PRELIMINARY STUDIES OF SALINITY ADAPTATION IN *FUNDULUS HETEROCLITUS* AND APPARENT CFTR mRNA EXPRESSION IN GILL TISSUE AND OOCYTES.

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The euryhaline killifish, *Fundulus heteroclitus*, readily tolerates transfer from saltwater to freshwater and can apparently survive indefinitely in either condition. The osmoregulatory mechanisms by which the killifish readily adapts to these different conditions are under intense study in a number of laboratories. For example, Singer et al., (Am. Jour. Physiol. 274:C715-C723, 1998) have shown by Northern blot analysis that the expression of mRNA for the chloride channel, the cystic fibrosis transmembrane regulator (CFTR), was increased 2-9 times in fish transferred to saltwater (SW) compared with freshwater (FW) fish. Kidder (Bull. Mt. Desert Island Biol. Lab 36:69, 1997) has shown that another possible mechanism for compensating for change in external osmotic conditions is "behavioral osmoregulation", that is, the fish actively seek media isotonic to their body fluids which minimizes the energy demand for osmoregulatory processes. Petersen and colleagues (personal communication) have been conducting preliminary population estimates in Northeast Creek on Mount Desert Island (MDI) and report that *Fundulus* may migrate between SW and FW fairly frequently. This suggests that the physiological acclimation state of fish "in the wild" may be intermediate in nature. It is also possible that an intermediate acclimation state would be optimal for fish exhibiting behavioral osmoregulation, i.e. those that seek conditions isotonic with their body fluids.

This report describes very preliminary data that employs PCR, with *Fundulus* specific primers, on cDNA produced from mRNA isolated from *Fundulus* tissues. The objective of these experiments was to detect and confirm the expression of CFTR mRNA in gill tissue from fish adapted to freshwater (0.5 ppt), saltwater (30 ppt) and in 5 ppt medium. In addition, we attempted to identify the presence of CFTR sequences in cDNA produced from mRNA from *Fundulus* oocytes as a reference point for future developmental studies. Killifish were trapped in Northeast Creek and transferred to MDIBL running SW tanks (for SW adapted fish) or to tanks containing 5 ppt SW or 0.5 ppt (FW). After acclimation periods of two or more weeks, the fish from each acclimation condition were sacrificed and gill tissue removed. Total RNA was isolated from these tissues by standard methods (RNAeasy, Qiagen, Inc.). Total RNA was also isolated from freshly harvested oocytes using the same procedure. cDNA synthesis used enhanced AMV-reverse transcriptase (Sigma, Inc.) and care was taken to adjust the initial RNA template for all samples to equivalent amounts (1 μg, measured spectrophotometrically).

PCR was conducted using published species specific primers (Singer et al., *op.cit.*), F: 5'-GCTTGGGCTTGGATCTTATGAC-3' ; R: 5'-GCCAGTATGCTATCTGAGTGAGC-3'. PCR controls consisted of cDNA prepared under similar conditions from tobacco mosaic virus RNA (TMV) using specific primers (Sigma, Inc.). Actin primers, F: 5'-TGAACCCCAAGGCCAACC GTGAGAAGATGA-3'; R: CCCAATCCAGACAGAGTATTTACGCTCAGG were also used to establish an internal control reference for the adaptation conditions. The PCR cycling conditions

were: 95°C, 3min; 95°C, 45 sec denaturation; 60°C, 30 sec annealing; 72°C, 90 sec elongation (36 cycles); 72°C termination for 10 min.

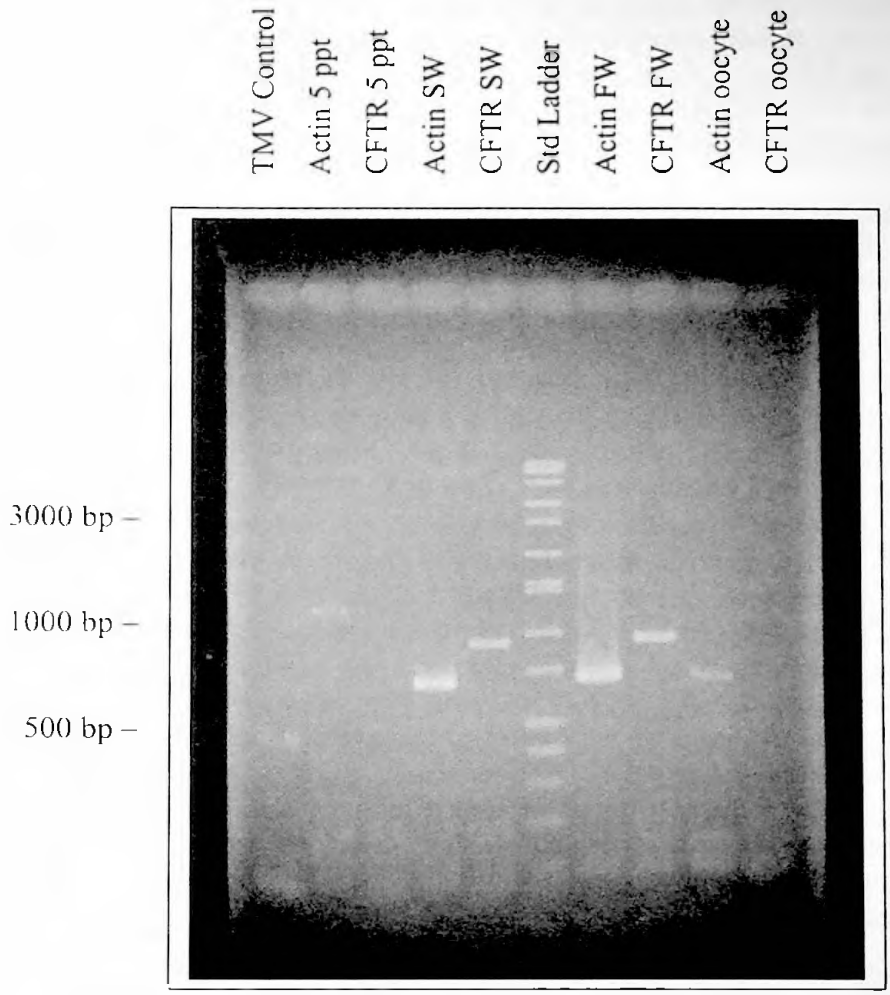

Fig. 1 shows the results of a typical gel. The sizes of the PCR products agree well with that expected from literature values (Lane 1: ~440 bp for the TMV control; Lanes 4, 7 and 9: ~700 bp for the actin control; Lanes 5 and 8: ~930 bp for CFTR). Lane 2 shows an anomalously large actin band (~1200 bp) which may imply some contamination by genomic DNA of this cDNA preparation.

The apparent relative expression of CFTR mRNA detected in this experiment suggests that little CFTR mRNA is present in 5 ppt adapted *Fundulus* gill tissue (lane 3) and in freshly harvested *Fundulus* oocytes (lane10). The apparent lack of CFTR mRNA in the 5 ppt condition is inconsistent with the detection of CFTR mRNA in FW and SW gill tissue (below) and it is possible that the results from the 5 ppt preparation was complicated by the presence of genomic DNA. This possibility is supported by the actin control for the 5 ppt condition which shows an anomalously large band size (~1200 bp vs 700 bp normally). Controls run on the FW, SW and oocyte cDNA preparations did not show genomic DNA contamination in these preparations. A significant amount of CFTR mRNA appears to be present in gill tissue from both FW and SW adapted fish. In comparison with the internal standard actin, the relative levels of CFTR in gills

from SW adapted fish is 2-4 times larger based on band area and densitometric measurements. This comparison must be regarded with some reservation since it is likely that the PCR conditions may have been well into the plateau phase (36 cycles). However, taken at face value, these data are consistent with the status of CFTR mRNA levels measured by Northern blotting by Singer et al. (*op. cit.)* in fully adapted killifish. It is of interest that oocytes do not show the presence of CFTR mRNA under these conditions. It might be expected that the expression of CFTR might increase with embryonic development until hatching (depending on local salinity) and it is not unreasonable that freshly harvested oocytes may not show detectable mRNA levels.

We have measured the osmotic pressure of oocyte cytoplasm by collecting oocytes by manual expression of 8 ripe female *Fundulus* netted in Northeast Creek. The oocytes were ruptured using a needle and then centrifuged at 14,000 x g for 5 min. The osmotic pressure of the supernatant was measured using a vapor pressure osmometer (Wescor, Inc.). To obtain sufficient volume to make these measurements 7 to 39 oocytes were used for each extract, the number used depending on the yield from each female. The measurements of osmotic pressure in the oocytes from individual females ranged from 390 mOsM to 440 mOsM. The mean was 415 $\pm$ 6 mOsM. This value is in reasonable agreement with the value for interocular fluid (368 $\pm$ 2.7 mOsM) in fish adapted to SW (Kidder, G.W., Bull. Mt. Desert Island Biol. Lab. 37:79. 1998). It is likely that freshly spawned oocytes resemble fairly closely those harvested from ripe females. It is also not surprising that the oocyte osmotic pressure is similar to the body fluids of the adult female. However, it is possible that oocytes could face considerable osmotic stress during later phases of development since Northern populations of *Fundulus* deposit their eggs in estuaries in algal mats or sand at the high water mark on exceptionally high spring tides (Taylor, M.H., Amer. Zool. 39:313-320, 1999). The oocytes may be exposed to prolonged periods of desiccation and to surges of freshwater and saltwater in such a location. One might predict that the developing embryos may show increasing expression of transport systems necessary for osmoregulation in hatchlings. Our preliminary data in this paper shows as a starting condition, freshly harvested oocytes do not show detectable levels of CFTR mRNA. We are, of course, interested in investigating in the future the question whether other osmoregulatory proteins are expressed in oocytes.

We are pursuing the development of a larger set of primers for markers of salinity adaptation state including the obvious selection of ion transport systems (Na/K ATPase, NHE, Na/K/2Cl) and other proteins that may be involved in regulation of these systems. We are also refining methods for real-time PCR which should provide more reliable measures of expression of factors correlated with osmoregulation in killifish oocytes and adults.

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