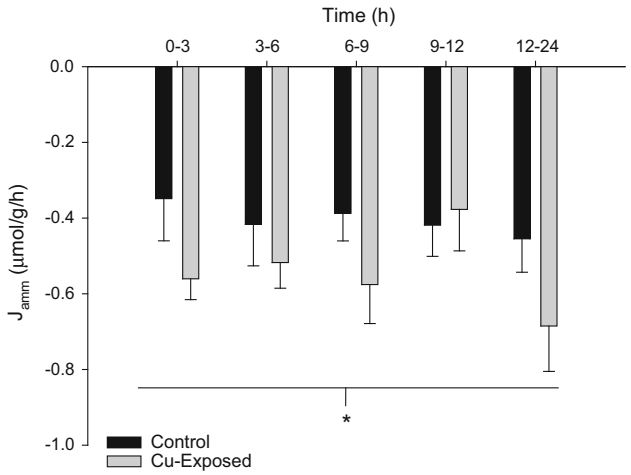

Fig. 2 Ammonia excretion (J_{amm}) over 24 h in control (nominal 0 µg/L) and Cu-exposed (nominal 100 µg/L) blue crabs (*Callinectes sapidus*). *Asterisk* represents an overall significant effect ($P = 0.025$) of Cu exposure over the entire 24-h exposure as determined by a two-way ANOVA; there was no significant interaction between Cu exposure and time ($P = 0.593$). Data are mean ± SE ($n = 6$)

significantly ($P = 0.002$) overall in response to Cu exposure (Fig. 4a). Terminal haemolymph [urea], similar to T_{amm} and [Na^+], was not significantly altered by Cu exposure (Fig. 4b).

12-h High External Ammonia Exposure

Upon exposure to HEA (2.5 mmol/L NH_4HCO_3), crabs began to take up ammonia from the environment such that J_{amm} over the first 3 h of exposure was 0.66 ± 0.17 µmol/g/h, which was significantly different from pre-HEA control J_{amm} of −0.23 ± 0.04 µmol/g/h (Fig. 5a). By 9–12 h HEA exposure, ammonia uptake was more than 50% lower than in the initial 3 h of exposure, although this was not statistically significant (Fig. 5a). Following transfer to ammonia-free water, ammonia excretion (negative J_{amm}) was re-established to a level significantly greater than that

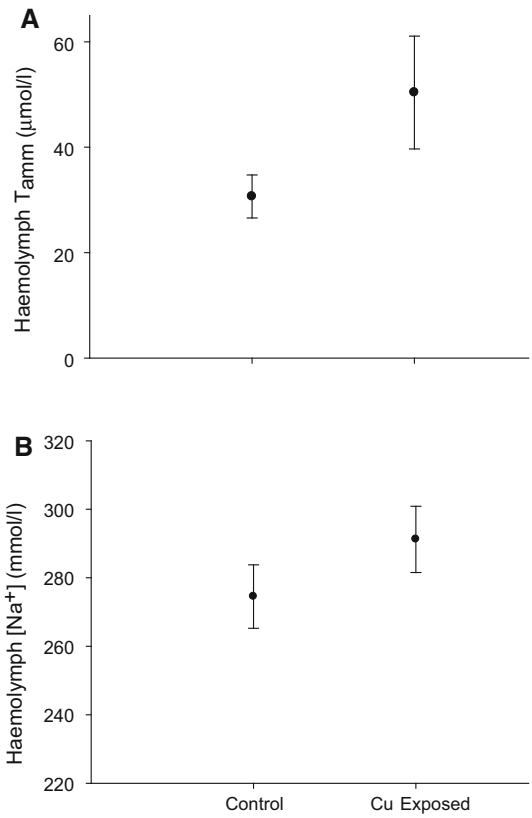

Fig. 3 **a** Terminal haemolymph total ammonia levels (T_{amm}) and **b** terminal haemolymph [Na^+] in control (nominal 0 µg/L) and Cu-exposed (nominal 100 µg/L) blue crabs (*Callinectes sapidus*) at 24 h. Data are mean ± SE ($n = 6$)

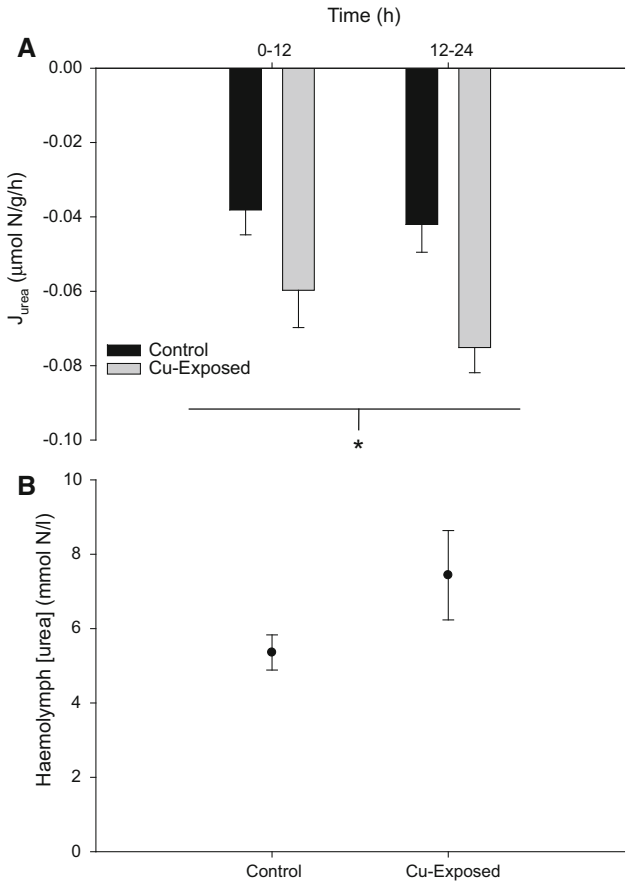

Fig. 4 **a** Urea-N excretion (J_{urea}) and **b** terminal haemolymph [urea] over 24 h in control (nominal 0 µg/L) and Cu-exposed (nominal 100 µg/L) blue crabs (*Callinectes sapidus*). *Asterisk* in panel A represents an overall statistically significant effect ($P = 0.002$) of Cu exposure over time as determined by a two-way ANOVA. There was no significant interaction between Cu exposure and time ($P = 0.472$). Data are mean ± SE ($n = 6$)

observed during the pre-exposure control period (Fig. 5a). By 3–6 h recovery, J_{amm} decreased by approximately 50% but remained significantly higher than the rate in the initial pre-exposure period for the duration of the 12-h recovery period (Fig. 5a). Exposure to HEA also led to a significant 3.5-fold elevation in haemolymph T_{amm}, which was maintained throughout the duration of the exposure (Fig. 5b). Interestingly, while ammonia uptake displayed an initially high rate at 0–3 h of HEA exposure followed by attenuation by 3–6 h of exposure, haemolymph T_{amm} did not show an initial transient increase but was maintained constant throughout the exposure. By 3 h of recovery, haemolymph T_{amm} was restored to control levels, consistent with the elevated J_{amm} observed over 0–3 h of post-HEA recovery (Fig. 5). Furthermore, exposure to HEA led to a transient, significant twofold increase in J_{urea} during the first 3 h of HEA, relative to the pre-exposure period (Fig. 6a), whereas haemolymph [urea] was not altered by HEA exposure (Fig. 6b). Unfortunately, urea could not be reliably detected in the recovery period due to the large volume used (800 mL), which was required to minimize the build-up of ammonia in the external medium. Thus, we could not report J_{urea} during recovery.

Discussion

Waterborne Cu Exposure Increases J_{amm} and J_{urea} by Blue Crabs

Exposure to nominal 100 and 200 µg/L Cu for 6 h caused a concentration-dependent increase in J_{amm} by blue crabs acclimated to 2 ppt sea water (Fig. 1). Moreover, exposure to nominal 100 µg/L Cu for 24 h caused an overall significant increase in J_{amm} (Fig. 2), whereas terminal haemolymph T_{amm} was not significantly affected by Cu (Fig. 3a). While these results do not support our initial hypothesis that Cu exposure would lead to hyperammonemia in crabs acclimated to 2 ppt, the increase in J_{amm} indicates that ammonia production was stimulated in response to Cu exposure, whereas excretion was not impacted but rather kept pace with elevated production. Notably, this response appeared to be more sensitive than the disruption of Na^+ balance typically observed in hyper-regulating crabs exposed to Cu (Boitel and Truchot 1990; Thurberg et al. 1973), as terminal haemolymph [Na^+] was not significantly affected (Fig. 3b). In fish, ammonia production in response to Cu exposure is hypothesized to be stimulated by increases in circulating levels of cortisol, the major stress hormone (Grosell et al., 2002). In crabs, the major stress hormone is the crustacean hyperglycemic hormone (CHH), the levels of which increased in the haemolymph of the shrimp *Paelaemon elegans* during exposure to waterborne Cu (Lorenzon et al. 2004). Although not assessed in that study, it is possible that the ensuing hyperglycemic response could reflect the stimulation of ammonia production via the catabolism of gluconeogenic amino acids.

Whereas Cu appeared to increase ammonia production in blue crabs acclimated to brackish water (2 ppt), the animals were able to excrete this excess ammonia to the surrounding environment (Fig. 2). In mysid shrimp (*Praunus flexuosus*) acclimated to sea water, exposure to 200 µg/L Cu for 24 h also resulted in significant increases in J_{amm} (Garnacho et al. 2001). In two species of Brazilian mangrove crabs (*Ucides cordatus* and *Callinectes danae*) also acclimated to sea water, J_{amm} was similarly higher in crabs that were collected from a polluted site relative to a clean reference site, in accordance with higher tissue burdens of Cu in crabs from the polluted site (Harris and Santos 2000). In contrast, Cu appears to have an inhibitory

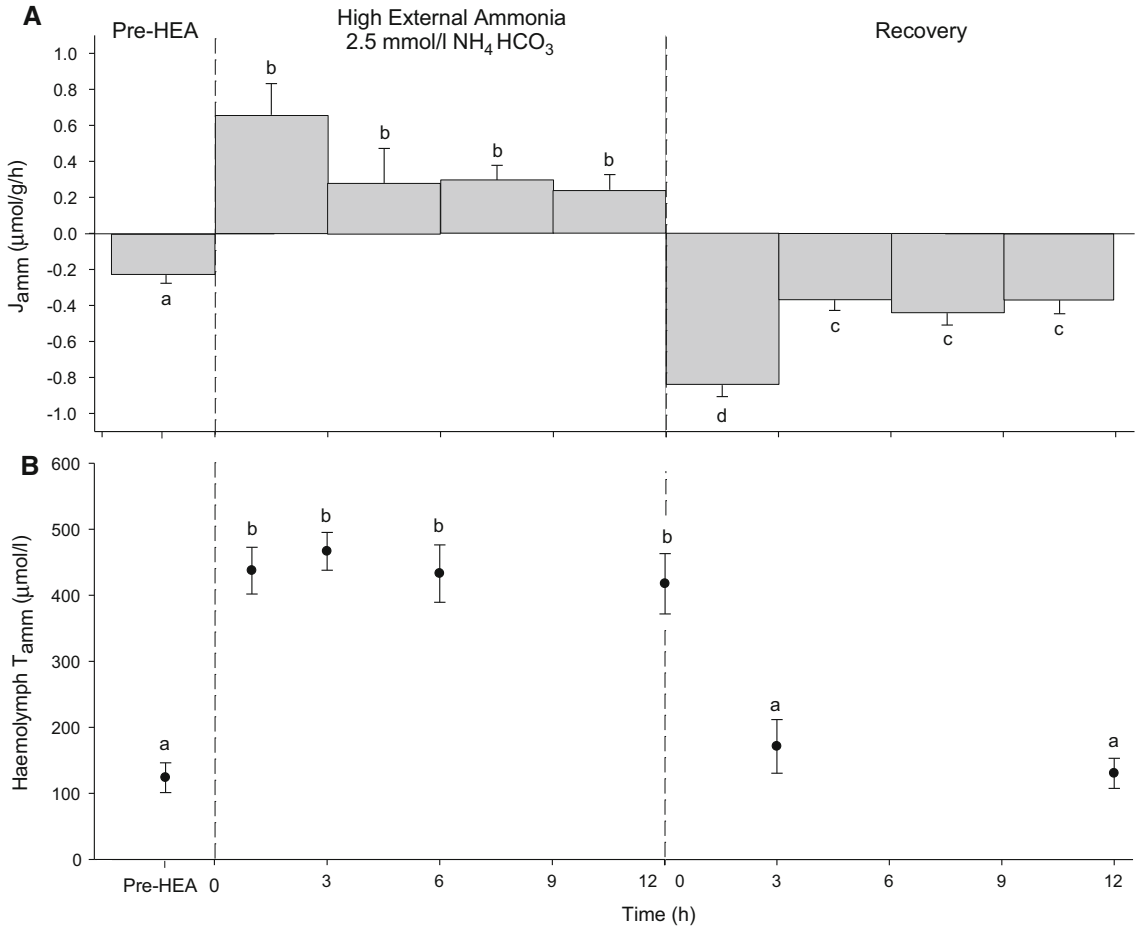

Fig. 5 **a** Ammonia excretion (J_{amm}) and **b** haemolymph total ammonia levels (T_{amm}) in blue crabs (*Callinectes sapidus*) under control conditions (pre-HEA), during 12 h of high external ammonia (HEA; 2.5 mmol/L NH_4HCO_3), and 12 h of recovery in control water. Means not sharing the *same letter* represent statistically significant differences over the experimental period as determined using a repeated measures ANOVA on ranks followed by a Student–Newman–Keuls method post hoc test or by a repeated measures ANOVA followed by a Holm–Sidak post hoc test, respectively. *Vertical dashed lines* mark the divides between the 3-h pre-HEA, 12-h HEA, and 12-h recovery periods. Data are mean ± SE ($n = 6$)

rather than stimulatory effect on J_{amm} in freshwater-acclimated crustaceans. In the freshwater shrimp *Macrobrachium carcinus*, exposure to 100 µg/L Cu for 6 h led to a 65% decrease in J_{amm} (Correa 1987) and exposure to 1 mg/L Cu for 14 d in the freshwater crab *Potamonautes warreni* led to a sustained 50% decrease in J_{amm} up to 21 d of exposure (Vosloo et al. 2002). The mechanism underlying this fresh versus sea water difference is unclear. In fish, Cu is believed to inhibit ammonia excretion by blocking key transporters and enzymes in the gill (Grosell 2012) with some studies suggesting Rh proteins (Lim et al. 2015) or carbonic anhydrase (Grosell et al. 2002; Zimmer et al. 2012) as potential targets. The latter appears to be independent of acclimation salinity (Zimmer et al. 2012), unlike the case of crustaceans. Inhibition of in vitro carbonic anhydrase activity has been demonstrated previously in a euryhaline crab (*Chasmagnathus granulata*; Vitale et al. 1999), potentially indicating this as a mechanism of J_{amm} inhibition in crustacean species. However, more work is needed to elucidate the basis of these differential responses to Cu in crustaceans.

We further hypothesized that if Cu were to cause hyperammonemia in *C. sapidus* that J_{urea} and/or haemolymph [urea] would increase as a function of ammonia detoxification, based on urea production in response to ammonia stress in other crustacean species (Chen and Cheng 1993a, b; Chen and Chen 1997; Chen et al. 1994; Cheng et al. 2004; Hong et al. 2007; Liu et al. 2014; Ren et al. 2015). Exposure to 100 µg/L Cu for 24 h led to an approximate 50% increase in J_{urea} during the 24-h period (Fig. 4a). Although crabs did not experience significant hyperammonemia in response to exposure to 100 µg/L Cu, this was nevertheless in general agreement with our hypothesis. Perhaps this increase in urea production, in addition to the increase in J_{amm}, acted to maintain haemolymph T_{amm} during Cu exposure. However, this increase

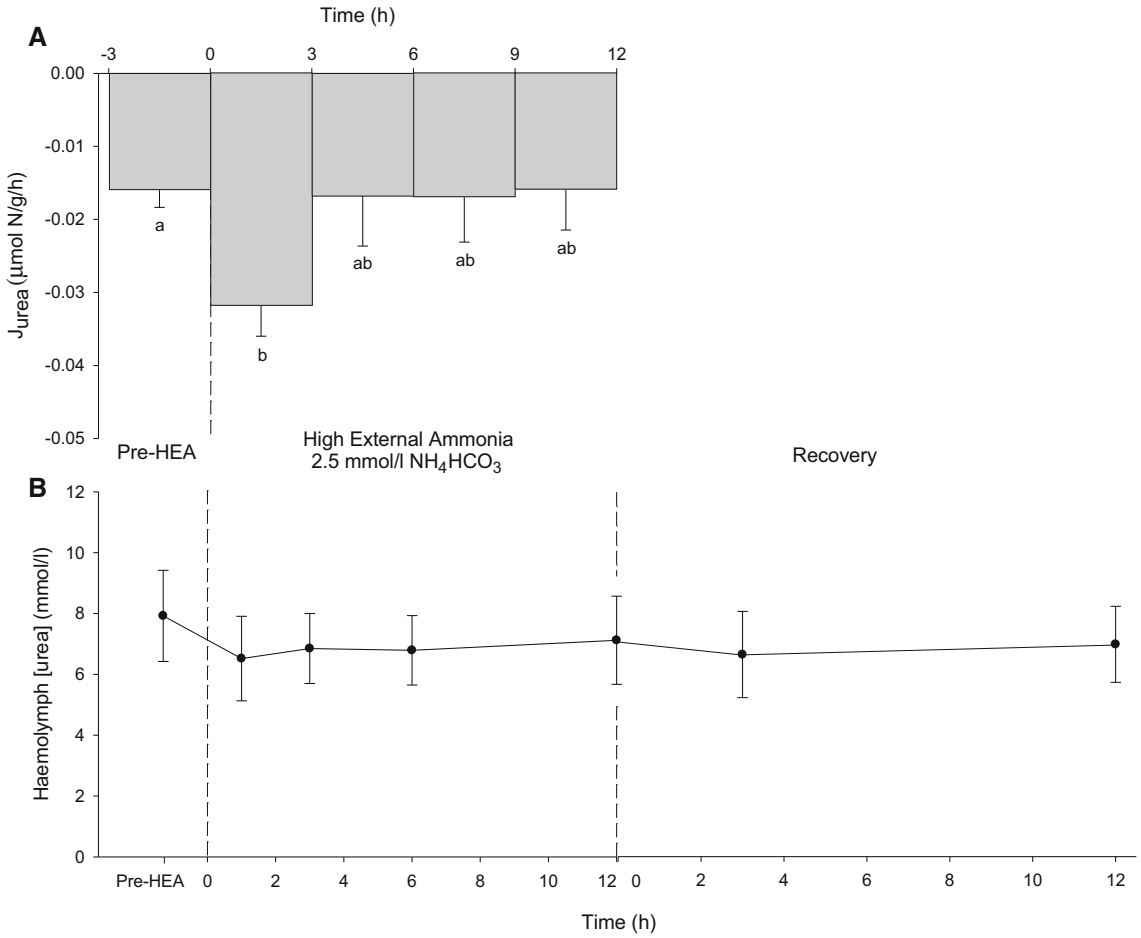

Fig. 6 **a** Urea-N excretion (J_{urea}) and **b** haemolymph [urea] in blue crabs (*Callinectes sapidus*) under control conditions (pre-HEA), during 12 h of high external ammonia (HEA; 2.5 mmol/L NH_4HCO_3), and 12 h of recovery in control water; note that only the pre-HEA and 12 h HEA periods are included for (**a**) J_{urea}. Means not sharing the *same letter* represent statistically significant differences between mean J_{urea} over the experimental period as determined using a repeated measures one-way ANOVA followed by a Holm–Sidak post hoc test. *Vertical dashed lines* mark the divides between the 3-h pre-HEA, 12-h HEA, and 12-h recovery periods. Data are mean ± SE ($n = 6$)

in J_{urea} only represented approximately 13% of the total increase in N excretion in response to Cu, whereas J_{amm} accounted for 87% of the increase. Therefore, it is unclear if this relatively small increase in J_{urea} contributed substantially to the detoxification of ammonia produced in response to Cu exposure. It is possible that this increase is a product of a general stress response, perhaps related to CHH, but more work would be necessary to address this possibility.

Hyperammonemia Does Not Lead to Substantial Urea Production in *C. sapidus*

To test the role of ammonia detoxification through urea production during Cu exposure, urea production in response to exogenous hyperammonemia induced by HEA exposure in blue crabs was assessed in a separate experiment. Upon exposure to HEA (2.5 mmol/L NH_4HCO_3), J_{amm} was reversed, representing a net uptake of ammonia from the external environment (Fig. 5a) that is typical of HEA exposure in blue crabs (Cameron 1986). Notably, ammonia uptake was highest in the first 3 h of HEA exposure, potentially indicative of an initial handling stress which could have led to alterations in ammonia excretion or production. Similarly, J_{urea} increased only transiently in response to HEA, reaching a rate no greater than that of control crabs in the Cu exposure experiments (Fig. 4a), and haemolymph [urea] also was unchanged (Fig. 5). These results do not support our secondary hypothesis that urea production is stimulated in response to ammonia stress. *C. sapidus* does however appear to be able to attenuate hyperammonemia during HEA by some unknown mechanism. Haemolymph T_{amm} was kept at levels far below that of the surrounding seawater (Fig. 5b) despite a sustained uptake of ammonia throughout the exposure. It is possible that conversion to other N-compounds, such as glutamine

(Liu et al. 2014), or perhaps sequestration of ammonia into muscle tissue, accounted for the attenuation of hyperammonemia during HEA exposure, but it is not known whether these mechanisms could be similarly recruited in response to Cu-induced increases in ammonia production. It also is possible that nitrogen metabolism was altered by the potential stress of repeated handling during haemolymph sampling, which was necessary in our time series study due to the coagulant properties of haemolymph that preclude the use of indwelling catheters. Nevertheless, we were able to achieve hyperammonemia in HEA-exposed crabs, which was the purpose of this experimental series.

In many crustacean species, J_{urea} or haemolymph [urea] does increase upon exposure to HEA, presumably to limit the accumulation of ammonia which can be toxic at elevated levels (Chen and Kou 1993). Blue crabs do not appear to possess this apparent ureagenic capacity (Fig. 6), and invertebrates in general are believed to produce urea via uricolysis or arginolysis but to lack the full complement of ornithine-urea cycle (OUC) enzymes necessary for the conversion of ammonia to urea (Wright 1995). The mechanism by which J_{urea} is increased in response to HEA in some crustaceans thus is not clear at present. HEA exposure has been demonstrated to increase urea-producing arginase activity in crustaceans (Chen and Chen 1997; Liu et al. 2014), but this process does not require ammonia as a substrate and would not contribute to ammonia detoxification. More work is needed to understand how and why urea production increases in response to HEA exposure in crustaceans, which are generally not believed to express a functional OUC.

Conclusions

In response to waterborne Cu exposure (100 μg/L), the excretion of two primary N wastes, ammonia and urea, increased in hyper-regulating blue crabs acclimated to 2 ppt, whereas haemolymph ammonia and urea concentrations did not increase significantly. Thus, the production rate of both ammonia and urea increased under Cu challenge. We had further hypothesized that Cu would stimulate urea production as a pathway for ammonia detoxification; however, it appears that blue crabs do not possess the capacity to produce substantial amounts of urea in response to elevations in haemolymph T_{amm} induced by experimental HEA exposure in contrast with previous work on crustaceans. Therefore, the increase in J_{urea} in response to waterborne Cu, and the small transient increase in response to HEA, are not likely a function of ammonia detoxification and may be related to some general stress response. Exposure to 100 μg/L Cu also had no effect on haemolymph [Na^+], suggesting that the stimulation of N excretion by Cu may be a more sensitive toxicological endpoint than the Cu-induced ionoregulatory disruptions generally observed in crustaceans. These findings may be particularly important in culture settings where ambient levels of Cu and ammonia can be elevated.

Acknowledgements The authors thank Dr. Josencler Luis Ribas Ferreira for technical assistance with experiments and analyses. This research was supported by an award granted by the International Development Research Centre (IDRC) to AB and CMW, by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to AB in the scope of the Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática (INCT-TA), by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) to AB in the scope of the Programa Ciências do Mar (CIMAR) and by the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS/Proc. 1700 12-2) to CMGM. AMZ was supported by an NSERC Canada Graduate Scholarship. AB is a research fellow from the Brazilian CNPq and supported by the International Canada Research Chair Program from IDRC.

References

Bambang Y, Thuet P, Charmantier-Daures M, Trilles J-P, Charmantier G (1995) Effect of copper on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus* Bate (Crustacea, Decapoda). Aquat Toxicol 33:125–139

Bamber SD, Depledge MH (1997) Responses of shore crabs to physiological challenges following exposure to selected environmental contaminants. Aquat Toxicol 40:79–92

Baumgarten MGZ, Niencheski LF. 1998. Avaliação da Qualidade Hidroquímica da Área Portuária da Cidade de Rio Grande (RS). Documentos Técnicos Oceanografi—vol 9. Editora da FURG, Rio Grande, Rio Grande do Sul, Brazil

Bianchini A, Martins SEG, Barcarolli IF (2004) Mechanism of acute copper toxicity in euryhaline crustaceans: implications for the Biotic Ligand Model. Intern Congr Ser 1275:189–194

Bidone ED, Laybauer L, Castilhos ZC, Maddock JL (2001) Environmental risk increase due to heavy metal contamination caused by a copper mining activity in Southern Brazil. An Acad Bras Cienc 73:277–286

Bjerregaard P, Vislie T (1986) Effect of copper on ion- and osmoregulation in the shore crab *Carcinus maenas*. Mar Biol 91:69–76

Boitel F, Truchot J-P (1989) Effects of sublethal and lethal copper levels on hemolymph acid-base balance and ion concentrations in the shore crab *Carcinus maenas* kept in undiluted sea water. Mar Biol 103:495–501

Boitel F, Truchot J-P (1990) Comparative study of the effects of copper on haemolymph ion concentrations and acid-base balance in shore crabs *Carcinus maenas* acclimated to full-strength or dilute seawater. Comp Biochem Physiol C 95:307–312

Brooks SJ, Mills CL (2003) The effect of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. Comp Biochem Physiol A 135:527–537

Cameron JN (1986) Responses to reversed NH_3 and NH_4^+ gradients in a teleost (*Ictalurus punctatus*), an elasmobranch (*Raja erinacea*), and a crustacean (*Callinectes sapidus*): evidence for NH_4^+/H^+ exchange in the teleost and the elasmobranch. J Exp Zool 239:183–195

Cameron JN, Batterton CV (1978) Antennal gland function in the freshwater blue crab, *Callinectes sapidus*: water, electrolyte, acid-base and ammonia excretion. J Comp Physiol 123:143–148

Chen J-C, Chen J-M (1997) Arginase specific activity and nitrogenous excretion of *Penaeus japonicus* exposed to elevated ambient ammonia. Mar Ecol Prog Ser 153:197–202

Chen J-C, Cheng S-Y (1993a) Haemolymph osmolality, acid-base balance and shift of ammonotelic to ureotelic excretory pattern of *Penaeus japonicus* exposed to ambient ammonia. Comp Biochem Physiol C 106:733–737

Chen J-C, Cheng S-Y (1993b) Urea excretion by *Penaeus japonicus* Bate exposed to different concentrations of ambient ammonia. J Exp Mar Biol Ecol 173:1–9

Chen J-C, Chia P-G (1996) Hemolymph ammonia and urea and nitrogenous excretions of *Scylla serrata* at different temperature and salinity levels. Mar Ecol Prog Ser 139:119–125

Chen J-C, Kou Y-Z (1993) Accumulation of ammonia in the haemolymph of *Penaeus monodon* exposed to ambient ammonia. Aquaculture 109:177–185

Chen J-C, Chen C-T, Cheng S-Y (1994) Nitrogen excretion and changes of hemocyanin protein and free amino acid levels in the hemolymph of *Penaeus monodon* exposed to different concentrations of ambient ammonia-N at different salinity levels. Mar Ecol Prog Ser 110:85–94

Cheng S-Y, Lee WC, Shieh W, Chen J-C (2004) Increased production and excretion of urea in the kuruma shrimp (*Marsupenaeus japonicus*) exposed to combined environments of increased ammonia and nitrite. Arch Environ Contam Toxicol 47:352–362

Correa M (1987) Physiological effects of metal toxicity on the tropical freshwater shrimp *Macrobrachium carcinus* (Linneo, 1758). Environ Pollut 45:149–155

Flemming CA, Trevors JT (1989) Copper toxicity and chemistry in the environment: a review. Water Air Soil Pollut 44:143–158

Garnacho E, Peck LS, Tyler PA (2001) Effects of copper exposure on the metabolism of the mysid *Praunus flexuosus*. J Exp Mar Biol Ecol 265:181–201

Grosell M (2012) Copper. In: Farrell AP, Wood CM, Brauner CJ (eds) Homeostasis and toxicology of essential metals, vol 31A., Fish physiologyAcademic Press, San Diego, pp 54–110

Grosell M, Nielsen C, Bianchini A (2002) Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp Biochem Physiol C 133:287–303

Hansen JI, Mustafa T, Depledge M (1992) Mechanisms of copper toxicity in the shore crab, *Carcinus maenas* I. Effects on Na, K-ATPase activity, haemolymph electrolyte concentrations and tissue water contents. Mar Biol 114:253–257

Harris RR, Santos MCF (2000) Heavy metal contamination and physiological variability in the Brazilian mangrove crabs *Ucides cordatus* and *Callinectes danae* (Crustacea: Decapoda). Mar Biol 137:691–703

Hong M, Chen L, Sun X, Gu S, Zhang L, Chen Y (2007) Metabolic and immune responses in Chinese mitten-handed crab (*Eriocheir sinensis*) juveniles exposed to elevated ambient ammonia. Comp Biochem Physiol C 145:363–369

Kormanik GA, Cameron JN (1981) Ammonia excretion in the seawater blue crab (*Callinectes sapidus*) occurs by diffusion, and not Na^+/NH_4^+ exchange. J Comp Physiol B 141:457–462

Lim MY-T, Zimmer AM, Wood CM (2015) Acute exposure to waterborne copper inhibits both the excretion and uptake of ammonia in freshwater rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol C 168:48–54

Liu S, Pan L, Liu M, Yang L (2014) Effects of ammonia exposure on nitrogen metabolism in gills and hemolymph of the swimming crab *Portunus trituberculatus*. Aquaculture 432:351–359

Lorenzon S, Edomi P, Giulianini PG, Mettulio R, Ferrero EA (2004) Variation of crustacean hyperglycemic hormone (cHH) level in the eyestalk and haemolymph of the shrimp *Palaemon elegans* following stress. J Exp Biol 207:4205–4213

Mangum CP, Silverthorn SU, Harris JL, Towle DW, Krall AR (1976) The relationship between blood pH, ammonia excretion and adaptation to low salinity in the blue crab *Callinectes sapidus*. J Exp Zool 195:129–136

Martins CMG, Almeida DV, Marins LFF, Bianchini A (2011a) mRNA expression and activity of ion-transporting proteins in gills of the blue crab *Callinectes sapidus*: effects of waterborne copper. Environ Toxicol Chem 30:206–211

Martins CMG, Barcarolli IF, de Menezes EJ, Giacomin MM, Wood CM, Bianchini A (2011b) Acute toxicity, accumulation and tissue distribution of copper in blue crabs *Callinectes sapidus* acclimated to different salinities: in vivo and in vitro studies. Aquat Toxicol 101:88–99

Millikin MR, Williams AB (1984) Synopsis of biological data on the blue crab, *Callinectes sapidus* Rathburn. NOAA technical report NMFS

Pinho GLL, Pedroso MS, Rodrigues SC, Souza SS, Bianchini A (2007) Physiological effects of copper in the euryhaline copepod *Acartia tonsa*: waterborne versus waterborne plus dietborne exposure. Aquat Toxicol 84:62–70

Rahmatullah M, Boyde TRC (1980) Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. Clin Chim Acta 107:3–9

Randall DJ, Tsui TK (2002) Ammonia toxicity in fish. Mar Pollut Bull 45:17–23

Regnault M (1987) Nitrogen excretion in marine and fresh-water crustacea. Biol Rev 62:1–24

Ren Q, Pan L, Zhao Q, Si L (2015) Ammonia and urea excretion in the swimming crab *Portunus trituberculatus* exposed to elevated ambient ammonia-N. Comp Biochem Physiol A 187:48–54

Spaargaren DH (1982) The ammonium excretion of the shore crab, *Carcinus maenas*, in relation to environmental osmotic conditions. Neth J Sea Res 15:273–283

Thurberg FP, Dawson MA, Collier RS (1973) Effects of copper and cadmium on osmoregulation and oxygen consumption in two species of estuarine crabs. Mar Biol 23:171–175

USEPA (2007) Aquatic life ambient freshwater quality criteria—Copper, EPA-822-07-001, 2007 revision. US Environmental Protection Agency, Washington

Verdouw H, van Echteld CJA, Dekkers EMJ (1978) Ammonia determination based on indophenols formation with sodium salicylate. Water Res 12:399–402

Vitale AM, Monserrat JM, Castilho P, Rodriguez EM (1999) Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulate* (Decapoda, Grapsidae). Comp Biochem Physiol C 122:121–129

Vosloo A, van Aardt WJ, Mienie LJ (2002) Sublethal effects of copper on the freshwater crab *Potamonautes warreni*. Comp Biochem Physiol A 133:695–702

Weeks JM, Jensen FB, Depledge MH (1993) Acid-base status, haemolymph composition and tissue copper accumulation in the shore crab *Carcinus maenas* exposed to combined copper and salinity stress. Mar Ecol Prog Ser 97:91–98

Weihrauch D, Wilkie MP, Walsh PJ (2009) Ammonia and urea transporters in gills of fish and aquatic crustaceans. J Exp Biol 212:1716–1730

Wright PA (1995) Nitrogen excretion: three end products, many physiological roles. J Exp Biol 198:273–281

Zimmer AM, Barcarolli IF, Wood CM, Bianchini A (2012) Waterborne copper exposure inhibits ammonia excretion and branchial carbonic anhydrase activity in euryhaline guppies acclimated to both fresh water and sea water. Aquat Toxicol 122–123:172–180