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Interrogation of the Gulf toadfish intestinal proteome response to hypersalinity exposure provides insights into osmoregulatory mechanisms and regulation of carbonate mineral precipitation

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ABSTRACT

Marine bony fish live in a hyperosmotic environment and maintain osmotic homeostasis by drinking seawater, and absorbing salt and water across their gastrointestinal tract. Although the ion and water transport mechanisms in the intestine have been the subject of much study, numerous questions remain unanswered. To address some of these questions, a shotgun proteomics methodology employing isobaric tandem mass tags (TMT) was used to interrogate the anterior intestine, posterior intestine, and intestinal fluid proteomes of Gulf toadfish (*Opsanus beta*) acclimated to normal (35 ppt) or hypersaline (60 ppt) seawater. Relative protein abundance between tissues was also investigated using label free quantitation. Protein products from nearly 3000 unique toadfish loci were identified and quantified between the tissues, and pathway analysis was performed to gain insight into biological significance. Numerous proteins potentially involved in ion transport, digestion, nutrient absorption, and intestinal $CaCO_3$ precipitation were found to respond to changing salinity, providing additional insight into the molecular mechanisms behind these processes. Intestinal protein heterogeneity was also observed with proteins involved in ion transport responding to hypersalinity exposure primarily in the anterior intestine, and proteins involved in digestion and nutrient absorption showing higher abundance in the anterior intestine, regardless of salinity.

1. Introduction

Marine fish live in a hyperosmotic environment which contains nearly three times as much salt as their body fluids. This leads to continual passive gain of excess salt and loss of water. Excess salt is excreted primarily through the gills via specialized mitochondrial rich cells, and to lesser extent, the urine (Hirano and Mayer-Gostan, 1976; Larsen et al., 2014). To battle dehydration, fish drink the only external source of water they have available – seawater. Imbibed seawater is heavily processed throughout the gastrointestinal tract, beginning with approximately 50% desalinization in the esophagus (Parmelee and Renfro, 1983). Most water absorption occurs across the intestinal epithelium via solute-coupled water uptake, which effectively moves large volumes of water, even in the absence of a significant osmotic gradient (Skadhauge, 1974; Genz et al., 2011; Grosell, 2011). To aid in the absorption of water across the intestinal epithelium, bicarbonate is transported into the intestinal lumen in exchange for Cl^-, which serves to increase luminal pH and bicarbonate concentration (Grosell and Taylor, 2007). This, along with the concentration of calcium in the intestinal fluid due to the relative impermeability of the gastrointestinal tract to Ca^{2+} and the removal of water from the lumen, leads to the precipitation of $CaCO_3$, which further lowers luminal osmotic pressure and allows for additional water absorption (Wilson et al., 2002; Whittamore et al., 2010). The precipitated $CaCO_3$ is eventually excreted to the environment, where it plays a substantial role in oceanic carbon cycling (Wilson et al., 2009).

The fish intestine is not an entirely uniform organ, as gene expression as well as physiological function is known to vary in different segments (Grosell, 2013; Ronkin et al., 2015). *In vivo*, the anterior intestine absorbs most of the water (Marshall and Grosell, 2006), despite

Abbreviations: AI, anterior intestine; PI, posterior intestine; IF, intestinal fluid; GIT, gastrointestinal tract; TMT, tandem mass tag; ppt, parts per thousand; LFQ, label free quantitation; ACE, angiotensin converting enzyme; HSC70, heat shock cognate 71 kDa protein; ANXA5, annexin A5; DPC, distance from perfect correlation; NKCC2, Na-K-Cl cotransporter 2; VHA, V-type proton ATPase; CFTR, cystic fibrosis transmembrane conductance regulator; IPA, ingenuity pathway analysis

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in vitro measurements showing that all portions of the intestine are capable of similar rates of water absorption (Grosell and Jensen, 1999). *In vivo* observations also suggest that much of the bicarbonate secretion occurs in the anterior intestine (AI), which is corroborated by higher transcription of SLC26a6 and NBC1 (two transporters involved in bicarbonate secretion) in the proximal compared to distal segments (Grosell et al., 2009; Taylor et al., 2010). In contrast, only the posterior intestine (PI) is responsive to guanylin peptides, a class of paracrine signaling peptides involved in the regulation of ion secretion (Ruhr et al., 2014).

Although the molecular mechanisms responsible for the osmoregulatory functions of the marine teleost intestine have been well investigated (reviewed in Grosell (2013)), numerous aspects remain poorly understood, especially regarding the regulatory mechanisms that ultimately control the rate of salt and water movement. Further, it has recently been discovered that the intestinal $CaCO_3$ precipitates forms in conjunction with an at least partially proteinaceous matrix, that controls the rate of mineral precipitation (Schauer et al., 2016). Although > 150 proteins have been identified in this matrix, the individual roles of the matrix proteins require further study (Schauer and Grosell, 2017). To address these questions, a shotgun proteomics experiment using isobaric tandem mass tags (TMT) was conducted here to determine how the AI, PI, and intestinal fluid (IF) proteomes in the Gulf toadfish (*Opsanus beta*) differ in fish acclimated to normal seawater (35 parts per thousand; ppt) versus hypersaline (60 ppt) seawater, which imposes a greater osmoregulatory challenge. The Gulf toadfish was chosen as a model for this study due to its euryhaline nature (2.5 to > 70 ppt) (McDonald and Grosell, 2006), their well characterized intestinal precipitation processes (Walsh et al., 1991; Heuer et al., 2016), and the availability of transcriptomic data for the species (Schauer et al., 2016). We hypothesized that proteins involved in osmoregulation would change in abundance during hypersalinity exposure due to the need for upregulation of osmoregulatory processes to maintain proper salt and water balance. Additionally, we confirm here previous speculations that the production of $CaCO_3$ precipitates increases during hypersalinity exposure (Genz et al., 2008), so we expected that proteins involved in regulating the precipitation reaction would also change in abundance when fish are exposed to hypersaline water. The relative abundance of individual proteins in the different tissues (AI, PI, and IF) was also investigated using label free quantitation.

2. Material and methods

2.1. Experimental animals

Gulf toadfish (*Opsanus beta*) were obtained from commercial bait shrimp fisherman operating out of Dinner Key, Miami, FL. Fish were fresh water dipped for 3 min and malachite green treated to remove ectoparasites upon arrival to the Rosenstiel School of Marine and Atmospheric Science where they were then kept in 60 l aquaria on flow-through, sand filtered seawater from Biscayne Bay (32–37 ppt, 21–26 °C) and fed once weekly to satiation with chopped squid unless stated otherwise. All experimental protocols were completed in accordance with and approved by the University of Miami Animal Care and Use Committee (protocol no. 13–225).

2.2. Measurement of precipitate production

In order to confirm previous reports showing a trend toward increased $CaCO_3$ production it Gulf toadfish during hypersalinity exposure (Genz et al., 2008), n = 40 toadfish (16–57 g) were randomly assigned to five, 30 l glass aquaria filled with 1 μm filtered, UV-sterilized seawater from Biscayne Bay that was adjusted to a salinity of 35 ppt by the addition of Instant Ocean marine salt (Spectrum Brands, Blacksburg, VA, USA) or reverse osmosis purified water. Precipitates from each tank were collected daily from the tank bottom by siphoning the water through a fine (~500 μm) mesh, after which a minimum 70% water change was completed. Collected precipitates were placed in pre-weighed 15 ml conical tubes, rinsed 2× with ultrapure water and bleached in a NaOCl solution containing 5% available chlorine for 24 h at 4 °C to remove contaminating organic material. Bleached precipitates were then rinsed again 2× in ultrapure water and weighed. $CaCO_3$ was dissolved in excess 0.5 M EDTA at 4 °C for 3 h, centrifuged at 3000*g* for 10 min, and the fluid was removed, leaving behind any residual contaminants. The tubes containing the contaminants were again weighed, and the mass of $CaCO_3$ was calculated by subtracting the weight of the tube plus contaminants, from the weight of the tube plus the collected precipitates. This process was repeated for a period of 7 days, after which the water in the tanks was replaced by 60 ppt seawater produced by the addition of Instant Ocean salts to filtered, UV-sterilized water. Fish were given 7 days to acclimate, after which the aforementioned precipitate collection procedure was repeated for another 7 days.

2.3. Sample collection and preparation

Toadfish (n = 18, 24–60 g) were randomly transferred to six, 15 l glass aquaria (n = 3 per tank), and each tank was randomly assigned a salinity of 35 or 60 ppt (n = 4 tanks per salinity). Tanks were filled with 1 μm, UV-sterilized seawater from Biscayne Bay, which was adjusted to the proper salinity via the addition of Instant Ocean marine salt or reverse osmosis purified water. Fish were acclimated to the different salinities for 10 days, during which time they were not fed. Temperature, salinity, and dissolved ammonia concentration measurements were taken each day throughout the acclimation period, and a minimum 70% water change was completed daily.

Upon completion of the acclimation period, intestinal epithelial tissue and IF samples were then collected from the fish and samples from two fish in the same tank were pooled. These pooled samples (n = 3 samples per treatment, each sample consisting of tissue pooled from 2 fish) were used for the mass spectrometry analysis (sample preparation for mass spectrometry analysis is describe in the Supplementary information). Tissues from the one remaining fish in each tank were retained for subsequent western blotting experiments. Proteins were extracted from the AI and PI epithelia in a homogenization buffer containing 1% octyl β-D-glucopyranoside and all samples were trypsin digested after which detergent was removed by ethyl acetate extraction (Yeung and Stanley, 2010). Digested peptides were labeled with TMT 6plex isobaric tags (Thermo Scientific) according to the manufacturer instructions and combined in equal amounts by sample type. To measure $CaCO_3$ production rates, excreted precipitates were collected from toadfish acclimated to normal seawater (35 ppt) and then from the same fish acclimated to hypersaline water (60 ppt) as described in the Supplementary information.

2.4. Mass spectrometry analysis

All mass spectrometry analysis was completed at the Colorado State University Proteomics and Metabolomics Facility (Fort Collins, CO, USA). TMT labeled, pooled peptides were fractionated using high-pH reverse phase LC as described in the Supplementary information. A volume of each pooled fraction was injected such that a mean of 1 μg of peptides was injected for each fraction of the sample type, but the injection volume was held constant across all fractions of the same sample type. Mass spectrometry analysis was performed on an Orbitrap Velos Pro (Thermo Scientific), using the acquisition parameters described in the Supplementary information.

2.5. Data analysis

Tandem mass spectra were extracted, charge state deconvoluted, and deisotoped using MSConvert (ProteoWizard). MS/MS data was