Effects of waterborne copper delivered under two different exposure and salinity regimes on osmotic and ionic regulation in the mudflat fiddler crab, *Minuca rapax* (Ocypodidae, Brachyura)

Mariana V. Capparelli\(^a\), John C. McNamara\(^ab\), Martin Grosell\(^c\)

\(^a\) Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-901 SP, Brasil

\(^b\) Centro de Biologia Marinha, Universidade de São Paulo, São Sebastião 11600-000 SP, Brasil

\(^c\) Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Marine Biology and Fisheries, Miami, Florida

**A R T I C L E   I N F O**

Keywords:
- Semi-terrestrial crab
- Heavy metal contamination
- Salinity exposure
- Gill K⁺-phosphatase activity
- Gill carbonic anhydrase activity
- Brachyura

**A B S T R A C T**

The effects of exposure to copper (Cu) on tissue Cu accumulation, on hemolymph osmotic, Na⁺ and Cl⁻ regulation, and on gill Na⁺/K⁺-ATPase (NKA) and carbonic anhydrase (CA) activities were evaluated in the fiddler crab *Minuca rapax*. Waterborne copper was delivered to the crabs at one of three salinities (seawater at 25% salinity [S] = isosmotic control; distilled water [ < 0.1% S] = hypo-osmotic medium; or 60% S = hyper-osmotic seawater) either for 5 days in a 0.5-cm water film containing 0, 50, 150, 250 or 500 µg Cu/L with free access to a dry surface, or in crabs fully submerged in 0, 250 or 500 µg Cu/L. In the crabs with free access to a dry surface, the highest Cu concentrations were found in the hemolymph and hepatopancreas with some accumulation in the gills; accumulation in the hemolymph and gills was enhanced in low salinity but was salinity independent in the hepatopancreas. Osmotic regulation was unaffected by Cu exposure; however Na⁺ and Cl⁻ hypo-regulation was impaired by Cu in 25 and 60% S. Gill NKA activity was stimulated 2-fold at 50 µg Cu/L and markedly inhibited at 150 µg Cu/L and above in 0 and 25% S. Gill CA activity was inhibited in < 0.1% S, but stimulated in 25 and 60% S. An inverse concentration-CA activity response was seen above 150 µg Cu/L for all salinities. In the submerged crabs, Cu accumulated in all tissues in 60% S; however, there was no clear-cut Cu concentration-accumulation relationship evident in any tissue for either exposure regime, likely owing to the crabs’ ability to regulate Cu. Copper exposure diminished osmotic, [Na⁺] and [Cl⁻] hypo-regulatory ability, especially in higher salinities. Gill NKA activity was markedly inhibited by Cu overall, and particularly above 250 µg Cu/L in < 0.1% S. Gill CA activity was inhibited in 25% S but inconsistently affected in 0 and 60% S. These findings show that *Minuca rapax* is affected both physiologically and biochemically by Cu contamination, although to different degrees, depending on the delivery regime, salinity, copper concentration and target tissue.

1. Introduction

While potentially toxic, copper (Cu) is an essential micronutrient required by all living organisms for myriad physiological and biochemical processes (Rainer and Brouwer, 1993; Rainbow, 1995; Martins et al., 2011; Grossel et al., 2002; Grosell et al., 2002). In crustaceans, Cu forms the oxygen-binding core of hemocyanin, the respiratory pigment that transports oxygen in the hemolymph to the tissues and cells (Engel and Brouwer, 1987; Rainer and Brouwer, 1993). At high concentrations (above 50 mmol Cu L⁻¹), Cu disrupts mitochondrial respiration in freshwater organisms, leading to mortality; however, at low [Cu], mortality results mainly from osmoregulatory disturbances (Arnold, 2005; Grossel et al., 2011). Copper is naturally present as a trace element in all aquatic environments; however, multiple anthropogenic sources from industries, agriculture and harbors discharge Cu into water bodies where it becomes potentially toxic to aquatic organisms (NiENCHESKI et al., 2006; D’ADAMO et al., 2008). Complexation of Cu with organic and inorganic ligands, and its competition with other cations for binding sites on enzymes and in pathways of metal ion uptake greatly influence the toxicity of Cu to freshwater organisms (GENSEMER et al., 2002; GROSSEL et al., 2007).

Copper may actively enter cells through the transport pathways involved in the ion exchange processes that underpin osmoregulation (VITALE et al., 1999; GROSSEL and WOOD, 2002; BIANCHINI et al., 2008). While such pathways show selectivity and allow the transport of ions of a specific ionic radius and charge, they are not selective with regard to...
metal ion specificity. To illustrate, Ca\(^{2+}\) has a similar ionic radius and charge density to Ca\(^{2+}\), and hence Cu\(^{2+}\) can pass through Cu\(^{2+}\) transport pathways (Rainbow, 1995). Since trace metals can be acquired through ion transport pathways, trace metal uptake in crustaceans may be mediated by their osmoregulatory mechanisms (Mantel & Farmer, 1983).

In osmoregulating freshwater and marine fish, and in invertebrates, the key manifestations of waterborne Cu toxicity derive from impairment of the physiological processes underpinning Na\(^+\) and Cl\(^-\) regulation (Grosell et al., 2007). In fish and crustaceans, metal uptake takes place across the epithelial surfaces that effect ion absorption and excretion, usually in the gills and intestine (Santore et al., 2001; Péqueux, 1995; McNamara et al., 2005). Copper can compete with Na\(^+\) (Grosell et al., 2002; Bianchini et al., 2008) and Ca\(^{2+}\) (Gensemer et al., 2002) for binding and uptake by gill ion transport proteins.

Changes in salinity affect the bioavailability and uptake of trace metals by euryhaline invertebrates. Salinity can affect metal uptake by altering chemical speciation and through competition, although uptake may be indirectly affected via osmoregulatory mechanisms (Grossel, 2011). The accumulation and toxic effects of metals appear to increase at low ambient salinity (Rainbow and Phillips, 1993; Bjerregaard and Depledge, 1994). Changes in salinity affect not only metal bioavailability but also the physiological adjustments responsible for osmotic and ionic equilibrium. Copper toxicity can vary depending on the osmoregulatory strategies employed by different species.

The effects of acute Cu toxicity in crustaceans are not well established. In the amphipod Gammarus pulex (Brooks and Mills, 2003), blue crab, Callinectes sapidus (Martins et al., 2011) and the copepod Acartia tonsa (Lauer and Bianchini, 2010), exposure to high (above 500 mmol Cu L\(^{-1}\)) Cu\(^{2+}\) reduces whole body [Na\(^{+}\)]. Further, despite extensive focus on freshwater and marine organisms, few investigations have addressed the responses of intertidal or estuarine crustaceans that are submerged only at high tide. Animals occupying such brackish-water ecosystems are influenced not only by spatial and temporal variations in hydro-chemical parameters and tidal dynamics, but also by the diverse toxicants that accumulate in estuaries (Witters, 1998). Thus, estuarine crustaceans may be exposed to stress from both salinity and contamination during different stages of their life cycle, emphasizing the need for investigations of these interface organisms.

Among the brachyuran crabs, the diverse species of fiddler crabs (Uca) exhibit remarkably well-developed capabilities of osmotic and ionic regulation (Zanders and Rojas, 1996b; Baldwin and Kirschner, 1976; Thurman, 2002; Faria et al., 2017). Tropical fiddler crabs occupy niches mainly in the sandy intertidal zone, and in mudflats in brackish or estuarine waters near mangroves (Thurman, 2003). Rivers discharging into such estuaries can rapidly spread pollutants that may potentiate the effects of other natural and/or anthropogenic stresses confronted by fiddler crabs and other crustaceans, fish and mollusks. Several Uca species are among the most abundant crustaceans that permanently inhabit such affected areas (Zanders and Rojas, 1996a).

The semi-terrestrial, mudflat fiddler crab, Minuca rapax (Crustacea, Ocypodidae) is distributed from Florida, through the Gulf of Mexico, the Antilles and Venezuela to Brazil (from Pará to Santa Catarina states) (Thurman et al., 2003; 2013) and inhabits burrows in muddy sand in estuarine mangrove environments. Minuca rapax is an excellent hyperosmotic regulator (Capparelli et al., 2016; Faria et al., 2017). When challenged by low ambient salinity, these crabs maintain ionic balance by up-regulating gill ion transport mechanisms, replacing ions lost by diffusion (Freire et al., 2008; McNamara and Faria, 2012). As seen in most brachyurans, the anterior gills are responsible mainly for gas exchange while the posterior gills constitute the primary site of uptake and secretion of Na\(^{+}\) and Cl\(^-\) and thus, of osmoregulation (Mantel and Farmer, 1983; Taylor and Taylor, 1991). Metal uptake likely takes place across the posterior gill epithelia, the most probable targets of metal toxicity (Péqueux, 1995). Further, given the increased transport related activity of posterior gill enzymes like the Na\(^{+}\)/K\(^{+}\)-ATPase in response to decreased ambient salinity (Vitale et al., 1999; Leone et al., 2012), metal uptake and toxicity may be exacerbated in dilute seawater.

Given that Minuca rapax is a euryhaline species typical of mangroves and estuaries, an interface environment characterized by frequent salinity changes and the accumulation of metal contaminants (Capparelli et al., 2016), the present study investigates the effects of exposure to Cu on tissue distribution and accumulation, and on physiological and biochemical parameters related to osmotic and ionic regulation, under severe hypo- and hyper-osmotic salinity challenges. We particularly evaluate the effects of long- (5 days) or short-term (5 h) Cu exposure regimes in crabs either allowed free access to a dry surface or fully submerged, respectively, to simulate the rigors of the intertidal habitat.

2. Materials and methods

Adult, intermolt specimens of Minuca rapax of either sex were collected from Virginia Key Beach (25° 44’ 28.17” N, 80° 0.8’ 50.74” W), Virginia Key, Miami, Florida and transported in plastic boxes containing sponge cubes moistened with local seawater to the Laboratory of Environmental Physiology and Toxicology, University of Miami, Florida. Only non-ovigerous, intermolt crabs of carapace width greater than 10 mm were used. To acclimatize to laboratory conditions before use, the crabs were maintained unfed for three days after collection at 25°C, with free access to a dry surface, in plastic boxes containing water from the collection site.

2.1. Contamination by waterborne copper – free access to a dry surface versus submersion

To examine the effects of exposure to Cu in different salinities (hypo-, iso- or hyper-osmotic media), crabs were maintained either with free access to water and a dry substrate, simulating ambient conditions at low tide, or they were completely submerged, simulating the high tide covering their burrows.

In the free access experiments, groups of 10 crabs each were held individually for 5 days at 3 combinations of salinity (< 0.1% S [distilled water], 25% S [control] or 60% S) and [Cu]. For each salinity, media were prepared using distilled water and Instant Ocean® seawater salts without copper (0 µg Cu/L [control]) or with copper (50, 150, 250 or 500 µg Cu/L) added as CuCl\(_2\). The media were left to stand and equilibrate for 8 h prior to use during which time all salts dissolved. Crabs were then exposed individually, in small plastic boxes containing 50 mL of control or copper-containing media in each salinity to a depth of a few millimeters, with free access to a dry surface. Every 24 h, the number of live crabs was counted and the medium was replaced.

To evaluate the effects of Cu exposure on submerged crabs, groups of 10 crabs each were held for 5 h at 3 different combinations of salinity (< 0.1% S, 25% S [control] and 60% S) and [Cu]. For each salinity, media were prepared using distilled water and Instant Ocean® seawater salts without copper (0 µg Cu/L [control]) or with copper (50, 150, 250 or 500 µg Cu/L) added as CuCl\(_2\). The media were left to stand and equilibrate for 8 h prior to use during which time all salts dissolved. Crabs were then exposed individually, in small plastic boxes containing 50 mL of control or copper-containing media in each salinity to a depth of a few millimeters, with free access to a dry surface. Every 24 h, the number of live crabs was counted and the medium was replaced.

To evaluate the effects of Cu exposure on submerged crabs, groups of 10 crabs each were held for 5 h at 3 different combinations of salinity (< 0.1% S, 25% S [control] and 60% S) and [Cu]. For each salinity, media were prepared using distilled water and Instant Ocean® seawater salts without copper (0 µg Cu/L [control]) or with copper (50, 150, 250 or 500 µg Cu/L) added as CuCl\(_2\). The media were left to stand and equilibrate for 8 h prior to use during which time all salts dissolved. Crabs were then exposed individually, in small plastic boxes containing 50 mL of control or copper-containing media in each salinity to a depth of a few millimeters, with free access to a dry surface. Every 24 h, the number of live crabs was counted and the medium was replaced.

2.2. Water chemistry analyses

Water samples for all treatments were taken at the beginning and end of each experimental period. Samples were acidified (1% HNO\(_3\), TraceMetal grade, Fisher Scientific, Waltham, Massachusetts, USA) and
stored in Falcon® tubes for later measurement of total (non-filtered samples) and dissolved (filtered samples) Cu by atomic absorption spectroscopy, employing a graphite furnace (Varian FS220Z atomic absorption spectrophotometer, Mulgrave, Victoria, Australia) using certified reference material. Matrix interference in 25 and 60% S was resolved using a solvent extraction technique (Blanchard and Grosell, 2006).

2.3. Measurement of hemolymph and tissue copper contents

Samples of hemolymph, gill and hepatopancreas were collected from each crab (N = 10) for all salinity and copper combinations under both experimental delivery regimes. To obtain hemolymph and tissue samples, the crabs were cryo-anesthetized in crushed ice for 10 min. A hemolymph sample of ~50 µL was then drawn into a 1-mL plastic syringe using a #25-7 gauge needle inserted into the arthrodial membrane at the base of the 3rd or 4th pereiopod, transferred to a micro-Eppendorf tube and frozen at ~20 °C. The crabs were then killed by destroying the cerebral and thoracic ganglia, after which all gill pairs and the hepatopancreas were dissected from each animal and held separately in micro-Eppendorf tubes in crushed ice for assays of enzymatic activities.

Tissue Cu content was measured by atomic absorption spectrophotometry as above after digesting the tissue samples for 24 h in 1 N HNO₃ (1: 10 w/v, TraceMetal Grade) at 60 °C. Samples were vortexed and centrifuged and the supernatant was collected for analysis after appropriate dilution.

2.4. Physiological and biochemical responses to copper contamination

Hemolymph osmolality was measured in 10-µL aliquots using a vapor pressure micro-osmometer (Model 5500, Wescor Inc., Logan, UT, USA). Hemolymph [Cl⁻] was quantified using anion chromatography (Dionex, DX-120 ion chromatograph, Dionex Corp., Sunnyvale, CA, USA, fitted with an AS40 automated sampler). Hemolymph [Na⁺] was measured using a Varian FS220Z atomic absorption spectrophotometer (Varian, Mulgrave, Victoria, Australia).

Gill Na⁺/K⁺-ATPase activity was measured as the K⁺-phosphatase activity of the Na⁺/K⁺-ATPase. After chilling briefly on ice, the 3 posterior gill pairs from each crab were pooled and homogenized in a dry ice/acetone bath in a homogenization bu vessel containing (in mmol L⁻¹) imidazole buffer 20, pH 6.8, sucrose 250, EDTA 6 and a protease inhibitor cocktail (Furriel et al., 2000). The hydrolysis of p-nitrophenylphosphate diethyl salt (pNPP) by each gill homogenate was assayed spectrophotometrically at 410 nm and 25 °C, continuously monitoring release of the p-nitrophenolate ion for 15 min using a microplate reader (SpectroMax Plus 348, Molecular Devices, Sunnyvale, CA, USA) under standard conditions as described by Furriel et al. (2000). K⁺-phosphatase activity was assayed by adding an aliquot of each gill homogenate to a reaction medium containing (in mmol L⁻¹) KCl 10, MgCl₂ 5, HEPES buffer 50, pH 7.5, pNPP 10 (for total K⁺-phosphatase activity) or the same medium containing 3 mmol L⁻¹ ouabain (for ouabain insensitive activity). The difference between the ouabain sensitive and insensitive activities represents the K⁺-phosphatase activity.

To assay carbonic anhydrase activity, the pooled anterior and posterior gills from each crab were homogenized using a Potter homogenizer in homogenization buffer (in mmol L⁻¹, mannitol 225, sucrose 75 and Trizma-base 10, adjusted to pH 7.4). The homogenate was then differentially centrifuged to separate the cytoplasmic and membrane-bound isoforms. Cytoplasmic carbonic anhydrase activity was measured using an electrometric method (Henry, 1991).

Protein concentration in all homogenates was measured by the Bradford method (Bradford, 1976) employing bovine serum albumin as the standard.

2.5. Statistical analyses

All data are presented as the mean ± SEM. Data were verified for normality of distribution and equality of variance, and where necessary were transformed using the square root of the arcsine (x) function.

Tissue copper concentrations in the hemolymph, gills and hepatopancreas were evaluated employing a three way analysis of variance (Statistica software package, Version 7, Tulsa, OK) with Salinity (< 0.1, 25 or 60% S), Exposure Regime (crabs with free access to a dry surface for 5 days, or submerged crabs) and Copper Concentration (0, 250 or 500 µg Cu/L) as the main factors. These data were adjusted to a balanced design using those copper concentrations common to both exposure regimes, with 5 replicate measurements in each combination of conditions.

Physiological (hemolymph osmolality, and sodium and chloride concentrations) and biochemical (gill Na⁺/K⁺-ATPase and carbonic anhydrase activities) parameters were evaluated using two-way analyses of variance (SigmaPlot, Systat Software, Inc., San José, CA) employing Salinity (< 0.1, 25 or 60% S), and Copper Concentration (0, 250 and 500 µg Cu/L [submerged crabs], or 0, 50, 150, 250 and 500 µg Cu/L [crabs with free access to a dry surface]) as the main factors. The Student-Newman-Keuls multiple means testing procedure was used to locate significantly different groups within each ANOVA performed. Effects and differences were considered significant at P ≤ 0.05.

3. Results

3.1. Water chemistry analyses

Table 1 provides the Cu contents in the experimental media after 5 days or 5 h. The most discrepant differences seen with respect to the nominal Cu concentrations prepared were noted at 250 and 500 µg Cu/L after 5 days exposure.

3.2. Effects of salinity, copper concentration and exposure regime on hemolymph and tissue copper accumulation

Table 1 provides the Cu contents in the experimental, gills and hepatopancreas of Minuca rapax after exposure to the experimental media. No mortality was recorded in M. rapax at any of the different salinity and copper combinations for either delivery regime. The three-way ANOVA revealed that hemolymph Cu accumulation was affected by Cu concentration alone (P = 0.01). Gill Cu accumulation was affected by exposure regime (P = 0.03), with an interaction effect between salinity and exposure regime (P = 0.02). Accumulation of Cu by the hepatopancreas was unaffected by the three main factors although there was an interaction effect between salinity and exposure regime (P = 0.02) and salinity and Cu concentration (P < 0.01), as well as among salinity, exposure regime and Cu concentration (P < 0.01).

Table 2 provides the Cu contents in the hemolymph, gills and hepatopancreas of Minuca rapax after exposure to the experimental media. Nominal Cu concentrations (in µg Cu/L) in the experimental copper containing media (nominal Cu concentrations of 0, 50, 150, 250 and 500 µg Cu/L at salinities of < 0.1% S [distilled H₂O, hypo-osmotic medium], 25% S [isosmotic control medium] and 60% S [hyper-osmotic medium], for the 5-day or 5-h exposure regimes. Solutions were allowed to equilibrate for 8 h after preparation prior to use.
media under the two exposure regimes, i.e., for 5 days with free access to a dry surface, or fully submerged for 5 h. Hemolymph, gill and hepatopancreas [Cu] ranged from 213 to 538, 15 to 200 and 126 to 556 μg Cu/L, respectively.

3.2.1. Free access to a dry surface (5 days)

Copper accumulation in the hemolymph was affected by both Cu concentration and salinity. Accumulation in the gills was dependent on salinity and Cu concentration, showing an interaction effect, while accumulation in the hepatopancreas was affected by salinity alone (two-way ANOVA, p < 0.05).

As shown in Table 2, in the isosmotic control salinity (25‰ S), for exposure concentrations of 250 μg Cu/L and above, hemolymph [Cu] was greater than at 0 μg Cu/L (control, 213 ± 1.0 μg Cu/L). Gill (Cu) at 150 μg Cu/L and above was less than or similar to the control (0 μg Cu/L, 39 ± 0.3 μg Cu/L). In the hepatopancreas, [Cu] was less than in the control (0 μg Cu/L, 330 ± 11.0 μg Cu/L) for all exposure concentrations except 250 μg Cu/L.

In the hyper-osmotic medium (< 0.1‰ S), hemolymph (336 ± 2.0 μg/L) and tissue (gill 27 ± 0.4; hepatopancreas 238 ± 4.0 μg/L) [Cu] were greater at exposure concentrations of 250 and 500 μg Cu/L than at 0 μg Cu/L (control).

In the hyper-osmotic medium (60‰ S), hemolymph (336 ± 2.0 μg/L) and tissue (gill 27 ± 0.4; hepatopancreas 238 ± 4.0 μg/L) [Cu] were greater at exposure concentrations of 250 and 500 μg Cu/L than at 0 μg Cu/L (control).

In the hyper-osmotic medium (< 0.1‰ S), gill (Cu) was higher in crabs exposed to Cu compared to the control (0 μg Cu/L, 15 ± 0.1 μg/Cu/L). Hepatopancreas [Cu] was greater than in the control (136 ± 2.0) in 250 μg Cu/L.

In the hyper-osmotic medium (60‰ S), hemolymph, gill and hepatopancreas [Cu] were greater than in their respective controls for all Cu exposure concentrations.

3.3. Effects of copper and exposure regime on osmotic and ionic regulation

3.3.1. Free access to a dry surface (5 days)

Hemolymph osmolality in Minuca rapax was affected only by salinity (2-way ANOVA, P < 0.05). Hemolymph [Na+] and [Cu+] were dependent on salinity, Cu concentration and their interaction (2-way ANOVA, P < 0.05).

Both unexposed control (0 μg Cu/L) and Cu-exposed Minuca rapax were slightly hyperosmotic (810 mmol/kg H2O), slightly hyper-natriuretic (360 mmol Na+/L), and slightly hypo-chloremic (380 mmol Cl-/L) in 25‰ S (approximately isosmotic control salinity) (Fig. 1A, B, C). All crabs strongly hyper-regulated hemolymph osmolality (≈ 600 mmol/kg H2O) and [Na+] (≈ 270 mmol/L) and [Cl−] (≈ 280 mmol/L) in distilled H2O, and strongly hypo-regulated osmolality and these ions in 60‰ S (≈ 1050 mmol/kg H2O, ≈ 470 mmol Na−/L, ≈ 550 mmol Cl/L) (Fig. 1A, B, C). Exposure to Cu had no effect on hemolymph osmolality in any salinity (Fig. 1A). In 25‰ S (control salinity), hemolymph [Na+] increased at most [Cu] (Fig. 1B), while [Cl−] increased in some [Cu] (Fig. 1C). In 60‰ S, hemolymph [Na+] was higher than all [Cu] while [Cl−] increased at 150 and 200 μg Cu/L (Fig. 1C).

### Table 2

Measured copper concentrations (in μg Cu/L) in the hemolymph, gills and hepatopancreas of Minuca rapax exposed to different salinities (< 0.1‰ S [distilled H2O, hypo-osmotic medium], 25‰ S [isosmotic control medium] or 60‰ S [hyper-osmotic medium] and Cu concentrations (in μg Cu/L furnished as CuCl2) after 5 days with free access to a dry surface, or after 5 h complete submersion. Data are the mean ± SEM (N=10).

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Salinity</th>
<th>Tissue</th>
<th>Copper content (μg Cu/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 (control) 50 150 250 500</td>
</tr>
<tr>
<td>Regime</td>
<td>(% S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 0.1</td>
<td></td>
<td></td>
<td>Hemolymph 336 ± 2.0 294 ± 1.3 288 ± 1.2 430 ± 2.0 513 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gill 27 ± 0.4 33 ± 0.2 24 ± 0.1 41 ± 0.5 67 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hepatopancreas 238 ± 4.0 216 ± 1.6 263 ± 0.6 464 ± 3.0 175 ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 days free</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heparopancreas 330 ± 11 150 ± 3.0 126 ± 0.3 302 ± 30 266 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hemolymph 340 ± 3.0 380 ± 1.7 245 ± 1.2 531 ± 4.0 353 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hepatopancreas 220 ± 1.7 245 ± 0.9 260 ± 1.5 170 ± 3.0 496 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hemolymph 488 ± 5.0 – – 431 ± 1.5 518 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.1</td>
<td></td>
<td>Gill 15 ± 0.1 – – 21.5 ± 1.0 42 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heparopancreas 136 ± 2.0 – – 244 ± 0.6 136 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hemolymph 538 ± 5.5 – – 293 ± 3.4 475 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>5 h</td>
<td></td>
<td>Gill 38 ± 0.4 – – 70 ± 3.4 40 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heparopancreas 273 ± 5.5 – – 202 ± 1.6 523 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hemolymph 290 ± 1.6 – – 444 ± 5.6 320 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hepatopancreas 238 ± 2.0 – – 273 ± 4.6 256 ± 2.0</td>
</tr>
</tbody>
</table>

* Significantly different from respective value for 25‰ S.
* Significantly different from 0 μg Cu/L Cu (control) in the same salinity (P ≤ 0.05).
kg H₂O) and [Na⁺] (≈ 225 mmol/L) and [Cl⁻] (≈ 275 mmol/L) in distilled H₂O, and strongly hypo-regulated osmolality and these ions in 60‰ S (≈ 1100 mOsm/kg H₂O, ≈ 455 mmol Na⁺ L⁻¹, ≈ 535 mmol Cl⁻ L⁻¹) (Fig. 2A, B, C). In 25‰ S (control salinity) hemolymph osmolality increased in all [Cu] (Fig. 2A). Exposure to Cu had no effect on hemolymph [Na⁺] but increased [Cl⁻] at 250 µg Cu/L (Fig. 2B, C). At < 0.1‰ S, Cu exposure had no effect on hemolymph osmolality, [Na⁺] or [Cl⁻]. In 60‰ S, hemolymph osmolality increased in both [Cu], [Na⁺] was highest at 500 µg Cu/L while [Cl⁻] decreased slightly (Fig. 2C).

3.4. Effects of copper and exposure regime on gill K⁺-phosphatase (Na⁺/K⁺-ATPase) and carbonic anhydrase activities

Gill K⁺-phosphatase (Na⁺/K⁺-ATPase) and carbonic anhydrase activities were affected by both salinity and Cu concentration (2-way ANOVA, P < 0.05).

3.4.1. Free access to a dry surface (5 days)

Posterior gill K⁺-phosphatase activity in unexposed control Minuca rapax in 25‰ S (control salinity) was 17 U/mg (Fig. 3A). In this salinity, activity increased 2-fold at 50 µg Cu/L but decreased in all other [Cu]. In 0‰ S, gill K⁺-phosphatase activity increased 2-fold in the unexposed control crabs (Fig. 3A). Activities in the Cu-exposed crabs followed a similar pattern, i.e., a 2-fold increase at 50 µg Cu/L, decreasing ≈ 3-fold at the other [Cu]. In 60‰ S, gill K⁺-phosphatase activity decreased 3-fold in the unexposed control crabs, with an increase in activity only at 500 µg Cu/L (Fig. 3A).

Carbonic anhydrase activity in the pooled anterior and posterior gills of unexposed control Minuca rapax in 25‰ S (control salinity) was 25 U/mg (Fig. 3B). In this salinity, activity increased 2-fold at 150 µg Cu/L decreasing at the higher [Cu]. In 0‰ S, carbonic anhydrase activity increased 2.6-fold in the unexposed crabs, decreasing progressively by up to 50% in the Cu-exposed crabs (Fig. 3B). In 60‰ S, control carbonic anhydrase activity was similar to 25‰ S, activities in the Cu-exposed crabs reaching a maximum at 150 µg Cu/L and then decreasing (Fig. 3B).

3.4.2. Submersion (5 h)

Posterior gill K⁺-phosphatase (Na⁺/K⁺-ATPase) activities were affected by both salinity and Cu concentration (2-way ANOVA, P < 0.05). Pooled anterior and posterior gill carbonic anhydrase activities were dependent on both salinity and Cu concentration (2-way ANOVA, P < 0.05) with a significant interaction effect (P < 0.05).

Posterior gill K⁺-phosphatase activity in unexposed control Minuca rapax in 25‰ S (control salinity) was 6 U/mg (Fig. 4A), less than half the activity seen in crabs with free access to a dry surface (Fig. 3A). In this salinity, there was no effect of Cu on enzyme activity. In < 0.1‰ S, gill K⁺-phosphatase activity increased in unexposed crabs and decreased 2-fold in the respective Cu-exposed crabs (Fig. 4A). In 60‰ S, control gill K⁺-phosphatase activity was similar to 25‰ S, and increased slightly at 500 µg Cu/L (Fig. 4A).

Carbonic anhydrase activity in the pooled anterior and posterior gills of unexposed control Minuca rapax in 25‰ S (control salinity) was 46 U/mg (Fig. 4B). In this salinity, activity decreased progressively at 250 and 500 µg Cu/L. In < 0.1‰ S, control carbonic anhydrase activity

Fig. 1. Osmolality (A), and Na⁺ (B) and Cl⁻ (C) concentrations in the hemolymph of Minuca rapax exposed to different salinities (< 0.1‰ S [distilled H₂O, hypo-osmotic medium], 25‰ S [isosmotic control medium] or 60‰ S [hyper-osmotic medium] and Cu concentrations (in µg Cu L⁻¹ furnished as CuCl₂) after 5 days with free access to a dry surface. Data are the mean ± SEM (N=10). *Significantly different from respective value for 25‰ S; †significantly different from 0 µg Cu L⁻¹ (control) within a salinity (P ≤ 0.05).
decreased, and also decreased in crabs at 250 µg Cu/L, with a 2-fold increase in activity at 500 µg Cu/L (Fig. 4B). In 60‰ S, control carbonic anhydrase activity decreased, showing an increase in crabs at 250 µg Cu/L (Fig. 4B).

4. Discussion

4.1. Copper accumulation

Copper accumulates in a concentration dependent manner in the
tissues of Minuca rapax above concentrations of 150 µg Cu/L, a titer at which Cu regulation begins to fail. At lower [Cu], this fiddler crab appears to regulate tissue Cu accumulation well.

The hemolymph and hepatopancreas showed the highest Cu titers, suggesting that these tissues respectively constitute sites of Cu transport and accumulation. Cu concentrations in these two tissues increase suddenly in crabs exposed to 250 and 500 µg Cu/L, the highest concentrations tested, although [Cu] in some salinity/Cu combinations were higher than in the control crabs. The elevated [Cu] seen in the hemolymph in both control and Cu-exposed crabs is not surprising since Cu is an essential element constituting hemocyanin, the main oxygen-carrying pigment found in decapod hemolymph (Péqueux, 1995) and is well regulated when accumulated from environmental sources (Rainbow, 1997).

Copper accumulation in the gills and hemolymph of Minuca rapax appears to be salinity independent. In low salinities, the uptake and toxicity of metals increases in estuarine crabs (Zanders and Rojas, 1996c) and in amphipods (Lockwood and Inman, 1973) and copepods (Wright and Frain, 1981). Estuarine and euryhaline marine crustaceans appear to be salinity independent. In low salinities, the uptake and accumulation. Cu concentrations in these two tissues increase suddenly in crabs exposed to 250 and 500 µg Cu/L, the highest concentrations tested, although [Cu] in some salinity/Cu combinations were higher than in the control crabs. The elevated [Cu] seen in the hemolymph in both control and Cu-exposed crabs is not surprising since Cu is an essential element constituting hemocyanin, the main oxygen-carrying pigment found in decapod hemolymph (Péqueux, 1995) and is well regulated when accumulated from environmental sources (Rainbow, 1997).

Copper uptake in Minuca rapax may derive from diffusive influx down its concentration gradient rather than by an active pumping mechanism. Subsequent binding to hemolymph proteins and transfer to organs such as the hepatopancreas (Bjerregaard, 1990) would reduce the Cu concentration in the extracellular fluid, maintaining a gradient for passive Cu influx from the external medium. Marine invertebrates can absorb toxic metals like Cu across the gills, or via the gut epithelia after food ingestion. In turn, such metals may be excreted across the same epithelia (Ahearn et al., 2004). Because the gill epithelium is in direct contact with Cu dissolved in the surrounding water, this is considered to be the main route of copper uptake from the dissolved phase. Consequently, the gill epithelium is also considered to be the first target interface of Cu toxicity.

Minuca rapax appears to employ a behavioral strategy to avoid Cu-containing media in free access experiments, which may explain why crabs exposed to 250 µg Cu/L and above for 5 days show no difference in Cu accumulation. To simulate the effect of submersion at high tide, we forcibly submerged crabs for 5 h at concentrations up to 500 µg Cu/L. However, Cu accumulated less in all tissues than in the 5-day free access exposure regime, likely reflecting the shorter effective immersion time. Nevertheless, the same pattern of Cu accumulation, i.e., higher Cu titers in the hemolymph and hepatopancreas compared to the gills, was seen independently of exposure strategy.

4.2. Physiological and biochemical responses to copper exposure

Minuca rapax is a strong euryhaline osmoregulator and maintains its hemolymph osmolality at around 750 mOsm/kg H2O over a wide range of environmental salinities (Baldwin and Kirschner, 1976; Thurman, 2002; Faria et al., 2017). At low salinities, in which active ion uptake is increased, metal exposure can damage the gill epithelium, impairing osmoregulatory ability as a consequence of the reduced ability to transport Na⁺ and/or Cl⁻ (Zanders and Rojas, 1996c; Pequeux, 1995; Hebel et al., 1997).

In the present study, hemolymph osmolality in Minuca rapax with free access to a dry surface was unaffected by Cu exposure for 5 days. However, Cu did affect hemolymph Na⁺ and Cl⁻ regulatory ability, especially in 60‰ S, in which the crabs hypo-regulated [Na⁺] and [Cl⁻] to a lesser degree than did unexposed crabs. Further, submersed M. rapax also showed a diminished ability to hyperegulate hemolymph osmolality and [Na⁺] and [Cl⁻] in 60‰ S. When in hypo-osmotic media below 25‰ S with free access to a dry surface, the semi-terrestrial fiddler crab U. pugilator can eliminate water gained by osmosis through evaporation (Dorazio and Holliday, 1985). Forcibly submerged crabs would be unable to use evaporation to aid in osmoregulation.

Uca pugilator, when exposed to salinities between 40 and 63‰ S (1200 and 1900 mOsm/kg H2O) in situ maintains hemolymph osmolality within a narrow range of from 1069 to 1085 mOsm/kg H2O, while submerged crabs osmoconform in these salinities (Zanders and Rojas, 1996b). Thus, maintaining crabs submerged affects osmoregulatory capability, and particularly hypo-osmoregulatory ability. This same pattern was seen here in submerged Minuca rapax, and was further affected by Cu exposure. High relative humidity in natural environments may minimize evaporative water loss and may explain why crabs
in natural high-salinity environments can regulate hemolymph osmolality better than crabs submerged at high salinity. However, regardless of whether a crab confronts a natural high salinity environment or a high salinity submersion environment, water lost by evaporation and/or osmosis must be replaced. Fiddler crabs may replace this lost water by imbibing seawater (Baldwin and Kirschner, 1976) and excreting the ingested salts. Fiddler crab urine is nearly isosmotic to the hemolymph but has a lower [Na⁺]. Thus, the antennal glands are not the sites of salt excretion. Salts ingested with seawater are likely excreted by the gills (Freire et al., 2008; McNamara and Faria, 2012), gut (McNamara et al., 2005) or some other extra-renal site.

Fiddler crabs are excellent hypo-osmoregulators (Holliday 1985; Zanders and Rojas, 1996; Lin et al., 2002; Thurman 2003ab, 2005; Faria et al., 2017), a capability essential to the evolution of their semi-terrestrial nature (Jones, 1941; Thurman, 2002) and that maintains hemolymph osmolality stable during aerial exposure. As semi-terrestrial crabs, Minuca rapax are fully submerged only at high tide for a maximum of about 5 h. In the present study, Minuca rapax exhibited impaired hypo-osmoregulatory ability only during submersion together with Cu exposure. Gill Na⁺/K⁺-ATPase and carbonic anhydrase activities increase in many crabs when in dilute seawater (Towle, 1981; Graszyński and Drews, 1981; Drews, 1987; Hale and Teller, 1983; Siebers et al., 1983; Holliday, 1985) as also seen here for Minuca rapax. However, in contrast to Uca pugilator (Holliday and Holliday, 1985) and Carcinus maenas (Hale and Teller, 1983), gill Na⁺/K⁺-ATPase activity in Minuca rapax did not increase at 60% S. Thus, salt secretion in this concentrated medium may not be underpinned by Na⁺/K⁺-ATPase activity, and may depend more on expression of the Na⁺/K⁺/2Cl⁻ symporter (McNamara and Faria, 2012).

Teal and Carey (1967) examining lactate metabolism in fiddler crabs showed that anaerobic metabolism predominates in submerged crabs. Gill Na⁺/K⁺-ATPase activity decreases with decreasing salinity in Uca pugilator (Dorazio and Holliday, 1985), which suggests that Minuca rapax may be unable to generate sufficient energy to resist an additional stress like exposure to Cu.

The increase in gill Na⁺/K⁺-ATPase activity in Minuca rapax exposed for 5 days to 50 µg Cu/L both in distilled H₂O and in 25% S with free access to a dry surface can be considered a hormetic response, with Cu exposure. Gill Na⁺/K⁺-ATPase and carbonic anhydrase impaired hypo-osmoregulatory ability only during submersion together with Cu exposure. Gill Na⁺/K⁺-ATPase and carbonic anhydrase activities increase in many crabs when in dilute seawater (Towle, 1981; Graszyński and Drews, 1981; Drews, 1987; Hale and Teller, 1983; Siebers et al., 1983; Holliday, 1985) as also seen here for Minuca rapax. However, in contrast to Uca pugilator (Holliday and Holliday, 1985) and Carcinus maenas (Hale and Teller, 1983), gill Na⁺/K⁺-ATPase activity in Minuca rapax did not increase at 60% S. Thus, salt secretion in this concentrated medium may not be underpinned by Na⁺/K⁺-ATPase activity, and may depend more on expression of the Na⁺/K⁺/2Cl⁻ symporter (McNamara and Faria, 2012).

Acknowledgments

This investigation received financial support from the Fundação de Amanpo à Pesquisa do Estado de São Paulo (FAPESP, grant 2011/22537-0 to JCM) from which MVC received doctoral scholarships (FAPESP 2011/08065-9 and 2013/10672-6). JCM received a research scholarship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 300662/2009-2). MG is a Maytag Chair of Ichthyology. This study is part of a doctoral dissertation submitted by MVC to the graduate program in Comparative Biology, FFL/EPUSP and received additional support from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, 330020930119).

References

Blanchard, J., Grosell, M., 2006. Copper toxicity across salinities from freshwater to seawater in the euryhaline fish Fundulus heteroclitus: is copper an ionoregulatory toxicant in high salinities? Aquat. Toxicol. 80, 131–139.


Grosell, M., et al., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquat. Toxicol. 84, 162–172.


