The fate of *Fundulus heteroclitus* embryos ingested by adult killifish

Arhea V. Marshall[1] and Robert L. Preston[2,3]
[1] Bowdoin College, Brunswick, ME 04011
[2]School of Biological Sciences, Illinois State University, Normal, IL 61790
[3]Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672

Northern killifish spawn in June and July at the margins of estuaries. Because of the 6-12 foot daily tidal range in Maine, not all embryos remain immersed in seawater and some are exposed to air and survive for long periods. Immersed embryos are exposed to aquatic predators. We previously showed that adult killifish readily eat immersed killifish embryos but it was possible that the embryos could survive passage through the adult GI tract. We show here that embryos do not survive ingestion. This supports the hypothesis that the unusual ability of embryos to survive in air provides a selective advantage under the potentially intense predation pressure of adult killifish.

Euryhaline northern killifish, *Fundulus heteroclitus macrolepidotus*, spawn in estuaries in Maine in June and July during peak high tides[7]. The eggs are deposited on vegetation and rocky substrates at the margins of streams. Because the low to high tidal range may vary from 6-12 feet on a daily basis, being highest at spring tides, some embryos may be stranded in air for extended periods of time. Investigations in this and other labs have shown that *F. heteroclitus* embryos can tolerate desiccation stress, and develop normally in air to hatching at maturity in ~14 days[3,4]. Immersion of these embryos in seawater (SW) triggers hatching[3,4]. Experiments have shown that aerial incubation favors increased embryo survival and the production of larger hatchlings compared with immersed embryos[3]. Immersed killifish embryos are subjected to a different suite of predators than aerially developing embryos. Immersed embryos may be ingested by larger aquatic organisms including killifish adults[5,8]. In a previous laboratory experiments we showed that the data strongly support the hypothesis that adult killifish may be a major predator of killifish embryos[5]. We suggested that the natural selection for desiccation resistance mechanisms that permit survival of killifish embryos in air removes the embryos from the potentially serious threat of predation by adult killifish as well as other aquatic predators.

A study on bass has shown that viable embryos may pass through their guts when ingested[6]. Penczak[8] also noted that killifish guts after feeding during spawning season contained a significant number of fish eggs, but did not rigorously categorize which fish prey species may be involved. Interestingly, a very early paper by Babkin and Bowie[2] showed that killifish apparently do not posses stomachs, and hence do not have acidic digestion enzymes (pepsin) or hydrochloric acid, although it is clear other digestive processes are certainly active. Such a condition might favor survival of killifish embryos after ingestion. The "hardening process[1]" that occurs in normal teleost embryo development might also confer some resistance to digestion after ingestion. In these experiments we analyzed the gut contents of adult killifish after they have ingested killifish embryos to test the hypothesis that the embryos can survive ingestion.

Fish were caught in minnow traps at Northeast Creek, Mount Desert Island, ME and kept in aquaria with natural running SW (~30 ppt). In a typical experiment, approximately 400 eggs were fertilized with milt from ten or more fish, in a beaker with 25 ml of 10 ppt artificial seawater (ASW; Instant Ocean, Mentor, OH). After sitting for 30 min, embryos were rinsed three times with 10 ppt ASW and then were placed on filter paper moistened with 10 ppt ASW in Petri dishes and cultured aerially to the desired age in a chamber whose humidity was equilibrated with 10 ppt ASW (see Chuaypanang et al.[4] for details). Opaque four-liter containers were used containing three liters of natural SW diluted with deionized water to 10 ppt. One male adult fish was added to each of three of the dark containers; and filter paper covers were added to cover the containers. The fish were acclimated to the containers for 20 min. Then, ten embryos were added and each fish was observed until eight or more of the embryos were ingested, usually a time period between five and ten hours. Embryos at four different developmental stages were used, one hour post-fertilization (hpf) and 2, 7 and 14 days post-fertilization (dpf). The different development embryo ages show significantly different resistance to desiccation stress (with maximum resistance at 7 dpf)[4] and therefore it was possible that development age could be a factor in survival after ingestion. The adult fish were then removed and the water in the container was strained through mesh to capture any surviving embryos and other matter. The adult fish were then euthanized in SW containing 1 g/L MS-222. The entire gut was removed and examined under dissecting microscope for the presence of embryos

and debris. The fecal matter was also microscopically examined for embryos and degraded embryo debris. Control experiments were used to confirm that the immersed embryos were viable for the entire experimental period before ingestion. In controls, ten embryos were placed within a dark container, under screen that excluded the fish from access to the embryos. For all conditions describe below, the controls confirmed that the embryos were viable until ingestion. Each experimental procedure was replicated four times.

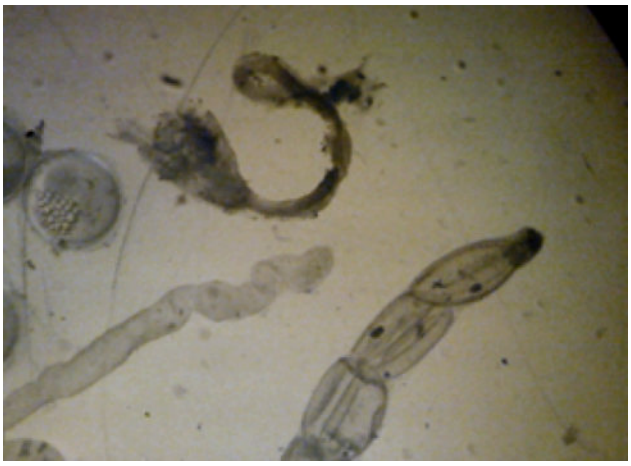

Figure 1: Microscopic view of 2 dpf killifish embryos after passage through adult gut. Three collapsed chorions encapsulated in a mucus sac can be seen at the lower right. An intact but degraded embryo is seen at the upper left. Other ingested matter is also present in the center area.

Those embryos that passed through the gut and were excreted were not viable and most were degraded presumably through the action of digestive enzymes. The criteria for viability included heartbeat, embryo fin movements in 7 dpf and 14 dpf embryos and distinct embryo tissue disruption and degradation in embryos of all ages. The tough outer envelope surrounding killifish embryos, the chorion[4], could be identified in most cases, as either collapsed spindle-like structures or spherical and apparently intact but with nonviable disrupted embryo cellular contents (Fig 1). In some instances, the excreted material contained a number of chorions enclosed in a mucus casing. Plant and algae debris was also occasionally found as part of the excreted material. The material dissected from intestinal tracts showed similar characteristics to the excreted material, collapsed "empty" spindle-like chorions, and degraded embryonic cellular matrix in those embryos that were spherical in conformation. The age of the embryos (1 hpf, 2 dpf, 7 dpf and 14 dpf) did not seem to make a difference except that the 1 hpf embryos appeared to have mostly intact chorions but with dead nonviable embryo material. More comprehensive experiments with a sizeable number of replicates may be needed to identify any embryo age related characteristics with regard to transient digestion resistance. However, the overall conclusion with these preliminary experiments is that we could not demonstrate in any case that viable embryos survived transit through adult killifish guts and further that all embryos remaining in the guts after five to ten hours were degraded and nonviable. These data support the hypothesis that the unique capacity of *Fundulus heteroclitus* embryos to survive for as long as 14 days or more exposed to air and that the strong expression of desiccation resistance in these embryos[4] favors enhanced survival in the presence of vigorous intraspecific oophagy[9].

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1. **Armstrong P, Child J.** Stages in the normal development *Fundulus heteroclitus*. *Biol. Bull*. 128:143-168, 1965
2. **Babkin BP, Bowie DJ.** The digestive system and its function in *Fundulus heteroclitus*. *Biol. Bull*. 54:254-277, 1928.
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*. 319A:179-201, 2013.
5. **Marshall AV, Preston RL**. Predation of killifish embryos by adult killifish (*Fundulus heteroclitus*). *Bull Mt. Desert Isl. Biol. Lab*. 50:48, 2011.
6. **Marteinsdottir G, Abel KW.** Influence of egg size on embryos and larvae of *Fundulus heteroclitus* (L.). *J. Fish Biol*. 41:883-896, 1992.
7. **Petersen CW, Salinas S, Preston RL, Kidder GW, III**. Spawning periodicity and reproductive behavior of *Fundulus heteroclitus* in a New England salt marsh. *Copeia*. 203-210, 2010.
8. **Penczak T.** Trophic ecology and fecundity of *Fundulus heteroclitus* in Chezzetcook inlet, Nova Scotia. *Marine Biol*. 89:235-243, 1985.
9. **Polis GA.** The evolution and dynamics of intraspecific predation. *Ann. Rev. Ecol. Syst*. 12:225-251, 1981.