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Fundulus heteroclitus acutely transferred from seawater to high salinity require few adjustments to intestinal transport associated with osmoregulation

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ABSTRACT

The common killifish, *Fundulus heteroclitus*, has historically been a favorite organism for the study of euryhalinity in teleost fish. Despite the species' large range of salinity tolerance, studies of osmoregulation in high salinity are rare, with most previous studies focused on fish transferred between freshwater and seawater. Similarly, while branchial transport properties have been studied extensively, there are relatively few studies investigating the role of the intestine in osmoregulation in killifish. This study sought to characterize the fluid and ion transport occurring in the intestinal tract of killifish adapted to seawater, and furthermore to investigate the adjustments that occur to these mechanisms following acute transfer to high salinity (70 ppt). *In vivo* samples of blood plasma and intestinal fluids of seawater-acclimated killifish indicated absorption of Na^+, Cl^-, and water, the relative impermeability of the intestine to Mg^{2+} and SO_4^{2-}, and active secretion of HCO_3^- into the intestinal lumen. The details of these processes were investigated further using *in vitro* techniques of isolated intestinal sac preparations and an Ussing chamber pH-stat titration system. However, these methods were discovered to be of limited utility under physiologically relevant conditions due to tissue deterioration. Results that could be validly interpreted suggested that there are few changes to intestinal transport following transfer to high salinity, and that adjustments to epithelial permeability occur in the first 24 h post-transfer.

1. Introduction

One of the characteristics distinguishing the killifish (*Fundulus heteroclitus*) as an exceptional model organism is its extensive range of tolerance to environmental variables, including salinity. Nearly two-thirds of killifish species are found in regions where they are exposed to a natural range of salinities spanning freshwater to seawater to even higher salinities. Under laboratory conditions, *F. heteroclitus* can be transferred from brackish water (29 ppt) to freshwater (FW) or up to 114 ppt seawater without mortality (Griffith, 1974). Furthermore, *F. heteroclitus* naturally inhabit coastal salt marshes in salinities ranging from 0.4 to 35 ppt (Griffith, 1974), and regularly experience large changes in salinity due to tidal fluctuations. Investigation of the response of killifish to the high end of their salinity tolerance range allows for better understanding of the regulatory systems that provide this species with its impressive environmental flexibility.

Although killifish are able to tolerate salinities much greater than seawater (SW), studies utilizing transfer to hypersalinity in this species are relatively rare. Most previous studies of the responses to salinity change in *F. heteroclitus* focused primarily on the physiological changes which occur during transfers between FW and SW. Work using other teleost species has demonstrated increased drinking rate, increased water uptake and related ion transport in the intestinal tract, and adjustment of branchial ion transport from absorbance to excretion in seawater-exposed animals (Marshall and Grosell, 2005; Evans, 2008). Furthermore, these impacts on ion and water transport by the gill and intestine are greater still in fish transferred from seawater to even higher salinities. For example, recent work on *Opsanus beta* in high salinity demonstrates increases in expression and activity of transporters essential to intestinal HCO_3^- secretion (Sattin et al., 2010; Taylor et al., 2010), which plays an important role in intestinal water absorption by fish exposed to marine environments.

In general, *F. heteroclitus* display similar physiological effects of FW to SW transfer as other euryhaline fish, but these adjustments occur more rapidly than in other species. In killifish transferred from FW to SW, plasma $[Na^+]$ and osmolality increase within the first 12–24 h (Jacob and Taylor, 1983; Marshall et al., 1999), and return to FW levels after 4–5 days of SW exposure. While branchial transporters are reasonably well-described for killifish in both FW and SW (Wood and Marshall, 1994), intestinal ion and water absorption have also been shown to be of great importance to ionic and osmoregulation in teleost fish (Grosell, 2006). Killifish use the strategy of being "at the ready" with respect to branchial ion transport in brackish water and seawater conditions. For example, cystic fibrosis transmembrane conductance regulator (CFTR) and Na^+–K^+–$2Cl^-$ cotransporter (NKCC) are redistributed to the opercular epithelial surface following

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transfer from FW to SW (Marshall et al., 2002b), rather than requiring transcription following salinity transfer, which would produce a substantially slower response. Both CFTR and NKCC are also expressed in enterocytes of both FW- and SW-acclimated killifish (Singer et al., 1998; Marshall et al., 2002a) and it is reasonable to speculate intestinal transporters may also be readily available to respond to changes in ambient salinity. An earlier study on killifish employing transfer from brackish water to FW indicated no transcriptional changes of ion transporters (Na^+–K^+-ATPase (NKA), carbonic anhydrase 2 (CA2), CFTR, NKCC2) in the intestine (Scott et al., 2006). These results may not reflect a lack of importance of these transporters in the killifish intestine, but rather that small salinity changes are not likely to reveal transcriptional changes because fish in estuaries inhabit brackish water and are regularly exposed to either freshwater or seawater when the tide changes. Exposure to 70 ppt seawater was used in this study to test the limits of the intestinal ion transport system to gain a better understanding of the characteristics most important to intestinal osmoregulation following increases in salinity.

The small size of *F. heteroclitus* sometimes becomes a difficulty for physiological measurements. For example, killifish cannot be cannulated for blood sampling. One possible way to expand beyond general *in vivo* sampling and gain more detailed information is to use *in vitro* methods, and a technique commonly used for killifish is the isolated opercular epithelium preparation. Since it was first described (Karnaky et al., 1977) the opercular epithelium has been used in many *in vitro* studies to examine branchial transport by means of the Ussing chamber method (reviewed by Wood and Marshall, 1994). However, the killifish gastrointestinal tract has been examined *in vitro* in only a handful of studies, and always under symmetrical (short-circuit) conditions (Marshall et al., 2002a; Scott et al., 2006, 2008; Wood et al., 2010). Even the extensively studied opercular epithelium only rarely has been examined using SW-like asymmetrical conditions in killifish (Degnan and Zadunaisky, 1979; Pequeux et al., 1988). While useful for transport studies, symmetrical conditions are not physiologically relevant with respect to ionic and acid/base parameters. To our knowledge, this study is the first to expose isolated intestinal tissue from *F. heteroclitus* to salines that mimic the natural composition of extracellular and luminal fluids (*i.e.* asymmetrical conditions). In this study, *in vivo* sampling, *in vitro* pH-stat titration coupled with Ussing chambers, and isolated intestinal sac preparations (under both standard symmetrical and more physiologically relevant asymmetrical conditions) were all used to examine hypo-osmoregulation in killifish.

2. Materials and methods

2.1. Experimental animals

Killifish (*F. heteroclitus*) were purchased from Aquatic Research Organisms Ltd. (Hampton, NH, USA), and held in 62 L aerated aquaria at a maximum density of 50 animals per tank with no attempt to separate the genders, according to an approved University of Miami animal care protocol (IACUC #09-001). Fish were held for at least 2 weeks in either 35 ppt filtered seawater from Biscayne Bay (Bear Cut, 34–37 ppt, 22–26 °C) or 70 ppt seawater (Instant Ocean sea salt added to seawater). Fish acclimated to 35 ppt seawater were fed daily and held in flow-through conditions, while fish acclimated to 70 ppt seawater were held in tanks circulated with a biofilter pump with weekly water changes and fed every other day. All fish were fed approximately 2% of their body weight, but starved at least 24 h prior to sampling. Sampling was carried out between June and October 2010.

2.2. In vivo sampling

Fish were sacrificed by a blow to the head. Blood was sampled by caudal puncture using a heparinized 22-gauge needle fitted to a 1 mL syringe (BD Syringe), and the sample immediately centrifuged to isolate the plasma. The gastrointestinal tract was exposed by a ventral incision, clamped at the anterior and posterior ends, removed from the body cavity, and the intestinal fluid was drained directly into a sample tube.

2.3. pH-stat general experimental protocol

Experiments performed using an Ussing chamber pH-stat system used the following protocol, modified from Grosell and Genz (2006). Fish were killed by a blow to the head and a segment (approx. 0.5 cm) of the anterior intestine was removed, cut longitudinally, and mounted on a holding slide (Physiologic Instruments, P2403) exposing 0.1 cm^2 tissue. The mounted tissue was then placed between two Ussing half-chambers (Physiologic Instruments, P4000) filled with 1.6 mL of appropriate saline circulated with the corresponding gas (Table 1). All salines were brought to room temperature and gassed at least 30 min prior to experimentation. The pH of the mucosal saline was monitored using a combination electrode (Radiometer, PHC4000.8), and secreted base was automatically titrated with 0.005 N HCl by a pH-stat titration manager system (Radiometer, TIM 854 or 856) to maintain the pH of the luminal chamber at 7.800. The rate of base secretion in $\mu mol\ cm^{-2}\ h^{-1}$ was calculated using the rate of HCl addition. Electrophysiology measurements were obtained by recording voltage using AcqKnowledge software (v.3.8.1) under current clamp conditions, with a 30 μA short-circuit pulse every 60 s. Conductance was calculated as the current divided by the recorded transepithelial potential (TEP) per cm^2 of exposed tissue. All treatments were run for 60 min, following an initial 90 min control period, except for salinity transfer and luminal bafilomycin experiments which had a 60 min control period.

2.4. pH-stat experiments

Killifish anterior intestinal tissue was prepared and assessed in the pH-stat system for 4 h of titration to determine tissue viability and typical base secretion rates under asymmetrical conditions. Several types of experiments were run on the pH-stat system to characterize transport of acid–base equivalents and ions by the intestinal epithelium. Buffered serosal saline without HCO_3^- was used to eliminate base transport across the basolateral membrane (particularly by Na^+–HCO_3^--cotransport, NBC). Thus the base secretion recorded under these conditions must be attributed to a HCO_3^- source endogenous to the epithelium (intracellular metabolic CO_2 hydration, as opposed to

Table 1
Concentration of ions (mM) composing mucosal and serosal salines used in pH-stat and intestinal sac preparation experiments. Osmotic pressure was adjusted when necessary with mannitol, and osmolality of serosal salines was measured without glucose. pH of serosal salines was adjusted to 7.8 with either HCl (regular and reduced Na^+) or NaOH (HCO_3^--free).

	Mucosal	Serosal	HCO_3^--free	Reduced Na^+
NaCl	69.0	144.1	144.1	–
KCl	5.0	5.1	5.1	5.1
$CaCl_2 \cdot 2H_2O$	5.0	1.6	1.6	1.6
$MgSO_4 \cdot 7H_2O$	72.5	0.9	0.9	0.9
$NaHCO_3$	–	11.9	–	11.9
NaH_2PO_4	–	2.9	2.9	2.9
$MgCl_2 \cdot 6H_2O$	22.5	–	–	–
Glucose	–	5.5[a]	5.5	5.5
N-methyl-D-glucamine	–	–	–	144.0
HEPES (free acid)	–	–	2.5	–
HEPES (Na^+-salt)	–	–	2.5	–
mOsm	296	292	291	293
Gas	O_2	0.3% CO_2 in O_2	O_2	0.3% CO_2 in O_2

[a] Replaced with mannitol in symmetrical intestinal sac filling (luminal) saline.