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Osmoregulation and branchial plasticity after acute freshwater transfer in red drum, *Sciaenops ocellatus*

Caroline J. Watson, Wiolene M. Nordi, Andrew J. Esbaugh *

University of Texas Marine Science Institute, Austin, TX 78373, USA

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ABSTRACT

Red drum, *Sciaenops ocellatus*, is an estuarine-dependent fish species commonly found in the Gulf of Mexico and along the coast of the southeastern United States. This economically important species has demonstrated freshwater tolerance; however, the physiological mechanisms and costs related to freshwater exposure remain poorly understood. The current study therefore investigated the physiological response of red drum using an acute freshwater transfer protocol. Plasma osmolality, Cl^-, Mg^{2+} and Ca^{2+} were all significantly reduced by 24 h post-transfer; Cl^- and Mg^{2+} recovered to control levels by 7 days post-transfer. No effect of transfer was observed on muscle water content; however, muscle Cl^- was significantly reduced. Interestingly, plasma and muscle Na^+ content was unaffected by freshwater transfer. Intestinal fluid was absent by 24 h post-transfer indicating cessation of drinking. Branchial gene expression analysis showed that both CFTR and NKCC1 exhibited significant down-regulation at 8 and 24 h post-transfer, respectively, although transfer had no impact on NHE2, NHE3 or Na^+, K^+ ATPase (NKA) activity. These general findings are supported by immunohistochemical analysis, which revealed no apparent NKCC containing cells in the gills at 7 days post transfer while NKA cells localization was unaffected. The results of the current study suggest that red drum can effectively regulate Na^+ balance upon freshwater exposure using already present Na^+ uptake pathways while also down-regulating ion excretion mechanisms.

1. Introduction

The red drum (*Sciaenops ocellatus*) is an estuarine-dependent fish highly valued by the recreational saltwater fishing industry (Murphy and Taylor, 1990). This sciaenid teleost is commonly found in most of the Gulf of Mexico and along the southeastern coast of the United States. Strict management actions have been taken in the United States to mitigate significant population declines observed since the late 1980s, including the designation of red drum as a federally protected game species in 2007. Nonetheless, low red drum stocks continue to generate concern for this economically important species (McEachron et al., 1998). Adults typically inhabit full strength seawater of near-shore waters and bay-gulf passes (Murphy and Taylor, 1990) and in late summer and early fall adults seek out lower estuaries, lagoons and inlets for annual spawning (Bachelor et al., 2012). Larvae and eggs are transported via surface and tidal currents to estuarine habitats with lower salinities, where they remain for 1–3 years until reaching sexual maturity (Rooker and Holt, 1997).

In general, moving from marine to freshwater habitats is coincident with an intense osmoregulatory and ionoregulatory challenge as the animal must defend against diffusive ion loss and water gain. This often results in transient decreases in plasma and muscle ion content as well as increased muscle water content (McDonald and Grosell, 2006; Tipsmark et al., 2008), which fish compensate for through various acclimation responses. These responses can include increased production of dilute urine to eliminate water (Fleming and Stanley, 1965; McDonald and Grosell, 2006) and reduced drinking rates (Scott et al., 2006) to limit intestinal water absorption. Prolonged exposure to freshwater will lead to gill re-modeling to change from ion excretion to absorption. The most common response includes down-regulation of genes associated with salt excretion including the *cftr* chloride channel and Na^+, K^+, $2Cl^-$ co-transporter (*nkcc1*); this has been demonstrated for a number of species (Pelis et al., 2001; McCormick et al., 2003; Scott et al., 2005; Tse et al., 2006; Hiroi and McCormick, 2007; Tipsmark et al., 2008; Tang et al., 2011). Conversely, ion uptake pathways are up-regulated in response to freshwater transfer. The most common response is an increase in Na^+, H^+ exchanger (*nhe*) expression (Edwards et al., 2005; Scott et al., 2005; Reilly et al., 2011) and Na^+, K^+ ATPase (*nka*) α subunit isoform switching (Richards et al., 2003; Bystriansky et al., 2006; Madsen et al., 2009; Bystriansky and Schulte, 2011; Tipsmark et al., 2011; Gilmour et al., 2012; Ip et al., 2012; McCormick et al., 2013; Urbina et al., 2013; Esbaugh et al., in press); however, an absorptive NKCC isoform – presumably NKCC2 (Brix and Grosell, 2012) – and Na^+, Cl^- co-transporters (NCC) (Hwang, 2009; Wang et al., 2009; Hwang

* Corresponding author. Tel.: +1 361 749 6835; fax: +1 361 749 6749.
E-mail address: a.esbaugh@austin.utexas.edu (A.J. Esbaugh).

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et al., 2011) have also been implicated in sodium uptake in freshwater fish. Relatively less is known about Na^+-independent Cl^- uptake pathways in euryhaline fishes; however, both pendrin (*slc26a4*) (Reilly et al., 2011) and the voltage gated Cl^- channel (*clc-3*) (Tang et al., 2010; Bossus et al., 2013) have been implicated while several bicarbonate exchange proteins have been implicated in freshwater stenohaline teleosts (Bayaa et al., 2009; Perry et al., 2009). Interestingly, some fish are unable to absorb Cl^- yet do not show a dramatically compromised ability to survive fresh water. Instead these fish maintain plasma Cl^- through the diet (Bucking et al., 2013), which highlights the potential for non-branchial ionoregulatory pathways.

It is well known that red drum exhibit freshwater tolerance beginning in the post-larval stage (9 mm standard length) (Crocker et al., 1981; 1983); however, the physiological costs and acclimation mechanisms associated with acute freshwater transfer in this species remain poorly understood. As such, the purpose of the current study was to investigate the physiological response of sub-adult red drum to an acute freshwater challenge over several short-term time points while monitoring plasma and muscle ion and water content. Furthermore, the time course of gill re-modeling was assessed by examining expression patterns, enzyme activity and immunohistochemical localization of genes relevant for Na^+, Cl^- excretion and Na^+ uptake.

2. Materials and methods

2.1. Experimental fish

All experimental procedures were performed under the auspices of the University of Texas at Austin Institutional Animal Care and Use Committee and unless specified all chemicals were obtained from Fisher Scientific (Pittsburg, PA, USA). The red drum (*S. ocellatus*) used in the current study were raised on-site at the Fisheries and Mariculture Laboratory (FAML) and the University of Texas Marine Science Institute (Port Aransas, TX, USA), according to previous established protocols (Holt et al., 1981a, 1981b). Sub-adult (103.4 ± 3.8 g ranging from 65.3 to 156.9 g) fish were held in recirculating in-door 150 L tanks supplied with filtered, running seawater originating from the Corpus Christi ship channel. The sub-adult life stage was defined based on the adult tail fin morphology (slightly concave with tail spot) in the absence of sexual maturity. The recirculation system was equipped with a common biological filter tank to control ammonia levels, which were monitored periodically using standard marine aquarium test kits. The water temperature was held at 25 °C and salinity was maintained at approximately 33 ppt with a 14:10 light dark cycle. Fish were fed daily with commercial fish pellets (AquaMax, Purina Mills LLC, St. Louis, MO, USA). Tanks were siphoned periodically to remove debris and partial water changes performed as necessary.

2.2. Experimental protocol

Exposures were performed in two 75 L water tables, with one maintained at full strength (33 ppt) seawater and the other with de-chlorinated Port Aransas tap water (mM; Na^+ 6.7, Cl^- 5.8, Ca^{2+} 9.1, Mg^{2+} 0.9, K^+ 0.3, titratable alkalinity 2.0, pH 8.1). Each table was aerated and equipped with a biofilter to control ammonia concentrations. Fish were isolated in plastic holding containers submerged in the respective water tables to eliminate the impacts of social interaction. All control fish were exposed to a similar transfer protocol to the seawater wet table and sampled after 24 h. Fish were fed ad libitum during the freshwater treatment; however, all fish were starved for at least 48 h prior to sampling to ensure the gut was clear for dissection. Individual holding chambers were inspected for food to ensure that fish were feeding appropriately post-transfer. Salinity and pH were monitored daily for both treatments. Fish were exposed to freshwater for 0 h (seawater control), 8 h, 24 h, 72 h and 7 days, after which individuals were removed from the holding containers and tissues and fluids sampled as described below.

2.3. Sampling techniques

Fish were euthanized by full immersion in a MS-222 bath (250 mg/L; 500 mg $NaHCO_3$ per L) followed by spinal transection. Blood was sampled by caudal puncture using a 21-gauge needle pre-rinsed in heparinized saline (50 units/mL). Red blood cells and plasma were separated by centrifugation (10,000 *g* for 1 min). To collect intestinal fluid for ion analysis, an incision was made above the anal vent and the body cavity opened to expose the intestine. The anterior and posterior ends of the intestine were clamped using hemostats, after which the tract was excised and the fluid emptied into 1.5 mL tubes. Intestinal fluids were centrifuged in order to separate solid matter from the sample. A portion of white muscle tissue (~0.5–1 g) was excised from the dorsal area for determination of water content and ion analysis. The lamellae from two gill arches were removed for enzyme and RNA analysis. Tissue samples for RNA analysis were placed immediately in 1 mL of RNAlater (Life Technologies, Grand Island, NY, USA) and stored at −20 °C, while enzyme samples were placed immediately on ice and subsequently frozen at −80 °C. Two additional gill arches were sampled from control and 7-day exposed individuals for immunohistochemical analysis. Samples were placed immediately in zinc formalin fixative (Z-Fix; Anatech Ltd, Battle Creek, MI, USA), stored overnight at 4 °C and subsequently transferred to 70% ethanol for long-term storage.

2.4. Analysis techniques

The wet mass of the muscle tissue was measured and placed in a dehydrating oven at 55–60 °C. Samples were weighed daily until they reached stable weights for three consecutive days. Muscle water content was calculated as the difference between the wet mass and final dry mass. 1 M HNO_3 (5 vol: weight) was used to dissolve the tissues overnight at 55–60 °C. The samples were subsequently vortexed and centrifuged (10,000 *g* for 3 min) and the supernatant was collected and refrigerated for later dilution for ion analysis. Prior to freezing, the intestinal fluid was tested for total CO_2 content using a carbon dioxide analyzer, model 965 (Olympic Analytical Services, UK). Fluid pH was measured using an Accumet Microprobe pH electrode with associated AB15 meter (Fisher Scientific) and osmolality was analyzed using a Vapro 5520 osmometer (Wescor, Logan, UT, USA). The concentration of cations (Mg^{2+}, K^+, Na^+ and Ca^{2+}) in blood plasma, muscle tissue and intestinal fluid were measured using a iCE 3000 atomic absorption spectrophotometer (Thermo Scientific, Waltham, MA, USA). The concentration of chloride anions in the same fluids was measured using a chloride analyzer, model 926 (Cole Parmer, Court Vernon Hills, IL, USA). Branchial NKA activity was measured at room temperature (23 °C) according to the method of McCormick (1993). NKA levels were normalized to total protein content as determined by the Coomassie Plus Protein Assay (Thermo Scientific) using albumin standards.

2.5. RNA analysis

Total RNA was extracted from red drum gill tissue using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to manufacturer guidelines, with homogenization performed using a motor driven tissue homogenizer. Total RNA was quantified using an ND-1000 (Thermo Scientific) spectrophotometer at a wavelength of 260 nm and sample purity was assessed using 260:280 ratios. A 1 μg sample of RNA was DNase-treated with amplification grade DNase I (Thermo Scientific; manufacturer specifications) to remove potential DNA contamination, which was subsequently used for cDNA synthesis. This was performed using RevertAid reverse transcriptase (Thermo Scientific), according to the manufacturer specifications. Real-time PCR was performed on an Mx3000P real-time PCR system (Stratagene, Santa Clara, CA, USA)