# Ion and water content of *Fundulus heteroclitus* embryos
## before and after desiccation stress

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Mummichogs spawn at the margins of estuaries. Stranded embryos can develop normally in air in about 14 days, although they are subjected to significant desiccation stress. We measured the water content and ion concentrations in control and desiccated embryos at 2, 7 and 14 days post-fertilization. These data should allow us to calculate the osmotic driving forces controlling water flow under desiccating conditions. One unusual observation was that the chloride concentrations in embryos were higher than expected and the reasons for this remain to be explained.

During the summer months in New England the mummichog or northern killifish, *Fundulus heteroclitus macrolepidotus*, spawns during daily high tides at the edges of estuaries in brackish water (about 10 ppt)[1-4,11]. The adults may migrate to and from full strength seawater (SW, ~30 ppt) to freshwater (FW, <1 ppt) and may survive in either salinity for indefinite periods[2-4,11]. The embryos may remain immersed, but as the tide ebbs some embryos might be stranded for long periods in air on bordering vegetation or on the rocky shoreline (up to 14 days, if spawning occurred at spring tide). This exposes the embryos to desiccation stress[3,4,7,9,12]. Aerial incubation may have some evolutionary selective advantage, because of the higher concentrations of oxygen available in air as compared to water. Embryos also are exposed to a different set of predators than those present in the aquatic environment[3,4]. We recently showed one of the most important predators may be adult killifish themselves[6]. Aerially incubated embryos also appear to have greater viability and hatching success[3]. In aerially incubated embryos, flooding with seawater at spring tides after 14 days triggers hatching[3,4]. It has been proposed that exposure to the relatively hypoxic seawater is a signal that initiates hatching[3,4]. During mid-stages of development (5-9 days post fertilization, dpf; stages 28-33)[1] killifish embryos can resist severe desiccation for short periods (2-6 hours), resulting in increased survival of stressed mid-stage embryos[9]. Mid-stage embryos can tolerate severe desiccation conditions (*e.g.,* 22% relative humidity, RH, for 2 hours) that always kill early stage (0-2 dpf, stages 1-20)[1] and late stage (12-14 dpf, stages 36-39)[1] embryos[9].

We have shown that certain physiological factors may play a role in the desiccation tolerance by mid-stage embryos including: down regulation of water channels proteins (specifically aquaporin 3, AQP3) in mid-stage embryos[10,12], the constitutive and/or induced expression of heat shock proteins (HSPs[10]; especially HSP 90) and the presence of high concentrations of compatible solutes that may help stabilize cellular native protein structure during desiccation[8]. In order to evaluate the driving forces for water gain or loss, a more accurately measurement of water content and major osmolyte content for killifish embryos. Our laboratory has measured free amino acid and protein content, as well as determined whether glucose or glycerol may contribute to the compatible solute [8,10]. It appears that careful measurements of killifish embryo major ion ($Na^+$, $K^+$ and $Cl^-$) content have not been done, especially in comparison of control and desiccated conditions. We also determined that water content of embryos before and after exposure to desiccation stress. The preliminary data presented here focus on mid-stage embryo ion content.

Mummichogs were collected from Northeast Creek, Mount Desert Island, ME, and held in 30-gallon aquaria with running natural SW (about 30 ppt). Eggs and milt were stripped manually into a beaker containing 10 ppt artificial seawater (ASW; Instant Ocean, Mentor, OH). 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[3]. The embryos were inspected daily and developed normally over 14 days. At maturity the embryos hatched normally when flooded with 10 ppt ASW.

**Table 1.** Water content of control and desiccated embryos (µl water/embryo; mean ± SE). Comparisons between control and desiccated embryos at 2 dpf, 7 dpf and 14 dpf showed a significant difference in water content ($p \leq$ 0.005). All comparisons among controls are not significantly different at the $p \leq 0.05$ level (Student's *t*-test). All comparisons among desiccated embryos are not significantly different at the $p \leq 0.05$ level.

| dpf | Control µl/embryo | % Water Content | Desiccated µl/embryo | % Water Content |
|-----|-------------------|-----------------|----------------------|-----------------|
| 2 | 2.96 ± 0.15 | 82.9 ± 0.80 | 1.96 ± 0.09 | 74.3 ± 0.19 |
| 7 | 2.95 ± 0.02 | 81.6 ± 0.10 | 2.05 ± 0.05 | 75.6 ± 0.56 |
| 14 | 2.84 ± 0.05 | 82.5 ± 0.17 | 1.85 ± 0.08 | 75.8 ± 0.73 |

To measure total water content, 20 embryos of 2, 7 or 14 dpf were removed from the incubation chamber and quickly blotted on filter paper before being transferred to a 25 mm Petri dish (no contact between embryos on dish). For desiccated embryo treatment, 20 embryos of 2, 7 and 14 dpf were exposed to a 23% RH chamber for 2 hours. RH was established in small chambers in which the air was equilibrated with saturated salt solutions at 20°C (23% RH, potassium acetate)[5]. With controls, 20 embryos were exposed for 2 hours in a chamber containing air that was in equilibrium with 10ppt ASW in the chamber. After treatment, the desiccated and control embryos were blotted very briefly on filter paper before being transferred to microfuge tubes. The tubes were immediately capped, and weight was recorded. After weighing, caps of tubes were opened and tubes were placed horizontally in a metal tray within a 70°C oven. Weight was measured at 30, 60, 90, 120, and 300 minutes and overnight. Triplicate measurements were taken at each stage of development (2, 7 or 14 dpf). Control experiments showed that empty microfuge tubes did not change weight under these conditions.

Table 1 shows that the water content in 2, 7 and 14 dpf embryos decreases from about 82-83% in control embryos to 74-76% in embryos desiccated for two hours at 23% RH. Embryos of all ages reached about the same endpoint under these conditions. We know from other experiments that mid-stage embryos (7 dpf) resist water loss initially, but by two hours at 23% RH this capacity for resistance usually fails (unpublished data). In spite of this water loss, some embryos if rehydrated may survive to hatchling[9].

**Table 2.** Ion activity (mM ± SE; n = 3) in 7 dpf control and desiccated whole embryos. Desiccated embryos were exposed to 23% RH for two hours.

| | CONTROL | DESICCATED |
|-----|---------|------------|
| Na$^+$ | 66.0 ± 4.5 mM | 75.2 ± 1.3 mM |
| K$^+$ | 73.9 ± 2.1 mM | 95.1 ± 7.6 mM |
| Cl$^-$ | 259.5 ± 27.2 mM | 351.0 ± 40.4 mM |

For ion measurements 200 7 dpf embryos for each condition were blotted on filter paper and treated as either a control, whereupon a dish of embryos were placed in a sealed chamber at equilibrium with 10ppt ASW (isotonic with fish bodily fluids[3]), or experimental condition, where the dishes were placed in chambers at 23% RH. After two hours the embryos were removed from treatment chambers and disrupted using hand homogenizers or a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenized embryos were then centrifuged (10,000 x *g* for 10 minutes), and the liquid supernatant was removed, and used for direct measurements (embryonic extract).

The ion activity in the extracts was measured using ion-selective electrodes (Lazar Research Laboratories, Los Angeles, CA). Before measuring control/desiccated extracts, a standard curve was obtained for each electrode by measuring a dilution series of either NaCl or KCl as appropriate. An equation then was obtained from the standard curve of the dilution series ionic measurements by logarithmic regression analysis. The

typical analysis procedure was: 200 $\mu$l of embryonic extract was pipetted into the cap of a microfuge tube and the tip of the appropriate electrode was lowered into the extract. After one minute of equilibration, the ionic activity (measured in millivolts) was recorded. This value was then inserted in the logarithmic standard equation to obtain the extract ion activity in milliequivalents (or millimoles for the monvalent ions measured here). All experiments were done in triplicate.

The extracts used were not diluted and therefore, the activities measured are those in whole embryos. As expected the ion activities in desiccated 7 dpf embryos were higher than in control embryos, but the ratios of increase were lower (1.14 for $Na^+$, 1.29 for $K^+$ and 1.35 $Cl^-$) than predicted by the ratio of water content in control to desiccated embryos (2.95/2.05 = 1.44). Whole embryos have two major compartments, the perivitelline space (PVS) that lies between the tough outer flexible covering, the chorion, and the embryo compartment surface membrane (ECSM) that encloses the developing embryo. It is generally thought that the chorion is relatively permeable to small molecules and water[7,9,12], and we have direct evidence to support this conclusion from single embryo desiccation studies (Preston, *unpublished data*). The water content of the PVS is estimated to be about 25% of the whole embryo[12]. Assuming that the PVS ion composition is in equilibrium with 10 ppt ASW in the culture substrate (134 mM $Na^+$, 3.0 mM $K^+$ and 155 mM $Cl^-$), it is possible to calculate the apparent ion activities in the embryo compartment (EC). In 7 dpf embryos the calculated ion activities were 43 mM $Na^+$, 98 mM $K^+$ and 294 mM $Cl^-$. It is to be expected that embryonic tissues are high in $K^+$ and lower in $Na^+$. However, the high $Cl^-$ activity is unusual and bears further verification. The mechanisms by which such high $Cl^-$ activities could be sustained include active $Cl^-$ pumping into the EC, complexation with cationic moieties or sequestration by binding and/or further compartmentation. The sum of the major ions in the PVS is 292 mM, while the sum of these components in the EC is 435 mM of which 141 mOsM is accounted for by $Na^+$ and $K^+$. The excess $Cl^-$ (153 mM) suggests that other counterions, perhaps some organic, must be present.

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