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Intraspecific variation in gene expression after seawater transfer in gills of the euryhaline killifish *Fundulus heteroclitus*

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Abstract

Previous research has suggested that northern populations of the euryhaline killifish (*Fundulus heteroclitus*) are better adapted to freshwater environments than their southern counterparts. In this study, we examined whether this adaptation has come at an ionoregulatory cost in seawater, by comparing published data for northern killifish to newly acquired data on the molecular responses of southern killifish to seawater transfer. After abrupt transfer from brackish water (10‰) to seawater, Na,K-ATPase activity, *Na,K-ATPase* α_{1a} mRNA expression, and *NKCC1* mRNA expression increased 1 and 4 days after transfer in the gills of southern fish (by 2–3-fold), but increased at 1 day and not 4 days after transfer in northern fish. Small increases in mRNA expression were observed in both populations at 14 days. *CFTR* expression also increased in southern and northern fish at 1 and 4 days into seawater, and was also elevated at 14 days in northern fish. Because fish from both southern and northern populations maintained plasma Na^+ and Cl^- balance after seawater transfer, the differences in activity and expression could not have been caused by differences in plasma ion levels. Instead, some other regulatory factor may account for the differences in expression between populations. This study shows that freshwater adaptation in northern populations of killifish has not necessarily come at a significant ionoregulatory cost in seawater, but has altered the molecular responses of their gills to seawater transfer compared to southern killifish.

Keywords: *Na,K-ATPase*; *NKCC*; *CFTR*; mRNA expression; Gills; Fish; Ion regulation; Evolution

1. Introduction

The euryhaline killifish *Fundulus heteroclitus* inhabits brackish water estuaries and salt marshes along the eastern coast of North America. The species is distributed from Newfoundland to Florida, and thus spans a cline of environmental temperatures. Correspondingly, many previous studies have investigated thermal adaptations in populations across the range. Differences between populations include latitudinal differences in glycolytic enzyme expression and activity (Powers et al., 1986; Pierce and Crawford, 1996), endocrinology (DeKoning et al., 2004), metabolism (Podrabsky et al., 2000), morphology, and behaviour (Powers et al., 1993). Unfortunately, only a few studies have assessed whether intraspecific differences exist between northern and southern populations of *F. heteroclitus* in physiology associated with other environmental factors (e.g., tidal cycle, DiMichele and Westerman, 1997).

We recently investigated the freshwater tolerance and ionoregulatory physiology of northern and southern killifish populations (Scott et al., 2004b). Interestingly, individuals from northern latitudes appeared better adapted to freshwater environments. For example, northern fish reduced Cl^- loss and paracellular permeability, reduced apical crypt abundance in the gills, and increased Na^+ influx to a greater extent than southern killifish in freshwater. These differences were likely due in part to differences in ion transporter expression patterns between populations, as northern killifish exhibited greater increases in *Na,K-ATPase* mRNA expression in their gills. In the present study, we address whether these potential freshwater adaptations in killifish

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from northern latitudes came at an ionoregulatory cost in seawater, or whether they influenced seawater ionoregulatory physiology in other ways.

Several recent studies have examined the molecular and cellular responses of fish gills to seawater transfer (reviewed by Sakamoto et al., 2001). Most of these studies have shown that expression of the principle seawater ion transporters, namely *Na,K-ATPase*, Na,K,2Cl-cotransporter (*NKCC*), and a Cl^- channel homologous to the cystic fibrosis transmembrane conductance regulator (*CFTR*) of humans, increases after seawater transfer, but the time course of the responses often differ between species. The reason for these temporal differences in expression after transfer is unclear, and it is not known whether they are related to differences in salinity tolerance between species. In this regard, an intraspecific comparison of molecular and physiological responses to seawater transfer may be valuable for evaluating this issue.

The objective of this study was to determine the molecular responses of individuals from a southern population of *F. heteroclitus* to abrupt transfer from near-isosmotic brackish water (10‰) to seawater, and compare them to previously collected data from a northern killifish population (Scott et al., 2004a). Near-isosmotic brackish water is the preferred salinity for *F. heteroclitus* (Fritz and Garside, 1974), and transfer from brackish water to seawater may be more environmentally representative of the conditions killifish naturally encounter in estuaries. The ionoregulatory ability of each population was assessed by measuring plasma ions, which were contrasted to the patterns of mRNA expression in the gills. In doing so, we have explored whether the potential freshwater adaptations in northern killifish affected this population's seawater tolerance and physiology, and whether any differences in the molecular responses to seawater transfer between populations are related to differences in salinity tolerance.

2. Materials and methods

2.1. Experimental animals

Adult killifish (*F. heteroclitus* L.) of the southern subspecies (*F.h. heteroclitus*) were captured from Whitney Island, Florida. Fish were maintained in indoor holding facilities in synthetic brackish water (10‰; Deep Ocean) made up in dechlorinated Vancouver city tap water ($[Na^+]$, 0.17 mM; $[Cl^-]$, 0.21 mM; hardness, 30 mg/L as $CaCO_3$; pH 5.8–6.4) in static filtered glass aquaria. Before sampling, fish were maintained for at least 30 days in this brackish water at an ambient temperature of 21–24 °C and a 14L:10D photoperiod. Fish were fed commercial trout chow (PMI Nutrition International) daily. Treatment of animals was conducted according to University of British Columbia animal care protocol #A01-0180.

2.2. Salinity transfer experiment

The methods for this salinity transfer experiment using the southern killifish subspecies is equivalent to those previously reported for the northern subspecies alone (Scott et al., 2004a). Eight southern control fish were sampled after acclimation to 10‰, after which fish from each population were quickly transferred by net to aquaria containing 35‰ seawater or back to brackish water (i.e., a sham treatment where the animals were simply transferred between two tanks of their same acclimation salinity). Individual fish were subsequently sampled by netting at 3, 8, 24, and 96 h, 14, and 30 days after transfer, for both brackish water and seawater transfers. The fish were stunned by cephalic blow, blood samples were collected in heparinized capillary tubes from the severed caudal peduncle, and the fish were then killed by rapid decapitation. Blood was centrifuged at 13,000 g for 10 min and plasma was frozen in liquid nitrogen. Second and third gill arches were immediately frozen in liquid nitrogen. All tissues were stored at −80 °C.

2.3. Total RNA extraction and reverse transcription

Total RNA was extracted from tissues (approximately 20 mg) using Tripure isolation reagent (Roche Diagnostics), following the manufacturer's instructions. RNA concentrations were determined spectrophotometrically and RNA integrity was verified by agarose gel electrophoresis (~1% [wt.:vol.] agarose:Tris–acetate EDTA). Extracted RNA samples were stored at −80 °C following isolation. First strand cDNA was synthesized by reverse transcribing 3 μg total RNA using 10 pmoles oligo(dT_{18}) primer and 20 U RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas) following the manufacturer's instructions.

2.4. Real-time PCR analysis of gene expression

Primers for killifish *Na,K-ATPase* α_{1a} (Acc. No. AY057072), cystic fibrosis transmembrane conductance regulator (*CFTR*) Cl channel (Acc. No. AF000271), Na,K,2Cl-cotransporter 1 (*NKCC1*; Acc. No. AY533706), and elongation factor 1α (*EF1α*, expression control; Acc. No. AY430091) were designed using Primer Express software (version 2.0.0, Applied Biosystems) and are reported in Scott et al. (2004a). Gene expression was quantified using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence analysis system (Applied Biosystems) as previously described (Scott et al., 2004a). Control reactions were conducted with no cDNA template or with non-reverse transcribed RNA to determine the level of background or genomic DNA contamination, respectively. Genomic contamination was below 1:49 starting cDNA copies for all templates.