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Characterization of developmental Na^+ uptake in rainbow trout larvae supports a significant role for Nhe3b

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ABSTRACT

Developing freshwater fish must compensate for the loss of ions, including sodium (Na^+), to the environment. In this study, we used a radiotracer flux approach and pharmacological inhibitors to investigate the role of sodium/hydrogen exchange proteins (Nhe) in Na^+ uptake in rainbow trout (*Oncorhynchus mykiss*) reared from fertilization in soft water (0.1 mM Na^+). For comparison, a second group of embryos/larvae reared in hard water (2.2 mM Na^+, higher pH and [Ca^{2+}]) were also included in the experiment but were fluxed in soft water, only. Unidirectional rates of Na^+ uptake increased throughout development and were significantly higher in embryos/larvae reared in soft water. However, the mechanisms of Na^+ uptake in both groups of larvae were not significantly different, either in larvae immediately post-hatch or later in development: the broad spectrum Na^+ channel blocker amiloride inhibited 85–90% of uptake and the Nhe-inhibitor EIPA also caused near maximal inhibitions of Na^+ uptake. These data indicated Na^+ uptake was Nhe-mediated in soft water. A role of Nhe3b (but not Nhe2 or Nhe3a) in Na^+ uptake in soft water was also supported through gene expression analyses: expression of *nhe3b* increased throughout development in whole embryos/larvae in both groups and was significantly higher in those reared in soft water. This pattern of expression correlated well with measurements of Na^+ uptake. Together these data indicate that in part, rainbow trout embryos/larvae reared in low Na^+ soft water maintained Na^+ homeostasis by an EIPA sensitive component of Na^+ uptake, and support a primary role for Nhe3b.

1. Introduction

In freshwaters, fish face a challenge to overcome unfavorable ion gradients to replenish ions, including sodium (Na^+), lost by diffusion. In adult fishes, ion uptake is primarily achieved at the gill, a multifunctional epithelium with additional coupled roles in gaseous exchange, acid-base balance and nitrogenous waste excretion (Evans et al., 2005). In larvae, these physiological processes are first performed cutaneously, with the role of the gill progressively increasing during development (Fu et al., 2010; Rombough, 2007; Zimmer et al., 2014a). Recently, it has been demonstrated that ion uptake shifts earliest to the developing gill with gaseous exchange following later, and this has been interpreted as the primacy of ion acquisition over respiration in driving the evolution of gill development in fish (Fu et al., 2010). Nevertheless, the molecular identities and cellular organizations of the proteins involved in ion uptake and especially their expression under variable environmental conditions have yet to be fully elucidated and are the subject of continuing debate.

Early models of acid-base balance and ion-regulation in fish postulated Na^+ uptake to occur in exchange for acidic equivalents (H^+) at the gill (e.g. Krogh, 1937, 1938). This has since been empirically demonstrated [e.g. in rainbow trout (*Oncorhynchus mykiss*); Goss and Wood, 1991]. Sodium uptake at the gill has also been suggested to be coupled to ammonia (total NH_3/NH_4^+) excretion (Liew et al., 2013; Wright and Wood, 1985; Zimmer et al., 2010). Although less studied, Na^+ uptake is also required for embryonic fish: Na^+ balance in chorionated embryos is acid-sensitive (Eddy et al., 1990) and the timing of the ontogenetic shift of Na^+ uptake and NH_4^+ excretion to the gill in larval trout is highly correlated (Zimmer et al., 2014a). The mitochondrion-rich ionocytes in the gill (MR cells; Perry, 1997) also appear early in fish development, being present on the yolk-sac membrane and prior to the development of gills (Nakada et al., 2007; Esaki et al., 2009).

Recently proposed models of Na^+ transport in adult rainbow trout gill MR cells, have a sodium transport protein potentially coupled to rhesus glycoproteins and V-type H^+-ATPases in a metabolon (Dymowska et al., 2012; Wright and Wood, 2009). Together, these act in concert to mediate NH_3 and H^+ extrusion and maintain favorable electrogenic gradients and boundary-layer acidification to facilitate Na^+ uptake. The electroneutral sodium/proton exchange proteins (Nhe) in the solute carrier (Slc) family of membrane transport proteins are currently considered primary candidates for this role (see reviews

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by Wright and Wood, 2009; Takei et al., 2014). First documented in rainbow trout by Lin and Randall (1991) and with localization at the branchial epithelial membrane confirmed later (Edwards et al., 1999), three isoforms have now been identified: Nhe2 (Slc9a2), Nhe3 [Slc9a3 (referred to hereafter as Nhe3a)] (Ivanis et al., 2008) and the more recently described Nhe3b (GenBank ID: NM_001160482.1). However the function of Nhe transporters in low Na^+ waters has been questioned due to thermodynamic constraints of combined low pH and low [Na^+] (Parks et al., 2008). Indeed, it has been demonstrated that acid sensing ion channels (Asic) may substitute for the function of a Nhe protein in juvenile rainbow trout under unfavorable conditions (Dymowska et al., 2014); Asics are voltage-insensitive Na^+ channels gated by extracellular H^+ and are expressed in the gill of juvenile trout.

The goal of this study was to investigate Na^+ uptake and the role of Nhe in rainbow trout embryos/larvae reared from fertilization in soft synthetic freshwater containing 0.1 mM Na^+ at pH 6. This [Na^+] and pH are below the point of transport equilibrium of Nhe (Parks et al., 2008). We utilized unidirectional whole embryo/larva $^{22}Na^+$ fluxes and dose-increments of pharmacological inhibitors of Na^+ uptake to determine the role of Nhe. Expression of *nhe* isoforms (*nhe2*, *nhe3a* and *nhe3b*) was also analysed in whole embryos/larvae. A second group of embryos/larvae reared in hard water (2.2 mM Na^+, pH 8, higher [Ca^{2+}]) was also included in the experimental design. Importantly, fluxes with hard water reared embryos/larvae were performed in soft water, only. The rationale behind this approach was that if rearing embryos/larvae in soft and hard waters leads to differential expression of *nhe* isoforms then embryos/larvae would display different Na^+ uptake profiles when fluxed under the same water conditions. These data could therefore add further weight to the roles of specific Na^+ uptake pathways in soft water. To complement these data and to address a data gap in the trout literature tissue specific expression analyses of *nhe* isoforms were also performed in juvenile rainbow trout; it was not possible to investigate tissue specific expression patterns in embryos/larvae. This is the first study to differentiate developmental expression patterns of isoforms of *nhe* in rainbow trout during early development and the data gathered suggest a predominant role of Nhe3b in Na^+ acquisition in low [Na^+] soft water.

2. Materials and methods

2.1. Experimental animals and environmental waters

All experiments were conducted under Animal Use Protocol #00000072 as approved by the University of Alberta and the Biosciences Animal Policy and Welfare Committee under the directions provided by the Canadian Council for Animal Care in Canada. Rainbow trout embryos ($n = 500$) were collected on the day of fertilization from Raven Brood Trout Station, Raven, Alberta and transported to the University of Alberta. Embryos were equally divided between Heath chambers in two continuously aerated and UV-treated dechlorinated recirculating water systems containing 130 L of either soft [low Na^+ (0.1 mM, nominal concentration)] or hard [high Na^+ (2.2 mM, nominal concentration)] synthetic freshwaters maintained at 10 °C. The chemical compositions of the synthetic freshwaters used correspond to those previously used in trout ionoregulatory research (Allin and Wilson, 2000; Fu et al., 2010) and standardized waters recommended for use by the U.S. Environmental Protection Agency (USEPA, 2002). Measured [Na^+] were (means ± S.D., $n = 26$): 0.105 ± 0.006 and 2.267 ± 0.201 mM for soft and hard waters, respectively. Soft and hard waters were pH 5.9 ± 0.2 and pH 8.1 ± 0.1, respectively. Concentrations of other elements in freshwaters were: Ca^{2+} 0.015 mM; Mg^{2+} 0.035 mM; K^+ 0.035 mM, in soft water and Ca^{2+} 0.9 mM; Mg^{2+} 1.0 mM; K^+ 0.1 mM, in hard water.

Immediately after the final flux (see Section 2.3), when complete reabsorption of the yolk sac (~22 d post hatch, dph) had occurred, excess fry were transferred to 120 L flow-through tanks supplied with aerated 10 °C re-circulating dechlorinated Edmonton City tapwater (Na^+ 0.46 mM; Ca^{2+} 0.99 mM; Mg^{2+} 0.65 mM; K^+ 0.02 mM) and maintained as stock fish. Later, and in light of gathered data of developmental expression of *nhe* isoforms in whole embryos/larvae (see Section 2.4 and results), it was apparent that these data could be better interpreted by complementing them with analyses of expression of *nhe* isoforms in specific tissues of juvenile trout. These expression analyses also addressed a data gap in juvenile rainbow trout. Therefore, at 6 months, a sub-sample (same cohort) of the juvenile trout (2.1 ± 0.3 g, $n = 6$) were re-acclimated for 7 d in aerated tanks containing 20 L of either soft or hard synthetic freshwater (chemistry as indicated above). Following the acclimation period, fish were euthanized in pH buffered MS222, and tissues excised and snap frozen in liquid N_2 for reverse transcriptase-PCR analysis (*nhe* isoforms).

2.2. Measurement of embryo/larva sodium concentration

At time points selected to span development from pre-hatch (chorionated embryos) to immediately post-hatch and then the period up until the absorption of the yolk sac, embryos/larvae ($n = 3$) were randomly selected and removed from Heath trays, and individually snap frozen in liquid N_2. Embryos/larvae were subsequently dried to constant weight at 80 °C, transferred to metal-free polyethylene tubes, digested in 250 μL trace analysis grade concentrated nitric acid and diluted to a known volume with ultrapure water for measurement of [Na^+] via atomic absorption spectrophotometry (Model 3300, Perkin Elmer, CT, USA). Embryo/larval [Na^+] was calculated as μmol Na^+ mg^{-1} dry weight.

2.3. Unidirectional $^{22}Na^+$ fluxes

Unidirectional $^{22}Na^+$ (in the form of $^{22}NaCl$, Perkin Elmer) fluxes in whole embryos/ larvae were conducted in soft water, only (water chemistry as described in Section 2.1). The broad spectrum Na^+ channel blocker, amiloride and the purported Nhe specific inhibitor 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) were used to examine the involvement of Nhe in Na^+ uptake in embryos/larvae (Kleyman and Cragoe, 1988). Embryos/ larvae ($n = 6$ per dose/treatment) were transferred in system water from the Heath chambers to individual 12 × 75 mm borosilicate tubes and placed in a constant temperature water bath at 10 °C. At $t = -30$ min, the system water was removed, and 1 mL of soft water (both treatment groups) premixed (vortexed) with pharmacological agents (0, 0.1, 1, 10, 100 μM amiloride or EIPA in 0.1% dimethyl sulfoxide (DMSO vehicle; all reagents from Sigma) was added. Previous studies have indicated that 0.1% DMSO does not interfere with ion uptake in trout embryos/larvae (Boyle et al., 2015) and comparison of Na^+ uptake rates in hard and soft water reared embryos/larvae exposed to 0.1% DMSO with controls (i.e. no DMSO), indicated no significant difference (Student's *t*-test, $p > 0.05$). Accordingly, flux measurements performed without DMSO are not shown in figures and were not included in further statistical analyses. After the pre-incubation period ($t = 0$), the solutions were removed and immediately replaced with 1 mL of soft water premixed (vortexed) with both the pharmacological agent (in 0.1% DMSO) and 0.0125 μCi $^{22}Na^+$ mL^{-1}, as appropriate. After $t =$ 5 min, a 100 μL sample was withdrawn from each tube and placed in a borosilicate tube for later counting to calculate the specific activity of $^{22}Na^+$ in the water. At the completion of the experimental flux period ($t = 60$ min), the flux media was removed with a Pasteur pipette and replaced with 1 g L^{-1} MS222 prepared in soft water and buffered to pH 6.0. Euthanized embryos/ larvae were then washed 3 times in ~4 mL ice-cold 2.9 g L^{-1} NaCl to displace residual unbound $^{22}Na^+$. A sub-sample (1 mL) of the final wash solution was also removed to borosilicate tubes for counting to measure carry-over of residual externally bound $^{22}Na^+$. The activities of $^{22}Na^+$ in all trout embryos/ larvae and water samples were counted with a Cobra Quantum gamma counter (model E5003, Packard Instrument Company Inc., Downers Grove,