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Effects of copper on osmoregulation in sheepshead minnow, *Cyprinodon variegatus* acclimated to different salinities

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ABSTRACT

The sheepshead minnow, *Cyprinodon variegatus* is a euryhaline fish that inhabits estuaries and coastal marshes where it encounters a wide range of salinities. Many of these areas also have elevated levels of contaminants, creating the potential for toxic ions to interfere with the uptake of ions for osmoregulation. To determine whether the effect of copper on osmoregulatory activity is dependent on the osmotic conditions that individuals have been living at, fish were acclimated for 14 days to 2.5, 10.5 or 18.5 ppt seawater and then exposed to a fixed free cupric ion level (14.6 μM Cu^{2+}) for 6 h. Plasma Na, plasma Cl, wet/dry weight ratio, transepithelial potential difference (TEPD) and branchial Na^+/K^+-ATPase activity were determined before and after copper exposure. We also computed Na and Cl equilibrium potentials. Following the salinity acclimation (in fish not yet exposed to copper), fish from the low salinity group (2.5 ppt) had lower TEPD, lower plasma Na levels and higher branchial Na^+/K^+-ATPase activity compared to the fish acclimated to higher salinities. No differences in plasma Cl and wet/dry weight ratio were detected. Copper exposure caused a significant decrease in plasma Na levels and Na^+/K^+-ATPase activity and an increase in wet/dry weight ratio, but these changes were limited to the 2.5 ppt salinity group. No significant changes in plasma Cl were detected. Copper treatment resulted in a small decrease in TEPD for all except the lowest salinity acclimation group. A comparison of equilibrium potentials with TEPD showed evidence of active transport of both Na and Cl in 2.5 ppt acclimated fish but not for the 10.5 or the 18.5 ppt acclimated fish. Our results show that effects of copper on osmoregulation are dependent on the fish' past salinity regime, and that these effects tend to be more pronounced for euryhaline fish that have been living under hyposmotic conditions.

1. Introduction

Fish inhabiting estuaries and coastal marshes have to be able to handle the frequent and large salinity fluctuations typical for these environments (Moyle and Cech, 1982). Euryhaline fish, such as the sheepshead minnow *Cyprinodon variegatus*, have efficient osmoregulatory mechanisms that have permitted them to thrive there (Barton, 1987; Haney, 1999; Pillard et al., 1999). These mechanisms involve active uptake of ions such as Na^+ and Cl^- at low salinities, but ion secretion at high salinities (Evans, 2008; Evans et al., 2005). Because estuaries are often sites of elevated metal levels (Ridgwaya and Shimmield, 2002; Wright and Mason, 1999), and because uptake pathways in teleost fishes may transport multiple ions involved in osmoregulation and unintentionally transport toxic ions (Grosell and Wood, 2002; Handy et al., 2002), there is the potential for interaction between these two groups of ions. This study focuses on one such specific interaction, investigating if the effect of copper exposure on osmoregulatory processes is dependent on the environmental salinity at which the fish have been living. Our prediction is that the impact of copper on osmoregulation will be less severe in fish acclimated to hyposmotic or hyperosmotic salinities than in fish acclimated to an isosmotic salinity.

While copper is considered an essential metal for its role as a co-factor for numerous enzymes, it interferes with ionoregulation when present at high levels (Blanchard and Grosell, 2006; Hall et al., 1997; Wilson and Taylor, 1993). Studies have shown that, even at concentration as low as 0.1 μM Cu^{2+}, Na^+/K^+-ATPase is inhibited (Lauren and McDonald, 1985; Li et al., 1996; Tate-Boldt and Kolok, 2008). Also, there is some evidence that copper competes with sodium at the apical Na channel, which may result in reduction of total body Na levels (Grosell and Wood, 2002). The Na^+/K^+-ATPase plays a central role in osmotic/ionic regulation in teleost fish, via active transport of Na^+ and Cl^- ions (Shikano and Fujio, 1998; Wilson et al., 2000; Withers, 1992). For example, in the European sea bass *Dicentrarchus labrax*, acclimation to freshwater or seawater resulted in an increase in the activity of Na^+/K^+-ATPase – which was accompanied by a 2- to 5-fold elevation of the gill α-subunit Na^+/K^+-ATPase mRNA level (Jensen et al., 1998).

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In this study, sheephead minnows were acclimated for 2 weeks to hyposmotic (2.5 ppt), isosmotic (10.5 ppt) or hyperosmotic (18.5 ppt) salinity, and then exposed to copper. These exposures were conducted at these different salinities, but at the same free cupric ion concentration – to control for the effect of salinity on Cu speciation and hence on Cu toxicity. The following osmoregulation-related variables were measured before and after the copper exposure: Plasma Na, plasma Cl, wet/dry weight ratio (a measure of tissue water content), transepithelial potential difference, and activity and expression of Na^+/K^+-ATPase.

2. Materials and methods

2.1. Fish collection

Fish used in this study were collected near Avery Island, Louisiana (N29°56.155′; W91°54.388′). The salinity here at the time of collection was 7 ppt and was measured using a salinometer (YSI 30). Fish were netted and transported to the laboratory in coolers containing aerated ambient water. To determine the salinity that corresponds to an isosmotic condition for these fish, a vapor pressure osmometer (Wescor Inc.) was used to determine the osmotic concentration of the fish plasma. The latter was determined to be 301 mOsm, equivalent to a 10.5 ppt salinity. Following the collection, the fish were maintained in the laboratory in water with a 10.5 ppt salinity and 23 °C temperature.

2.2. Experimental protocol

After 4 weeks of acclimatization to laboratory conditions, fish were divided into 3 groups and transferred gradually (in 4 steps) to water of 2.5 ppt, 10.5 ppt and 18.5 ppt salinity to start their 2-week salinity acclimation. The acclimation was done in three glass aquaria per acclimation group; each aquarium filled with 30 L aerated filtered seawater. A 50% water change was done every other day and fish were fed twice daily with Tetramin flake food. Fish experienced a 16 h/8 h light/dark cycle throughout acclimation and exposure. Waters of different salinities were prepared by diluting natural seawater with deionized water. Salinities were measured with a salinometer (YSI 30).

After 14 days exposure to the three salinities, 24 fish (8 from each aquarium) were removed for each salinity group and used for various analyses (see below). The remaining 24 fish were exposed to 14.6 μM free Cu (Cu^{2+}) for 6 h. Because copper complexes with ions in seawater and since copper toxicity is generally a function of free cupric ion levels rather than total copper levels (Burke et al., 2003; Engel and Sunda, 1979; Sola et al., 1995), we exposed fish in all the groups to the same free cupric ion concentration. The amount of $CuSO_4$ to be added to each experimental medium in order to achieve the desired Cu^{2+} concentration was calculated with the software Visual MINTEQ. The total copper concentrations were 10, 15.3 and 16.85 mg/L, respectively for the 2.5, 10.5 and 18.5 ppt salinity groups. Total copper concentrations were confirmed using flame atomic absorption spectrophotometry (Perkin-Elmer 1100B).

2.3. Transepithelial potentials

Before and after 6 h of copper exposure, five or six fish from each salinity treatment were anesthesized with 0.1% tricaine solution buffered with sodium bicarbonate. Agar bridges (2.5 M KCl in 2% agar) were attached to silver–silver chloride electrodes. The transepithelial potential difference (TEPD) was measured by the insertion of one end of an agar bridge into the body cavity of the fish; the other end was immersed in the water surrounding the animal. The difference in potential was measured with a voltage clamp set in potential mode (WPI model DVC 1000). Potential values were recorded only when the readings on the voltmeter were stable and the accuracy of the readings was confirmed by return to a voltage of zero once the bridge was removed from the fish and immersed in the medium. All of the experimental fish recovered within 3 min, once removed from the anesthesia.

2.4. Plasma sodium and chloride levels analyses

Fish were euthanized with an overdose of tricaine buffered with sodium bicarbonate and blood was collected by cutting through the caudal region of the animal. The blood was centrifuged at $5000 \times g$ for 5 min in a microcentrifuge (Beckman microfuge E) to separate the plasma from the erythrocytes; the plasma was stored at −20 °C until analysis. The sodium concentrations of the plasma and the experimental media were measured directly by furnace atomic absorption spectrophotometry (Perkin-Elmer 1100B equipped with 700 HGA graphite furnace). The chloride concentrations of plasma and media were determined by titration (Aminco J4-4417). Equilibrium potentials were calculated from these data using the Nernst equation, $E = RT/ZF \ln([\text{ion}]_{\text{outside}}/[\text{ion}]_{\text{inside}})$ where E is the equilibrium potential, R is the gas constant, T is the temperature (298 K), Z is the charge on the ion and F is the Faraday's constant. The values were compared to the measured TEPD to determine if there was active transport of ions (Table 1). A difference of about 5 mV between the calculated equilibrium potential and the measured TEPD for an ion is generally taken as evidence of active transport (Evans et al., 1976).

2.5. Wet/dry weight ratio

The wet/dry weight ratio is a measure of tissue water content, and osmoregulatory success is indicated by a constant wet/dry weight ratio. After the 14-day salinity acclimation, and at the end of 6-h copper exposure, nine fish were removed from each experimental treatment. Each fish was euthanized in tricaine, blotted on laboratory tissue paper and weighed. The fish were weighed again after drying in an oven at 65 °C for 48 h.

2.6. Branchial Na^+/K^+-ATPase activity assay

Gills were dissected out, homogenized in ice-cold sucrose buffer (250 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.4), and branchial Na^+/K^+-ATPase activity was determined according to Hylland et al. (1997), with minor modifications. In our assay, p-nitrophenylphosphate was used as the substrate for the ATPase and the amount of the formed product, p-nitrophenol, was quantified spectrophotometrically at 410 nm using a UV–vis spectrophotometer (Ultrospec 3000, Pharmacia Biotech). Na^+/K^+-ATPase activity was obtained by subtracting the activity in a ouabain-blocked mixture from the total ATPase activity. Na^+/K^+-ATPase activity was expressed as μM p-nitrophenol/mg protein of the homogenate, and the molar extinction coefficient used was 13,200 M^{-1} cm^{-1}. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories).

2.7. Na^+/K^+-ATPase Western blot

The supernatant of the gill homogenates (obtained during activity determinations described above) and molecular weight standards (New England Biolab) were heated at 37 °C for 5 min. We loaded 20 μg of protein into each well and separated by electrophoresis on SDS-containing 10% polyacrylamide gels. Separated proteins were transferred from unstained gels onto a nitrocellulose membrane (Bio-Rad) at room temperature for 1 h, using a tank transfer system (Bio-Rad, Mini Protean 3). Blots were preincubated for 2 h in PBST buffer (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4,