The presence of the cyanide detoxification enzyme, rhodanese, in *Fundulus heteroclitus* embryos

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Adult killifish reproduce and thrive in estuaries with high levels of pollution including the presence of cyanide. We investigated the capacity of aerially incubated embryos to recover from transient exposure to cyanide. With mature embryos (14 days post-fertilization, dpf) 1 mM cyanide speeds up the rate of hatching although the embryos are immobile, but after 24 hrs embryos recover mobility and appear normal. We detected the presence of the cyanide detoxification enzyme, rhodanese, and showed that its measurable activity increased with embryo age, reaching its highest level at 14 dpf. These data showed for the first time that rhodanese is present in killifish and that it may be an important mechanism for cyanide detoxification.

Northern killifish, *Fundulus heteroclitus macrolepidotus*, spawn at the edges of estuaries in June and July during daily high tides[1-6,10]. The adults may migrate to and from full strength seawater (SW, ~30 ppt) to freshwater (FW, <1 ppt) but spawn in brackish water (~10 ppt)[1-6,10]. As the tide ebbs, some embryos may remain immersed and some embryos may become stranded in air for long periods (up to 14 days) on bordering vegetation or on the rocky shoreline[1-4,6,10]. In spite of enduring significant transient desiccation and thermal stress[3,4,6,11,12] aerially incubated embryos develop normally and hatch when flooded by high tides after 12-14 days[1-4,6,11,16].

DiMichele and Taylor[6] have shown in mature aerially incubated killifish embryos (14 days post fertilization, dpf) hatching was triggered by immersion in SW. They concluded that an important signal that triggered hatching was hypoxia. In previous experiments, we were able to accelerate hatching rates of killifish embryos (compared with controls) by exposing immersed 14 dpf embryos to a nitrogen atmosphere. Two-hour exposure of 14 dpf embryos in 10 ppt ASW (artificial seawater, ASW; Instant Ocean, Mentor, OH) with added 1 mM sodium cyanide also accelerated hatching (Preston, unpublished data), although the hatchlings appeared unresponsive and immobile. After transferring these hatchlings to 10 ppt ASW, the majority of the hatchlings were swimming normally after 24 hrs. We concluded that killifish embryos and hatchlings have considerable resistance to cyanide toxicity and can recover readily after being exposed to it. Other early studies also noted this cyanide resistance. Crawford and Wilde[5] evaluated cyanide effects on early development and showed that 2 mM sodium cyanide had no effect on embryos until developmental stages[1] 10-12 were reached. After stage 12 or later cyanide caused immediate arrest of development. But if the cyanide was removed, development proceeded normally after a 1-day lag period. They speculated that the 1-day lag period may be the time for all the cyanide to be removed from the embryos, but they did not determine the possible metabolic mechanisms of recovery.

Other investigators have suggested that rhodanese is an important mechanism for cyanide detoxification in a variety of organisms, including plants, fungi, mammals and fish[13,15] although there have been no previous experiments done with *Fundulus heteroclitus*. We hypothesize that rhodanese activity is likely to be an important cyanide detoxification mechanism in killifish. The objectives of these experiments were to determine whether rhodanese activity is present in killifish embryos and to test the prediction that the rhodanese activity should be at high levels in embryos at maturity.

Killifish were collected from Northeast Creek, Mount Desert Island, ME, and held in 30-gallon aquaria with running natural SW (~30 ppt). Eggs and milt were manually stripped into a beaker containing 10 ppt ASW. After 30 minutes, the embryos were placed on filter paper moistened with 10 ppt ASW for aerial culture at 20˚C in a closed plastic chamber whose vapor phase was in equilibrium with 10 ppt ASW[8]. The embryos were inspected daily and developed normally over 14 days. At maturity the embryos hatched normally when flooded with 10 ppt ASW.

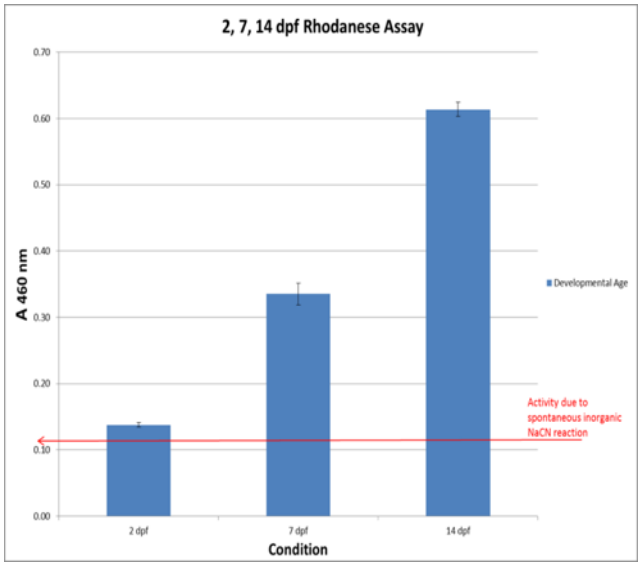

Figure 1. Apparent rhodanese activity increases with developmental age in killifish embryos. The amount of thiocyanate formation is shown as measured by a colorimetric assay (see text). The horizontal line shows the baseline level of non-enzymatic thiocyanate formation. The means ± SE for 2 dpf, 7 dpf and 14 dpf embryos are significantly different from each other using one-way ANOVA Tukey multiple comparisons test ($n = 4$; $p < 0.05$).

Rhodanese activity in embryo extracts was measured by a modification of the methods of Saidu[13] and Sorbo[14]. The rhodanese catalyzed reaction of thiosulfate with cyanide produces the considerably less toxic product, thiocyanate. Typical conditions were as follows: Enzyme extracts were prepared by homogenizing 300 mg of embryos in 1.0 ml deionized water using disposable 1.5 ml homogenizer pestles and tubes, followed by centrifugation at 4000 x g for 5 minutes. The supernatant was used for assays. The rhodanese assay reagents (50 μl each of 250 mM K phosphate buffer pH 8.6, 250 mM NaCN, 250 mM Na thiosulfate) were combined in 1.5 ml microcentrifuge tubes. The embryo extract (100 μl) was added to the assay mixture and incubated for 20 minutes. To determine a standard curve for the assay, 100 μl of various concentrations of sodium thiocyanate in deionized water was used in place of the enzyme extract. Formaldehyde (50 μl of 37%) was added to blanks before the extract was added in order to stop enzyme activity. After 20 minutes of incubation, all samples with active enzyme were stopped with 50 μl of formaldehyde. The enzyme product (thiocyanate) was detected by adding 250 μl of 250 mM ferric nitrate in 14% nitric acid, and then the sample was centrifuged at 10,000 x *g* for 10 minutes. An aliquot (250 μl) of each sample was transferred to a 96-microwell plate. The plate was read at 460 nm with a PowerWave Spectrophotometer (BioTek, Winooski, VT). Additional controls were conducted in which the time course of the slow non-enzyme catalyzed reaction of thiosulfate with cyanide was determined to allow for correction of the data for this basal component (see Fig 1). The mean and standard error (SE) for each data point was calculated ($n = 4$). Statistical testing used a one-way ANOVA Tukey multiple comparisons test. Embryo extracts from 2 dpf, 7 dpf, and 14 dpf embryos were used in order to compare the amount of rhodanese activity at different developmental stages. Since a constant amount of tissue (300 mg) was used for each extract preparation, it was assumed that the A_{460} was proportional to enzyme activity per unit tissue.

Rhodanese activity increased as developmental age increased in embryos, although in 2 dpf embryos the apparent rhodanese activity was only slightly above that for the spontaneous non-enzyme basal component (Fig 1). The highest level of rhodanese activity occurred in mature late-stage embryos as might be expected if the hatchlings may be facing toxic environments as they swim from their hatching site. Cyanide occurs in natural waters at low levels (0.003-0.02 mg/L) but may be an important pollutant in ponds near mining sites (200-700 mg/L)[7]. Cyanide can persist in the environment but does not biomagnify[7]. Rhodanese activity most likely adds an additional advantage to the well-known capacity of killifish to tolerate highly polluted environments[9]. Crawford and Wilde[5] showed that cyanide arrests development, and after one day in control medium embryo development proceeds normally. Their studies also reported an increase in lactate levels in the presence of cyanide and a decrease upon its removal, indicating that anaerobic metabolism was sufficient to maintain the embryo in the "suspended" state. They did not further investigate the possible mechanisms of cyanide detoxification. A literature search suggested that our experiments are the first to detect rhodanese in *Fundulus heteroclitus*. The presence of rhodanese supports the hypothesis that rhodanese is likely to be important in cyanide detoxification in *Fundulus* embryos. We also conducted preliminary experiments using degenerate primers sets that clearly detected conserved teleost rhodanese sequences in *Fundulus* embryo cDNA. We plan to conduct further experiments to analyze rhodanese expression at the molecular level.

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1. **Armstrong PB, Child, JS.** Stages in the normal development of *Fundulus heteroclitus*. *Bio. Bull.* 128: 143-168, 1965.
2. **Baldwin JL, Goldsmith CE, Petersen CW, Preston RL, Kidder GW.** Synchronous hatching in *Fundulus heteroclitus* embryos: Production and properties. *Bull. Mt. Desert Isl. Biol. Lab.* 43: 110-111, 2004.
3. **Baldwin JL, Petersen CW, Preston RL, Kidder GW.** Aerobic and submerged development of embryos of *Fundulus heteroclitus*. *Bull. Mt. Desert Isl. Biol. Lab.* 45: 45-46, 2006.
4. **Chuaypanang S, Kidder GW, Preston RL.** Desiccation Resistance in Embryos of the Killifish, *Fundulus heteroclitus*: Single Embryo Measurements. J. Exp. Zool. (in press).
5. **Crawford RB, Wilde Jr, CE.** Cellular differentiation in the anamniota: II. Oxygen dependency and energetics requirements during early development of teleosts and urodeles. Exp. Cell Res. 44: 453-470, 1966.
6. **DiMichele L, Taylor MH.** The environmental control of hatching in *Fundulus heteroclitus*. *J. Exp. Zool.* 214:181-187, 1980.
7. **Eisler, R.** Cyanide hazards to fish, wildlife and invertebrates: A synoptic review. U.S. Wildlife Serv., Biol. Rep. 85 (1.23), 1991.
8. **Greenspan L.** Humidity fixed points of binary saturated aqueous solutions. *Res. Nat. Bureau Stand.* 81A:89-96, 1977.
9. **Nacci DE, Coiro L, Champlin D, Jayaraman S, McKinney R, Gleason TR, Munns WR, Specker JL, Cooper KR**. Adaptations of wild populations of the estuarine fish *Fundulus heteroclitus* to persistent environmental contaminants. *Mar Biol.* 134: 9-17, 1999.
10. **Petersen CW, Salinas S, Preston RL, Kidder III, GW.** Spawning periodicity and reproductive behavior of *Fundulus heteroclitus* in a New England salt marsh. *Copeia* 203-210, 2010.
11. **Preston RL, Flowers AE, Lahey BC, McBride SR, Petersen CW, Kidder GW.** Measurement of the desiccation of *Fundulus heteroclitus* embryos in controlled humidities. *Bull. Mt. Desert Isl. Biol. Lab.* 45: 101-103, 2006.
12. **Preston RL, Petit MK, Fontaine EP, Clement EM, Ruensirikul S.** Factors contributing to desiccation tolerance by *Fundulus heteroclitus* embryos. *Bull. Mt. Desert Isl. Biol. Lab.* 49: 53-55, 2010.
13. **Saidu Y.** Physicochemical features of rhodanese: A review. African J. *Biotechnol.* 3: 370-374, 2004.
14. **Sorbo, BH.** Crytalline rhodanese – I. Purification and physicochemical examination. *Acta Chem. Scand.* 7: 1129-1136, 1953.
15. **Tayefi-Nasrabadi H, Rahmani R.** Partial purification and characterization of rhodanese from rainbow trout (*Oncorhynchus mykiss*) liver. *The Scientific World Journal* 2012: 1-5, 2012.
16. **Tingaud-Sequeira A, Zapater C, Chauvigné F, Otero D, Cerdà J.** Adaptive plasticity of killifish (*Fundulus heteroclitus*) embryos: dehydration-stimulated development and differential aquaporin-3 expression. *Am J Physiol Regulat. Integr. Comp Physiol.* 296: 1041-1052, 2009.