Osmoregulation in *Fundulus heteroclitus* oocytes and embryos measured by sedimentation pycnometry.

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In teleost fish, maturing oocytes are in contact with maternal fluids which are isosmotic with the maternal blood[3,5]. After spawning and fertilization, most teleost eggs become much less permeable to water which allows them to maintain osmotic balance during development. Killifish, *Fundulus heteroclitus*, spawn in estuaries at intermediate salinities and the embryos, which ordinarily require about 14 days to develop, are exposed to large changes in salinity due to periodic tidal inflow of seawater (SW) and subsequent flushing by fresh water (FW) outflow. In addition, killifish embryos are capable of full development aerially in moist environments[2]. Guggino[3,4] showed that the water permeability of killifish embryos is sufficiently high that active osmoregulatory mechanisms are necessary and this is consistent with our previous findings that embryos showed increasing expression of cystic fibrosis transmembrane regulator (CFTR) mRNA as development proceeds[6]. We also showed that unfertilized oocytes were responsive to osmotic changes in the external medium using sedimentation pycnometry (SP) a method that measures oocyte density changes as an index of water gain or loss[6,7]. In this study, we extend our SP measurements to killifish embryos to test the hypothesis that killifish embryos should show increased regulatory capability compared with unfertilized oocytes,

Oocytes were manually expressed from females adapted to SW (922 mOsM) and fertilized *in vitro* in 10 o/oo medium (325 mOsM). The embryos were held at 20° C in air at 100% humidity. Oocyte and embryo density was measured by SP as follows[7]: Freshly collected oocytes or embryos (stages 29 through 36)[1] were placed in SW or diluted SW of the following osmolarities: 10 mOsM, 296 mOsm and 917 mOsM. After incubation periods of one and four hours, single oocytes or embryos were transferred to 1.5 ml microfuge tubes containing 0.3 ml of the appropriate SW and 0.5 ml of phthalate mixtures. By varying the quantities of two phthalates of differing densities, phthalic acid diethyl ester and phthalic acid bis (3,5,5-trimethylhexyl) ester, a 12-member density step gradient was formed with densities ranging between 1.03 g/ml and 1.06 g/ml. The tubes were centrifuged at 14,000 x *g* and the tubes inspected for floating or sedimented oocytes. A modification of our earlier procedure[7] was used to assure consistent sedimentation behavior with embryos. Tubes containing the oocytes or embryos plus phthalates were centrifuged in additive increments for 5, 15, 30, 45, 60 and 300 sec and sedimentation or lack thereof was noted at each time. It was concluded that the best representative total centrifugation period was 50 sec (5+ 15 + 30 sec increments). Each measurement was done at least 6 times.

Figure 1 shows the apparent osmotic behavior of killifish embryos (stages 29-36) compared with that of unfertilized oocytes. The embryos or oocytes were exposed to hypotonic (10 mOsM), isotonic (296 mOsM) or hypertonic media (917 mOsM) for one and four hours and then the density change was determined by SP. The data show that as the osmotic pressure of the medium changes the embryos gain water (at 10 mOsM) or lose water (at 917 mOsM) compared with the isotonic condition (296 mOsM). It is apparent that there is a greater change in density in unfertilized oocytes compared with embryos, a result that is consistent with the hypothesis that active osmoregulatory mechanisms are being expressed as embryos develop. The amount of density change when comparing FW (10 mOsM)

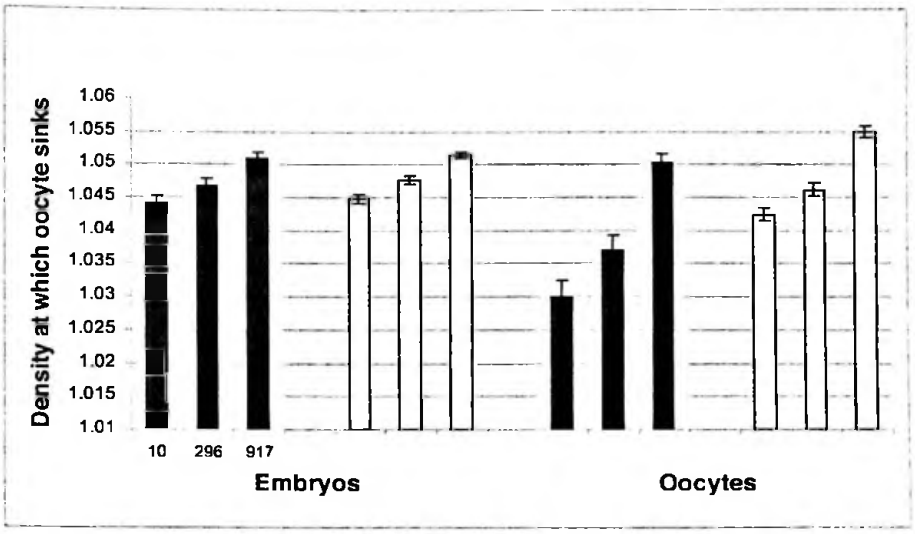

Figure 1: Mean minimum densities measured by sedimentation pycnometry for single *Fundulus heteroclitus* oocytes incubated in 10, 296, and 917 mOsm/kg SW solutions for one hour (solid bars) or four hours (open bars). Data are displayed as means ± standard errors, n=6. Significant differences are present between the densities at the three salinities in each experimental group, $p<0.05$ (t-test).

and SW (917 mOsM) is two to three times greater in unfertilized oocytes than embryos (Table 1). These results support our hypothesis that increasing osmoregulatory capacity is to be expected during killifish embryonic development.

In other experiments, we measured the quantitative expression of Na/K ATPase alpha subunit 1a mRNA using real-time PCR, which increases as development proceeds. Although preliminary in nature, the results are consistent with our earlier findings of increasing CFTR mRNA expression during killifish embryonic development[6]. It has also been noted that chloride cells appear early in development in the yolk sac of embryos before their appearances in embryonic gills[4]. These data support the hypothesis that killifish embryos actively osmoregulate during development and that they do not simply resist osmotic stress by remaining completely impermeable to water and solute transfer.

Table 1: Change in density of *Fundulus* embryos and oocytes after one hour and four hour exposure to FW (10 mOsM), isotonic (296 mOsM) and hypertonic (917 mOsM) medium. The density changes were calculated as follows: $(\rho_{917} - \rho_{10})/(917\ mOsM - 10\ mOsM)$, where ρ_{917} and ρ_{10} are the apparent oocyte densities in 917 mOsM and 10 mOsM medium respectively.

	One hour incubation Density change $g\ cm^{-3}\ osmole^{-1} \times 10^{-6}$	Four hour incubation Density change $g\ cm^{-3}\ osmole^{-1} \times 10^{-6}$
Embryos	7.39	7.28
Oocytes	22.5	13.9

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