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Effects of the acclimation to high salinity on intestinal ion and peptide transporters in two tilapia species that differ in their salinity tolerance

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ABSTRACT

Tilapiine species, widely distributed across habitats with diverse water salinities, are important to aquaculture as well as a laboratory model. The effects of water salinity on two tilapia species, that differ in their salinity tolerance, was evaluated. *Oreochromis niloticus* reared in brackish-water, showed a significant decrease in growth and feed efficiency, whereas *O. mossambicus* reared in seawater did not show any significant changes. The expression and activity of Na^+/K^+-ATPase (NKA), V-type H^+-ATPase (VHA) and carbonic anhydrase (CA), as well as expression levels of genes encoding two HCO_3^- and three peptide transporters (*nbc1*, *slc26a6*, *slc15a1a*, *slc15a1b* and *slc15a2*) were measured in three intestinal sections of these two species, grown in freshwater and brackish/sea-water. Overall, the spatial distribution along the intestine of the genes examined in this study was similar between the two species, with the exception of *tcaIV*. The salinity response, on the other hand, varied greatly between these species. In *O. mossambicus*, there was a salinity-dependent increased expression of most of the examined genes (except *slc26a6* and *slc15a2*), while in *O. niloticus* the expression of most genes did not change, or even decreased (*tcaIV*, *nbc1* and *slc15a1b*). This study highlighted differences in the intestinal response to salinity acclimation between closely- related species that differ in their salinity tolerance. *O. mossambicus*, which has a high salinity tolerance, showed expression patterns and responses similar to marine species, and differed from the low-salinity-tolerance *O. niloticus*, which showed a response that differed from the accepted models, that are based on marine and diadromous fishes.

1. Introduction

Salinity plays a pivotal role among the physical features of the aquatic environment that govern the distribution of aquatic species in nature (Whitehead et al., 2011). Most fish species have to adapt to an environment with different salinity levels than their own tissues and cells, presenting an osmotic challenge to the fish. In the freshwater environment, fish gains excess water from, and lose salt to, the low ionic and hypo-osmotic environment. With regard to ion-regulation, the fish gain ions by active uptake via the gills, and by absorption of food-originated ions via the gastrointestinal tract (GIT). In seawater, fish lose water to and gain salt from the high-ionic and hyper-osmotic environment. Seawater-acclimated fish need to replace water loss by imbibing salty water and absorbing the ions and water via the GIT, whereby water is retained in the body and excess sodium and chloride is extruded via the gill (Marshall and Grosell, 2006).

The intestine of seawater-adapted fish is the site for water absorption, required to maintain homeostasis in the hyperosmotic environment. It is also the location for elimination of excess salts, mainly divalent ions and HCO_3^-, which become concentrated in the intestinal fluid due to the preferential absorption of Na^+ and Cl^-. In contrast, the intestine of freshwater fish must take up ions, mainly from food, and limit water absorption (Ferreira and Baldisserotto, 2007; Grosell, 2010). Salt and water balance is maintained to a great extent by intestinal ion channels, transporters and exchangers. Some of these transporter proteins conjugate ion-transport with peptide and amino acids transport, and thus, their activity is tightly linked to food digestion.

African cichlids, including tilapias, are widely distributed among habitats with diverse water salinities. Mozambique tilapia (*Oreochromis mossambicus*) is native to estuaries and lower reaches of rivers from the Zambezi River to the southeast coast of South Africa and generally are not found beyond a mile from the tidal ebb and flow. Nile tilapia (*O. niloticus*), on the contrary, is found in river basins and lakes of western,

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northern and central Africa (Trewavas, 1983). Reflective of these distinct distributions, Mozambique tilapias are strongly euryhaline, thriving in a wide range of water salinities, up to the excess of 70‰ (Kültz et al., 1992; Uchida et al., 2000), whereas Nile tilapias are far less tolerant to high salinities and do not easily acclimate to salinities exceeding 25‰ (Watanabe et al., 1985). Due to its physiological characteristics, *O. mossambicus* emerged as a model for comparative research aiming at assessing salinity effects on fish physiology (Kültz et al., 2007). *O. niloticus* is the world's most widely cultured species and is of great importance to human protein supply and thus, to the global economy (FAO, 2015). There are only a few comparative studies on the physiological response to increased salinity in these two species (Nakano et al., 1997; Breves et al., 2010; Velan et al., 2011; Ronkin et al., 2015; Yamaguchi et al., 2017).

The important role of the intestine in fishes' osmoregulation has long been recognized (Marshall and Grosell, 2006; Grosell, 2010), however, there is still a major lack of knowledge regarding genes and pathways associated with intestinal osmoregulation. The rapid development of molecular and immunological tools has facilitated the assessment of specific target genes for several transporters involved in the intestinal ion coupled fluid transport. The tilapias' response to salinity challenge at the gene expression level along the GIT has been characterized only recently (Li et al., 2014; Seale et al., 2014; Ronkin et al., 2015). In a previous study, we compared the intestinal transcriptome of *O. mossambicus* and *O. niloticus*, a work that presented a broad view on gene expression patterns, but statistical constraints of multiple comparisons limited the significance only to transcripts with at least a 3.5-fold change in response to salinity (Ronkin et al., 2015). The aim of present study was to examine specific genes that are ion-driven transporters, channels and exchangers, known to be involved in teleosts intestinal ion regulation. Therefore, we monitored their salinity-dependent differential expression and activity in the salinity tolerant and sensitive tilapiine species.

2. Materials and methods

2.1. Experimental animals and salinity challenge

The tilapia stocks used in this study were maintained at the aquaculture facilities of the Agricultural Research Organization (ARO), Israel. The *O. mossambicus* stock originated in Natal, South Africa and brought to Israel in the 1970s. The *O. niloticus* is the Thai Chitralada strain that is farmed in Israel. The experimental setting for the fish high-salinity growth trial was similar to the one described by Nitzan et al. (2017). Two consecutive experiments were conducted, one with *O. mossambicus* (24 ± 0.6 g) and the other with *O. niloticus* (62 ± 0.9 g). In each experiment, thirty-six male fish were kept in 40 liter aquaria, one fish per aquarium, with 12:12 h light–dark photoperiods, and water temperature 24 ± 2 °C. Fish were maintained in freshwater, before increasing the salinity in 18 aquaria gradually (5 ppt/day) using marine salt (Red Sea salt). Salinity was increased to 30 ppt in the experiment with *O. mossambicus*, and to 20 ppt in the experiment with *O. niloticus*. The fish were fed to satiety twice a day at 8:00 AM and 2:00 PM, with commercial tilapia food that contained 35% protein, 3.5% fat, 10% moisture, 5% cellulose, 7% ash, 1.2% calcium, 1.2% phosphorus and 0.7% salt (4662, Zemach feed mill, Israel). Food residues were removed 30 min after feeding, dried and weighed, to measure specific growth rate (SGR) and feed conversion ratio (FCR). After six weeks, fish were sacrificed and sampled 24 h after the morning feeding. Tissue samples from three intestinal segments, the anterior, middle, and posterior intestine were dissected and kept in RNAlater© buffer (Ambion®) at −20 °C until used for qPCR studies. At the same time, tissues from three intestinal segments were snap frozen using liquid nitrogen and kept separately at −80 °C in SEI buffer for ATPase assay and in phosphate buffer (25 mM, pH 7.4) for carbonic anhydrase assay.

The study was approved by the ARO Committee for Ethics in Using Experimental Animals (Approval number: IL-568/15), and carried out in compliance with the current laws governing biological research in Israel.

2.2. Growth performance and feed utilization

The growth performance and feed utilization were calculated according to the following equations:

$$SGR\ (\%day^{-1}) = 100 \times (\ln Wt - \ln Wi)/t$$

Wt is the final body weight (g), Wi is the initial body weight (g), and t is the experimental duration in days.

FCR = feed consumed (g, dry weight)/weight gain (g, wet weight).

2.3. ATPase assay

An ATPase assay, based on a method developed by McCormick (1993) and modified by Hawkings et al. (2004), was adapted to determine both the ouabain (Na^+/K^+-ATPase inhibitor)- and bafilomycin (V-type H^+-ATPase inhibitor)-sensitive ATPase activities. The ouabain-sensitive hydrolysis of adenosine triphosphate is enzymatically-coupled to the oxidation of nicotinamide adenine dinucleotide (reduced form), which is directly measured in a microplate reader. All intestinal sections were stored in SEI buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2EDTA, 50 mmol l^{-1} imidazole and adjusted to pH 7.4) at −80 °C until assays were performed. Tissues were thawed and homogenized with 0.5% sodium deoxycholic acid on ice and immediately centrifuged at 5000 × *g* for 30 s to remove insoluble material. Homogenate (10 µl) from each sample was added to nine wells in a 96-well plate. This provided three treatments for each sample [control, ouabain (500 µmol l^{-1}) and ouabain (500 µmol l^{-1}) + bafilomycin (50 nmol l^{-1})] with triplicate measurements of each treatment. 150 µl of assay mixture [50 mmol l^{-1} imidazole buffer, 2 mmol l^{-1} phosphoenol pyruvate (PEP), 0.16 mmol l^{-1} NADH, 0.5 mmol l^{-1} ATP, 3.3 U ml^{-1} lactate dehydrogenase (LDH), 3.6 U ml^{-1}phosphokinase (PK)] was added to each well with appropriate drug treatment, and 50 µl of salt solution (50 mmol l^{-1} imidazole, 189 mmol l^{-1} NaCl, 10.5 mmol l^{-1} $MgCl_2$, 42 mmol l^{-1} KCl). Absorbance was read at 340 nm in a microplate reader (Epoch™ microplate spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA) at 15 s interval for 20 min. The average rate for each treatment was taken from the stable slope and calculated from a standard curve generated prior to the assay. Na^+/K^+-ATPase activity was obtained by subtracting the ouabain-treated ATPase activity from control ATPase activity (McCormick, 1993). H^+-ATPase activity measured by calculating the difference in ATPase activity between the ouabain- and the ouabain + bafilomycin-treated samples (Hawkings et al., 2004). Total protein content was determined using Bio-Rad protein assay dye reagent (500-0006), employing bovine serum albumin (Sigma) as standard. The assay conditions were validated with mid intestinal tissues of *O. mossambicus* and *O. niloticus* for different protein concentrations, temperature (20–30 °C), incubation time (0–40 min), pH, ATP (0–2 mmol l^{-1}), NADH (0–40 nmoles l^{-1}), concentration of inhibitors ouabain (200–1000 µmol l^{-1}) and bafilomycin (10–100 nmol l^{-1}). Inter-assay variation was determined from five assay reactions (triplicates) conducted on three different days and intra-assay variation was determined from five reactions in triplicate on the same day, using the same set of reagents.

The ATPase activity did not vary significantly in the range of 20–30 °C. ATPase activity was linear up to 20 min and nearly linear up to 30 min of incubation at 20 °C. The activity increased with increasing concentrations of homogenate, up to 20 µl. ATPase activity was linear for the range of concentrations used in assay for ATP and NADH. Na^+/K^+-ATPase activity was maximally-inhibited between 400 and 800 µmol l^{-1} of ouabain and H^+-ATPase activity was maximally inhibited between 10 and 100 nmol l^{-1} of bafilomycin. Therefore,