Measurement of the desiccation of *Fundulus heteroclitus* embryos in controlled humidities

Robert L. Preston[1], Amy E. Flowers[1], Bridget C. Lahey[1],
Sean R. McBride[1], Christopher W. Petersen[2] and George W. Kidder[3]

[1]Department of Biological Sciences, Illinois State University, Normal, Illinois 61790,
[2]College of the Atlantic, Bar Harbor, Maine 04609,
[3]Mount Desert Island Biological Laboratory, Salisbury Cove, Maine 04672

The euryhaline killifish (or mummichog), *Fundulus heteroclitus*, spawns in estuaries at spring tides, scattering its eggs at the margins of the stream among rocks and bordering vegetation[1]. The embryos incubate in air for 14 days or more until the next spring tide floods them, which triggers a nearly synchronous hatching[1,2]. It is generally accepted that one of the key stimulus that initiates hatching is anoxia that develops in the embryos after flooding[1,2]. Experiments in our laboratories have shown that the embryos of the northern subspecies, *F. heteroclitus macrolepidotus*, the indigenous species in Maine and northern New England, also develops while immersed in water, but may develop more slowly and have smaller hatchling size (see Kidder *et al.* in this issue[3]).

It is generally thought that aerial incubation of *Fundulus* embryos provides a much larger supply of oxygen to the embryos than in the immersed state[1,2]. On the other hand, aerial incubation also could easily expose embryos to fluctuating air temperatures and to stress from desiccation. One would predict that aerially incubated *F. heteroclitus* embryos should show resistance to both temperature and desiccation stress, perhaps by expression of cellular stress proteins (*e.g.* heat shock proteins, HSPs) and by maintaining low water permeability in the embryo chorion, the nonliving external capsule that incases and protects the vitelline membrane and embryo. Guggino[4] directly measured water permeability by measured tritiated water turnover by immersed embryos and concluded that although the permeabilities were fairly low compared to other organisms ($0.4 - 1.6 \times 10^{-6}$ cm·sec^{-1} depending on developmental stage), they were not low enough to maintain water balance in immersed embryos. In this preliminary study, we sought to determine water loss in aerially incubating embryos exposed to air of various fixed relative humidities (RH) to test the hypothesis that *F. heteroclitus* embryos will resist rapid desiccation at low RHs.

Fish were collected from Northeast Creek, Mount Desert Island, ME and were stripped of eggs and milt into diluted artificial seawater (ASW, 10 ppt), and the embryos were placed on moist filter paper in 3.5 cm Petri dishes for incubation at 20° C in an atmosphere equilibrated with 10 ppt ASW. The embryos developed normally and late stage embryos (stage 36 or later)[5] were used for the ensuing experiments. Before the experiments began, the embryos were blotted for a few seconds to remove any adherent water film and then transferred to the chambers described below. We constructed a replacement weighing pan in an enclosed "humidity chamber" for a Sartorius Model MC1 balance. This allows us to measure weight loss ± 0.01 mg from a single embryo (hydrated weight ~4 mg) for long periods in air of different RHs. The RH within the chamber was established by equilibrating the air in the chamber with desiccant, room air, water or saturated solutions of various salts[6]: 0% RH, Drierite (anhydrous $CaSO_4$) desiccant; 33% RH, $MgCl_2$; 62% RH, ambient room air measured with an hygrometer; 84% RH, KCl; and 100% RH, deionized water. To test the physiological consequences of dehydration on hatching capability, 4 groups of 15 stage 36 embryos were blotted very briefly on filter paper and placed in jars containing RHs set, as described above, by saturated salt solutions at 0%, 22% (K Acetate), 43% (K_2CO_3), 75% (NaCl) and 100% RH for two hours (at 23°C). Embryos of stage 36 under normal conditions will usually hatch within 24 hours of flooding. After two hours five embryos

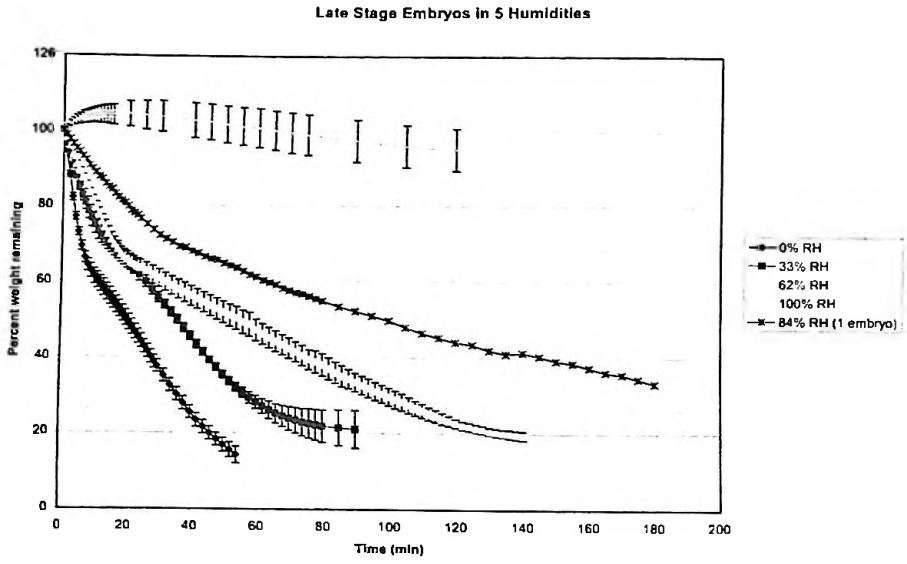

Figure 1: Desiccation of *F. heteroclitus* embryos in air of different relative humidities (RH). Single embryos (stage 36-37) were transferred from the chamber in which they had developed on moist filter paper for 10-14 days at 20°C in air in equilibrium with 10 ppt ASW, blotted for a few seconds on filter paper and transferred to a modified weighing pan that was enclosed in a humidification chamber (Sartorius MC1 microbalance, nominal resolution 0.01 mg). A typical hydrated embryo at this stage weighed about 4 mg. That data shown are normalized to percent water loss and are the mean ± SE of measurements on three separate embryos (with the exception of the 84% RH data which is for 1 embryo).

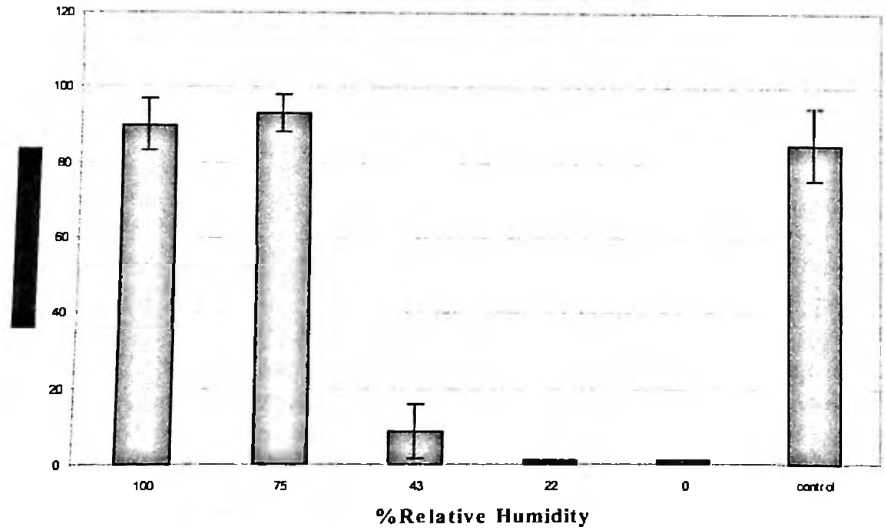

Figure 2: Hatching percentage of *F. heteroclitus* embryos (stage 36-37) after a two hour exposure to various relative humidities (RH) followed by flooding with 10 ppt ASW. The hatching percentages were determined 24 hours after flooding. Each bar shows the mean ± SE for 4 experiments, each employing 10 embryos each. In the control condition, the embryos were flooded immediately with 10 ppt ASW without prior incubation in the controlled humidity chamber. There was no successful hatching (0%) at 22% RH and 0% RH. The hatching percentage at 100% RH and 75% RH are not significantly different from the control or each other (t-test, $p < 0.05$).

were transferred to RNAlater (Qiagen, Valencia, CA) for later molecular analysis and the remaining 10 embryos were flooded with 10 ppt SW bubbled with nitrogen (to assure the water was initially anoxic).

In 100% RH, using late stage embryos (stages 36-37), water uptake is observed (as might be expected) for over 30 minutes followed by a very slow decline (Figure 1). At all lower RHs tested, the embryos rapidly lost weight in at least two phases, a rapid decline for 10 – 30 min depending on the RH followed by a slower prolonged weight loss for an additional 30 min (0% RH) to about 120 min for higher RHs. At some RHs (33% and 62%) a third phase may be present as the embryo reaches equilibrium with the air. In general, as might be expected, over two hours the lower the RH the more rapid the rate of water loss from the embryo. We speculate that the first phase represents water loss from the external surface of the chorion and the peri-vitelline space. The second phase, the slower rate of weight loss (typically ½ to ¼ that of the first phase) could be due water loss from the embryo tissue itself.

One surprising result was that embryos rapidly lose water at all RH tested below 100% and that there is rather short-term "resistance" to dehydration (Fig. 1). After two hours at RH tested below 84% the embryo has lost nearly all its cellular water (75% weight loss). Since the embryos in the field may be exposed to air for 14 days, it seems that there is a dramatic difference between these properties and those needed to sustain the embryos aerially in the field. The effect of a two hour exposure to low humidities on hatching efficiency of stage 36 embryos is shown in Figure 2. Note that hatching was successful in embryos exposed to 100% and 75% RH (95 – 97% hatching rate after 24 hours). In the control that was not subjected to the two hour aerial incubation, but flooded immediately, the hatching percentage was 93 ± 3 (n = 4 groups of 10 embryos). At 43% RH, 3 embryos hatched in one experiment; none in the other two. At 22% and 0% RH, no embryos hatched.

These data clearly demonstrate that *F. heteroclitus* embryos cannot tolerate long-term desiccation stress since they lose water rapidly over the course to two hours. Whatever "resistance" to desiccation exists must be limited to rather short-term effects (less than two hours and more likely in the range of 30 min or so). The nearly complete successful hatching at 75% RH after two hours of exposure and then flooding with 10 ppt ASW suggests that there may be some compensation for stress at the cellular level, since we would predict from Figure 1 that the embryos have lost about 70% of its cellular water. A possible explanation for this hatching success could be that there was the presence or induction of stress proteins, presumably HSPs. To test this hypothesis, we designed primers for a short *Fundulus* HSP 70 and HSP 90 sequence and tested them on cDNA prepared from total RNA (see methods in Preston *et al.*[7]) from embryos exposed for two hours to the five RHs. Subsequent qualitative PCR and preliminary real-time PCR experiments have shown that both HSP 70 and HSP 90 mRNA are expressed in these embryos at all RHs. Further work is continuing in our lab in this area.

In summary, we have shown that single *F. heteroclitus* embryos rapidly desiccate over the course of two hours in RHs of 84% or lower. These results do not support the hypothesis that these embryos are uniquely impermeable to water and thus may survive aerially in nature for 14 days or more. It is possible that in the short-term there is induction of stress proteins (HSPs), but this seems to fall far short of the desiccation resistance need for long-term survival. We may speculate that long-term survival may be critically dependent upon the microenvironment in the field in which the embryos find themselves. Presumably local pockets of high RH and microfilms of adherent water to rocks, vegetation or other substrates must maintain embryo hydration. We are pursuing this line of research in future investigations.

Supported by NSF C-RUI 0111860.

1. **DiMichele, L. and M. H. Taylor.** The environmental control of hatching in *Fundulus heteroclitus*. *J. Exp. Zool.* 214:181-187, 1980.
2. **Baldwin, J.L., C.E. Goldsmith, C. W. Petersen, R. L. Preston and G. W. Kidder.** Synchronous hatching in *Fundulus heteroclitus* embryos: Production and properties. *Bull. Mt. Desert Isl. Biol. Lab.* 43: 25-27, 2004.
3 **Baldwin, J.L., C. W. Petersen, R. L. Preston and G. W. Kidder.** Aerobic and submerged development of embryos of *Fundulus heteroclitus*. *Bull. Mt. Desert Isl. Biol. Lab.* 45 (this issue).
4. **Guggino, W.B.** Water balance in embryos of *Fundulus heteroclitus* and *F. bermudae* in seawater. Am. J. Physiol. 238: R36-R41, 1980.
5 **Armstrong, P. B., and Child, J. S.** Stages in the normal development of *Fundulus heteroclitus*. *Biol. Bull.* 128:143-168, 1965.
6. **Greenspan, L.** Humidity fixed points of binary saturated aqueous solutions. Res. Nat. Bureau Stand. 81A:89-96, 1977.
7. **Preston, R. L., R. J. Clifford, J.A. Thompson, D.L. Slager, C. W. Petersen and G. W. Kidder.** CFTR mRNA expression in developing *Fundulus heteroclitus* embryos. *Bull. Mt. Desert Isl. Biol. Lab.* 43: 25-27, 2004.