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INTRODUCTION

The Mount Desert Island Biological Laboratory (MDIBL) is an independent, non-profit marine and biomedical research facility and international center for comparative physiology, toxicology and marine functional genomic studies. The Laboratory is located on the north shore of Mount Desert Island, overlooking the gulf of Maine about 120 miles northeast of the Portland near the mouth of the Bay of Fundy. The island, well known for Acadia National Park, provides a variety of habitats including shallow and deep saltwater, a broad intertidal zone, saltwater and freshwater marshes, freshwater lakes and streams, forests and meadows.

The Laboratory is among the oldest cold-water research facilities in the Eastern United States, and its unique site provides an outstanding environment for studying the physiology of marine and freshwater flora and fauna. During 2009, the scientific personnel included 76 doctoral level scientists (including 63 Investigators), plus 75 students, and technical staff, representing 81 institutions in 21 US states, Canada, Croatia, Denmark, Germany, India, and The Netherlands.

HISTORY AND ORGANIZATION

MDIBL was founded in 1898 at South Harpswell, Maine by J.S. Kingsley of Tufts University. The Wild Gardens of Acadia donated its present site at Salisbury Cove, and relocation was completed in 1921. The Wild Gardens of Acadia, a land-holding group headed by George B. Dorr and John D. Rockefeller, Jr., who was instrumental in the founding of Acadia National Park.

In 1914, the Laboratory was incorporated under the laws of the State of Maine as a non-profit scientific and educational institution. Founded as a teaching laboratory, MDIBL is now a center for marine research and education that attracts investigators and students from across the U.S. and around the world. Since the pioneering work of H.W. Smith, E.K. Marshall and Roy P. Forster on various aspects of renal and osmoregulatory physiology of local fauna, the Laboratory has become known worldwide as a center for investigations in electrolyte and transport physiology, developmental biology, electrophysiology and marine molecular biology.

The Mount Desert Island Biological Laboratory is owned and operated by the Board of Trustees and Members of the Corporation; at present, there are 236 members. Officers of the Corporation - Chair, Vice-Chair, Director, Secretary, Treasurer, Clerk - and an Executive Committee are elected from among the Trustees. The Chair and Executive Committee oversee and promote long-range goals of the Laboratory. The Director, with the aid of a full-time Administrative Director, staff and a Scientific Advisory Committee is responsible for implementing the scientific, educational and public service activities of the Laboratory.
The Center for Comparative Toxicology (CCT), formerly known as the Center for Membrane Toxicity Studies (CMTS), was established at the Mount Desert Island Biological Laboratory (MDIBL) in 1985. The purpose of this Center has been to involve a group of internationally recognized investigators, who are primarily experts in mechanisms of epithelial transport, to study the biological effects of environmental pollutants on cell and membrane transport functions. The primary emphasis of this research effort has been to elucidate the mechanisms of toxicity of environmental pollutants at the cellular and molecular level, using novel aquatic models developed at this laboratory.

The focus of the research programs of the Center has broadened in the last several years from the more narrow objective of identifying the molecular targets for the effects of heavy metals (or metal compounds) on cell functions, to include the effects of a broader range of environmental toxicants (including marine toxins) and the mechanisms by which the organism takes up and eliminates a wide range of xenobiotics and environmental pollutants. However, the concept that a "membrane lesion" accounts for the cellular toxicity of many environmental toxins still remains as a paradigm.

**Research Cores:** The Center consists of two highly integrated research cores or themes consisting of:

- Signal Transduction and Ion Transport
- Xenobiotic Transport and Excretion

Investigators in the Signal Transduction and Ion Transport Core are examining the basic mechanisms concerning the cell's signaling response to changes in its external environment, particularly as related to environmental stress, heavy metal exposure, marine toxins and environmental estrogens. These signaling pathways often involve mechanisms of homeostasis of ion transport, pH and cell volume regulation. Investigators in the Core are interested in determining the fundamental mechanisms by which cells regulate their cell volume, maintain internal pH and secretory functions and how these processes are disturbed by environmental influences. Investigators in the Xenobiotic Transport and Excretion Core are examining the processes that are used by various epithelial tissues such as the liver and kidney to take up and excrete drugs and xenobiotics and other toxic compounds that enter from the environment and to study the effects of toxicants on this process. Investigators in this Core also interact with investigators working in the signal Transduction and Ion Transport Core.

**Facilities Cores:** The Center provides for five facility cores for Center investigators. These include:

- an Animal Core that is responsible for the acquisition, and maintenance of the many marine species available to investigators at this Center;
- an Instrumentation and Facilities Core that maintains the basic laboratory equipment that investigators would not otherwise be able to easily bring to the laboratory (a fully equipped cell culture and molecular biology facility, Marine DNA Sequencing Center, and an electrophysiology facility);
- a Cell Isolation, Culture and Organ Perfusion Core that provides isolated cells and tissues from marine species to Center investigators;
- an Imaging Core that maintains and operates a confocal fluorescent microscope as well as providing other imaging technology including epifluorescence and video-enhanced microscopy;
- a Bioinformatics Core that is the site of development of a national Comparative Toxicogenomics Database and webpage design. This core incorporates molecular data on marine sequences with a
highly annotated database and provides comparative information with human genes of toxicologic interest.

All Center members and pilot recipients have free access to these core facilities. Non-Center members who utilize these facilities are charged appropriate fees.

Community Outreach and Education Program: The Center's outreach program involves community education on water monitoring programs. This is directed primarily at high school and college students in the immediate area of the laboratory. However, an extensive summer research educational program includes high school students from both regional and national sites, the latter emphasizing minority student education as well as college and postdoctoral fellowship training.

Pilot Projects: The Pilot Project Program provides support for investigators who are interested in pursuing a new project related to environmental toxicology in one or more of the Center's Research Cores. The purpose of these Pilot grants is to obtain preliminary data to facilitate new grant submissions. Grants are awarded competitively and successful applicants receive up to $10,000/season.

APPLICATIONS AND FELLOWSHIPS

Research space is available for the entire summer season (June 1 - September 30) or a half-season (June 1 - July 31 or August 1 - September 30). Applications for the coming summer must be submitted by February 15th each year. Investigators are invited to use the year-round facilities at other times of the year, but such plans should include prior consultation with the MDIBL office concerning available facilities and specimen supply.

A number of fellowships and scholarships are available to research scientists, undergraduate faculty and students, and high school students. These funds may be used to cover the cost of laboratory rent, housing and supplies. Stipends are granted with many of the student awards. Applicants for fellowships for the coming summer research period are generally due in early January.
For further information on research fellowships, please contact:

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ACKNOWLEDGEMENTS

The Mount Desert Island Biological Laboratory is indebted to the National Institutes of Health and National Science Foundation and for substantial support. Funds for building renovations and new construction continue to permit the Laboratory to expand and upgrade its research and teaching facilities. Individual research projects served by the Laboratory are funded by private and government agencies, and all of these projects have benefited from the NSF and NIH grants to the Laboratory. For supporting our educational initiative, MDIBL acknowledges the National Science Foundation Research Experience for Undergraduates, Maine IDeA Network for Biomedical Research Excellence (NCRR/NIH), Cserr/Grass Foundation, Milbury Fellowship Fund, Northeast Affiliate of the American Heart Association, Cystic Fibrosis Foundation, Blum/Halsey Fellowship, Stanley Bradley Fund, Stan and Judy Fund, Adrian Hogben Fund, Bodil Schmidt-Nielsen Fellowship Fund, Maine Community Foundation, the Hearst Foundation, the Betterment Fund and many local businesses and individuals.
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My Time at MDIBL

Leon Goldstein
Emeritus Professor
Brown University

I first visited MDIBL in the summer of 1956 and was struck by the scientific atmosphere and scenic beauty. I returned in the summer of 1957 to work in the laboratory of Dr. Roy P. Forster, a collaboration which was to last 25 years. Dr. Forster had me work on a project dealing with the source of ammonia excreted by fishes. The summer was full of frustration, and I was ready to abandon the project. However, the next summer when I returned to the lab, the results of my experiments turned out to be more positive, and I found that the source of gill ammonia for excretion was central and not the gill tissue itself. The results were obtained by a combination of techniques involving the measurement of the gill blood flow and sampling blood going to and coming from the gill.

The next experiment was a collaborative effort between me, Forster and Bodil Schmidt-Nielsen on the relation of renal tubular transport of ammonia to its biosynthesis in the metamorphosing tadpole. We obtained the tadpoles in Lake Wood, an act that, had it been discovered by the park rangers, could have resulted in a fine. Interestingly, we found that the tadpole switched from ammonia to urea biosynthesis just as the tadpole was ready to metamorphose to an air breathing, semi-aquatic frog.

Following these studies Forster and I turned our attention to the African lungfish (Protopterus sp.). Our interest in the fish was due to its ability to aestivate and survive in the mud for years during dry spells. The question that we asked was how does the aestivating fish cope with ammonia if it is excreted by the gills as it is when the fish is in the water? We put the lung fish in one leg of a pantyhose and placed it in a bucket of mud obtained from Hamilton Pond across the street from the Lab. We allowed the mud to harden and kept the fish in the dried mud for about a month. We then broke open the mud and placed the fish back into water. A short period later the fish was moving and seemed no worse the wear for its ordeal. Upon sacrificing the lung fish we found that the fish had coped with the ammonia problem by converting it to urea (presumably in the liver). What was surprising was that the fish was able to recover its mobility after lying dormant for a month in the dry mud. In contrast to what happens in humans who are immobilized for long periods the lung fish appeared not to have lost a significant amount of its muscle. In view of the long periods of confined space that astronauts will undergo during travel to planets such as Mars, it is of practical interest to discover how the lung fish keeps its muscle tissue intact.

In related studies, Forster and I examined the relation between the aquatic nature of the environment and urea biosynthesis in the aquatic Australian lung fish (Neoceratodus) and the semi-aquatic, South America lungfish (Lepidosiren). We found a correlation between the lack of water in the environment and the biosynthesis of urea.

Forster and I then turned our attention to osmoregulation and urea excretion in the osmoconforming skate (Raja erinacea). We found that most of the urea was excreted by the kidneys, and that the gill was relatively impermeable to urea.

We were fortunate to obtain a piece of liver from coelacanth Latimeria chalumnae. We found that this ancient fish (thought at one time to be extinct and indirectly related to the fish giving rise to land-dwelling vertebrates) possesses the ability to synthesize both urea and trimethylamine oxide (TMAO), a compound found in elasmobranchs which along with urea is used to raise the osmotic pressure in the blood to that of salt water. Since seawater elasmobranchs such as the dogfish (Squalus acanthias) employ the same strategy, it is interesting to speculate on an evolutionary relation between the coelacanth and elasmobranches. However, each could have developed the ability of synthesize TMAO with no evolutionary link.

In a departure from studying extracellular osmoregulation, Forster and I turned our attention to intracellular osmoregulation in elasmobranchs. In a series of studies we examined the role of osmolytes (amino acids, polyols and amino compounds) in regulation of the osmotic activity of the intracellular fluid. We found that elasmobranch muscle accumulated high concentrations of these osmolytes
inside their cells. When the fishes undergo dilution of their environment a drop in osmotic pressure of the blood was seen. This resulted in an imbalance by releasing osmolytes from the intracellular fluid. Prominent among the osmolytes were the amino acids taurine and β-alanine, together accounting for the bulk of the osmolytes released by the cells. We also found that the brain cells of skates release osmolytes following dilution of the extracellular fluid. These results are similar to what has been observed in the brains of humans when dilutions of the extracellular fluid causes brain swelling and is corrected in part by release of osmolytes from the brain cells.

Pat King (a graduate student) and I continued to investigate cell volume regulation and osmolytes in fish and, in a collaborative effort with Rolf Kinne, we found taurine transport in brush border membrane vesicles of the flounder kidney. Continuing with taurine transport we studied the transport of the compound in the flounder intestine. Having established the importance of osmolytes in cell volume regulation we sought to find the mechanism of release of the osmolytes during hypotonic stress. First we determined the stimulus for the release. The question we asked was whether cell volume expansion or a drop in intracellular osmotic pressure was the stimulus. We expanded cell volume osmotically using NH₄Cl, failed to stimulate the release of osmolytes. Therefore we concluded that cell volume expansion was not the stimulus, rather a drop in intracellular osmotic pressure was responsible.

In a collaboration study with Dr. J.K. Haynes, we examined the specificity of the transport system involved in the release of osmolytes during hypotonic stress. Since we had previously established that the transport system is bidirectional, we measured the rate of uptake of a variety of osmolytes, ranging in size and charge, into skate erythrocytes. We found that the determining factors were the size and lack of net charge of the osmolytes. Therefore we concluded that cell volume expansion was not the stimulus, rather a drop in intracellular osmotic pressure was responsible.

In a study of taurine efflux and band 3 in the hagfish RBC we found that the hagfish RBC did not release osmolytes and lacked band 3. We concluded that band 3 could be involved in the osmolytes transport process. Since all vertebrates with the exception of the hagfish have RBC with band 3 (used for anion exchange), in order for band 3 to function as the osmolytes transporter it must have some unique properties. In collaboration with Dr. Mark Musch of the University of Chicago, we found that unlike human RBC where band 3 exists as monomers and dimers under normal and hypotonic conditions, in skate RBC hypotonicity induces a shift to tetramers. Furthermore we found that tyrosine kinases were used in the conversion of monomers to tetramers and that inhibition of the kinases blocked osmolytes transport. In addition we discovered that the osmolytes channel was regulated by ionic strength.

In collaboration with graduate students and Mark Musch we showed that hypotonicity induces exocytosis of the osmolyte channel in skate RBC. We found that tyrosine kinase and cell membrane surface expression regulate the osmolyte transport. Finally, we discovered that there is cycling of the channel protein between the interior of the cell and the cell membrane. The signal for endocytosis is monoubiquitin tag of the protein. It is possible that there is cycling of the channel protein between the interior and the cell membrane. Furthermore, hypotonicity could result in more channel protein at the cell surface.

This is a very brief account of my scientific activities at the MDIBL for the past 50+ years, which was made possible by grants from the National Science Foundation. I now turn to the personal side of these years. First and foremost I owe a great deal to the late Roy Forster. He was my mentor, collaborator and friend. He helped shape my career. He taught me the scientific approach to problem solving. He taught me more about physiology than I ever learned in a text. He taught me how to write scientific reports and always to ask “what is the question I am trying to answer”. Other friends I met along the way were: Tom Maren, whose wisdom and generosity I will always cherish and Frank Epstein, a stimulating intellectual and caring friend. Dave Evans has been a warm and generous friend. Finally, I have to thank my wife Barbara who has been a constant source of support.
Quantitative differences in the expression of ion transporter mRNA in the gills and intestinal tissue in ammocoetes from two populations of sea lamprey (*Petromyzon marinus*)

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With the exception of the hagfishes, all fishes studied display plasma salt concentrations different from that of their surrounding environments, forcing these animals to regulate their internal fluid and salt balance. The two populations of sea lamprey found in North America present us with a unique model to study internal salt regulation, as one population is land locked in the freshwater great lakes whilst the other moves as juveniles to sea then back to freshwater as adults. This study found that in the larval stage of these two populations there is a distinct difference in gene expression of salt transporter proteins

Modern lampreys are basal vertebrate organisms whose ancestors were the first vertebrates to successfully colonize a freshwater environment. The sea lampreys of North America (NA) are an ideal model species for osmoregulatory studies due to their four stage life cycle and the unique separation that exits between populations within the species. The anadromous sea lamprey undergo two migrations during their life cycle; i) as juveniles they migrate downstream encountering an increase in salt gradient until they reach the sea. ii) As mature adults they migrate back upstream facing a decrease in salinity gradient from seawater to freshwater. The second NA population within this species is the sea lampreys that occupy the Great Lakes, these animals are landlocked and inhabit freshwater exclusively throughout their entire life cycle. The ammocoetes from both populations are stenohaline freshwater animals that are able to regulate blood and tissue ions very efficiently while they live in a very dilute environment(1). This project aimed to examine expression of osmoregulatory transporter specifically Na⁺/K⁺-ATPase (NKA), cystic fibrosis transmembrane conductance regulator (CFTR) and Na⁺,K⁺ 2Cl⁻ cotransporter (NKCC), mRNA in gill, and intestinal tissues.

Wild-caught, ammocoete (larval) lampreys were purchased from Acme Lamprey, Harrison, Maine. Great lakes ammocoete tissues were obtained from the laboratory of Dr Michael Wilkie. Total RNA from the gills and intestines of ammocoetes (n=5 for each population) was isolated by homogenization in Tri-Reagent (Sigma, St Louis, MO). First strand cDNA was synthesized from 4µg of gill total RNA with oligo-dT using Superscript™ II RNAse H-reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). To determine the expression of gill and intestinal NKA, CFTR, and NKCC mRNA levels, quantitative real-time PCR (qRT-PCR) was performed. Non-degenerate primers were designed to amplify a product between 50–100 bp across a predicted intron–exon boundary. House keeping gene L8 was used as an internal control gene. Each sample was run in triplicate using 2 µl of 1/10 diluted original cDNA, 7.4 pmol of primers and SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl. Each primer pair’s efficiency was determined by performing a ten-fold dilution curve using plasmid cDNA. Efficiency (E) for each primer pair was calculated using the equation \( E = -1+10^{-\text{slope}} \) where “slope” was the slope of the dilution curve. Melting curve analysis ensured only one product was amplified resulting in a ΔCT, and were analyzed using the Pfaffl equation: ratio= \( \frac{E^{ΔCT\text{target}}}{E^{ΔCT\text{L8}}} \). Each Pfaffl ratio was then standardized to the average Wild gill Pfaffl ratio. Statistical significance was determined using ANOVA, significance was set at \( \alpha=0.05 \). All statistics were run using SPSS (v.11, Chicago, IL).

Gill and intestinal L8 mRNA levels did not alter within populations. L8 mRNA expression in the FW was significantly (\( P=<0.033 \)) increased in comparison to Wild gill L8. This result requires further investigation; we currently hypothesize that it may be the result of developmental differences between the populations. Our original hypothesis was that at this stage of the life cycle with both wild anadromous and Great Lake animals habituating similar freshwater environments there would be not difference in the expression of osmoregulatory genes. However, the results demonstrated that there are significant decreases in NKA (\( P= 0.001 \)) and NKCC (\( P= 0.003 \)) mRNA expression in both the gill and intestine of FW Great lakes animals in comparison to the Wild anadromous populations. CFTR was also down regulated, however the results were not significant (Fig 1).
We suggest that the significant difference in NKA & NKCC mRNA expression in the wild versus FW Great lakes animals may be due to the anadromous nature of the wild population and a possible preprogramming of the genes in preparation for migration to sea following transformation.

While these results are preliminary, they do highlight the need to further investigate the physiological differences between these two populations of Sea lamprey. Currently we are conducting experiments to determine if mRNA expression is translated into functional protein expression. Future work would involve an examination of the overall impact altered gene/protein expression has on the ability of the two populations to physiologically tolerate osmoregulatory challenges.

Supported by NSF REU site at MDIBL (DB10453391) to Salvatore Blair & MDIBL NIA and Appalachian State University URC grant to Susan L. Edwards.

PiT-like transporters are associated with inorganic phosphate transport by choroid plexus of spiny dogfish shark (Squalus acanthias)

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Cerebrospinal fluid (CSF) nourishes neurons, and its phosphate concentration affects cellular metabolic capability, calcium as well as pH. CSF has very little inherent buffering capacity to control acidity; thus, small changes in ions such as phosphate may be critical to neuronal function. We have found the tissue in the brain that controls the phosphate concentration bathing neurons.

Inappropriate increase of inorganic phosphate (Pi) (hyperphosphatemia) generally causes hypocalcemia due to excessive precipitation of calcium phosphate in soft tissues, including muscle, as well as bone and scales. This is a result of the calcium x phosphate ion product, and thus, there is no reason to doubt the possibility of a similar relationship in all vertebrates, cartilaginous skeleton or not. Excess Pi must be removed since attempts to alleviate the hypocalcemia by increasing calcium uptake or mobilization will only lead to acceleration of calcium phosphate deposition in the tissues. Therefore, even sharks, which live in an inexhaustible supply of calcium (sea water concentration is 10 mM), must mount a regulatory response to Pi surfeit. In humans, the concentration of Pi in cerebrospinal fluid (CSF) is about 0.4 mM while plasma is ~1 mM. Our measurements of inorganic ion levels in dogfish CSF indicate these properties hold for both humans and sharks. The choroid plexus (CP) secretes 70-80% of the CSF, and likely has a significant role in control of CSF [Pi] since the 33P-labelled Pi unidirectional fluxes across freshly dissected dogfish shark IVth CP revealed a flux ratio of 30:1 in the CSF-to-blood direction. The latter measurements are possible because in the dogfish shark the IVth CP is a single epithelial cell layer that forms the roof of the IVth ventricle and is large enough (~15 x 25 mm) that paired halves can be mounted in Ussing chambers. In this apparatus, as in situ, the single epithelial sheet of the IVth CP physically separates the CSF and blood compartments, given that the capillaries of this tissue are fenestrated.

The active Pi absorption from CSF was inhibited 5-fold by 10 mM arsenate, 3-fold by depolarization with 100 mM K+, 2-fold by 0.2 mM ouabain and 6-fold by Na+-free medium. Li+ stimulated transport 2-fold compared to Na+-free medium. Phosphonofomic acid (PFA, 1 mM) had no effect on active Pi transport. The ability of Li+ to substitute for Na+ on the transporter and its insensitivity to PFA are features that distinguish the PiT1 and 2 transporters from NaPi-2 type transporters. RT-PCR revealed both PiT1 and PiT2 (SLC20 family) gene expression, but no NaPiII (SLC34 family) expression, in the shark CP. PiT2 immunoreactivity was shown by immunoblot, and immunohistochemistry localized the PiT2-like transporter in the CP apical region. In conclusion, the CP actively removed Pi from the CSF. This process has transport properties consistent with a PiT-type, Na+-dependent transporter and is located in the apical region of the CP. Supported by NSF.

The effect of temperature on gill aquaporin 3 (AQP3) mRNA expression in killifish (Fundulus heteroclitus): Correlations with branchial osmotic water permeability

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The idea was tested that killifish gill cell surface membrane water channel proteins (aquaporin 3) might be responsible for water flows into and out of the gills, that increase with temperature. There are several factors that determine the level of gill water flow: these include the measurement temperature used and the temperature to which fish are acclimatized. Changes in aquaporin 3 may account for changes in gill water flow occurring due to differences in fish acclimation temperature.

Studies in eels have shown previously that the aquaporin water channel protein isoform, aquaporin 3 (AQP3) is expressed in the gill and that its mRNA and protein expression decrease markedly when eels are acclimated from freshwater to seawater. Correlated with this, at least in eels, osmotic water permeability is also higher in freshwater compared to seawater acclimated fish. It is not yet known whether AQP3 plays a role in branchial osmotic water permeability and therefore whether changes in AQP3 expression are responsible for the difference in osmotic permeability between environmental salinities. However, temperature has also been shown to affect gill osmotic water permeability, where generally higher permeability occurs at higher temperatures. The object of this study was to test the hypothesis that killifish (Fundulus heteroclitus) branchial AQP3 mRNA expression should correlate with changes in osmotic water permeability (that were determined in a previous study), if water flows through AQP3 are involved in osmotic water fluxes across the gill epithelium.

As described previously, prior to the experiment fish were acclimated to seawater at 13°C, then groups of six fish were acclimated to either 0°C or 13°C for 10 days. The animals were then sacrificed and the gills were removed by dissection, incubated in ‘RNA later’ solution (Ambion) and stored frozen at -20°C. The gill epithelial tissue was then scraped from the gill arches using a razor blade and tissue was homogenized in RNA extraction solution (Solution D) using a 1ml syringe and a 16 gauge needle. RNA was extracted as described previously. RNA concentrations were determined using a Nanodrop 2000 microvolume spectrophotometer (Thermo Scientific) and RNA levels were further normalized using RNA agarose gel electrophoretic analysis of the samples (18S and 28S rRNAs were measured). Complementary DNA (cDNA) was made from equal amounts of each RNA sample, using Superscript III reverse transcriptase (Invitrogen), by incubation at 50°C for >1hr, using 0.5 units Supersase.In RNase Inhibitor (Ambion), but otherwise according to manufacturers instructions except that initial RNA denaturation was performed at 65°C for 10 minutes. The 20µl cDNA reactions were diluted to 100ul with H2O and 5µl was then used for each QPCR assay. Samples were analyzed using a Brilliant II SYBR Green QPCR Mix and a MX4000 QPCR system (Stratagene) as per manufacturer’s instructions. PCR primers (200nM) were designed to be located across conserved gene intron-exon splice junctions based on genomic sequences from other species. This prevents the amplification of genomic DNA during PCR. One of the cDNA samples was used as a reference standard and this was diluted serially three times using a one in ten or a one in five dilution. These diluted cDNA samples were used to create a log10 relative cDNA concentration scale to measure each of the samples against. Once QPCR was performed and cycle threshold (Ct) numbers were obtained, the values of the standards were plotted using the Ct number against log10 relative cDNA concentration, a straight line was fitted to the data using linear regression. The Ct values of each unknown QPCR sample were then used to determine the relative concentration of each gene’s expression using the standard graph. Each cDNA sample was measured in triplicate in each assay. The three relative concentration values for each cDNA sample were averaged. In addition to AQP3, primers for the putative housekeeping gene, encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also designed for comparison purposes. Prior to QPCR each primer set was used to amplify its product and this was visualized on an agarose electrophoresis gel to show that only one product was made. Melting point analysis was also performed during QPCR and this showed only a single peak (with each primer set), suggesting that only one product was produced.
The results of this study show that far from there being a correlation between the increase in killifish gill osmotic water permeability (with increasing temperature) and AQP3 mRNA expression, the opposite was in fact true (Figure 1.). AQP3 mRNA expression was actually significantly (6.9-fold) higher in the cold-acclimated fish compared to the warm-acclimated fish (normalized to a relative expression level of 1). For the sake of completeness a putative housekeeping gene, GAPDH, was also measured and this gene had a somewhat elevated (+89%; but not statistically significantly higher) level of mRNA expression in warm-acclimated fish (Figure 2.). This suggests that the difference in AQP3 mRNA abundance between the groups was unlikely to be due to a systematic error in the level of total RNA or cDNA used in the QPCR assay.

On the face of it this result was interesting but puzzling. However a perusal of the literature provides a possible explanation that could still allow a role for AQP3 as a component of gill osmotic water permeability. Most of the studies concerning osmotic water permeability essentially use fish acclimated to a particular temperature but assayed for osmotic water permeability at different temperatures (due to acute temperature changes). Increases in permeability under such circumstances are probably to be expected, due to kinetic effects such as those measurable for example using a $Q_{10}$. However one study has tried to tease out, and make a distinction between, the effect of having fish-acclimated long-term to different temperatures and the effect of utilizing different acute fish incubation temperatures for the water permeability assay. As expected fish acclimated to a specific temperature, as previously found, had higher permeabilities at higher assay temperatures. However, both trout and tilapia gills had lower levels of osmotic water permeability in warm-acclimated fish (compared to cold-acclimated fish), when compared using the same assay temperature, i.e. the effect of varying the acclimation temperature of the fish was the opposite of the effect of varying the acute assay temperature used. The lower gill water permeabilities found in the study of Robertson and Hazel in warm-acclimated fish, concur with the changes in AQP3 mRNA expression found in this study. In hindsight, this suggests fish acclimated to different temperatures should be assayed at the same temperature (i.e. 0˚C acclimated-fish assayed at 0˚C etc), although as in the study of Robertson and Hazel, the assay could be repeated at each of the temperatures the fish were acclimated to (i.e. 0˚C acclimated-fish assayed at 0˚C and 13˚C; 13˚C acclimated-fish assayed at 0˚C and 13˚C).
Robertson and Hazel suggested that the lower level of osmotic water permeability of warm-acclimated fish was due to transmembrane water permeation of gill cells, and they also showed an effect on water permeability due to manipulation of the level of membrane cholesterol. However changes in the level of cholesterol affect membrane fluidity, which in turn will likely affect the function of membrane proteins. The data in the study of Robertson and Hazel is therefore also not inconsistent with the transmembrane permeation of water occurring through transmembrane proteins such as AQP3. Changes in the biochemical permeability characteristics of gill cell membranes (such as decreases in the level of AQP3 water channels present) may be made at higher temperatures to offset the effect of higher levels of water flow caused by physical (kinetic/thermal) factors.

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Nystatin permeabilization of the basolateral membrane reveals a direct stimulatory effect of C-type natriuretic peptide (CNP) and phosphodiesterase III inhibitors on apical CFTR chloride channels in rectal gland epithelial cells of the spiny dogfish (Squalus acanthias)

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Chloride-secreting epithelial cells from the shark rectal gland, in contrast to mammalian intestinal epithelial cells, can be cultured as flat sheets on filters without loss of key transport properties. By studying the electrical properties of the monolayers in Ussing chambers, detailed information about the molecular mechanism by which cardiac hormones elicit salt secretion in the shark rectal gland could be obtained. The outcome of this study may help to understand how microbial enterotoxins, by exploiting a similar mechanism, could provoke diarrheal disease in humans, and why cystic fibrosis patients are resistant to the action of these enterotoxins.

C-type natriuretic peptide (CNP) is the dominant cardiac peptide in the shark heart and is a major physiological activator of CFTR-mediated chloride secretion in the shark rectal gland (SRG)6. CNP acts by binding to a CNP-selective receptor guanylyl cyclase at the basolateral membrane of the epithelial cells (designated NPR-B) and activating cyclic GMP (cGMP) production1. Among the potential pathways by which cGMP signaling could affect the ion transporters in the SRG, activation of a specific membrane-bound isoform of cGMP-dependent protein kinase (cGK type II9-11) was initially considered as the most plausible mechanism, in view of the prominent role of this enzyme in phosphorylation and gating of the CFTR chloride channel in mammalian epithelial tissues4,11. However, our previous analysis of CNP signalling in SRGs strongly argued against the importance of cGKII in this tissue5,8.

First, our attempts to clone dogfish orthologs of cGKI or cGKII from the rectal gland using degenerate primer sets designed to bind to the most conserved sequences, i.e. the cGMP binding sites and part of the kinase domain consensus sequence, succeeded in the partial cloning of 2 different S. acanthias cGKI isoforms but failed to identify a dogfish ortholog of cGKII8. Moreover, experiments designed to detect trace amounts of cGK protein in lysates of cultured SRG epithelial cells by cGMP-or cAMP-affinity chromatography, autoradiography of autophosphorylated protein kinase, or western blotting were likewise negative8. Finally, none of the membrane-permeant and phosphodiesterase (PDE) resistant cGMP analogs tested, including 8-Br-cGMP, the cGKII-selective analog 8-pCPT-cGMP and the cGKI-selective analog 8-Br-PET-cGMP, were able to mimic CNP-induced Cl− secretion, neither in perfused rectal glands5 nor in filter-grown cultures of SRG epithelial cells8.

Additional studies suggested that CNP acts through cAMP-dependent protein kinase (PKA) rather than through cGK or protein kinase C (as suggested in a previous model7): first, the PKA inhibitor H89 and the broad spectrum kinase inhibitor staurosporine, but not the relatively selective cGK inhibitor H-8, prevented both CNP- and forskolin/cAMP-provoked Cl− secretion in SRG epithelial cells8. Moreover, neither the PKC activator PMA alone, nor the combination of 8-Br-cGMP and PMA was able to stimulate SRG Cl− secretion to a significant extent8. Furthermore, exposure of SRGs to CNP resulted in a modest but significant increase in cAMP levels7. Finally, pharmacological blockade of the cGMP-inhibitable PDE3 isoform by amrinone or milrinone fully mimicked the effect of CNP on Cl− secretion, and prevented a further increase in Cl− secretion by this agonist, both in intact glands and in cultured epithelial cells5,8. Taken together, these data provide strong functional evidence for the expression of a shark ortholog of PDE3 in SRG epithelia, and support a model in which CNP elicits Cl− secretion through cGMP inhibition of PDE3, resulting in reduced degradation of tonically produced cAMP and subsequent activation of PKA. The failure of PDE-resistant cGMP analogs to mimic CNP action can be explained by their inability to interact with PDE3, in contrast to cGMP itself.

Hypothetically, in a polarized epithelium the key transporters activated through the cGMP/PDE3/PKA pathway may differ from those targeted by the cGMP/cGKII pathway. Whereas cGKII is localized apically in close vicinity of its major target, the CFTR Cl− channel4,11, PDE3 is likely to be distributed more evenly, or
predominantly basolaterally within the SRG epithelial cells. Consequently the pool of cAMP/PKA elevated by PDE3 inhibition could be compartmentalized basolaterally, apically, or on both sides. In addition, cGMP gradients in the cell may also have opposite polarity, depending on whether the cGMP agonist has its receptors in the luminal membrane (as in case of the guanylin/GC-C/cGKII pathway) or in the basolateral membrane (CNP/NPR-B in SRGs)\(^1\).

A convenient tool to discriminate between effects of regulators on ion conductances in the basolateral membrane and the apical membrane is the nystatin permeabilization technique\(^2\). In this approach, either the apical membrane or the basolateral membrane is permeabilized for monovalent ions by the ionophore nystatin. By imposing a mucosal to serosal Cl\(^-\) or K\(^+\) gradient across the epithelium and clamping the transepithelial potential to zero, the short circuit current (Isc) becomes a reliable indicator of the activity of CFTR and K\(^+\) channels in the apical and basolateral membrane, respectively. Here we used this approach to analyze the effects of CNP and pharmacological PDE3 inhibitors on basolateral versus apical ion conductive pathways, and to compare their actions with those of cAMP agonists (VIP, forskolin). SRG tubular epithelial cells were isolated and cultured on CoStar Transwell filters for 6-22 days as previously described\(^12\). Confluent monolayers were mounted in a modified Ussing chamber and bathed with solution A containing 268 mM NaCl, 6 mM KCl, 3 mM MgCl\(_2\), 5 mM CaCl\(_2\), 20 mM NaHCO\(_3\), 350 mM urea, 5 mM glucose at pH 7.5. The chamber was kept at 20\(^\circ\)C and was continuously gassed with 95% O\(_2\)/5% CO\(_2\). The voltage clamp and data acquisition equipment was designed and constructed by W. van Driessche (Catholic University, Louvain, Belgium) and has been described in detail previously\(^3\). Following stabilization of the basal Isc, a steep mucosa-to-serosa Cl\(^-\) gradient was imposed by replacing NaCl and KCl in solution A on the serosal side by Na-glutonate and K-glutonate, respectively, with additional buffering with 0.4 mM NaH\(_2\)PO\(_4\), 0.33 mM Na\(_2\)HPO\(_4\), and 10 mM HEPES. Nystatin was added serosally (0.72 mg/ml; to measure apical Cl\(^-\) absorption) or mucosally (0.14 mg/ml; to measure basolateral K\(^+\) absorption) from a 1000-fold concentrated stock solution in DMSO followed by brief sonication. After stabilization of the Isc, test compounds were added to the serosal bath and their effect on the Isc was monitored. Data shown are Isc tracings, representative of 10-12 experiments.

![Figure 1. Stimulation of electrogenic Cl\(^-\) secretion by cGMP and cAMP agonists in filter-grown SRG epithelial cells.](image-url)

For comparison, we first verified the responsiveness of the monolayer to the secretagogues under non-permeabilized conditions at symmetrical buffer conditions (buffer A on both sides), and confirmed that the cGMP agonist CNP, the PDE3 inhibitor milrinone, and the cAMP agonist forskolin were all capable of eliciting active Cl\(^-\) secretion that was inhibitable by the NKCC inhibitor bumetanide and the K\(^+\) channel inhibitor Ba\(^{2+}\) added at the basolateral side (Figure 1).
Figure 2. Stimulation of electrogenic Cl\(^{-}\) absorption by cGMP and cAMP agonists in filter-grown SRG epithelial cells is dependent on permeabilization of the basolateral membrane by nystatin.

As shown in Figure 2, following basolateral permeabilization of the SRG cell monolayers with nystatin and after imposing a mucosal-to-serosal Cl\(^{-}\) concentration gradient, CNP provoked a large increase of an absorptive Cl\(^{-}\} current across the apical membrane that was further enhanced by the cAMP agonist forskolin but not, or only very modestly by the selective PDE3 inhibitor milrinone. In reverse, when milrinone was added first, it fully mimicked the action of CNP. Furthermore, in the absence of nystatin, (buffer only) the Isc was very small and unresponsive to the secretagogues, indicating that none of the agonists changed the paracellular current flow, i.e. affected the Cl\(^{-}\) permeability of the tight junctions (Figure 2, upper tracing).

These findings clearly demonstrate that (1) the cGMP agonist CNP and the PDE3 inhibitor milrinone are both capable of stimulating the Cl\(^{-}\) conductance of the apical membrane (but not the paracellular Cl\(^{-}\) permeability) in a non-additive fashion, supporting a common mechanism of action; (2) both agonists act through direct activation of apical Cl\(^{-}\) channels, most plausibly CFTR, in addition to their potential effects on the electrochemical driving force for Cl\(^{-}\} exit; (3) CNP-triggered cGMP signalling at the basolateral membrane is clearly capable of evoking a distal response at the apical membrane, most plausibly by inhibiting PDE3-catalyzed cAMP breakdown in the vicinity of the adenylyl cyclase(s), which are likewise positioned basolaterally. Apparently PDE3 inhibition then allows cAMP to spread out to the apical border and to trigger PKA-mediated phosphorylation and activation of CFTR, suggesting that the type 3 isoform is a major PDE accounting for cAMP compartmentation in SRG epithelial cells.

As a first step in the molecular cloning of PDE3 from SRGs, a ‘blastn’ search of shark EST sequences using the human PDE3a or PDE3b protein as the query sequence was performed and yielded several hits. Two hits appeared valid: (1) DV496524, a 628 bp EST cDNA clone from the embryo-derived cell line SAE; bp 37-627 is more similar to PDE3a than to PDE3b (63 vs. 44%), encoding amino acid 540-724. A 443 bp PCR fragment from this EST sequence was amplified using cDNA isolated from total rectal gland, from isolated epithelial cells and from cultured epithelial cells. The sequence of the PCR products was confirmed by Baseclear (Leiden, The Netherlands). (2) EE886450, a 583 bp EST from the shark rectal gland cDNA library; bp 73-522 is more similar to PDE3b than PDE3a (52% vs. 35%) and encodes amino acid 3-170. Primers were used to successfully amplify a 389 bp PCR fragment with a confirmed sequence, using cDNA isolated from total rectal gland, from isolated epithelial cells and from cultured epithelial cells.
Though preliminary, these data support the results of the functional studies and suggest that orthologs of mammalian PDE3a and/or b isoforms are expressed in SRG epithelium.

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Cholinergic and adrenergic responses of beating strips from the systemic heart of the Atlantic hagfish 
(Myxine glutinosa)

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We propose that important features of cardiac function may be clarified by charting the electrical and mechanical properties of cardiac tissues from an evolutionary sequence that includes tunicates, hagfish, lamprey, elasmobranchs, amphibians, and mammals. Here we have examined the heart of the hagfish, which has descended from one of the earliest offspring diverging from our vertebrate lineage. We found specialized atrial and ventricular tissues, as well as unexpected responses of a type that in higher vertebrates are contingent on autonomic innervations.

Extant chordate species suggest an evolutionary sequence where a primitive aneural heart (tunicate, hagfish) successively acquired parasympathetic (lamprey) and sympathetic (elasmobranchs) innervation. Yet it is also known that hagfish exhibit sympathetic vascular responses⁵ and that its myocardial cells are interdispersed with cells that contain dense-core bodies¹,⁵ and therefore are likely to release catecholamines. Here we measured isometric force and trans-gap action potentials in ventricular and atrial strips from the systemic heart of the Atlantic hagfish (Myxine glutinosa) to characterize its electrical and mechanical activity and elucidate the evolution of autonomic cardiac reflexes.

Hagfish were anesthetized (0.2% 1-phenoxoy-2-propanol in sea water) until a ventral incision could be initiated without eliciting reflex contractions. The heart was excised and cut in annular sections (<1 mm thick) in planes perpendicular to the longitudinal axis of the animal and heart. The ring-shaped sections were opened, trimmed to a diameter of 0.5-1 mm by fine dissection, and mounted in a single sucrose gap perfusion chamber with one end attached to an isometric force transducer, while the other was pulled through holes in adjustable latex membrane to facilitate electrical stimulation and measurements of attenuated action potentials across a sucrose gap. The standard superfusing solution contained: 504 mM NaCl, 8 mM KCl, 5 mM CaCl₂, 3 mM MgSO₄, 9 mM MgCl₂, 5 mM glucose, and 10 mM HEPES titrated to pH 7.4 with NaOH⁶.

The intact hearts, the separated atria and ventricles, and the mounted strips all beat spontaneously (Fig. 1). The beat interval and the duration of the action potential (APD) increased with cooling, and was ~30% faster for atrial than for ventricular tissue. Electrical stimulation of strips at frequencies higher than the inherent, demonstrated capture and refractoriness consistent with the atrial pacing observed in the intact heart. Active force development increased with stretch as passive force rose concurrently (data not shown). In spite of the low blood pressure of hagfish⁵, the maximal contractile force (~100 mN/mm²) was comparable to that of higher vertebrates. The following experiments were performed at 2-6 °C.

Figure 1. Temperature-dependence of the beat interval and the duration of the ventricular action potential (APD). The beat interval was measured in intact excised hearts, the separated atrium and ventricle (top), and ventricular strips.

Ventricular strips had trans-gap action potentials (TG) with well developed plateaus and durations that decreased by elevation of temperature (Fig. 2A, top) and during partial depolarization with K⁺ (Fig. 2B, top). Simultaneously recorded isometric twitches were maintained for the duration of the action potential and had
amplitudes that decreased when heating produced very short action potentials (Fig. 2A, bottom), but were insensitive to changes in $[K^+]_o$ (Fig. 2B, bottom) and a number of other interventions (5 vs. 10 mM $[Ca^{2+}]_o$, pacing at 10 vs. 30 beats/min, 5 μM isoproterenol, reduced $[Na^+]_o$) that did not compromise the long duration (> 0.5 s) of the ventricular action potentials. However, considering the strong force development, it is possible that this lack of responsiveness may reflect experimental conditions where the contractile filaments generally were fully activated by saturating concentrations of intracellular Ca$^{2+}$.

Figure 2. Effect of temperature (A) and $[K^+]_o$ (B) on transgap action potentials (TG, top) and isometric twitch force (bottom).

Figure 3 shows an experiment that tested this possibility. A 7 min exposure of a ventricular strip to Ca$^{2+}$-free solution with 10 mM EGTA produced a slow decline in the amplitude of the twitch force that was accompanied by a prolongation of the action potential and a marked slowing of the spontaneous rhythm of beating. Since the increase in APD allowed longer time for force development, we also plotted the rate of force development (dF/dt) and found that this parameter was more sensitive to Ca$^{2+}$-free solution than peak force. Similar differences were also seen during re-equilibration where dF/dt continued to increase after the peak force had reached a relatively constant level. These findings are consistent with the notion that many of our measurements of twitch force may reflect saturating $[Ca^{2+}]_i$ and that dF/dt under these conditions may be more sensitive to physiological interventions than the force itself.

Figure 3. Effect of Ca$^{2+}$-withdrawal on action potentials (TG, top), twitch force (middle), and the rate of force development (dF/dt, bottom). Panel A shows sample recordings during individual beats (a, b, c, d) at the times indicted on a slower time scale in panel B. The upper graph in panel B shows the time course of the changes in the beat interval and the APD.

Having surveyed major effects of ionic interventions on excitation-contraction coupling, next we examined the effects of compounds that mimic autonomic stimulation in higher vertebrates. Thus, Figure 4 illustrates an experiment where a ventricular strip was exposed to 10 μM acetylcholine in a successful attempt to simulate parasympathetic responses: the upstroke of the action potential was slowed, but its duration increased, the beat interval prolonged, and dF/dt decreased without decreasing the twitch amplitude. Of these results, it is the prolongation of the beat interval that is reminiscent of higher vertebrates where vagal stimulation causes the sino-atrial node to decrease the heart rate. Since the ventricle of the hagfish heart is also paced by the atrium (Fig. 1), we tested for cholinergic effects in strips from this tissue.

Figure 4. Effect of 10 μM acetylcholine on action potential (TG, top), force (middle), and dF/dt (bottom) of a ventricular strip. A: sample records before (Control), during (10 μM Ach), and after the drug exposure (Wash). B: Time course of the Ach-induced effects.
Figure 5 shows results from an atrial strip that was exposed to different concentrations of K⁺ (panel A) and to 10 μM carbachol (panel B). It was noticeable that the atrial action potential had a less pronounced plateau, was shorter than that of the ventricle, and showed little change in duration during partial K⁺-depolarization (Cf. Figs. 2B and 5A). However, cholinergic effects were clearly present since here carbachol also increased the duration of the beat interval and strongly suppressed dF/dt without changing peak force (Fig. 5B). In fact, dF/dt was so large in the absence of carbachol, that the twitch force showed signs of saturation early during the action potential (within ~200 ms) and appeared to be maintained after complete repolarization had occurred.

Figure 5. Effects of [K⁺]₀ (A) and 10 μM carbachol (B) on the action potential (top), twitch force (middle) and dF/dt (bottom) in an atrial strip.

Figure 6 illustrates an experiment where we tested for β-adrenergic effects by exposing a ventricular strip to 5 μM isoproterenol. While this produced no systematic variations in twitch force or APD, we found a strong and reversible suppression of KCl-induced contractures. These findings suggest that the positive inotropic effect of β-adrenergic stimulation, that in higher vertebrates is mediated, in part, by enhancement of IᵥCa, may be absent in the ventricle of the hagfish. On the other hand, the relaxation of the contracture force was similar to that found in higher vertebrates where it has been associated with suppression of the influx mode of the cardiac Na⁺-Ca²⁺ exchanger (NCX1.1)⁴. Therefore, we tested if some of the molecular determinants of β-adrenergic regulation of NCX1.1 might also be present in the hagfish.

Figure 6. Effect of 5 μM isoproterenol on the twitches (A) and contracture force (B) in a ventricular strip paced at 12 beats per minute. The contracture responses were elicited by elevating [K⁺]₀ from 8 to 512 mM for 30 sec. The responses were measured under control conditions (C) after a 3 min exposure to isoproterenol (Iso) after washing out this drug for 10 (W10) and 20 minutes (W20).

Using mRNA extracted from the hagfish heart, we sequenced 1077 bp of the cardiac NCX. Degenerate CODEHOP primers were determined from known marine species Na⁺-Ca²⁺ exchangers. Figure 7A shows the deduced AA-sequence, aligned with corresponding sequences from dog, frog, shark, and tunicate (*Ciona intestinalis*). Unlike the tunicate sequence, that from hagfish resembles the typical cardiac splice variant of higher vertebrates since it included both a putative PKA site and the stretch that in these animals is coded by exons C, D, E, and F. Irrespectively, the hagfish sequence did not include the inserts that in frog (exon X) and in shark (shark I and shark II) have been associated with specific modes of cAMP-dependent regulation of NCX.

The measurements on strips of hagfish myocardium presented here complement previous *in situ* measurements showing tolerance of the vascular system to hypoxia², sympathetic tone⁵, and responses to osmotic stress³. We found that the slow but strong force development of ventricular strips was maintained for the duration of the long lasting action potential as seen e.g. in amphibian and elasmobranch hearts. In contrast, atrial strips from hagfish had shorter action potentials and generated force at a faster rate. Considering the early divergence of the hagfish from other vertebrates, it is plausible that such specialization of atrial and ventricular tissues may be common to all vertebrates. At any rate, it is a pattern that has no counterpart in the tubular hearts of tunicates and the embryos of higher chordates. In comparison, the automaticity of the hagfish heart presented
some unique features since strips cut from all parts (frontal-, mid-, and caudal sections) of the heart all paced with an inherent frequency that was only slightly slower than in the atrium (Fig. 1) and was strongly dependent on extracellular Ca$^{2+}$ (Fig. 3). Therefore, it is possible that the hagfish heart may lack specialized pacemaker tissues (such as sino-atrial and atrio-ventricular nodes), and that the mechanism of pacing may depend critically on tidal changes in [Ca$^{2+}$]. To test this hypothesis it would be of interest to: 1) measure of rates of pacing more extensively at low temperatures (2-6 °C, Cf. Fig. 1) and at physiological pH (7.9-8.2), 2) perform fluorometric measurements of [Ca$^{2+}$], and voltage-clamp studies using enzymatically dispersed single myocardial cells from different parts of the hagfish heart, and 3) compare their mechanism of pacing to the Ca$^{2+}$-driven pacing found in mammalian embryonic hearts.

Although the hagfish heart lacks autonomic innervations, we found that isoproterenol suppressed KCl-contractions (Fig. 6), possibly by decreasing Ca$^{2+}$-influx via NCX (Fig. 7), and that cholinergic compounds slowed the frequency of beating in both ventricular (Fig. 4) and atrial strips (Fig. 5). It remains to be determined if these responses may be more pronounced at physiological pH (7.9-8.2 vs. 7.4 used in the reported experiments), are elicited by paracrine activity or humoral factors, and play a physiological role.

Figure 7. Comparison of the AA-sequences of cardiac NCX from dog, frog, shark, hagfish, and tunicate. A: Alignment of AA-sequences representing the long regulatory cytoplasmic loop that in vertebrate NCXs is found between terminal clusters of membrane-spanning α-helices. B-E: Schematic representation of functional motifs (PKA site, integrin-like motifs (β1 and β1) composed of strands a-f, α-catenin like motif composed of α-helices A-D, variably spliced exons A-F, shark inserts I and II).

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CFTR from divergent species (human, pig, *Fundulus heteroclitus*, and *Squalus acanthias*) expressed in *Xenopus* oocytes respond differently to channel inhibitors

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CFTR, the protein responsible for the human disease cystic fibrosis, is a chloride channel whose atomic structure is unknown. We studied the CFTR protein from four different species and their response to channel inhibitors to gain further insights into the structure of the channel. Our results suggest that the marked species differences observed in response to these inhibitors cannot be explained by previous mutagenesis studies.

Cystic fibrosis (CF) results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial chloride channel that is expressed in secretory and absorptive epithelia. CFTR is composed of 12 transmembrane domains (TMDs), two nucleotide binding domains (NBDs) and a cytosolic regulatory region (R region) that contains multiple sites for protein kinase A (PKA) dependent phosphorylation. Transport of ions through the helices is controlled by the NBDs. It is believed that those structures interact with ATP to form a dimer and that binding and subsequent hydrolysis of ATP regulate CFTR channel opening. Ion permeation in channels is influenced by the presence of charged amino acid side chains around the entrances of the channel pore. These residues attract oppositely charged ions from the solution, increasing their effective local concentration, while at the same time repelling ions of like charge. Functional evidence suggests that permeant anions bind to discrete sites within the CFTR channel pore. These binding sites may be involved in attracting chloride ions into the CFTR pore and in coordinating ion-ion interactions that are necessary for rapid ion movement through the pore.

Inhibitors of CFTR have been employed as tools to investigate further the role of different key amino acids in the CFTR channel pore to predict the structure of CFTR. Chloride ion binding sites within the CFTR pore may also be sites at which substances bind to occlude the pore and inhibit chloride permeation through the channel. A range of organic anions have been shown to inhibit chloride transport by such a mechanism. Among those most extensively studied are the sulfonylurea glibenclamide, the thiazolidone CFTRinh-172, and the glycine hydrazide GlyH-101. It is suspected that glibenclamide and GlyH-101 work as open channel blockers, glibenclamide from the inside and GlyH-101 from the outside, whereas CFTRinh-172 is suggested to bind at regulatory regions and does not function as an open channel blocker. However, despite numerous site specific mutagenesis studies, the location and number of those inhibitor binding sites remain unclear.

Studies comparing species have been a powerful tool to study the structure and function of CFTR. The evolutionary distance between the species and the conservation of certain motifs provide an opportunity to study structure-function relationships without site specific mutagenesis. Here we investigated the response of different CFTR species to multiple inhibitors of the channel using the *Xenopus laevis* oocyte expression system.

CFTR orthologues (human pig, killifish and shark) obtained from different labs were cloned into pcDNA3.1, whereas kfCFTR was obtained in pGEMTeasy. Expression vectors were grown up in 150ml cultures of TOP10 E. coli (Invitrogen, Carlsbad, CA) and maxipreped using Pure Yield Maxi prep Systems (Promega, Madison, Wisconsin). Full length sequence was obtained from each clone to confirm the integrity of the CFTR open reading frame. CFTR DNA was linearized with XhoI and purified by PCR purification (Qiagen, Alameda, CA). Capped cRNA was synthesized using T7 RNA polymerase and in vitro transcription (Ambion, Austin, TX). The reaction products were precipitated using lithium-chloride precipitation and tested with the Agilent Bioanalyzer system (Agilent, Santa Clara, CA).

Mature female *Xenopus laevis* (Xenopus I, Dexter, Michigan) were anesthetized in a 0.15 % cold solution of tricaine and several ovarian lobules were removed through a sterile abdominal incision per a protocol approved by the MDIBL and Yale University IACUC. The ovarian lobules were manually dissected in smaller pieces and
kept in calcium free ND96 (96mM NaCl, 1mM KCl, 1mM MgCl₂·6H₂O, 5mM HEPES (1/2 Na equilibrated to pH 7.5) (all from Sigma Chemical Co., St. Louis, MO). Oocytes were defolliculated by incubating in a 2.5mg/ml solution of type I collagenase for 2 h and subsequently treating with a hypertonic potassium phosphate solution. Mature stage V and VI oocytes were selected and stored in MBS (88mM NaCl, 1mM KCl, 2.4mM NaHCO₃, 0.82mM MgSO₄·7H₂O, 0.33mM Ca(NO₃)₂·4H₂O, 0.41mM CaCl₂·H₂O, 10mM HEPES (1/2 Na) and 1% penicillin/streptomycin equilibrated to pH 7.4 (all from Sigma Chemical Co., St. Louis, MO) at 18°C. After 12h oocytes were injected with 10ng cRNA/50nl or an equivalent volume of water and stored in MBS at 18°C. Using a two electrode voltage clamp (TEV-200, Dagan Instruments, Foster City, CA) current-voltage (I-V) curves were obtained 2-3 days after injection by clamping the voltage from -120 to +60 mV at a rate of 100mV/s. After correcting for capacitance currents, reversal potentials were determined and the conductance was calculated over a range of ±20mV. I-V ramps were taken under basal conditions and during stimulation by forskolin (10µM) and IBMX (1mM). When the stimulation reached a steady state, inhibitors were added, beginning with the smallest inhibitor concentration. When currents reached a steady state, the next higher concentration of the same inhibitor was used. IBMX and forskolin were continually perfused during the inhibitor studies. Inhibitors and concentrations perfused were: CFTRinh-172 (Sigma, Cystic Fibrosis Therapeuticals), GlyH-101 (Cystic Fibrosis Therapeuticals) and Glibenclamide (Sigma) at concentrations of 5, 10 and 20 µM. Inhibition was determined by the ratio of the conductance measured at the steady state of IBMX/forskolin stimulation and the conductance obtained at the steady state of inhibition by the specific dose of the specific inhibitor. Data were analyzed with pCLAMP software (Axon Instruments). Results are expressed as micro Siemens (µS) ± SEM. Statistical significance was determined by Student’s t test.

One hundred and five (105) oocytes were examined, including hCFTR (n=20), kfCFTR (n=25), pCFTR (n=21), sCFTR (n=15) un.injected (n=12) and water injected (n=12). hCFTR, kfCFTR, pCFTR and sCFTR had basal conductance of 11.9 ± 1.4 µS, 20.5 ± 1.9 µS, 13 ± 3.3 µS and 6.7 ± 1.3 µS, respectively. Uninjected and water injected control oocytes had a significantly lower baseline conductance of 7.2 ± 1.2 µS and 6.2 ± 0.6 µS, respectively (P < 0.05 compared with hCFTR, kfCFTR, pCFTR). After addition of 10 µM forskolin and 1mM IBMX to the perifusate, hCFTR, kfCFTR, pCFTR and sCFTR had similar steady state conductances (206 ± 21.9 µS, 218.7 ± 14.3, 160.1 ± 29.5 µS and 160.7 ± 34 µS, respectively, while conductances in uninjected and water injected oocytes did not change.

CFTRinh-172 inhibited hCFTR significantly (15.9 ± 2.9% inhibition at 5µM, 39.8 ± 3.7% at 10µM and 61.2 ± 3.2% at 20µM) (Figure 1). However, at high concentrations of the inhibitor (20 µM), kfCFTR and pCFTR were much less inhibited compared to hCFTR (p < 0.05 for kfCFTR and p < 0.001 for pCFTR. sCFTR was unresponsive to CFTRinh-172 (1.5 ± 0.2% inhibition at 5µM, 5 ± 0.8% at 10µM, 8 ± 1.4% at 20µM)(p <0.001 for each compared to hCFTR).

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Fig 1. Dose response (% inhibition) of CFTRinh-172 on different CFTR species. sCFTR was unresponsive to CFTRinh-172 (< 10% inhibition at all concentrations, p <0.001 for each compared to hCFTR). At 20 µ M kfCFTR and pCFTR were significantly less inhibited than hCFTR (p <0.05 and p <0.001 respectively).
Figure 2 illustrates the response of the different CFTR species to increasing concentrations of glibenclamide (5, 10 and 20 µM). Glibenclamide inhibited hCFTR significantly (29.7 ± 3.7% inhibition at 5 µM, 44.6 ± 1.9% at 10 µM and 61.3 ± 3.7% at 20 µM). sCFTR was highly responsive to glibenclamide (30.2 ± 4.5% inhibition at 5 µM, 40 ± 8.6% at 10 µM and 52.2 ± 7.7% at 20 µM). However, pCFTR was insensitive to glibenclamide (4.9 ± 1.7% inhibition at 5 µM, 10.2 ± 4.2% at 10 µM and 12.8 ± 4.2% at 20 µM, all p < 0.001 compared to hCFTR).

In contrast to CFTRinh172 and glibenclamide, all species were responsive to GlyH-101 at 20 µM. (hCFTR: 47.8 ± 4.7%; kCFTR: 58.1 ± 3.9%; pCFTR: 80.2 ± 3.6%; sCFTR: 70.1 ± 3.8%). Nevertheless, species differences were observed at all concentrations, with pCFTR and sCFTR showing greater inhibition (p < 0.001 compared to hCFTR).

The “charged vestibule model” is widely used to explain structure-function relationships of CFTR. According to this model, the CFTR channel pore consists of three essential elements: an outer vestibule, pointing towards the outside of the membrane, a narrow region, that represents the rate limiting area of the selectivity filter and a inner vestibule that is open towards the cytoplasm. Positive charged amino acids within the pore are thought to promote the accumulation of Cl⁻ and create a conditioned environment adjacent to the rate-limiting section of the pore. Studies using site-specific mutagenesis have proposed single positively charged residues to be essential components of the outer and inner vestibule. Site directed mutagenesis studies have suggested that CFTRinh-172 acts by binding to a regulatory region at TM6, with R347 and R352 proposed to be especially important in this interaction. Previous work employing site specific mutagenesis with glibenclamide suggested that K95 and R303 are important in binding to the inner vestibule region of the CFTR pore. GlyH101 is thought to act as an open channel blocker by binding to the outer vestibule region of the CFTR pore with positively charged residues R334, K335, R104 and R117 considered essential for this interaction. However, our analysis of all residues implicated in these studies shows that each amino acid is entirely conserved across the different species of CFTR.

We conclude that the striking species differences to inhibitors observed in our study cannot be explained by the residues implicated in mutagenesis studies. We speculate that the species differences to these inhibitors among human, killifish, pig and shark CFTR are due to charges on structurally adjacent amino acids.

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Effects of type specific phosphodiesterase inhibitors on chloride secretion in the perfused rectal gland of the dogfish shark (*Squalus acanthias*)

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In the shark, a peptide released from the heart, C-type natriuretic peptide, activates salt secretion by interacting with a cell surface receptor and increasing the content of an intracellular second messenger, the cyclic nucleotide cyclic GMP. Cyclic nucleotides levels in the cell are reduced by the action of phosphodiesterase enzymes of which there are 11 types. Using the perfused shark rectal gland, we employed type specific phosphodiesterase inhibitors to identify which type(s) may play a physiological role in the gland when cyclic GMP levels are increased by C-type natriuretic peptide.

C-type natriuretic peptide (CNP), the dominant peptide in the shark heart and the major activator of CFTR-mediated chloride (Cl-) secretion in the shark rectal gland (SRG), activates a CNP-selective receptor guanylyl cyclase (NPR-B) at the basolateral membrane of rectal gland tubular cells. The mechanism by which the resulting elevation in cyclic GMP (cGMP) activates Cl- secretion in SRGs has been poorly defined. Recent work in our laboratory suggests that endogenous cGMP inhibits a type III phosphodiesterase (PDE-3) that subsequently raises intracellular cAMP levels. Type III PDE is the only PDE isoform that inhibits the breakdown of cAMP and is itself inhibitable by cGMP.

To better define the pathway linking NPR-B-cGMP signalling to Cl- secretion in the SRG, we carried out perfusion studies in the intact rectal gland measuring chloride secretion after adding type specific PDE inhibitors to the perfusate. The PDE inhibitors that were individually perfused were seven type specific inhibitors including: vinpocetine (50 µM), a type I PDE inhibitor; EHNA (50 µM), a type II inhibitor; amrinone (50-600 µM) and cilostamide 1 µM, both type III PDE inhibitors; rolipram (20 µM), a type IV inhibitor; a PDE V type II inhibitor, and dipyridamole (20 µM), an inhibitor of type VIII and XI PDE.

Freshly excised rectal glands were perfused in vitro using methods previously described. Glands were first perfused to basal levels with shark Ringer’s for 30 min before various PDE inhibitors were added to the solution for an additional 15 min. Cl- secretion was measured at 10 min intervals for the first 30 min and at 1 min intervals thereafter. At the end of the experiments rectal glands were snap frozen in liquid nitrogen and stored at -80°C. To extract cyclic cAMP from tissues, a small portion of the gland was homogenized in a 6% TCA solution. The protein pellet was separated from the solution by centrifugation and saved for protein assay. The TCA solution was then extracted with 1,2,2-trichlorofloroethane, and the aqueous layer was saved for the cAMP assay. A cAMP EIA assay kit was used from biomedical technologies. The non-acetylated protocol was followed using an incubation period of 20 to 22 hours. The protein pellet was dissolved in sodium hydroxide overnight in a 37°C water bath. Protein concentration was determined using a Lowry type protein assay from Bio-rad (500-0111). All values are expressed as mean ± SEM.

After 30 min of basal perfusion, Cl- secretion was 155 ± 43 µEq Cl/hr/g (n=16). When vinpocetine, EHNA, rolipram, and PDE V type 2 were infused after 30 min of basal secretion, no significant rise in secretion occurred. The maximum values for secretion were vinpocetine 178 ± 33 µEq Cl/hr/g, EHNA 105 ± 76 µEq Cl/hr/g, rolipram 101 ± 27 µEq Cl/hr/g, and PDE V type II inhibitor 160 ± 79 µEq Cl/hr/g (n=3-4 per group). These results strongly suggest that PDE types I, II, IV, VII and XI do not regulate Cl- secretion through CFTR and may be absent in this tissue.

In contrast, both PDE type III specific inhibitors, amrinone and cilostamide were able to markedly stimulate Cl- secretion. Cilostamide (1 µM) stimulated to 1451 ±186 µEq Cl/hr/g (n=3). Amrinone stimulated secretion in a dose dependent manner, achieving values of 893 ± 96, 1182 ± 215, 1348 ± 211, 2110 ± 237 and 1974 ± 395 µEq Cl/hr/g at concentrations of 50,100, 200, 400 and 600 µM (all p<0.01 compared to basal...
secretion, n=3-7 per group). The combination of CNP (10 nM) + amrinone resulted in Cl⁻ secretion that was identical to CNP alone (1809 ± 49 μEqCl/h/g). Thus, the effects of a specific type III phosphodiesterase inhibitor were nearly identical to CNP and the addition of this inhibitor to CNP did not result in further Cl⁻ secretion.

Finally, to establish that cGMP inhibition of PDE III in the gland indeed elevates cellular levels of cAMP in rectal gland tissue, we measured the tissue content of cAMP after stimulation of chloride secretion by amrinone. In glands perfused under basal conditions (without PDE inhibitors), basal tissue cAMP was 1.71 ± 0.25 pmol/mg protein. During stimulation with amrinone, tissue cAMP content increased to 10.7 ± 2.41 pmol/mg protein (p<0.01 compared to basal values, n=6-7 per group).

Based on these data and previous studies from our laboratory², we conclude that CNP activates Cl⁻ secretion by stimulating endogenous cGMP that then inhibits a type III PDE. This results in a local increase of cAMP which in turn activates PKA mediated phosphorylation of apical CFTR, thus increasing transepithelial Cl⁻ secretion.

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Some regions of messenger RNAs (mRNAs) do not code for protein. We found evolutionarily conserved regions in noncoding areas (3'UTRs) of mRNAs from two genes (\textit{ppp4r}, \textit{ctbp}) that help regulate expression of these genes.

A variety of regulatory signals representing numerous mechanisms control gene expression. Among these are regulatory signals in 3'UTRs of mRNAs. One means to identify potential sites of noncoding regulatory sequences is to look for areas of nucleotide (nt) identity conserved among a wide range of organisms. We took this approach to compare 3' UTR sequences from transcripts from the dogfish shark, \textit{Squalus acanthias}, with those of homologous genes from other vertebrate classes, and identified lengthy, highly conserved 3'UTR sequences present in cartilaginous and bony fish, amphibians, birds and mammals.1 Sharks first appeared approximately 400 million years ago, and the conservation of these 3'UTR regions over such a long period suggests functional significance. Out of 600 genes examined, we identified eight with conserved sequences in the 3' UTRs. Although the search was not biased to identify any particular type of gene activity, we surprisingly found that all eight were related to control of growth or differentiation.

Preliminary evidence indicates that 3'UTRs for two of these genes affect gene expression. These are protein phosphatase 4 regulatory subunit 2 (\textit{ppp4r}) and C-terminal-binding protein 1 (\textit{ctbp}). Ppp4r modulates DNA repair and acts on centrosomal microtubule assembly. Ctbp inhibits growth factor function and is centrosome-associated during mitosis. The conserved region in the 3' UTR of shark ppp4r mRNA is a 3'-terminal 203 nt sequence 87% identical to the human sequence and including a polyadenylation [poly(A)] signal, along with a second upstream sequence 55 nt long and 92% identical to the human sequence. The conserved region in the 3' UTR of ctbp is 80 nt long, includes a poly(A) signal, and is 98% identical to the human sequence.

We cloned into the pMIR plasmid the conserved regions from the two shark genes. pMIR contains a luciferase reporter gene and a multiple cloning site allowing inserted sequences to be expressed in the 3'UTR of luciferase mRNA. The plasmid also contains the SV40 poly(A) signal. As a control for UTR length, plasmids were also constructed in which the sequences were cloned into pMIR in the reverse orientation. We separately transfected these plasmids, and control pMIR with no insert, into human embryonic kidney cells. As a control for transfection efficiency, we cotransfected in each experiment a plasmid with the same backbone as pMIR but with beta-galactosidase (b-gal) as the reporter gene and no 3' UTR insert, Assay was carried out by simultaneous measurement of luciferase and b-gal, with results expressed as the ratio of luciferase to b-gal activity. 

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<tr>
<th>Gene</th>
<th>UTR</th>
<th>Ratio reporter/transfection control</th>
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<tr>
<td>Ctbp</td>
<td>Correct orientation 3'UTR</td>
<td>3.32 ± 0.03</td>
</tr>
<tr>
<td>Ctbp</td>
<td>Inverted orientation 3'UTR</td>
<td>2.31 ± 0.06</td>
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<tr>
<td>Ppp4r</td>
<td>Correct orientation 3'UTR</td>
<td>3.11 ± 0.22</td>
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<tr>
<td>Ppp4r</td>
<td>Inverted orientation 3'UTR</td>
<td>2.45 ± 0.02</td>
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<td>No insert control</td>
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<td>2.45 ± 0.05</td>
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Inclusion of the ctbp 3' UTR in the correct orientation resulted in 44% increased expression relative to the inverted orientation. Inclusion of the ppp4r 3' UTR resulted in 27% increased expression. These results indicate that the 3'UTR regions of ctbp and ppp4r influence gene expression, possibly through enhancement of polyadenylation. Supported by NIH grants R01-RR019732, P20-RR016463, and P30-ES03828.

The use of fibrinogen, riboflavin and UVA to immobilize the LASIK flap in corneas of spiny dogfish shark (*Squalus acanthias*)

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In human corneas that have undergone LASIK, the flap normally lays very smoothly on the surface of the laser-modified stroma, but it never actually attaches to it, even several years after the surgery, thus constituting a hazard in case of physical trauma to the eye, such as contact with an automobile air bag. Work performed here on shark corneas suggests that such flaps might be glued firmly to the stroma if a solution of fibrinogen were applied to the interface between the flap and the stroma at any time following the surgery.

Laser-Assisted In Situ Keratomileus (LASIK) is a common procedure used to correct eye conditions such as nearsightedness, farsightedness and astigmatism. One liability that results from this procedure is that the permanent flap that results from cutting into and exposing the middle layer of the cornea (the stroma), forever remains non-attached to the underlying laser-modified stroma. Such a potentially loose layer represents a medical risk. To decrease the risk of re-exposure of the stroma and immobilize this LASIK flap, a protocol using fibrinogen, riboflavin and UVA light (RF+FIB+UVA) was tested for its ability to adhere the layers of the stroma resulting from LASIK surgery.

To represent the LASIK flap, a model flap was created in the isolated corneas of the Spiny dogfish shark (*Squalus acanthias*). Then, experimental and control solutions were applied between the stromal flap and underlying stroma. Protocol controls included varying the solution applied between the stromal layers to contain either riboflavin only (RF), fibrinogen only (FIB) or both (RF+FIB). Experimental corneas received RF+FIB and long wavelength (356nm) ultraviolet light (UVA). To quantitatively measure the adhesion strength, corneas were clipped to a digital force gauge and constant force was applied. The data produced by the force gauge was interpreted by computer software to record the peak tension as the stromal flap was pulled from the underlying stroma surface at a constant rate.

The experimental RF+FIB+UVA protocol generated adhesion that reached an average peak tension of 2.01 Newtons (N) while controls, such as RF only, reached an average peak tension of only 0.26N. Similarly, the current LASIK protocol, which uses no RF, FIB or UVA to seal the LASIK flap, produced an average peak tension of only 0.24N. From the data collected, the RF+FIB+UVA protocol generates an average of an 8-fold increase in adhesion strength. Further data are being collected from other controls.

These results suggest that some molecules currently in clinical trials for treating keratoconus, may also be used to immobilize the LASIK flap onto its laser-modified stroma, thus reducing risk of flap dislodgement. Thank you to all who supported and funded this work: NIH EY0000952; K-INBRE (P20-RR16475); Terry C. Johnson Cancer Center, Kansas State University; and NCRR M-INBRE (P20-RR016463).

Testing the effects of pyridoxal-5'-phosphate on riboflavin-ultraviolet-A (UVA)-induced crosslinking of the corneas of spiny dogfish sharks (*Squalus acanthias*) for the treatment of keratoconus

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A clinical protocol for treating a cornea disease is currently in trials in the U.S. and is based upon a procedure already approved in Europe. When applied to the cornea of patients with the progressive degenerative disease called “keratoconus”, it almost stops further degradation of the cornea. However, that protocol requires a 60 min exposure of the cornea to long wavelength ultraviolet light (UVA), therefore also exposing important tissues within the eye, e.g., the retina. Here, an amendment to the current RF-UVA crosslinking protocol is proposed that could allow use of reduced UVA exposure times, while still producing desired increases in corneal stroma strength, thereby reducing apoptosis (cell death) of the main connective tissue cells of the cornea, the stromal keratocytes.

An amendment to the current treatment of keratoconus by riboflavin-ultraviolet-A cross-linking (RF-UVA) is proposed to reduce ultraviolet-A irradiation time.

Current treatment entails removing the epithelium from the cornea, followed by two consecutive thirty-minute topical applications of a 0.1% riboflavin (RF) solution to the cornea. During the second topical application of RF, the cornea is irradiated with long-wavelength UV light (UVA), 365 nm. To test the effects of pyridoxal-5'-phosphate (PLP) on this procedure, corneas were isolated from spiny dogfish sharks (*Squalus acanthias*) and de-epithelialized; 1% PLP was added to the current 0.1% RF treatment solution and used in either both or just the first topical application. Strengthening effects were quantified with a digital force gauge to measure the tensile force required to pull apart 2 mm-wide strips of central cornea after treatments.

The additional application of a 1% PLP solution during both treatments blocks the crosslinking and strengthening effects of RF-UVA. However, application of PLP in only the first of the two topical applications, followed by sufficient rinsing to prevent singlet oxygen quenching by PLP, yields a 131% increase in strength over the current RF-UVA treatment.

PLP can non-enzymatically convert native primary amine groups in the cornea to carbonyl groups, providing more sites for cross-linking, thus allowing possibly shorter exposures to UVA. The ability of PLP to quench reactive oxygen species provides more evidence for the role of PLP as a biological antioxidant.

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Comparative genomics of arginine kinase from the green crab (*Carcinus maenas*)

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The organization of the single gene for arginine kinase was studied for the green crab, blue crab, rock crab and lobster. It is similar to that seen for the same gene in insects. This is consistent with a shared ancestry between insects and decapods.

The homologous phosphagen kinases from vertebrates (creatine kinase) and insects (arginine kinase) have had strikingly different evolutionary histories1,2,3. The vertebrate enzyme is encoded by at least four, duplicate loci while the insect enzyme is encoded by a single locus with multiple, alternative transcripts. In *Drosophila melanogaster* this single locus consists of one large exon that encodes the bulk of the catalytic portion of the enzyme. In other insects studied, this large exon is split by a single small intron. There are an additional five exons 5' to this that contribute to seven alternative transcripts3.

The green crab, *Carcinus maenas*, was shown previously2 to possess a single copy of a gene for arginine kinase with a single intron within the coding sequence that fell at the same position as the intron seen in some insect genes for this enzyme. If decapods and insects share similar gene organization, there should be additional exons to the 5' side of the region encoding the main catalytic portion of the decapod enzyme. This prediction was investigated using Genome Walking approaches in the green crab (*Carcinus maenas*), blue crab (*Callinectes sapidus*), Atlantic rock crab (*Cancer irroratus*) and lobster (*Homarus americanus*).

Genomic DNA was isolated from gill tissue using Genome-tips 20/G (Qiagen), checked for quality on 0.5% agarose gels, and used to prepare libraries using the GenomeWalker™ Universal Kit (Clontech) following the manufacturer's protocol. These libraries were used in two rounds of amplification using nested sets of gene specific primers and adaptor primers. The most prominent band seen on an agarose gel after the secondary PCR was either used directly for sequencing or was gel purified using a QIAEX II Gel Extraction Kit (Qiagen). Sequencing was performed by the DNA Sequencing Center at MDIBL. Sequence from the distal end of the fragment was used to design the next round of gene specific primers for the next step of the walk.

Walking libraries were prepared for each of the four decapods. Three steps were taken to the 5' side and one step to the 3' side of the *Carcinus maenas* coding sequence encompassing a total of 8 kb of genomic DNA. One step to the 5' side of the coding sequence was taken for each of the libraries for *Callinectes sapidus*, *Cancer irroratus* and *Homarus americanus*. Figure 1 is a summary of this work.

![1 kb](image)

Figure 1. Organization of *C. maenas Argk*. Filled line – coding sequence; diagonal – UTR; dashed - not sequenced.

The small leftmost exon is separated from the bulk of the gene by over 3kb. This is similar to the arrangement seen in *Drosophila melanogaster*. A similar arrangement of exons was seen for the other three decapods. Additional 5' exons are expected upon further rounds of walking. These preliminary data support the premise of a similar gene organization between insects and decapods.

This work was supported by a New Investigator Award from MDIBL, Oklahoma INBRE, and the Office of Research of the University of Tulsa.

Toward a mono-disperse CFTR protein preparation

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The CFTR ion channel regulates salt and liquid balance in tissues of fish and animals including humans. To understand how normal CFTR works and the effects of mutations in it which cause cystic fibrosis, it is necessary to determine its 3 dimensional structure. The objective of this project is to purify a crystallizable form of the protein to enable this structure determination.

CFTR was first purified from a total particulate fraction of insect cells in which the protein was expressed at high level using baculovirus ¹. This homogeneous preparation after reconstitution enabled demonstration that the polypeptide alone was sufficient to generate ATP and protein kinase A dependent chloride channel activity with properties similar to that in the apical membrane of many epithelial cells. However, the relatively large amounts of protein produced in these cells was only soluble in strong detergents and had a strong tendency to aggregate. Therefore, it was not suitable for crystallization or other biophysical studies. Similar problems have plagued other CFTR over-expression efforts. Thus far CFTR crystals have been obtained only after low level expression in mammalian cells ². However the quantities of these preparations have not been sufficient for the large scale 3D crystallization trials expected to be required with a large dynamic membrane protein like CFTR. Therefore we have returned to the insect cell system with the aim of obtaining soluble mono-disperse CFTR from a subcellular fraction after expression under variable conditions. After protein expression and subsequent membrane fractionation using sucrose gradients, we found that CFTR could be recovered from a light membrane fraction that was more amenable to detergent solubilization than other, heavier fractions. This increased solubility also occurred in detergents more compatible with protein crystallization (Fig 1.)

Consistent with this, the CFTR in the plasma membrane enveloping budded virus was also quite soluble and non-aggregating. Co-infection with one baculovirus containing human CFTR tagged with a biotin acceptor peptide, and a second with biotin ligase, enabled the purification of soluble CFTR from light membrane as well as the membrane surrounding purified viral particles utilizing immobilized avidin (Fig 2.). To test our hypothesis that this portion of the total CFTR pool in these cells may be mono-disperse, size exclusion chromatography and other analyses will be performed. These methods will be further optimized,
scaled up and extended to CFTR from shark and other species at MDIBL in the coming year. (Supported by the NIH and CFF)


Neuropeptide F is a small peptide hormone that stimulates certain neurons involved in regulating feeding behavior in a wide variety of organisms including humans. We have cloned and sequenced the NPF gene from the pond snail *Helisoma trivolvis*. This information will allow us to detect NPF expression in specific cells in the snail nervous system and to make synthetic hormone for studies on the role of the nervous system in feeding.

Neuropeptide phenylalanine (NPF) is a member of a family of peptide hormones that are found in a wide variety of species from invertebrates to mammals. NPF is known to affect feeding behavior across taxa, and recent data indicates that NPF modulates feeding behavior in the snail *Helisoma trivolvis* by regulating the central pattern generator in the buccal ganglia.

To facilitate studies on the function and expression of *Helisoma* NPF we cloned the entire NPF cDNA sequence. The initial primers used were based on gastropod NPF/NPY nucleotide sequences (*Lymnaea, Helix, and Aplysia*) encoding the mature hormones (Fig. 1). The strategy used to clone NPF cDNA in the absence of any prior sequence information is shown in Figure 2. Using unique primer sequences based on conserved gastropod NPF/NPY cDNA sequences we generated a 100 bp PCR amplicon from oligo(dT)-primed *Helisoma* brain cDNA. The sequence of the fragment was most similar to *Lymnaea* NPY in a BLASTx analysis. This partial sequence was extended using 5’- and 3’-RACE protocols.

**Figure 1.** Phylogenetic relationships of selected NPF-related peptides in the GenBank database. Synthetic oligonucleotide primers were designed from conserved gastropod NPF/NPY cDNA sequences.

5’-RACE and 3’-RACE primers were designed from the putative NPF cDNA fragment, which generated overlapping PCR amplicons of 618 and 444 bp, respectively. The contig generated by sequencing multiple 5’-RACE and 3’-RACE clones was validated by amplifying the coding region using primers from each RACE amplicon to give the 465 bp HF1R7 fragment (Fig. 2).

**Figure 2.** Strategy used to determine the full-length sequence of *Helisoma* NPF cDNA and to clone the complete coding region (F1R7).
The 855 bp full-length *Helisoma* NPF cDNA encodes a 90 amino acid precursor that contains a 39 amino acid mature hormone (Fig. 3). Mature *Helisoma* NPF is 92.3% (36/39 residues) identical to *Lymnaea* NPY, 71.8% (28/39 residues) identical to *Helix* NPF, and 64.1% (25/39 residues) identical to *Aplysia* NPY.

![Alignment of the deduced amino acid sequence of *Helisoma* NPF with NPF/NPY from three other gastropods. Asterisks denote identical residues, and colons denote similar residues. The mature neuropeptide hormones are outlined. Q-9 in the published *Lymnaea* mature NPY sequence has been corrected to E as in *Helix* and *Helisoma* NPFs.](attachment:image.png)

**Figure 3.** Alignment of the deduced amino acid sequence of *Helisoma* NPF with NPF/NPY from three other gastropods. Asterisks denote identical residues, and colons denote similar residues. The mature neuropeptide hormones are outlined. Q-9 in the published *Lymnaea* mature NPY sequence has been corrected to E as in *Helix* and *Helisoma* NPFs.

The full-length cDNA will be used to synthesize anti-sense cRNA probes to localize NPF gene expression by *in situ* hybridization, and the deduced amino acid sequence will be used to synthesize mature NPF peptide for electrophysiological studies.

We thank Christine Smith of the MDIBL Marine DNA Sequencing Center for DNA sequencing. This research was supported by Maine IDeA Network of Biomedical Research Excellence grant P20-RR016463 from NCRR, and by National Science Foundation grant RUI 0744305 to NK. SBE was an Aspirnaut scholar supported by INBRE grant P20-RR016463.

1. **Sousa, G.L.** Investigating the distribution of NPF within the alimentary system and CNS of the pond snail *H. trivolvis* and determining its impact on the buccal feeding circuitry. Thesis (B.S. in Biology), Bates College, Lewiston, ME, 2008.
Immunofluorescent localization of voltage-gated sodium channels to identify node-like structures in nerve fibers of the sand shrimp (Crangon septemspinosa)

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Voltage-gated sodium channels (Naᵥ) are necessary for nerve cell signaling and are often located in nodes, which are areas lacking myelin, the fatty tissue that insulates nerves. In this study, we localized Naᵥ in a local sand shrimp species to putative nodes on nerve axons in the posterior nervous system. This result supports the hypothesis that Naᵥ are highly conserved and that Naᵥ dense node-like regions are present on nerve cells in this species of shrimp.

Myelin is best known and characterized in vertebrates, and while many taxa of invertebrates have myelin²³, it is studied to a much lesser extent. One of the characteristics of a myelinated nerve fiber is a gap in the myelin (node). At nodes, nerve fibers are dense with organelles and transmembrane proteins, including voltage-gated sodium channels (Naᵥ)⁵. Several shrimp species have been shown to possess myelinated nerve fibers with nodes², but the presence of Naᵥ, while assumed, has never been tested.

Here we utilized immunohistochemical methods to visualize the presence of Naᵥ within the ventral nerve cord (VNC) of the Caridean shrimp Crangon septemspinosa. An antibody generated against a highly conserved region of the Naᵥ was used in this project due to its ability to label Naᵥ in other invertebrates⁴ and vertebrates⁵. Another antibody generated against acetylated α-tubulin, a component of the neuronal cytoskeleton, was used for visualization of entire nerve fibers. Using previously described methods¹, the combination of Naᵥ (1:100 dilution; Sigma-Aldrich; s6936) and acetylated α-tubulin (Santa Cruz Biotech, Inc.; sc-23950) antibodies allowed for localizing Naᵥ dense regions to single nerve fibers. Images were collected using an Olympus Fluoview 1000 confocal microscope system.

Images of the VNC revealed many dense Naᵥ-containing, node-like structures within individual intersegmental nerves (as indicated by acetylated α-tubulin labeling; Fig. 1). Such staining suggests some nerve fibers were myelinated. Peripheral nerves originating from the segmental ganglia showed similar punctate Naᵥ immunoreactivity, but also included nerve fibers that showed full (non-punctate) Naᵥ immunoreactivity, suggesting non-myelinated axons (data not shown).

This is the first preliminary evidence for the presence of Naᵥ on nerve fibers in this species, and the first use of this Naᵥ antibody in decapods for the localization of node-like structures. To validate the location of Naᵥ to nodes, immuno-transmission electron microscopy is planned.

This work was supported by the Maine IDeA Network of Biomedical Research Excellence (2-P20-RR016463; Patricia Hand, PI) to HK, MDIBL institutional funds to AC and a New Investigator Award to CW.

Neuroanatomy of a copepod, *Calanus finmarchicus*, using acetylated α-tubulin immunohistochemistry

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The nervous system of a small planktonic crustacean (copepod) was mapped using nervous system-specific fluorescent antibodies. This revealed a quantitative constancy in the pattern of nerves emerging from the central nervous system as well as several previously undetected nerves. The study paves the way to accurate neuroanatomical studies in an ecologically important animal.

*Calanus finmarchicus* is the most abundant copepod of the northern Atlantic, forming a major food source for fishes and whales. Despite its importance, little is still known about its nervous system. In order to pursue neuroanatomical studies on *C. finmarchicus*, it is necessary to first map the nervous system. In this study, we traced axon tracts and their projections into peripheral nerves using immunohistochemical techniques.

Animals were collected in the Gulf of Maine using vertical net tows, dissected to expose the nervous system, fixed in 4% paraformaldehyde in 0.1M phosphate-buffer (PB) for 2-3 hours at 5°C, rinsed at 22-25°C in 0.1M PB with 0.3% Triton X-100 (PBTX) (15 min; 6 changes), incubated at 5°C in anti-acetylated α-tubulin antibody overnight (Santa Cruz Biotechnology, catalog # sc-23950) diluted 1:25 in 10% normal donkey serum (NDS) and 0.1 M PBTX, rinsed again (22-25°C, 15 min x 6) in PBTX, incubated overnight in 1:300 donkey anti-mouse IgG secondary antibodies conjugated to Alexa 594 or Alexa 488 fluorophors (Invitrogen) in 10% NDS and 0.1M PBTX. After a final rinse (22-25°C, 15 min x 6) animals were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories) and imaged on an Olympus Fluoview 1000 confocal microscope. Using Image J software (W. Rasband), 2D x-y coordinates of different features in the images were mapped relative to the midpoint between the protocerebrum and deutocerebrum.

Quantitative analysis was made of three copepods, with qualitative confirmation from several additional preparations. The emergence of nerves from the central nervous system (CNS) was highly consistent among all three specimens. The standard deviation of nerve position averaged 20 mm, comparable to that found for identified aminergic somata in the same species. Nerve roots, their projections and the internal structures of the nervous system compared relatively well to Lowe’s results. However, it appears that one intersegmental nerve (Figure 1: “dlm1 n.”) that innervates dorsal longitudinal muscles was missed. Thus, every intersegmental nerve root supplies both ventral and dorsal longitudinal muscles. Two previously unidentified nerves emerged from the deutocerebral region and a third emerged posterior to the roots innervating the first pereiopods (P1n). This study provides a framework for precisely localizing features within copepod nervous systems, including somata and fiber tracts labeled immunohistochemically.

We thank Drs. B. Beltz and A. Christie for help with immunohistochemical procedures and Capt. A. Peterson, R/V *Indigo* (College of the Atlantic) for animal collections. Supported by NSF OCE-0451376, P. Lenz, P.I, and NSF REU site at MDIBL DBI 0453391.

1. **Hartline, D.K. and A.E. Christie** Immunohistochemical mapping of histamine, dopamine and serotonin in the central nervous system of the copepod *Calanus finmarchicus* (Crustacea; Maxillopoda; Copepoda) (in preparation)
Cofilin 1 is essential for zebrafish (Danio rerio) survival

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The dynamic actin cytoskeleton forms the scaffold of all eukaryotic cells. This structure is responsible for the cell's shape, movement, cell division and transport of molecules throughout the cell. Cofilin 1, an actin binding protein, plays a critical role in the regulation of actin dynamics in eukaryotic cells. We have determined cofilin 1 is required for normal development and survival of zebrafish.

Actin dynamics regulate a variety of key functions in eukaryotic cells such as cell motility, neuronal pathfinding, membrane dynamics, cytokinesis, cell polarity and tension. The dynamic nature of actin is controlled by the distribution of monomeric (G-actin) and filamentous actin (F-actin) in the cell. The cellular location and function of actin is primarily regulated by the proteins that bind actin. The actin-depolymerizing factor (ADF)/cofilin (AC) protein family plays a crucial role in actin regulation. Cofilin 1, an 18 kDa non-muscle AC protein isoform, preferentially binds ADP charged actin monomers. When cofilin binds along the actin polymer, it alters the helical filament twist which leads to filament severing and pointed end depolymerization. Cofilin binding to actin is inhibited by phosphorylation of serine 3 on cofilin. Several cofilin-null model organisms have been characterized including Saccharomyces cerevisiae, Schizosaccharomyces pombe, Dictyostelium discoideum, Drosophila melanogaster, Caenorhabditis elegans and Mus musculus. All of these model systems have severe defects in development that result in early death. These cofilin-null systems have led to the discovery of the key roles AC proteins play in many devastating diseases such as cancer, Alzheimer’s disease, immune diseases, and several developmental disorders. In addition previous studies demonstrated the AC protein family plays a critical role in rat proximal tubule apical microvilli microfilament breakdown in response to ischemia induced acute kidney injury.

To further investigate the role of cofilin 1 in kidney function, we acquired a mixed population of zebrafish embryos from the Zebrafish International Resource Center (ZIRC) (Eugene, Oregon) that were either wildtype (cfl1+/+) or heterozygous (cfl1+/hi3736aTg) for a mutation in the cofilin 1 gene. The affected embryos contained the hi3736aTg proviral insertion downstream of the first exon of the cofilin 1 (cfl1) gene (5). The embryos were reared in the University of Maine zebrafish facility. Unfortunately, the heterozygous mutant embryos did not show an obvious abnormal phenotype. Therefore, the cfl1+/hi3736aTg mutant zebrafish were identified using PCR technology (5). Once the zebrafish were mature, DNA extracts of fin clips were prepared. Three different primer sets were used to confirm the cfl1+/+ zebrafish. Primers were made against the hi3736aTg proviral sequence, the cofilin-hi3736aTg proviral sequence and the cofilin sequence. The identified cfl1+/hi3736aTg

Figure 1. PCR products of cfl1+/+, cfl1 +/- or cfl1 -/- zebrafish. The proviral sequence, hi3736aTg, was inserted downstream of the first cfl1 exon (5). To identify the genotypes of zebrafish spawns, we used PCR primers to the cfl1 gene sequence (lanes 3, 6, 9), to the cfl1-hi3736aTg sequence (lanes 2, 5, 8), and to the hi3736aTg sequence (lanes 1, 4, 7). Genotypes of a wild-type zebrafish (lanes 7-9), a cfl1 +/- zebrafish (lanes 4-6), and a cfl1 -/- zebrafish (lanes 1-3) were determined. The lower amplicons represent primer dimer artifacts.
zebrafish were then mated to produce a mixed embryo spawn. We observed a 3:1 ratio of wildtype to mutant phenotypes and confirmed a 1:2:1 ratio of wildtype, heterozygous and homozygous zebrafish respectively by PCR of larval DNA extracts from five day post fertilization zebrafish (Figure 1). The cofilin 1 mutant embryos from this spawn generally survived 8-12 days post fertilization (dpf). No observable developmental abnormalities were apparent until approximately 48 hours post fertilization (hpf) when pericardial edema was noted. At 72 hpf several significant abnormalities were documented. Pericardial edema increased and the heart structure was abnormal with a slow heart rate. Both head and jaw development were atypical and smaller eyes with deformed pupils were observed. In addition, the cofilin 1 mutant embryos did not develop an observable swim bladder. By 5 dpf, the cofilin 1 mutant phenotype was more pronounced with the already identified abnormalities intensified and the backs of the embryos arched (Figure 2). Future studies will investigate the role of actin in the eyes, heart, head and pronephros structures by transmission electron microscopy and confocal laser scanning microscopy to determine the role of cofilin 1 in these abnormal cofilin 1 mutant structures.

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*Authors contributed equally
Sharks, skates and rays appeared approximately 500 million years ago and have undergone very little morphological change since. Thus, studying these animals provides a snapshot into animal history. We are interested in finding the evolutionary origin of the colon, a digestive tract organ whose main function is to absorb water and prevent dehydration. We found that the skate intestine has the ability to absorb water. This is an important clue to understanding the origin of the colon and the adaptation of vertebrate animals from aquatic to terrestrial life.

Marine elasmobranchs retain high levels of urea and TMAO (trimethylamine oxide) in their body to remain slightly hyper-osmotic to their ocean environment. As a result, elasmobranchs living in seawater experience very little to no water loss due to osmosis. Water absorbed through the gills is countered by an increase in renal excretion. Thus, marine elasmobranchs do not actively drink seawater to maintain osmotic consistency as is experienced by marine teleosts. Despite this, we have histological and immunohistochemical evidence that a region of the spiral intestine in marine elasmobranchs can, in fact, absorb water. In the vertebrate digestive tract, acid mucins are associated with regions of high water absorption. High levels of acid mucins are found in the distal region of the spiral intestine, suggesting that this region may absorb water. In addition, water-selective aquaporins are expressed in the spiral intestine. Together, these data suggest that despite the unique osmoregulatory nature of elasmobranchs, they may have regions of their digestive tract that can absorb water. To test this hypothesis, we investigated the ability of the little skate, *Leucoraja erinacea*, digestive tract to absorb water by directly measuring water absorption in different regions of the digestive tract under different osmotic pressures.

To measure water absorption, digestive tracts from adult male little skates were harvested and flushed clean with running marine water followed by Elasmobranch Ringer’s solution. The stomach and spiral intestine were dissected from the digestive tract, and the intestine was further divided into proximal and distal halves. Each tissue section was filled with Elasmobranch Ringer’s solution to a pressure of 1.0 kPa, tied at each end, and measured for length (cm) and weight (mg). The tissue was kept in a stirring bath of Elasmobranch Ringer’s with an aerator. The intestine or stomach was weighed every 30 minutes for a total of 3 hours. The change in mass corresponded to the amount of water absorbed by the organ over a given time. For experiments examining the effects of osmotic pressure on water absorption, the pressure of Ringer’s inside the tissue was either maintained at 1.0 kPa or increased to 3.0 kPa. This protocol was adapted from a similar method reported on *Anguilla japonica*.

Figure 1. Water absorption in the stomach, proximal and distal spiral intestines of *L. erinacea* at 1.0 kPa. Water absorption was calculated as the change in mass per length of tissue (cm) over 3 hours.

Water absorption occurs in both the stomach and spiral intestine of *L. erinacea*. Net water absorption is linear with time and is higher in the spiral intestine than the stomach (Fig. 1). There is no significant difference in water absorption between the proximal and distal regions of the spiral intestine (Fig. 1). To determine if
water absorption in the spiral intestine is pressure-dependent, we investigated the amount of water absorbed in spiral intestines at 1.0 kPa and 3.0 kPa. Interestingly, water absorption is unaffected by an increase in pressure inside the intestine (Fig. 2). This suggests that water transport across the membrane is not due to changes in osmotic pressure, but is the result of facilitated diffusion.

Figure 2. Water absorption measured at 1.0 kPa and 3.0 kPa in the spiral intestine.

In previous studies, we demonstrated the expression of acid mucins and a water-selective aquaporin (AQP4) in the *L. erinacea* spiral intestine 6,7. From this data we hypothesized that the elasmobranch spiral intestine has the ability to absorb water. Our present findings support our hypothesis and further suggest that water absorption in the elasmobranch spiral intestine is likely facilitated by channel proteins such as aquaporins, which are specifically expressed in the intestine.

This work was supported by a MDIBL New Investigator Award and the Skidmore-Union Network, a project established with an NSF ADVANCE Partnerships for Adaptation, Implementation and Dissemination (PAID) grant to N.A.T. Support for undergraduate research came from the REU Site at MDIBL (NSF DBI-0453391) and a Sciortino Cancer Research Fellowship from Union College to A.S.

Identification of a voltage-gated sodium ion channel gene in the copepods *Calanus finmarchicus*, *Bestiolina similis*, *Undinula vulgaris*, and *Parvocalanus crassirostris*

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Nerve impulses are caused by sodium ions penetrating nerve cell membranes through special protein pores, known as voltage-gated sodium channels (Naᵥ). The shape and function of these channels is determined by their amino acid sequence. We obtained partial amino acid sequences of the Naᵥ channel in two copepods in order to better understand special properties they are thought to have, including fast action and resistance to red tide.

Nerve signals are propagated throughout the nervous system by the action of voltage-gated sodium ion channels (Naᵥ). Naᵥ kinetics contributes to the speed of nerve impulse conduction, as well as firing frequency constraints. Interestingly, some calanoid copepods have mechanoreceptors in their antennules that fire at extraordinary high frequencies, which may suggest that the kinetics of their Naᵥ differs from other invertebrates¹. Calanoid copepods are crustacean zooplankton and are key components of ocean foodwebs². In this study, we started to characterize copepod Naᵥ by obtaining partial cDNA sequences of the channel.

Total RNA was extracted from whole organisms of four species of calanoid copepod (*Calanus finmarchicus*, *Bestiolina similis*, *Parvocalanus crassirostris* and *Undinula vulgaris*) using Qiashredder and RNaseasy kits (Qiagen). After testing for quality (Bioanalyzer 2100, Agilent) and quantity (Nanodrop, Thermoscientific), total RNA (≤ 1mg) was reverse transcribed into cDNA (Quantitect Reverse Transcription kit by Qiagen). Nav are highly conserved, especially in the transmembrane regions of the α subunit (60kDa) which is flanked by two smaller β subunits (36 kDa and 33 kDa). A search of the NCBI database located only two confirmed sequences for the α-subunit for two crustaceans: *Cancer borealis* (EF089568) and the parasitic copepod, *Lepeophtheirus salmonis* (AJ812299) as of 12/10/2009. Degenerate primers (Table 1) were designed by aligning these and other full-length sequences of Naᵥ and identifying conserved areas of high homology, which typically corresponded to areas in the transmembrane regions of the protein.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
<th>Expected size</th>
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<tbody>
<tr>
<td>F2</td>
<td>AARTAYTAYTTYCARGARGGNTGG</td>
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</tr>
<tr>
<td>R4</td>
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</tr>
<tr>
<td>DF1*</td>
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</tr>
<tr>
<td>DR1*</td>
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</tr>
<tr>
<td>DF2*</td>
<td>AARATGGGNAAYTAYTTYTYYAC</td>
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</tr>
<tr>
<td>DR2*</td>
<td>ACNCKRAANACDATCATRAA</td>
<td>480bp</td>
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</tbody>
</table>

Table 1: Degenerate primers for *C. finmarchicus* (*) and other copepod Naᵥ partial sequences. For *C. finmarchicus*, DF1/DR1 were used to obtain PCR products that were then amplified using the 2nd set of primers (DF2/DR2).

Putative Naᵥ cDNA was amplified by PCR using pairs of degenerate primers tested at a range of annealing temperatures. PCR products were analyzed by agarose gel electrophoresis, bands with expected sizes were excised and DNAs were extracted (MinElute gel extraction kit, Qiagen) and prepared for sequencing. In some cases, the PCR product was cloned into a TOPO TA pCR®2.1 vector (Invitrogen, K4510-20) prior to sequencing. Sequences were then checked by BLAST analysis for identification.

Two separate sets of degenerate primers were used to successfully amplify a product in the four species. The first set (F2 and R4, Table 1) amplified a ~300 bp product in *B. similis*, *P. crassirostris* and *U. vulgaris*. In *B. similis*, a high quality sequence was obtained directly from the PCR product. The forward (207 bp) and reverse (228 bp) sequences were nearly identical (204 out of 207 bp) and the consensus sequence is shown in Fig. 1A. The other set of primers (DF1, DR1, DF2, DR2, Table 1) was used to amplify a PCR product in *C. finmarchicus*. Following cloning, the forward and reverse sequences were identical (475bp) and had an 83% identity to the *B. similis* sequence (Fig. 1). Quality sequences were not obtained from the remaining two copepods’ PCR products.

The consensus DNA sequences were translated into amino acid sequences and aligned with each other and other aNaᵥ (Fig. 2). The alignment revealed only a 4% difference in the copepod sequences (3 amino acids).
two cases (marked as ‘:’ in Fig. 2), the differences were conserved substitutions: 1) a lysine [K] was substituted for an arginine [R], and both are positively charged; 2) an isoleucine [I] was substituted for a valine [V], and both are nonpolar. The final amino acid difference was more notable, as the B. similis K residue was found to be replaced by a nonpolar methionine [M] in C. finmarchicus. Whether these amino acid differences confer a functional difference in the Na$_V$ kinetics is yet to be determined.

Next, the translated amino acid sequences were compared to α-Na$_V$ from other species (Fig. 2). The C. finmarchicus Na$_V$ amino acid sequence exhibited the highest degree of similarity to a human louse, Pediculus humanus corporis ([BAC67159.1]; identity: 86% C. finmarchicus, 90% in B. similis), while the B. similis sequence was most similar to the C. borealis Na$_V$ ([ABL10360.2]; 93% identical; 82% in C. finmarchicus). Both copepod sequences were also similar to another sequenced crustacean Na$_V$ from L. salmonis ([CAH23473.1]; 85% and 89% identity to C. finmarchicus, and B. similis, respectively). Only two amino acid substitutions were found to be non-conserved (Fig. 2).

Finally, in order to determine what portion of the α-Na$_V$ was sequenced, translated amino acid sequences were also aligned with full length Na$_V$ sequences from two insects (Blatella germanica [U73584] and Aedes aegypti [XM001657311]). From the multiple sequence alignment, we deduced that the partial sequences for the two copepod species represent an overlapping region in the channel domain II4-6. The II4 region likely confers voltage-sensitivity to the channel and is rich in R and K residues.

Future projects include designing gene-specific primers preceding and following the found segment in order to extend the known sequence in both directions. In addition, 5' and 3' RACE PCR will be used to elucidate the full length cDNA of the gene. Also, the sequence will be compared with those from animals vulnerable to red tide.

This work was funded by: REU site at MDIBL (NSF DBI-0453391) and a Denison University Battelle fellowship to Kaitlin Costello; Cades Foundation, Honolulu to Svenja Skarke; NSF OCE 0451376 to Petra Lenz; New Investigator Awards to J. Sook Chung and Caroline Wilson; and Denison University institutional funds to Caroline Wilson. The authors wish to thank Andrew Peterson, Captain R/V Indigo, College of the Atlantic.


Arginine vasotocin constricts aortic vascular smooth muscle from the dogfish shark, *Squalus acanthias*

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The brain-produced, peptide hormone vasopressin is known to produce vasoconstriction in mammalian blood vessels. We have tested the vasoactivity of arginine vasotocin (AVT; fish equivalent of vasopressin) on ventral aortic rings from the dogfish shark. The rings responded with a concentration-dependent constriction, which was not changed by removal of the endothelial layer, the site of potentially confounding vasoactive substances.

The teleost homologue of mammalian vasopressin is arginine vasotocin, and this neurohypophysial nonapeptide has been shown to play a significant role in various aspects of fish physiology, including: stress, metabolism, reproduction, cardiovascular functions and osmoregulation.\(^1\) Since we have recently generated partial sequences for two AVT receptors: V1A1\(^2\) and V1A2\(^3\), from killifish (*Fundulus heteroclitus*) gill tissue, it is appropriate to test the vasoactivity of AVT in fish vascular smooth muscle (VSM). To begin our study, we used VSM from the ventral aorta of the spiny dogfish.

Spiny dogfish were purchased from local fishermen, maintained in running seawater, and sacrificed by pithing. Ventral aortic rings were prepared, mounted in myobaths, and their tension monitored as previously described.\(^4\) Aliquots of standard AVT solutions were added to produce a cumulative range of concentrations from \(10^{-10}\) to \(10^{-6}\) M. To test for any endothelium-generated responses, paired rings were either left intact or stripped of the endothelium by gentle rubbing with roughened PE 50 tubing. Resulting changes in tension (vasoconstriction) were analyzed by repeated measures ANOVA, followed by a Bonferroni Multiple Comparisons post test, using InStat (Graphpad Software, San Diego, CA).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>(10^{-10}) M AVT</th>
<th>(10^{-9}) M AVT</th>
<th>(10^{-8}) M AVT</th>
<th>(10^{-7}) M AVT</th>
<th>(10^{-6}) M AVT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact VSM</td>
<td>5.8 ± 1.8</td>
<td>17.2 ± 5.6</td>
<td>38.4 ± 8.3(^*)</td>
<td>77.6 ± 2.1(^*)</td>
<td>100(^*)</td>
</tr>
<tr>
<td>Rubbed VSM</td>
<td>3.8 ± 1.8</td>
<td>10.6 ± 3.5</td>
<td>32.1 ± 4.5(^*)</td>
<td>75.9 ± 3.6(^*)</td>
<td>100(^*)</td>
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</table>

Table 1. Data are expressed as X ± SE % of maximal tension, at \(10^{-6}\) M AVT. N = 5. \(^*\) p < 0.01 compared with previous concentration. None of the means was different between intact and rubbed rings (paired t-test; two-tailed).

It is clear from Table 1 that AVT produces a concentration-dependent constriction of elasmobranch aortic vascular smooth muscle, and that this response is not modulated by the presence of an intact endothelium. Since the concentrations used were at least 10X that described in elasmobranch circulation (e.g., Ref. 5), it is not clear if circulating AVT is importance in maintenance of systemic blood pressure in elasmobranchs. However, it may play a role in regional blood flow distribution to specific tissues\(^1\). This work was funded by NSF IOB-0519579 to DHE.

Partial cloning of two receptors for neurohypophysial hormones from the gill of the killifish, *Fundulus heteroclitus*

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Peptide hormones from the posterior pituitary gland (specifically: arginine vasotocin and isotocin) control various aspects of cardiovascular physiology and salt and water balance in vertebrates, including fishes, where the relevant receptors are designated V1, V2 and IT. In this study, we have generated partial clones for a second AVT receptor (V1A2) and an isotocin receptor (IT). The AVT receptor is expressed in relatively high concentrations in osmoregulatory tissues like the gill and opercular epithelium, but the IT receptor is predominantly in the brain and kidney.

In fishes the neurohypophysis synthesizes and secretes two peptide hormones: arginine vasotocin (AVT) and isotocin, which are homologues of mammalian vasopressin and oxytocin, respectively. A role for AVT in fish cardiovascular control and osmoregulation is well established, and there is also some evidence that isotocin (IT) may be involved in at least osmoregulation in teleosts. AVT receptors are designated V1A1, V1A2, V1B and V2, and the isotocin receptor is designated IT. Since we were able to generate a partial sequence of V1A1 from the killifish gill last summer, it is appropriate to seek the other receptors in the same tissue.

Total RNA was extracted and cDNA was reverse transcribed as previously described. Degenerate primers were designed against highly conserved regions of the AVT and IT receptor sequences available on NCBI:

AVTRF1: 5’-CARATHGAGATHATGGTGCTGAG-3’

AVTRR1: 5’-CAACAAATAATATAGGCTAGCACGATACRAANGTCAT-3’ nested with AVTRR2: 5’-CCATGGTTTTCCGCTTTTTGTAYTDDATRRT-3’

ITRF1: 5’-GCAGATCTAGTTGCTGCCTGGTYCARGTNYT

ITRR1: 5’-GGAAGAAAAGGCAGTGTAAATCCANGGRTT nested with ITRF2: 5’-GCTGTAAAATATCTACAGATGGTGGGNATGTWYGC. With the AVT primers, we obtained a product of 550 nucleotides that was cloned (using Invitrogen’s TOPO TA kit) and sequenced (MDIBL); the IT sequence we obtained was 650 nucleotides. Both sequences were analyzed using NCBI BLAST and had a high degree of homology to V1A2 and IT receptors, respectively. Further confirmation was obtained from a maximum likelihood tree of the vertebrate AVT/vasopressin and IT/oxytocin receptors (Fig 1).

Figure 1. Maximum likelihood phylogeny of the AVT/vasopressin and isotocin/oxytocin (OT) receptor family. Numbers at nodes represent percent bootstrap values.
To examine the distribution of these two receptors among killifish tissues, we used semi-quantitative duplexing PCR. Killifish V1A2 is expressed in the greatest abundance in the brain, stomach, and kidney, with moderate expression in the gill, opercular epithelium, and intestine (Fig. 2, left panel). Isotocin receptor was expressed highly in the brain and stomach with moderate expression in the gill and opercular epithelium but only low expression in the intestine and kidney (Fig. 2, right panel). In agreement with our previous finding of high V1A1 expression in osmoregulatory tissues, these results suggest a role for AVT in regulating killifish gill functions. Further studies of the relationship between AVT/IT and gill ion regulation would be of interest. This work was funded by NSF IOB-0519579 to DHE.

Rhesus ammonia transporter proteins in freshwater goldfish (*Carassius auratus*) and koi (*Carassius carassius*)

Bradley Wilbur, Andrew Diamanduros, and James Claiborne
Department of Biology, Georgia Southern University, Statesboro, GA 30460

Ammonia is a natural waste product of animals, and is excreted directly across the gills into the environment by most fish. If ammonia levels build up too high in the animal, it will be fatal. We have begun a study of the proteins (called Rh glycoproteins) in the gill which may be the mechanism of excretion. Here, we have located three different genes in the gills of goldfish (*Carassius auratus*) and koi (*Carassius carassius*) which allow the fish to make these proteins.

Rhesus (Rh) proteins are membrane-spanning passive transport macromolecules thought to be responsible for facilitating the movement of ammonia across cell membranes in a variety of tissues including the fish gill. Early work demonstrated Rh proteins in human erythrocytes. Following initial studies on the Rh proteins found in sculpin, a saltwater species, we have begun a parallel effort on goldfish (*Carassius auratus*) and koi (*Carassius carassius*), freshwater fishes of the carp family. In order to determine the genes for these proteins, RNA extraction, reverse transcription, and PCR with primers designed against *Danio rerio* Rh proteins were used to create amplified cDNA.

Goldfish and koi were purchased locally, sacrificed by pithing, and tissues were removed and stored in RNAlater (Sigma). Gills were homogenized with a Polytron probe in a Trizol RT reagent solution (50mg/1mL). Sterile RNA isolation methods were observed, using only DEPC-treated water and cleaning all surfaces with a sodium dodecyl sulfate solution. RNA was purified using bromoanisole (Sigma), isopropanol, ethanol, and an Eppendorf cooled centrifuge. Generation of cDNA from RNA used the Reverse Transcription reaction, using Superscript III (Invitrogen), and oligo dT primers. Amplification of cDNA was carried out using PCR, including betaine for added primer specificity. Visualization of the amplicons used gel electrophoresis. Each gel used consisted of a 1% agarose in 1x TAE solution with ethidium bromide to stain DNA under UV light. Successful PCR attempts were prepared for sequencing using the ExoSap-IT reagent. Each sample was recombined with a separate upstream and downstream primer to allow for the sequencing of both 5’ and 3’ DNA strands. All samples were delivered to the Mount Desert Island Biological Laboratory sequencing center.

Gene amplification attempts across two koi and three goldfish yielded a consistent pattern of 3 PCR products (Figure 1). All RhA products were measured to be ~1000 bp in length, RhB: ~500 bp and RhC2a: ~220 bp. RNA controls were added to ensure that any PCR products were a result of cDNA created from the transcriptome and not the genomic DNA. These sequences were compared to the genome of *Danio rerio* and were verified to be the Rh genes previously described. Gene homology between the goldfish and koi species was > 95%. All Rh sequences were isolated from gill tissue. Other tissues attempted included liver, heart, intestine, and muscle, all with negative results.

With the description of three unique Rh genes expressed in the gills of goldfish and koi, it is likely that they are participating in the transfer of ammonia across the gill epithelium, as in marine sculpin or pufferfish. Future work will utilize these sequences in a quantitative PCR approach to examine the following ammonia loading. Full sequence determination will require 5’ and 3’ RACE. Funded by NSF IOB-061687 to JBC and REU site award at MDIBL (NSF DBI-0453391).

Secretion of chloride and mechanism of transport of glucose in the rectal gland of *Squalus acanthias*

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2Department of Medicine Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215
3Department of Medicine Temple University School of Medicine, Philadelphia, PA 19140

Most cells use exogenous glucose to fuel their energy requirements. This report shows that glucose supports only in part the energy requirements of the cells of the gland that maintains the salt balance of *Squalus acanthias*. It also shows that glucose gets into the cells through a specialized transport molecule.

All reported *in vitro* experiments with intact rectal glands, rectal gland slices, isolated rectal gland tubules, or isolated rectal gland cells, have used carbohydrates to supply the energy requirements of the cells. Most have used glucose as the sole energy providing substrate. Virtually all cells have in their plasma membranes transporters that allow glucose to get into the cells. Facilitative glucose transporters, glucose uniporters, allow the passive diffusion of glucose into and out of the cell down its chemical gradient. These transporters are inhibited by phloretin. Sodium-dependent glucose transporters, sodium symporters, indirectly powered by Na, K-ATPase, allow the secondary active movement of glucose into the cells. These transporters are inhibited by phloridzin. There is no knowledge of the kind of glucose transporters that must be present in the cell membranes of rectal gland cells. The present experiments were designed to study the requirement for glucose of perfused rectal glands and the type of glucose transporter that mediates the movement of glucose into the rectal gland cells.

Isolated rectal glands of *S. acanthias* were perfused through their single artery by gravity at 16°C and 40 mm Hg pressure with oxygenated shark Ringer’s solution containing 5 mM glucose, and 5 x 10^-4M theophylline, in a single pass perfusion. Venous effluent and duct fluid were collected separately from PE-90 catheters placed in the vein and duct of the gland. Collections were made every ten minutes. After thirty minutes of perfusion with theophylline, the perfusate was changed to an experimental perfusate and collections continued at ten minute intervals. In the experiments where phloretin or phloridzin were used, the glands were perfused with these agents for ten minutes before any additional experimental maneuvers. Reagents were purchased from Sigma-Aldrich (St. Louis, MO). Chloride was measured using a Buchler-Cotlove chlorhidrometer. Chloride secretion was calculated from the chloride concentration in the duct fluid, the volume of the fluid, the collection time, and the weight of the gland and expressed as µEq per gram of gland per hour. Statistical analysis was done using Student’s “t” test, or “paired t” test as applicable.

Perfusion without glucose resulted in an immediate and significant drop in the rate of chloride secretion, Figure 1. The magnitude of the fall in the secretion rate did not increase by prolonging the duration of the time of collection.

Perfusion without glucose resulted in an immediate and significant drop in the rate of chloride secretion, Figure 1. The magnitude of the fall in the secretion rate did not increase by prolonging the duration of the time of collection.

Figure 1. Effect of removing glucose from the perfusate. Data is shown as percentage of the rate of chloride secretion at time 30 minutes, when glucose was removed from the perfusate. The rate of chloride secretion fell immediately after removal of the glucose from the perfusate. Chloride secretion after that was not statistically lower. Symbols are mean ± SEM, n= 8 for controls and 7 for no glucose.

Notably, there was significant residual secretion that was observed in the absence of glucose in the
perfusate. The residual rate amounted to about 50% of the rate of secretion in the presence of glucose. This residual secretion of chloride could be almost completely inhibited by KCN, indicating that it is energy dependent and must be fueled by an endogenous substrate (data not shown).

The rate of chloride secretion depended on the concentration of glucose in the perfusate as shown in Figure 2, where only the glucose-dependent portion of chloride secretion is shown. A Lineweaver-Burk plot is given as an inset in Figure 2. From this plot, an apparent Km for glucose for the glucose-dependent secretion of chloride was calculated to be 1.1 mM that agrees well with the observed data.

Figure 2. Effect of glucose concentration in the perfusate on the secretion of chloride in the isolated perfused rectal gland of the shark. The secretion of chloride increased progressively as the concentration of glucose increased in the perfusate. The inset shows a Lineweaver-Burk plot of the data. The apparent Km for the glucose-dependent secretion of chloride is 1.1 mM.

As shown in Figure 3 Phloretin, at a concentration of $10^{-4}$M, had no effect on the secretion of chloride in the presence or absence of glucose (data for the latter is not shown).

However, phloridzin, at a concentration of $10^{-4}$M, reduced the secretion of chloride by about 30%, similar in magnitude to the reduction observed by perfusing without glucose.

Figure 3. Effect of phloretin and phloridzin on the secretion of chloride by the isolated perfused rectal gland of the shark. Phloretin, at a concentration of $10^{-4}$M had no effect on the secretion of chloride. Phloridzin, at a concentration of $10^{-4}$M, reduced the secretion of chloride by about the same amount as removal of glucose from the perfusate. Columns represent the drop in the secretion of chloride given in $\mu$Eq/h/g. The height of the column represents mean ± SEM, n= 8 for control, 7 for phloretin, 8 for phloridzin, and 11 for no glucose.

To test for the possibility that the movement of glucose into the cells is mediated by a myo-inositol carrier, that as SGLT1 is inhibited by phloridzin, glands were perfused with myo-inositol at a concentration of 2 x $10^{-2}$M. Myo-inositol had no effect on the rate of secretion of chloride. Although this suggests that glucose does not enter the cell through the myo-inositol carrier, it is possible that larger concentrations of myo-inositol are necessary to demonstrate a competition with glucose.

Rectal glands require exogenous energy fuels to fully support the stimulated secretion of chloride. Glucose appears to be sufficient to support stimulated secretion of chloride even at high rates of secretion.3 But a significant proportion of the secretion of chloride is sustained in the absence of glucose. The fuel that supports this glucose-independent component is not known. The observation that KCN reduces the secretion of chloride
in the absence of glucose suggests that there is additional fuel available to the rectal gland within its cells. Published reports indicate that rectal gland cells contain glycogen granules that presumably could be the endogenous source of fuel.\textsuperscript{2}

The use of phloretin and phloridzin illuminates the mechanism that mediates the entry of glucose into the cells. Phloretin, that inhibits the facilitated glucose uniporter, had no effect on the secretion of chloride. Therefore, it is unlikely that facilitated glucose transporters like GLUT2 mediate the entry of glucose into the cell. Phloridzin, on the other hand, reduces the secretion of chloride to about the same extent as removing glucose from the perfusate, suggesting that the movement of glucose into the cells is mediated by a sodium-dependent glucose symporter.

These experiments show that the rectal gland cells utilize glucose to fuel the secretion of chloride and the mode of entry of glucose into the rectal gland cells appears to be mediated by a sodium-dependent glucose transporter.

Molecular identification of a sodium-glucose cotransporter in the rectal gland of *S. acanthias*

Patricio Silva,1 Katherine C. Spokes,2 and Rolf Kinne.3

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2Department of Medicine Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215
3Max-Planck-Institut für molekulare Physiologie, Dortmund, Germany

Glucose cannot get into cells because it cannot cross the lipid envelope of the cells, it requires specialized molecules that can convey it into them. This report shows that the cells of the gland that maintains the salt balance of *Squalus acanthias* have a transport molecule that moves glucose into the cells.

Glucose and other sugars are hydrophilic substances that are not able to cross the lipid bilayer that envelops the cell. For these substances to enter the cell a transport system is required. There are two types of glucose transporters, glucose uniporters and sodium glucose symporters.1 The present series of experiments was designed to identify the type of transporters present in the rectal gland cells.

A rectal gland and a piece of spiral valve from a single dogfish were homogenized separately in lysis buffer (QIAGEN) using a Tekmar tissue homogenizer. The homogenate was passed through a QIAGEN shredder column. mRNA was prepared using QIAGEN Rneasy minikit, and treated with DNAase. Single strand cDNA was prepared using an Invitrogen First-Strand synthesis kit. PCR amplification was done using RedTaq ready mix (Sigma) and the primers shown in Table I. The amplified products were separated in a 2% agarose gel in TAE. The products were eluted using MinElute Gel extraction kit (QIAGEN), purified and sequenced in the MDIBL Marine sequence center.

<table>
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<th>Table I</th>
<th>Primer sequence</th>
<th>Predicted # bases</th>
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<tr>
<td></td>
<td>Right cttggtcgggtgatggaagtt</td>
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An initial PCR amplification using a pair of primers designed for sodium-glucose cotransporter (AM184079.1), Glut-2, and two additional pairs of primers for Na-K-ATPase and the Na-K-Cl cotransporter as controls, shown in Table I, resulted in a product of about the expected 200 base size for the sodium-glucose cotransporter in both the rectal gland and the intestine. Products of the expected size for Na-K-ATPase were obtained for both the rectal gland and intestine samples. Na-K-Cl cotransporter was only run for the rectal gland and yielded a product of the expected size. No products were obtained for Glut-2 either in the rectal gland or intestine samples.
Because of the positive result for the initial amplification for the sodium-glucose cotransporter, four additional pair of primers were designed to span most of the length of the known sequence. The primers used are also shown in Table I. Four bands of the expected size were obtained. They are shown in Figure 1. The bands were cut out of the agarose gel, eluted, purified and submitted for sequencing to the MDIBL Marine sequence center using the same primers. The resulting sequences were spliced together and compared with the original sequence for intestinal sodium-glucose cotransporter. The final sequence is shown in Figure 2.

Figure 1. Agarose gel showing the products of four PCR reactions using four different pairs of primers for four different portions of the SGLT1 cDNA. The primers used and labeled 1 through 4 are shown in Table I. All primers yielded products of the expected number of bases. Primers for Na-K-ATPase were used as control and also resulted in a product of the expected number of bases.
Figure 2. This figure shows the alignment of the sequence for the SGLT1 of the rectal gland obtained by combining the different sequences obtained from each of the four different products of the PCR reactions. The sequence obtained is virtually identical to the SGLT1 sequence reported from intestine of S. acanthias.

The sodium-glucose cotransporter identified in the rectal gland of S. acanthias is identical to that previously described in the intestine of the same species. They are members of the solute carrier family 5, and homologous to the sodium-glucose cotransporter 1, the high affinity type. Sodium-glucose cotransporters are inhibited by phloridzin, and phloridzin blocks the uptake of glucose, as measured by its inhibition of the capacity to sustain chloride secretion in the rectal gland of the shark. Thus, it would appear that this cotransporter is located in the basolateral membrane of the rectal gland cells if it mediates the transport of glucose into the cells. A similar location has been described for salivary and mammary glands\(^1\),\(^2\), that exhibit, like the rectal gland, sudden increases in high volume fluid secretion.

The failure to identify GLUT2 in the rectal gland of the shark may be due to various possibilities. There might be indeed no GLUT2 expressed in the rectal gland. The primers designed to amplify GLUT2 may be the wrong primers, although they were designed using the portions of GLUT2 conserved across many different species. Resolving this question may require the use of degenerate primers.

This report shows that rectal gland cells display in their cell membranes a member of the solute transport family 5, a sodium-glucose cotransporter, type 1. This cotransporter may be the molecule that mediates the movement of glucose into the rectal gland cells.


Cells maintain their volume and the intracellular composition of their fluid using a variety of mechanisms. A crucial mechanism is the sodium pump. This report shows that the composition of the sodium pump of the little skate *Leucoraja erinacea* is different from that of the related spiny dogfish *Squalus acanthias*.

Na-K-ATPase energizes directly and indirectly the movement of ions and other substances into cells. It is abundant in the cells of the rectal gland of *S. acanthias* and other elasmobranchs. The molecule has been sequenced in *S. acanthias* and other members of the shark and ray family but not skates. The present series of experiments was designed to identify the partial sequence of Na-K-ATPase in the rectal gland of the skate and compare it to the reported sequences for elasmobranchs.

A rectal gland from a single skate, and a single dogfish were homogenized separately in lysis buffer from QIAGEN using a Tekmar tissue homogenizer. The homogenate was then passed through a QIAGEN shredder column. mRNA was then prepared using QIAGEN Rneasy minikit, and treated with DNAase. Single strand cDNA was then prepared using an Invitrogen First-Strand synthesis kit. PCR amplification was then done using RedTaq ready mix from Sigma and the primers shown in Table I. The amplified products were separated in a 2% agarose gel in TAE. The products were then eluted using MinElute Gel extraction kit from QIAGEN, purified and sequenced in the MDIBL Marine DNA sequencing and analysis center.

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<td></td>
<td><strong>Right</strong> cattggctgggatgaaagtt</td>
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</tbody>
</table>

PCR primers were designed for Na-K-ATPase using previously obtained partial sequence of the alpha subunit of Na-K-ATPase of the rectal gland of *S. acanthias* (unpublished results). Primers designed for the reported sequence of NKCCT were used as controls. The primers used are shown in Table 1. The PCR amplification products are shown in Figure 1. The rectal glands of *S. acanthias* and *L. erinacea* yielded products of the expected number of bases.

Figure 1. Agarose gel showing the products of PCR amplifications for Na-K-ATPase and NKCCT. The primers used are shown in Table I. All primers yielded products of the expected number of bases. Primers for NKCCT were used as control and also resulted in a product of the expected number of bases.

The amplified products were cut out of the agarose gel, eluted and submitted for sequencing to the to the MDIBL Marine DNA sequencing and analysis center using the primers used for amplification. The sequences are shown in Figure 2. The sequence for the alpha subunit of *S. acanthias* rectal gland Na-K-ATPase proved to be identical to that of a previously published
The sequence for the alpha subunit of rectal gland of *L. erinacea* was significantly different from that of *S. acanthias* alpha subunit (AJ81093.1) and more closely resembled that of the reported sequence for *Torpedo californica* (X02810.1).

The amino acid sequence translated from the base sequence in Figure 2, is shown in Figure 3. This sequence also resembled more closely that of *T. californica* (X02810.1), than that of *S. acanthias* (AJ81093.1) and this difference was not due to a shift in the reading frame.

Partial sequence for the alpha subunit of Na-K-ATPase from the rectal gland of *L. erinacea* is reported here. The partial nucleotide sequence of the alpha subunit of *L. erinacea* proved to be closer to that of *T. californica* than *S. acanthias*. The translated amino acid sequence followed the same pattern. The difference in amino acid sequence could not be due to a shift in the reading frame because shifting the frame to the second or third positions resulted in loss of translation. Although it is unlikely that these differences are physiologically significant, this is not known.
Factors contributing to desiccation tolerance by *Fundulus heteroclitus* embryos

Robert L. Preston¹, Michaela P. Petit², Edal P. Fontaine³, Ethan M. Clement⁴ and Sirilak Ruensirikul¹

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²Arcadia University, Glenside, PA 19038
³Rockland District High School, Rockland, ME 04841
⁴Sheridan High School, Sheridan, AK
⁵Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672

Killifish spawn at the margins of estuaries and stranded embryos can develop normally in air in about 14 days. Hatching is triggered by immersion in seawater at spring tides. Aerially developing embryos may be severely desiccated but under some conditions can develop and hatch normally. We found that these embryos resist desiccation by decreasing water loss pathways (channels) in their tissues and increasing molecules (compatible solutes and stress proteins) that help prevent desiccation damage to cellular proteins.

Northern killifish, *Fundulus heteroclitus macrolepidotus*, spawn on spring tides at the margins of estuaries in intermediate salinities (about 10 ppt). The adults may live for extended periods in salinities ranging from full strength seawater (SW; 30 ppt) to freshwater (FW; <1 ppt). Embryos stranded by the ebbing tide tend to develop aerially more successfully than those embryos remaining immersed in shallow water. Aerial incubation is advantageous because of the higher concentrations of oxygen available in air as compared to water and aerially incubated embryos have greater viability and hatching success³,10. In aerially incubated embryos, flooding with seawater at spring tides after 14 days triggers hatching and it has been suggested that the relatively anoxic seawater is the signal that initiates hatching²,4.

Aerially developing embryos are exposed to desiccation stress⁷. During mid-stages of development (5-9 days, stages 28-33)¹ killifish embryos can tolerate and to a certain extent, resist severe desiccation for short periods⁹. For example, our measurements of water loss from single embryos⁹ showed that late-stage embryos (12 days, stage 36)¹ showed continuous water loss representing 80% of embryo wet weight over eight hours in 75% relative humidity (RH). On the other hand, 5 day old embryos in 75% RH lose about 40% of the embryo wet weight in one hour, and maintain relatively constant weight for about 4 additional hours, after which relatively rapid weight loss (water loss) commences⁹. This tolerance for and resistance to desiccation is reflected in dramatically increased survival of stressed mid-stage embryos as determined by hatching success compared with early stage embryos (0-2 days, stages 1-20) and late stage embryos (12-14 days, stages 36-39)⁹. Mid-stage embryos can tolerate rather severe desiccation conditions (22% RH for 2 hours) that always kill early and late stage embryos.

We proposed that several physiological factors may play a role in enhanced desiccation tolerance by mid-stage embryos: constitutive and/or induced expression of stress proteins (heat shock proteins, HSPs) especially HSP 70 and HSP 90; down regulation of aquaporins (AQPs) in mid-stage embryos (especially AQP3); and high levels of compatible solutes that may help stabilize cellular native protein structure during water loss⁹. The preliminary data presented here focus on two of these factors, AQP3 mRNA expression as a function of embryo developmental age and the possible presence of compatible solutes other than amino acids in embryo tissues⁶,10-12. We previously showed that free amino acids are found in concentrations of at least 40mM - 60mM in killifish embryos at all developmental stages and at these concentrations they are likely to be of major importance as compatible solutes⁸. However, in other animal species other compatible solutes also play a role and these experiments were conducted to determine the levels of glycerol and glucose in embryos⁶,11,12.

Killifish were collected from Northeast Creek, Mount Desert Island, ME, and held in aquaria with running natural SW (about 30 ppt). In typical control conditions, eggs and milt were expressed manually into a vial containing 10 ppt artificial seawater (Instant Ocean, Mentor, OH; ASW). The embryos were placed on filter paper moistened with 10 ppt ASW for aerial incubation at 20°C²,³ in a closed chamber whose vapor phase was in equilibrium with 10 ppt ASW. Under these conditions the embryos developed normally over 14 days and hatch after flooding with 10 ppt ASW.
For AQP3 experiments a species specific primer pair was designed from the AQP3 sequence provided by C. Cutler. These primers specify a gene segment 211 bp long (AQP3-211). RNA was isolated from the preserved embryos using RNeasy Plus Midi Kit (Qiagen, Valencia, CA). RNA concentration was analyzed spectrophotometrically (NanoDrop, Wilmington, DE). cDNA was synthesized from the isolated RNA using the Quantitech reverse transcriptase (RT) Kit (Qiagen, Valencia, CA). Real Time PCR was performed on a Stratagene MX4000 real-time PCR machine. The following reaction conditions were typically employed: 12.5 µl Stratagene SYBR Green mix, (Stratagene, La Jolla, CA) 0.75 µl forward and reverse primers, 0.38 µl ROX reference dye, 9.62 µl nuclease-free water for each reaction, which were all performed in triplicate. The thermocycling conditions were: initial denaturation at 95°C for 10 min, denaturation for 45 sec at 95°C, annealing for 30 sec at 50°C, elongation for 90 seconds at 72°C for 40 cycles followed by 30 sec at 55°C (41 cycles).

The relative expression of AQP3 mRNA in developing embryos using Fundulus heteroclitus ornithine decarboxylase as a internal reference is shown in Fig 1. Expression of AQP3 mRNA decreased in 5 day-old and 8 day-old embryos, as compared to the early stage (2 day-old) and late stage (14 day-old) embryos. This correlates well with the fact that mid-stage Fundulus embryos show a resistance to desiccation that is not seen in early-stage and late-stage embryos. The observed down-regulation of AQP3 expression in mid-stage embryos could be a possible mechanism for water retention. These data are also consistent with the data of Tingaud-Sequeira et al. that showed down regulation of AQP3 in aerially incubated embryos compared with immersed embryos.

Detection of compatible solutes - glycerol: Typical experimental conditions are described. Fifty 7-day old embryos were aerially incubated in 22.5% relative humidity (RH) in Petri dishes. The RH in the incubation chamber was maintained under the standard incubation conditions in air in equilibrium with 10 ppt ASW. Measurements were done in triplicate. The desiccated embryos all had significantly higher concentrations of glucose than the control (p < 0.05 or less; Student’s t-test). The 22.5% RH condition was significantly different from the other two desiccation conditions (p < 0.001).

Figure 1. Expression of AQP3 mRNA compared with ornithine decarboxylase in Fundulus embryos of various developmental ages. The values are mean ± SE for triplicate measurements (see methods). The values for the 2 day and 14 day old embryos are significantly different from the 5 day and 8 day old embryos (p < 0.05, Student’s t-test).

Figure 2. Apparent glucose concentration in Fundulus embryos after exposure to desiccating conditions for two hours. The control was maintained under the standard incubation conditions in air in equilibrium with 10 ppt ASW. Measurements were done in triplicate. The desiccated embryos all had significantly higher concentrations of glucose than the control (p < 0.05 or less; Student’s t-test). The 22.5% RH condition was significantly different from the other two desiccation conditions (p < 0.001).
colorimetric coupled enzyme microplate assay (EnzyChrom™ Glycerol Assay Kit; BioAssay Systems, Hayward, CA). An appropriate glycerol standard curve (0.1 to 1 mM) was employed and to compensate for endogenous inhibitors of the coupled-enzyme assay, standard addition methods were used. All assays were done in quadruplicate. The results (data not shown) indicated very low levels of glycerol were present (< 1 mM) in these experiments.

Detection of compatible solutes - glucose: Control embryos (9 days old) were incubated in air in equilibrium with 10ppt SW and these were compared with three experimental conditions in which separate groups of 20 embryos were exposed to a 22.5% RH, 43.2%RH and 75.3%RH for two hours. The embryos from each condition were homogenized and then centrifuged at 10,000 x g for 5 minutes. Triplicate measurements of each condition were made. The glucose concentration in the supernatants were measured using a ReliOn® glucometer and glucose test strips (Solartek Products Inc, Alameda, CA). A standard curve using appropriate glucose solutions was used to calibrate the instrument for use with killifish. Preliminary standard addition experiments indicated no apparent effect of biological extracts with added glucose compared glucose solutions alone. The apparent glucose concentrations ranged from 4 mM to 9 mM under the various conditions of the experiments. These apparent concentrations were calculated based on the assumption that glucose was distributed throughout the entire water volume of the embryos (including the perivitelline space). It is likely the concentration would be higher (perhaps 2 to 4 fold) if the glucose was confined to cellular compartments. Under these conditions it is possible glucose might play at least a partial role in stabilizing cellular proteins during desiccation stress. In conclusion, the data presented here are consistent with the hypotheses that the regulation of AQP3 expression and the presence of compatible solutes play roles in the survival of killifish embryos when exposed to transient desiccation stress.

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Preliminary observations on the effect of desiccation stress on heart rates in *Fundulus heteroclitus* embryos

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During spawning, killifish may deposit their fertilized eggs in areas of estuarine stream margins that expose the developing embryos to long periods of desiccation stress. Aerially developing embryos under some conditions may nonetheless develop and hatch normally. In these experiments we measure the effect of desiccation stress on embryo heart beat rates and show that killifish hearts can continue beating for 1.5 to 5 hours until stopping. In some embryos, hearts stopped for an hour or more may resume beating after the embryos are immersed in seawater.

During June and July in Maine killifish, *Fundulus heteroclitus macrolepidotus*, spawn in estuaries with fluctuating salinity and tidal heights (tidal range may be as much as 4 meters from high to low tides). Fertilized eggs are deposited at the edges of the estuary and some embryos are left exposed to air for long periods. The embryos may develop normally aerially or in shallow water for ~14 days before hatching\(^2\). In aerially incubating embryos, the chorion and perivitelline membrane are permeable to water and the embryos are susceptible to desiccation\(^5\). During desiccation stress, the concentration of ions in the blood needed to maintain correct balance for cardiac function and blood circulation is likely to be increased; therefore the *Fundulus* heart rate is likely to be affected. Earlier research in this laboratory has shown that mid-stage (4 to 10 days old) *Fundulus* embryos can survive severe hydration stress and hatch normally\(^6\). We hypothesize that cardiac function in mid-stage embryos must also tolerate desiccation stress. These preliminary experiments on five to seven day old embryos (stages 27-31)\(^1\) characterize the effect of desiccation stress on cardiac function by measuring heart rate in “unstressed” (control), stressed embryos and rehydrated embryos following desiccation stress.

Oocytes and milt were expressed manually from ripe fish (held in tanks in 30 ppt running seawater), mixed *in vitro* and incubated aerially in the relative humidity (RH) resulting in air in equilibrium with 10 ppt artificial seawater (ASW; Instant Ocean, Mentor, OH) until the embryos matured to a developmental age of five to seven days. All experiments were conducted at 20°C. Eight 25 mm Petri plates were fitted with a central plastic pedestal. A solution of either saturated NaCl, which established a 75% RH atmosphere\(^4\) at 20°C or 10 ppt ASW (control condition; RH > 98%) was placed in plates and the embryos positioned on the pedestals. The covered Petri dish was placed under a dissecting microscope and the embryo’s ventricular heart rate was recorded every 10 minutes until the heartbeat stopped. Once the heart has stopped the embryos were left in that environment for 30 minutes. The embryos were then submerged in a 10 ppt ASW. In some cases, the heart resumed beating and the heart rate was further recorded every 10 minutes.

In control condition, the heart rate of the embryos tested remained fairly constant at about 100 beats per minute for at least four hours (Fig. 1). In embryos exposed to 75% RH the hearts eventually stopped, but at different times for each embryo (Figure 2A). Based on our earlier observations, mid-stage embryos exposed to 75% RH lose up to 40% of their water in one hour, but then resist water loss for another four to five hours before the water loss continues\(^6\). Mid-stage embryos exposed to 75% RH (and lower) for two hours also show high survival rates (70% to 100%) to

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**Figure 1.** Heart rate in four *Fundulus* embryos incubated in air in equilibrium with 10 ppt ASW.
hatching at 14 days. It is expected therefore that cardiac function should be able to withstand this type of desiccation stress readily. Figure 2A shows that some embryos hearts cease beating after 1.5 to 2.5 hours of desiccation stress, and the hearts of some embryos continue for more than 5 hours. We attribute the differences to biological variability among embryos.

After immersing the embryos with stopped hearts in 10 ppt ASW, two resumed beating after 60 to 80 minutes (Figure 2B). The other two embryos did not recover and their hearts remained stopped for the 2.5 hour observation period. The surprising finding that killifish embryo hearts stopped by desiccation stress may resume beating after immersion in 10 ppt ASW suggests that these embryos may have unique tolerance to stress of this type. It is likely that the small size of the embryos (2 mm diameter) may allow for sufficient oxygen diffusion to the developing tissues and therefore the heart may not be critical for cell survival in an aerial killifish embryo. In addition, the embryo tissues very likely tolerate some local anoxia without severe damage. Killifish embryos have been shown to be tolerant of anoxia and even withstand prolonged exposure to cyanide without developmental effects. These data support the idea that Fundulus heteroclitus embryos are extremely tolerant of desiccation stress and may maintain high-level (cardiac) physiological function during severe desiccation and may be capable of recovery after rehydration.

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Chemical and molecular chaperones in the red blood cells of the spiny dogfish, *Squalus acanthius*

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Many marine elasmobranchs use organic compounds to osmoconform to their environment and some of these compounds are also known to stabilize protein folding. Our goal was to determine if cells containing these compounds also require the protective heat shock proteins to stabilize proteins during heat and osmotic stress. We have shown that heat shock proteins are induced in dogfish shark red blood cells following heat shock but only in cells without the addition of the organic chemical chaperone, trimethylamine oxide. These cells maintain cell membrane viability suggesting that the expression of heat shock proteins may be critical for elasmobranchs as ocean temperatures rise.

Osmoconforming marine vertebrates, such as elasmobranchs, keep the osmotic concentration of their internal fluids essentially the same as their environment by using organic osmolytes, such as trimethylamine oxide (TMAO) and urea to osmoconform. There is some evidence that the stress-induced production of heat shock proteins (HSPs) is suppressed by the presence of the osmolyte and chemical chaperone, TMAO in cells of the spiny dogfish shark. In this work, we were interested to know if TMAO affects the ability of dogfish red blood cells to induce molecular chaperones (e.g. HSPs) with high temperatures and osmotic stress and how the presence of TMAO and/or HSPs impacts cell viability during environmental stress. We hypothesized that TMAO would attenuate the induction of HSPs during stress but that cell viability would be compromised without the protection of HSPs.

We sampled whole blood from pithed spiny dogfish in a procedure approved by the MDIBL IACUC. Blood was washed in elasmobranch saline, resuspended at a hematocrit of 20% and stored at 4°C for ~18 h. Red blood cells were washed and incubated in saline with and without TMAO, placed in a shaking water bath at 13°C for 1 h and then subjected to either a 1 h heat shock at 25°C or a 1 h hypoosmotic shock (at 50% NaCl concentration). We sampled cells at 13°C (control), immediately (0 h) and 2, 8 and 20 h following the stress. We measured cell viability via Trypan Blue dye exclusion and HSPs with immunoblotting.

Our early results show that a 1 h acute heat shock resulted in a significant induction of HSP70 in spiny dogfish red blood cells 8 h following the stress and this induction was dramatically attenuated by TMAO (Fig. 1A). Interestingly, in red blood cells incubated with TMAO, and thus exhibiting a muted heat shock response, cell membrane viability was compromised 20 h following the stress (Fig. 1B). A hypoosmotic stress did not induce HSP70 in these cells regardless of the presence of TMAO (data not shown).

![Figure 1. Relative HSP70 band density (A) and percent cell viability (B) in red blood cells of the spiny dogfish at 13°C (control) and 0, 2, 8 and 20 h following a 1 h heat shock. An asterisk indicates a significant (p < 0.05) difference between cells incubated with (dark bars) and without (light bars) TMAO.](image-url)
From these pilot studies, we conclude that the chemical chaperone, TMAO, inhibits the induction of HSPs in elasmobranchs cells possibly by preventing heat-induced protein denaturation. Moreover, this reduced heat shock response appears to compromise cell membrane viability. Given that global sea surface temperatures are the highest they have been in 130 years, these results indicate that ocean warming may become particularly problematic for animals such as elasmobranchs, fish that thrive in narrow, relatively stable temperature ranges and use TMAO as an osmolyte. This study was supported by an MDIBL New Investigator Award and NSERC to SC and an NSERC Undergraduate Research Award to CER.

Heat shock proteins in the Atlantic hagfish, *Myxine glutinosa*

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The blood plasma of the ancient hagfish has essentially the same osmotic and ionic composition as seawater. Salts will damage cellular proteins and we sought to determine whether or not the protective heat shock proteins are expressed in hagfish cells under normal conditions and following heat and low oxygen stress. We determined that muscle tissue and red blood cells constitutively express heat shock protein 70 and 90 but we did not detect an induction of these proteins after heat shock or anoxia.

The osmoconforming hagfish are unique among vertebrates in that their plasma has essentially the same ionic composition as seawater. Given that high levels of Na⁺ and Cl⁻ severely damage cellular proteins, it is puzzling how hagfish blood cells maintain such high concentrations of these inorganic ions, particularly since the blood is not protected by the osmolyte and chemical chaperone, trimethylamine oxide (TMAO). We predicted that heat shock proteins (HSPs) may have an important protective role in hagfish cells but to date, we know almost nothing regarding the heat shock response in this ancient fish. Thus, we were interested in describing the heat shock response in hagfish and to compare this response in tissues with and without endogenous levels of TMAO (e.g. muscle and blood, respectively). We hypothesized that hagfish red blood cells would have high levels of constitutive HSPs relative to muscle tissue with moderate levels of TMAO and that both heat and anoxia stress would result in an increase in HSPs in red blood cells.

We sampled whole blood from anesthetized hagfish (*n* = 19). Blood was washed in heparinized hagfish saline, resuspended at a hematocrit of 10 - 15% and stored at 4°C for ~ 18 h. Red blood cells were incubated for 1 h at 13°C in a shaking water bath and subjected to a 1 h heat shock at 25°C or a 2 h period of anoxia (by bubbling cells with N₂ gas). Cells were sampled under control conditions and 2, 8 and 20 h following the stress. We also sampled hagfish white muscle tissue from animals taken directly from the holding tanks. We measured red blood cell membrane viability via Trypan Blue dye exclusion and HSPs with immunoblotting with commercially available HSP70 and HSP90 antibodies. All procedures were approved by the MDIBL IACUC.

Hagfish red blood cells had high constitutive levels of both HSP90 (data not shown), and HSP70 and did not appear to induce these proteins with heat (Table 1) or anoxic stress (data not shown). However, because our HSP70 antibody did not distinguish between constitutive and inducible isoforms, it may be that high constitutive levels are masking any induction of HSP70, but this possibility awaits further analysis. It is noteworthy that neither heat nor anoxia stress affected red blood cell membrane viability (data not shown). In contrast to our prediction, HSP70 levels in the muscle of hagfish were significantly higher than in the red blood cells (Table 1); an interesting finding given that hagfish muscle has appreciable levels of TMAO whereas in blood, this chemical chaperone is virtually undetectable. These preliminary data indicate that hagfish muscle and blood constitutively express HSP70 and HSP90, but red blood cells likely rely on protective mechanisms other than TMAO and HSPs to protect their proteins from potential damage by inorganic ions. This work was supported by an MDIBL NIA and NSERC to SC and an NSERC Undergraduate Research Award to DEB.

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Values are expressed mean ± SE (*n*); different letters indicate a significant (*p* < 0.05) difference (t-test).

Sea lamprey (*Petromyzon marinus*) - a unique cholestatic animal model

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The lamprey loses its bile ducts and gallbladder during the process of metamorphosis, a process that resembles biliary atresia in human infants. In contrast to patients with biliary atresia who develop progressive cholestasis and liver failure, lampreys continue to grow although it is not known whether they are cholestatic or how they accommodate the inability to secrete bile. In these initial studies, we assessed bile salt composition and excretion in adult migrating lampreys and examined the cytotoxicity of lamprey bile salts. Our results indicate that the adult lamprey is a unique cholestatic model that appears to develop novel mechanisms to adapt to the loss of the hepatic biliary tree.

Sea lamprey (*Petromyzon marinus*) is a jawless vertebrate. It is one of the most evolutionarily primitive vertebrates having evolved about 500 million years ago. The life cycle of lamprey is divided into four stages, *i.e.* larva, metamorphosis/transformation, juvenile, and adult/migratory. Lamprey larvae are similar to other vertebrates. They have an intact biliary system that drains bile into the intestine. However, during metamorphosis, the biliary tree becomes atretic, and the gallbladder disappears too. After metamorphosis, lampreys not only survive but grow to adulthood. This is strikingly different from human infants with biliary atresia who are progressively cholestatic and will not survive without a surgical by-pass (Kasai procedure) or liver transplantation. After transformation, lampreys begin a stage of feeding and rapid growth during which they parasitize other fish. Once they develop to adults after several years, they stop feeding and migrate back to rivers where the males release 3 keto-petromyzon sulfonate (3k-PZS, a C24 bile salt) as a pheromone that attracts the females. After spawning, they die. It is not known if the post-metamorphosis lampreys are in a cholestatic state and if so, how they adapt to this process and continue to grow. Our current aim in these preliminary investigations is to begin to address these questions. Here we report initial findings concerning the composition and cytotoxicity of bile salts in the liver and plasma of the migrating lampreys, as well as the ability of these adult lampreys to excrete an intravenous injection of ³H-taurocholate (³H-TCA) and brosulphathalein (BSP), a prototype organic anion.

Upstream migratory lamprey was caught in the Connecticut or Kenebunk Rivers, and maintained in 11 °C fresh water tanks (males and females were kept separated) with 12 hours light-dark cycle. Bile salts were extracted from liver and serum, and analyzed using mass spectrometry. The cytotoxicity of two major species of bile salts, 3k-PZS and petromyzonol sulfate (PZS) was determined by performing cell lysis experiment using human red blood cells as previously described. All animals were anesthetized in 0.1g/L Tricaine prior to intravenous injections, blood sampling or sacrifice. Clearance studies were performed with ~ 30 μCi ³H-TCA or 8 mg BSP injected intravenously via the caudal tail vein. The ³H-TCA injected animals were maintained in a plexiglass holding chamber as previously described for ~ 12 hours. The BSP injected animals were immediately returned to their fresh water tanks, and blood samples were collected during a 72-hour period at various time intervals. After exsanguinations by cardiac puncture, the intestine was ligated and the contents of the proximal and distal intestine were collected by inserting a cannula and irrigating the luminal contents with 1.0 ml saline. Each of the renal tubules was gavaged in a similar manner via external openings in the anal pore. Liver, intestine, kidney and muscle samples were removed and homogenized in PBS/1%Triton X-100. 50 μl aliquots were assayed for radioactivity by liquid scintillation. BSP concentration was assayed as we have previously reported.

Bile salt concentrations in the liver and serum of a male were 1.77 mM and 19 μM, respectively, whereas these two concentrations were 0.7 mM and 1.6 μM in a female, respectively. The millimolar levels of bile salts in the livers of lamprey are reminiscent of severe cholestasis in patients. In contrast, the serum bile salt levels in lamprey are substantially lower than these levels in humans or rodent models of cholestasis, where they can
reach levels of ~1 mM. Furthermore, in the male serum, the percentage of C24 and C27 bile salts were 79% and 21%, respectively. In contrast, the percentage of C24 and C27 bile salts in the liver averaged 25% and 75%, respectively. Similar ratios were also obtained in the liver of the female lamprey. These findings indicate that the liver of migrating lamprey is cholestatic, and suggest that lamprey has developed a unique mechanism to selectively retain C27 bile salts in the liver. These data also suggest that post-metamorphosis lampreys may develop a yet to be determined mechanism for efficiently eliminating bile salts from the blood.

As C24 bile salts are the major bile salt in the male serum and they are released as a pheromone, we assessed the cytotoxicity of PZS (the major bile salt in larval bile) compared to 3k-PSZ and several mammalian bile acids. As shown in Figure 1, 3 mM and 10 mM PZS lysed 10% and 110% red blood cells, respectively (by setting cells lysed by distilled water as 100%). In contrast, 10 mM 3k-PZS had no significant cytolytic effect. As controls, taurodeoxycholic acid (TDCA), taurocholic acid (TCA), and tauroursodeoxycholic acid (TUDCA) demonstrated different levels of cytolysis as previously observed. These results indicate that 3k-PZS is markedly less cytotoxic than PZS. These findings suggest that the adult lamprey has developed novel mechanisms to reduce bile salt toxicity by metabolizing PZS into 3k-PZS that it then secretes as a sex pheromone.

To determine if the liver of the post-metamorphosis cholestatic lamprey can still take up bile salts into the liver as well as excrete bile salts, we injected 3H-TCA into the caudal vein of lamprey. Scintillation counting data demonstrate that almost 100% of the injected 3H-TCA was recovered in all six injected lampreys. As shown in Figure 2, the highest concentrations of 3H-TCA were detected in serum, followed by intestine and liver, indicating that the liver can only partially remove bile salt from plasma over this time period. This is in striking difference from similar studies in the marine skate and rodents, where the majority of the labeled bile salt is removed from plasma and enriched in liver and gallbladder two hours after injection. In the current studies, substantial amounts of injected 3H-TCA were also found in the muscle, serum, liver, and tail water. The detection of 3H-TCA in the tail water indicates that lampreys may excrete bile salt via the intestine and/or kidney. However, a high concentration of 3H was detected in the intestinal washes but not in the ureter washes, suggesting that bile salts can be excreted through the intestine. It remains to be determined if the intestinal 3H-TCA is directly excreted by intestine from the blood or through a previously unrecognized minute bile duct remnant from the liver. Of note, intestinal contents were completely absent in these fasting migratory lamprey.

To further assess the ability of the adult lamprey to transport organic anions, we performed a BSP clearance experiment. As demonstrated in Figure 3, BSP was slowly removed from plasma with an initial t1/2=4.5±0.2 hr, and the second t1/2=52±19 hr (n=3). These slow rates of removal are in contrast to our early studies in both marine skate and dogfish, where much faster removal rates were observed (initial t1/2 was 12.5 minutes and the second t1/2 was ~ 2 hrs). The tissue distribution of BSP in these animals is slightly different from 3H-TCA injected animals, where the highest concentration of BSP was detected in the liver, followed by serum and intestine. Again, there were significant amounts of BSP in the washes of both proximal and distal intestine, reflecting an ability to excrete this compound.

In summary, these initial studies indicate that the adult lamprey is a unique cholestatic model that may possess novel mechanisms to maintain bile salt and organic anion homeostasis. Consistent with an earlier report...
that the bile pigment, biliverdin, is present in the distal intestine of the adult lamprey\(^4\), our findings also suggest a role for the intestine in the elimination of bile acids and a prototype organic anion (BSP). Future studies will determine whether there are specific organic anion transport systems for these substrates in the lamprey intestine as well as examine mechanisms in the adult lamprey liver that maintains cholestatic levels of bile salts without tissue injury.

**Figure 2.** Tissue distribution of \(^3\)H-TCA in adult female lampreys. Concentration (A) and % of total injected amount (B). n=4.

**Figure 3.** Plasma BSP concentration from a representative female lamprey.

These studies were supported by National Institutes of Health Grants DK34989, and DK25636 and an NIEHS new investigator award (ES03828) from MDIBL to W.L.

Blockage of arteries, e.g. by blood clots, results in acute oxygen deprivation with devastating effects on heart and brain. Here we have examined how the ion channels that generate the electrical activity of the heart and coordinate the contraction of its different parts initially respond to the loss of oxygen. Our results suggest that these responses depend on the examined animal (rat or shark), but in either case may constitute a first line of defense.

The first description of O₂-sensing by ion channels was made more than 20 years ago on rabbit carotid body cells, where K⁺ currents were reversibly reduced by 25 to 50 % by hypoxia ⁷. Such effects have been confirmed in cardiomyocytes from different animals including dog, where the transient outward K⁺ current, I_{to}, was suppressed by acute hypoxia ³, and guinea pig, where and the inward rectifier K⁺ channel, I_{K1}, was activated by prolonged (12 minutes) inhibition of the respiratory chain by cyanide ¹⁰.

Similarly, hypoxia has been shown to suppress Ca²⁺ currents, I_{Ca}, in carotid body and vascular smooth muscle cells ⁶,⁸ and the acute effect has in humans been linked, by mutation analysis, to the C-terminal of the α₁c subunit of the cardiac L-type Ca²⁺ channel ⁴,⁵.

Here we compared the effects of acute hypoxia on K⁺ currents in ventricular cardiomyocytes from rat and shark, which differ with respect the complements of expressed K⁺ currents, the shape and duration of the cardiac action potential, and metabolic rate. The I_{to} was found only in the rat myocytes where it was strongly suppressed by hypoxia. The I_{K1} was observed in both types of cell, but showed signs of hypoxic suppression only in shark, where it is instrumental in terminating the plateau of the long lasting action potential. In addition, hypoxia also suppressed I_{Ca} in shark with the same potency (~30%) as we have previously observed in rats ⁹. We suggest that modulation of ion channels by hypoxia may be species dependent and may have evolved in parallel with differences in electrophysiological characteristics.

Ventricular cardiomyocytes from rat and shark were dispersed enzymatically and were voltage-clamped in the whole-cell configuration using patch pipettes with 2-10 MΩ resistance as previously described (e.g. ¹¹). As indicated in the legends of the figures, the extra- and intracellular solutions were tailored to meet the different ionic and osmotic requirements of rat and shark. Cells were superfused by a constant stream of extracellular solution that was saturated with 100% O₂ or N₂ and could be switched in ~50 ms ². Although the solution that was bubbled with 100% N₂ may be described as “anoxic”, we recognize that some atmospheric O₂ may have reached the examined cells and have cautiously labeled the exposure to such solution as “hypoxia”.

Fig.1 shows recordings of I_{to} in rat ventricle cardiomyocytes exposed to either O₂ or hypoxia (N₂). The external solution contained 0.2 mM Cd²⁺ and the cell was held at a relatively depolarized potential (~50 mV) to block inward Ca²⁺ and Na⁺ currents. As may be observed, rat cells showed a fast activating and inactivating I_{to} when exposed to a depolarizing pulse to +50 mV (panel A). These I_{to} currents were blocked approximately 50% by 60 s of hypoxia. A paired t-test showed a significant effect of hypoxia for the peak current (Panel B, p<0.001), but not for the steady state current, indicating a major effect on the fast component of the outward K⁺ currents.

Hyperpolarizing pulses from -50 mV (see protocol on the top of the figure) induced a strong I_{K1} current (Fig. 2) in rat cells. These currents were blocked by 50 µM of Ba²⁺ (data not shown), which is a pharmacological hallmark of the I_{K1}. Panels A and B show representative traces from a cell exposed either to O₂ or N₂, showing no apparent effect on the I_{K1}. In some cells, we found either a little blocking or enhancing effect during hypoxia, but the most predominant observation was a lack of any effect. This is summarized by the current-voltage relation averaged from 5 cells (panel C) showing an absence of effect for all of the pulse stimuli used. Furthermore, a paired t-test of the peak current at -120 mV yielded no statistical significance for a hypoxia effect (panel D).
Figure 1. The transient outward $K^+$ current ($I_{to}$) in rat ventricular cells is strongly suppressed by hypoxia. A: Sample traces from a cell subjected to 200 ms pulses to $+50 \text{ mV}$ from a holding potential of $-50 \text{ mV}$. Cells were exposed to solutions bubbled with 100% O$_2$ (normoxia) or N$_2$ (hypoxia) for 60s. B: Bar representation of normalized currents of 4 cells exposed to O$_2$ or N$_2$ at the peak or the steady state current. The measured currents were normalized relative to the membrane capacitance ($n=4$). Paired t-test: *** $p<0.001$ and ns (no significant) $p=0.995$. The standard external solution contained (mM): 5.4 KCl, 137 NaCl, 2 CaCl$_2$, 10 HEPES, 1 MgCl$_2$ at pH 7.4. In these experiments 0.2 mM CdCl$_2$ was added to block $I_{Ca}$. Internal solution (mM): 126 KCl, 10 NaCl, 14 EGTA, 10 HEPES, 10 glucose, 5 MgATP, at pH 7.2.

Hypoxia effects were compared using similar measurements in rat and shark ventricular cardiomyocytes. Fig. 3A illustrates suppression of the peak inward $I_{Ca}$ during hypoxia (blue vs. red traces). Due to rundown, $I_{Ca}$ did not recover completely when the cell was re-equilibrated in fully oxygenated solution (O$_2b$ vs. O$_2a$). For this reason we used bracketed measurements and compared the current during hypoxia (N$_2$) to a value of $I_{Ca}$ that was obtained by interpolation between the values measured before and after (O$_2b$ and O$_2a$) with O$_2$-saturated solution. As indicated in panel F, the average suppression of $I_{Ca}$ in shark cardiomyocytes was 28.0±2.5 % (SEM, $n=3$). This is comparable to the effects of O$_2$-deprivation on $I_{Ca}$ we have measured routinely in rat cardiomyocytes (data not shown) and therefore, validates the experimental approach, and demonstrates the responsiveness of shark cardiomyocytes to acute hypoxia.

Figure 2. The inward rectifier $K^+$ current ($I_{K1}$) in rat ventricular cardiomyocytes is insensitive to hypoxia. A and B: Sample traces from a cell subjected to 500 ms pulses ranging from -120 to -70 from a holding potential of -50 mV (as showed in the above protocol) either exposed to solution saturated with 100% O$_2$ or N$_2$ for 60 s. C: Average current-voltage relations of normalized membrane currents (pA/pC) from 5 cells that were examined as shown in panels A and B. D: Bar diagram of normalized currents at -120 mV ($n=5$). Paired t-test showed no significant (ns) effect of hypoxia ($p=0.717$). The same solutions were used as in Fig. 1, but without the addition of Cd$^{2+}$. 
Compared to rat ventricular cardiomyocytes, shark cells showed no significant \( I_{\text{to}} \) (data not shown). This difference is reflected in the shape of their action potentials, which in rat consists of a brief spike (~20 ms), but in shark has a well maintained, long lasting plateau (~1 s) without the early repolarization associated with \( I_{\text{to}} \) in mammalian species (e.g. human) with a long lasting action potentials. We found that the \( I_{\text{K1}} \) was well developed in shark cells and showed signs of hypoxia-induced suppression measured both with a ramp clamp protocol (Fig. 3BC) and voltage-clamp depolarizations to a fixed potential (~120 mV, Fig. 3DE). In either case, we took advantage of the property of \( I_{\text{K1}} \) that it is blocked both in the inward and outward direction in the absence of extracellular \( K^+ \). Thus, Fig. 3C shows that the \([K^+]_o\)-dependent component of the membrane current during the ramp clamps (Fig. 3B) has the characteristic shape and reversed potential (~ \( E_{\text{K}} \)) of \( I_{\text{K1}} \), and is suppressed both in the inward and outward direction by hypoxia. This component of the current showed no significant time-dependence (Fig. 3D). Fig. 3E shows the time course of repeated changes in \( O_2 \) tension, and nearly complete block in \( K^+ \)-free solution. The average suppression of \( I_{\text{K1}} \) by hypoxia was relatively small and somewhat variable (11.6±3.6%, SEM, n=13) yet highly significant. Switching between identical \( O_2 \)-saturated solutions also produced small positive or negative changes in the measured membrane currents that may relate to mechanical switching transients or different rates of flow in different barrels of the perfusion system. On average such changes may be expected to cancel out, but their absolute value (15.7±2.5 %, n=5) was comparable to the \( O_2 \)-effects and may therefore contribute to the observed variability. Another possible confounding factor is the strong inward rectification of \( I_{\text{K1}} \), that at potentials below ~80 mV produces inward currents of such magnitude (Cf. Fig. 3DE) that the measurements may reflect, not only the conductance of the \( K^+ \) channels, but also the various components of “access resistance” such as uncompensated pipette resistance and the axial resistance of the long (~100 \( \mu \)m) and slender (~2-5 \( \mu \)m) shark ventricular cells. Thus, it is plausible that the actual effect of hypoxia on \( I_{\text{K1}} \) of shark may be larger than indicated by the measurements presented here.

Figure 3. Suppression of \( I_{\text{Ca}} \) and \( I_{\text{K1}} \) in voltage-clamped shark ventricular cardiomyocytes by hypoxia. A: \( Ca^{2+} \) current measured during step depolarization from -60 to 0 mV in a cell that was superfused sequentially, at 2 min intervals, with solution saturated with 100% \( O_2 \) (\( O_2a \), red trace), \( N_2 \) (blue trace), and again with \( O_2 \) (\( O_2b \), red trace). B: Membrane currents (bottom) measured with a ramp clamp protocol (top) in a cell exposed to solutions saturated with \( O_2 \) (red trace), \( N_2 \) (blue trace) and \( O_2 \) again, but with 0 instead of 6 mM \([K^+]_o\) (black trace); \( C_m = 60 \) pF. C: Current-voltage relation for \( I_{\text{K1}} \) in the absence and presence of \( O_2 \) derived as the \( K^+ \)-sensitive components of the membrane currents during the ramp clamps in panel B. D: Bracketed measurements of \( I_{\text{K1}} \) by step depolarization from -60 to -120 mV in a cell exposed \( O_2 \) (\( O_2a \) and \( O_2b \), red traces), \( N_2 \) (blue trace), and \( K^+ \)-free solution (0K, black trace); \( C_m = 50 \) pF. The time course of the observed changes is shown in panel E. F: Average reduction in \( I_{\text{Ca}} \) and \( I_{\text{K1}} \) in anoxic solution (SEM, n = number of independent measurements). The extracellular solution contained (mM): NaCl 270, KCl 6 (or 0) urea 350, MgCl\(_2\) 10, Na\(_2\)SO\(_4\) 0.5, CaCl\(_2\) 5, HEPES 10, glucose 10 (pH 7.4, 968 mOsm). The dialyzing pipette solution contained (mM): NaCl 60, CsCl 200, Urea 300, MgATP 5, EGTA 10, CaCl\(_2\) 5, HEPES 10, TEA-Cl 10 (pH 7.2, 885 mOsm).
Here we have surveyed the effects of hypoxia on Ca\textsuperscript{2+} and K\textsuperscript{+} currents in ventricular cardiomyocytes from rat and shark. Our results suggest that I\textsubscript{Ca} is equally suppressed in rat and shark, but that both the complement of expressed K\textsuperscript{+} currents and their sensitivity to O\textsubscript{2} may be species dependent. The most intriguing observation is that I\textsubscript{K1} appears to be insensitive to hypoxia in rat, but is suppressed by hypoxia in shark. To consolidate this finding, it would be useful to conduct more extensive voltage-clamp experiments with low access resistance and with greater attention to I\textsubscript{K1} in its outward direction where it is associated with the phase of rapid repolarization that terminates the plateau of the action potential and thereby contribute to the control of the duration of the action potential and of the ventricular contraction in primitive vertebrate species, like shark, without significant releasable intracellular SR Ca\textsuperscript{2+} stores. Similarly it is possible that the repolarizing phase of the much briefer ventricular action potential of rat may be altered significantly by the O\textsubscript{2}-sensitivity of I\textsubscript{to} found in this species. The effects of oxygen on ionic channels may be mediated by hemeoxygenase (data not shown) or other oxygen-binding proteins and are likely to precede significant metabolic changes such as altered concentrations of ATP, ADP, and reactive oxygen species.

Considering also the suppression of I\textsubscript{Ca} in both rat and shark, it may be hypothesized that the longer lasting ionic currents that shape the ventricular action potential of the heart respond to acute hypoxia in a manner that would tend to lessen the requirements for metabolic energy to maintain the underlying ionic gradients. In addition our findings suggest that effects of hypoxia on ionic currents found in one vertebrate species may not be directly applicable to another.

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Aryl hydrocarbon receptor (AhR) upregulation of multidrug resistance protein 2 (Mrp2) expression at the blood-brain barrier of killifish, Fundulus heteroclitus

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Xenobiotic efflux pumps are key elements of the vertebrate blood-brain barrier. Here we show using a novel killifish brain slice preparation that protein expression and transport activity of one of those pumps, Mrp2, is increased in tissue exposed to AhR ligands. These results suggest alteration of barrier function in animals exposed to persistent environmental toxicants that activate AhR.

The vertebrate brain capillary endothelium forms the blood-brain barrier, which protects the brain from neurotoxicants, removes metabolic wastes from the CNS and limits CNS entry of therapeutic drugs. ATP-driven xenobiotic export pumps, e.g., P-glycoprotein and Mrp2, expressed on the luminal (blood-facing) surface of the endothelium, are important contributors to this barrier in all vertebrates1, including teleost fish2. In barrier and excretory tissues, transporter expression is upregulated by drugs, toxicants, dietary constituents and bile acids acting through xenobiotic-activated nuclear receptors1,2. Recent studies have identified AhR as a modulator of export pump activity and expression in killifish renal proximal tubules (3 and Mahringer et al, this volume) and rat and mouse brain capillaries (Wang and Miller, unpublished data). The present report summarizes results of initial studies focused on AhR ligands as modulators of Mrp2 expression at the teleost blood-brain barrier.

We previously described a protocol to measure efflux transporter activity in slices of killifish brain using fluorescent substrates, confocal microscopy and quantitative image analysis4. Images of brain tissue showed strong bath-to-capillary lumen transport of sulforhodamine 101 (Texas red, TR) mediated by Mrp2. In addition, bath-to-endothelial cell transport of fluorescein (FL) was mediated by an organic anion transporter previously characterized in teleost kidney5. In the present experiments, we exposed killifish brain slices for 3-6 h to the AhR ligands, β-naphthoflavone (BNF) a flavonoid, or 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) a widespread and persistent environmental contaminant that is a high-affinity ligand. We measured TR accumulation in capillary lumens over the last hour of exposure, which provided a measure of steady state transport capacity for Mrp2. Exposure to 1 µM BNF or 1 nM TCDD roughly doubled luminal TR. The increase in accumulation was abolished when the brain tissue was pretreated with the AhR agonist, resveratrol (1 µM) or with the protein synthesis inhibitor, cycloheximide (100 µg/ml). Transport of FL was not increased by 1 µM BNF. Upregulation of Mrp2-mediated transport was accompanied by a 85 ± 8% increase in Mrp2 immunoreactivity within capillaries, a finding consistent with the observed increase in transport activity.

The present study provides the first demonstration of regulation of any transporter in fish blood-brain barrier. The high efficacy of TCDD and the resveratrol reversal of effects are diagnostic for involvement of AhR. Because so many widespread aquatic pollutants, e.g., dioxins, polychlorinated biphenyls, crude oil constituents, are AhR ligands, our results suggest altered blood-brain barrier transport function may be common in the field. The in vivo consequences of blood-brain barrier transporter upregulation through AhR have yet to be explored in any species. Supported by the NIEHS Intramural Research Program.

Detection of oxidative stress as a response to environmental toxins using the transgenic EPRE-reporter zebrafish (*Danio rerio*)

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Toxic chemical contaminants in water resources have detrimental effects on wildlife and pose health hazards to humans. One of the effects of toxins on living organisms is the generation of reactive oxygen species (ROS) that can lead to damage on nucleic acids, proteins and lipids. The transgenic EPRE-reporter zebrafish makes it possible to visualize and quantify the damage caused by oxidative stress in a living organism. Surprisingly, we found that this transgenic zebrafish was not capable of detecting hazardous chemicals in the water at very low concentrations that are labeled “safe” by the Environmental Protection Agency (EPA).

The activation of the electrophile responsive element (EPRE) by the transcription factor Nrf2 and the subsequent induction of phase II detoxifying and oxidative stress enzyme genes is a defense mechanism of terrestrial organisms against damage by electrophiles and reactive oxygen species (ROS)¹. It has been shown previously that the EPRE reporter zebrafish is capable of detecting water contamination with HgCl₂ in concentrations as low as 0.1 µM by expression of a luciferase-green fluorescent protein (LUC-GFP) fusion protein, which is expressed under conditions of oxidative stress². This novel technique could serve as a high-throughput screening system to detect oxidative stress in a living organism caused by water contamination.

In this project we tested the effect of different environmental toxins such as the metalloid arsenic, the organic compound bisphenol A, the ester phthalate, the pesticide atrazine and HgCl₂ (positive control) on the functionality of the EPRE reporter fish (obtained from A. Udvadia, Great Lakes WATER Institute, University of Wisconsin-Milwaukee, WI). All dilutions were prepared in E3 (embryo media). Embryos were kept and handled in 12-well culture plates in groups of 8-10 fish and 1 ml of media. Media was exchanged daily for survival studies in which the embryos were incubated at 0 hours post fertilization (hpf) for a maximum of 72 hours. For functional studies with measurements of luciferase- and GFP-expression embryos were incubated at 48 hours post fertilization for 24 to 72 hours. Toxic effects of the substances on the development and morphology of the zebrafish embryos were documented.

In the survival study we found that zebrafish embryos are surprisingly resistant to toxins in the embryo media tolerating 500 ppm sodium arsenite (parts per million, maximum water concentration of 10 ppb allowed by EPA) and 1000 ppb HgCl₂ (parts per billion, maximum allowed water concentration of 2 ppb by EPA) during the first 72 hpf without significant visible developmental delays on most incubated embryos (Fig.1).

![Fig.1: Comparison of zebrafish embryos after incubation for 24 hours with 2000 ppb HgCl₂ (left) and 1000 ppm sodium arsenite (middle) to the control (right) at 24 hpf.](image-url)
Phthalate, bisphenol A or atrazine in the embryo media did not have any visible effect on embryonic development or survival at all tested concentrations compared to the controls (excerpt shown in Fig.2A). In addition, we found that the heart-rate of the zebrafish embryos is physiologically ranging between 125-150 bpm (beats per minute) at 48 hpf to 175-200 bpm at 72 hpf. The heart-rate of the zebrafish embryos did not change after incubation with phthalate, bisphenol A or atrazine and decreased slightly at higher/lethal concentrations of HgCl₂ and sodium arsenite (data not shown). Furthermore we found that high concentrations of the chemicals mentioned above could increase the luciferase expression and therefore indicated an induction of the electrophile responsive element in the zebrafish but that the expression of luciferase was altogether very weak and hardly above the expression in the controls (Fig.2B).

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**Fig.2: Survival and luciferase expression of zebrafish embryos treated with different toxins.** A) displays the survival rates of embryos treated with 1000 and 200 ppb (n=24 and 42) HgCl₂, 1000 ppm and 10 ppb (n =36 and 32) sodium arsenite, bisphenol A (BPA; n=24), 6000 ppb phthalate (n=16), 100 ppb atrazine (n=24) compared to the control. (n=47). B) depicts the luciferase expression of the EPRE-reporter zebrafish embryos (n=8 each) after 24 hours incubation at 48 hpf.

In summary, we were able to show that the EPRE-reporter zebrafish can detect HgCl₂ contamination in aquatic environments as previously shown by Kusik et al.², but failed to detect other tested contaminants in the water at low concentrations. We could only demonstrate luciferase expression at very high doses of the tested chemicals, which are far above the concentrations that are considered “safe” by the EPA. This may be due to a difference in the mode of action of the compared toxins, with a difference between the more acute toxicity of HgCl₂ as compared to bisphenol A or phthalate whose toxic effect become more apparent after chronic exposure. We conclude that these findings render this system not useful for detection of the unapparent aquatic pollution with toxins such as sodium arsenite. Further modification e.g. by glutathione depletion of the embryos prior to treatment (hereby reducing the antioxidant capacity and hence sensitizing the embryos³) may be needed before this transgenic fish could be applied as a screening tool in environmental testing.

**Funding:** Prof. Dr. Hermann Haller

1. Kobayashi M., Yamamoto M. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxidants & Redox Signaling* 7(3-4): 385-394, 2005
Detection and quantification of EDC/PPCPs in source waters containing dissolved organic matter

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A wide range of trace organic contaminants is emerging in water sources as a result of expanding human usage of pharmaceuticals, hormones and personal care products that are incompletely destroyed during wastewater treatment. Detecting and quantifying these contaminants in natural waters is a bit like searching for a needle in a haystack because of co-associated organic molecules that derive from natural processes such as the decomposition of vegetation and microbial turnover. This project is using state-of-the-art methods to extract the target compounds from surface waters in the Charles River Watershed (MA) and also from specified locations on Mount Desert Island (ME) prior to employing tandem mass spectrometry to quantify their concentrations, in order to determine controls on their environmental mobility.

A wide range of “emerging contaminants” including pharmaceuticals and personal care products (PPCPs) and endocrine disrupting compounds (EDCs) have been measured in water sources throughout the US¹. The presence of PPCPs and EDCs in streams has been attributed to widespread domestic use of these compounds, their eventual appearance in municipal wastewater, and wastewater treatment that is only partially effective in their removal prior to effluent discharge into surface waters. Thousands of different pharmaceutical substances are used daily including painkillers, antibiotics, antidiabetics, β-blockers, contraceptives, lipid regulators, antidepressants, and impotence drugs. Effluent and biosolids from wastewater treatment plants are primary entryways for PPCPs and EDCs into the environment. As part of a larger project, herein we aim to provide our Water Research Foundation research project partners (City of Cambridge and Charles River Watershed Authority) with a preliminary assessment of trace organic levels in their source waters and treated wastewaters, including information regarding the persistence of trace contaminants in waters affected by wastewater effluent. Trace inorganic constituents have potential use as surrogates for medical waste. For example, rare earth element (REE) data can be used to screen for gadolinium (Gd) anomalies, which provide an indication of hospital waste-derived contamination.²

Water samples were collected for trace organic and inorganic chemistry analyses from ten New England locations during July 2009 (see Table 1, Figure 1). For analysis of trace organic compounds (PPCPs and EDCs), four replicate water samples were collected in 1 L amber, glass bottles (I-CHEM certified to meet or exceed US EPA contaminent-free specifications; from VWR, cat. # IR249-1000). Water collections were acquired as grab samples or 24 hour drip composites. For trace inorganic solution

Table 1. Water sample collections in New England, 2009.

<table>
<thead>
<tr>
<th>Site No.</th>
<th>Location</th>
<th>Sampling method</th>
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<tbody>
<tr>
<td>1</td>
<td>Milford WWTP</td>
<td>24 hr comp.</td>
</tr>
<tr>
<td>2</td>
<td>Charles River Water Pollution Control District WWTP</td>
<td>24 hr comp.</td>
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<tr>
<td>3</td>
<td>Wrentham WWTP</td>
<td>grab</td>
</tr>
<tr>
<td>4</td>
<td>Medfield WWTP</td>
<td>24 hr comp.</td>
</tr>
<tr>
<td>5</td>
<td>Charles River Lock</td>
<td>grab</td>
</tr>
<tr>
<td>6</td>
<td>BVD West Well</td>
<td>24 hr comp.</td>
</tr>
<tr>
<td>7</td>
<td>BVD East Well</td>
<td>grab</td>
</tr>
<tr>
<td>8</td>
<td>MDIBL WWTP</td>
<td>grab</td>
</tr>
<tr>
<td>9</td>
<td>Bar Harbor WWTP</td>
<td>24 hr comp.</td>
</tr>
<tr>
<td>10</td>
<td>Frenchman’s Bay</td>
<td>grab</td>
</tr>
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WWTP = waste water treatment plant effluent
chemistry analysis, water samples were collected in duplicate using 60 mL HDPE bottles, and then filtered through 0.45 μm nylon membrane filters prior to ICP-MS analyses. All water samples were held at 4 °C until processing.

Water samples collected for trace organic compounds analysis were filtered through PALL 0.7 μm glass fiber filters (VWR, cat. # 28149-456) before solid phase extraction (SPE) using either a hydrophobic lipid balance (Oasis HLB, 6 ml, 150 mg Waters Corp, cat. # 186003365) or an octadecyl reverse phase matrix (Strata C18-U, 6 ml, 500 mg, Phenomenex Inc., cat. # 8B-S002-HCH). All SPE cartridges were conditioned with 7 mL acetonitrile, 5 mL methanol, and 5 mL water. Filtered water samples were loaded under vacuum at ca. 10 mL min⁻¹ followed by air drying under vacuum for ca. 20 min. Cartridges were eluted with 2 x 5 mL acetonitrile. Eluents were subsequently evaporated to dryness and re-dissolved in 1 mL 50% aqueous methanol for LCMS injection. All solvents were liquid chromatography grade. Liquid chromatography was performed with 5 μL sample injections on a Waters Acquity UPLC system with an Acquity UPLC BEH C18 column (1.7 μm, 2.1 x 50 mm ) and a gradient mobile phase of water and acetonitrile for 30 min. Ionization and detection of each indicator compound has been optimized resulting in cone voltage, capillary voltage, and drying gas flow settings optimized for electrospray ionization (ESI), and collision energy and collision gas pressure optimized for multiple reaction monitoring (MRM) by tandem mass spectrometry. Calibration curves consisting of at least seven points were developed for each contaminant analyte. Bisphenol A-d₁₆ and diazepam-d₅ were used as internal standards in negative and positive ion modes, respectively.

![Figure 2](image-url). Concentrations of rare earth elements (as measured by ICP-MS) in filtered source waters in the Charles River Watershed MA (left) and Mount Desert Island ME (right) at sites depicted in Table 1.

As shown in Figure 2, Gd anomalies are present for three sites in the Charles River Watershed, whereas they are absent for the other two Charles River sites, and also for all of the Mount Desert Island sites. Gd anomalies are indicated by the spikes in Gd concentration in the left side of Figure 2 for the Milford WWTP, Charles River WPCD, and Medfield WWTP sites. Such anomalies are tracers of contrasting agents used in magnetic resonance imaging² and, therefore, have potential as indicators of medical waste in general.

Data pertaining to the concentrations of EDC/PPCP compounds sampled from the same set of sites in MA and ME are shown in Figure 3. Emphasis in the initial sampling was on collection of treated wastewater effluents since this is considered an important source of these compounds to surface waters. Data for 10 compounds are plotted from A to J in order of increasing water solubility (decreasing compound hydrophobicity or octanol-water partition coefficient). In addition, the graphs show a comparison of compound recoveries made using a hydrophobic (C18) resin versus a hydrophilic-lipophilic balance (HLB) resin. The relative efficacy of recovery by the two SPE methods depends on the compound type. For example, higher concentrations of nonylphenol and ciprofloxacin were measured using the C18 resin, whereas higher concentrations of salicylic
acid and carbemazepine were recovered using the HLB resin. This finding is consistent with the relative hydrophobicities of these compounds and their corresponding affinities for solid phase extraction media. The HLB resin includes both hydrophilic and lipophilic components and the C18 resin is lipophilic only.

Tonalide (a fragrance contained in numerous PPCPs) was found at relatively high concentrations throughout the sampling region and presumably enters wastewater via gray water incorporation (Fig. 3A). Estrone, a constituent of birth control medication, was also commonly observed in wastewaters, and was particularly

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**Figure 3.** Emerging contaminant concentrations: (a) tonalide, a fragrance, (b) nonylphenol, a surfactant, (c) estrone, an estrogen, (d) bisphenol A, a plasticizer, (e) carbemazapine, an anti-epileptic pharmaceutical, (f) DEET, an insect repellent, (g and h) ibuprofen and salicylic acid, analgesics, (i) TCEP, a flame retardant, and (j) ciprofloxacin, an antibiotic, measured in water samples from nine different New England locations (Table 1) using two different extraction methods (HLB and C18).
elevated in the grab sample taken from the MDIBL treatment plant effluent. Carbamazepine (anti-epileptic drug), ciprofloxacin (antibiotic), and TCEP (flame retardant), were all elevated in the Wrentham WWTP effluents. Although our data indicate elevated concentrations of several EDC/PPCPs in treated wastewater effluents that are released into the environment, the fate and transport of these compounds in the environment is not well known. It is not clear, for example, that these contaminants persist as treated wastewater migrates downstream in surface waters or infiltrates through sediments to ground water. This issue is the subject of ongoing research.

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) upregulates FoxQ1b in the developing jaw primordium of zebrafish (Danio rerio)

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The ubiquitous environmental contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; popularly referred to as dioxin) is a known carcinogen and teratogen that disrupts craniofacial development through an unknown mechanism. We identified a novel developmental target of TCDD, FoxQ1b, which is expressed in a developing jaw structure that is conserved in all jawed vertebrates and develops abnormally in the presence of TCDD. This finding will provide novel insights into the toxic actions of TCDD and the role of Fox1b in vertebrate jaw development.

TCDD is a potent activator of the aryl hydrocarbon receptor (AhR), a transcription factor required for transducing the toxic effects of this chemical. Developmental exposure to low levels of TCDD has been shown to disrupt jaw and craniofacial development in evolutionarily divergent vertebrates. For example, TCDD exposure is associated with cleft palate in mice 1-3, and it induces beak malformations representative of severe cleft lip and palate in chicken 5.

We used zebrafish (Danio rerio) embryos and microarray analysis to uncover a novel target of TCDD-activated AhR belonging to the evolutionarily conserved family of forkhead box transcription factors4. We validated this observation by quantitative polymerase chain reaction (qPCR). Pooled embryos were exposed from 6-7 hours post-fertilization (hpf) to either 0.1% DMSO or 1 nM TCDD. RNA was isolated at 12, 24 and 48 hpf, reverse-transcribed and expression values for four genes were measured: Cytochrome P4501A (Cyp1a; positive control for TCDD induction), FoxQ1a (a zebrafish paralog of FoxQ1b), FoxQ1b and glyceraldehyde-3-phosphate dehydrogenase (Gapdh, normalizer). Statistically significant induction was observed for Cyp1a and FoxQ1b at all timepoints except for 12 hpf (Figure 1). No significant induction was observed for FoxQ1a at any time point. Timepoints are the average of 3 biological replicates of approximately 20 embryos each. Error bars are standard deviations of the mean (** indicates p < 0.01).

We confirmed that TCDD-mediated induction of FoxQ1b is AhR dependent. We exposed 48 hpf embryos to 0.1% DMSO or 1 nM TCDD in the presence or absence of the AhR antagonist, α-naphthoflavone and measured induction at 60 minutes post-exposure. Induction of FoxQ1b and Cyp1a was reduced approximately 5-fold in the presence of ANF (Figure 2A). In addition, we injected one to 4-cell stage embryos with a an Ahr or control morpholino antisense oligonucleotide and exposed them to 0.1% DMSO or 1 nM TCDD from 6-7 hpf. At 48 hpf, RNA was extracted, reverse transcribed and the Gapdh-normalized levels of Cyp1a and FoxQ1b were measured by qPCR. Cyp1a and FoxQ1b transcript levels were approximately 70% (p=0.03) and 90% (p=0.05) lower, respectively, in Ahr MO-injected embryos compared to control-injected embryos (Figure 2B). Remaining embryos were allowed to develop further and notably, Ahr MO-injected embryos exposed to TCDD did not exhibit jaw abnormalities.

To identify the jaw structures that are targets of TCDD toxicity, zebrafish embryos were exposed to 0.1% DMSO or 1 nM TCDD from 6-7 hpf, fixed at approximately 144 hpf and stained with alcian blue to reveal cartilaginous structures. Significant changes in the morphology of Meckel's and ceratohyal cartilages were apparent. To determine whether FoxQ1b was expressed in these structures, we performed in situ hybridization with a probe derived from the FoxQ1b open reading frame. At 24 hpf we observed a general increase in the intensity of FoxQ1b expression in TCDD-exposed embryos consistent with our qPCR analysis but expression was not discretely localized. By 48 hpf strong FoxQ1b expression in TCDD-exposed embryos was clearly seen in structures of the lower jaw, particularly Meckel's cartilage and associated structures (data not shown).
We hypothesize that misexpression of *FoxQ1b* may play an important role in TCDD-mediated craniofacial abnormalities. To investigate this hypothesis we are further characterizing the role of *FoxQ1b* in this process using knock-down and over-expression techniques.

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Figure 2. TCDD induction of *FoxQ1b* requires AhR.

The Comparative Toxicogenomics Database (CTD)

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Environmental compounds play a critical role in the etiology of most chronic human diseases. CTD is a publicly available resource that provides manually curated and integrated data to promote understanding and hypothesis development about the effects of environmental chemicals on molecular pathways and human disease.

The Comparative Toxicogenomics Database (CTD; http://ctd.mdibl.org/) is a publicly available resource that promotes understanding about the effects of environmental chemicals on human health. Currently, CTD provides over 200,000 molecular interactions involving more than 5,300 chemicals and 17,000 genes/proteins as well as over 6,000 and 9,500 substantiated chemical-disease and gene-disease relationships, respectively. Integration of these curated relationships enables users to infer novel networks between chemicals, gene and proteins, and diseases.

CTD data and analysis features expanded substantially since our last report. In addition to ongoing improvements in data visualization, major advances in CTD development included the following: incorporation of similarity indices that are used to produce lists of comparable genes (“GeneComps”) or chemicals

Figure 1. CTD ChemComps for bisphenol A.
(“ChemComps”) based on shared toxicogenomic profiles (Figure 1)\(^1\); creation of a “MyGeneVenn” tool in which users can compare their genes of interest to genes associated with up to two chemicals or diseases in CTD; substantial additions to the manually curated data in CTD; integration of molecular pathway data from the Reactome database; further development of a text-mining application to enhance manual curation\(^2\); publication of 5 manuscripts reporting on unique aspects of CTD data and analysis capabilities ([http://ctd.mdibl.org/about/publications/](http://ctd.mdibl.org/about/publications/)).

Future development of CTD will include: a) statistical analyses of inferred disease and pathway relationships; b) gene ontology enrichment analyses for chemicals and diseases, allowing users to identify biological functions that are affected by chemical exposures or diseases; c) implementation of a text-mining workflow that we developed to enhance the efficiency of manual curation; d) development of an online curation application to facilitate off-site data curation; and e) initiation of collaborative projects with domestic and international entities involving curation of dietary compounds and exposure data for integration in CTD.

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Rapid, non-genomic regulation of multidrug resistance protein 2 (Mrp2) by glucocorticoids in killifish (Fundulus heteroclitus) renal proximal tubules

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In kidney tubules, multidrug resistance-associated protein 2 (Mrp2) is a transport protein that drives active excretion of many foreign chemicals from blood to urine. Here we show that dexamethasone, a synthetic, anti-inflammatory steroid, rapidly increases Mrp2 transport function in fish kidney tubules. Dexamethasone does this by acting through a glucocorticoid receptor, but by a mechanism that does not involve new RNA or protein synthesis.

Mrp2 (ABCC2) is expressed at the luminal membrane of renal proximal tubules, where it mediates ATP-dependent efflux of anionic xenobiotics and metabolic wastes into the urine. Previous studies showed that Mrp2-mediated transport activity in killifish renal proximal tubules is rapidly reduced by endothelin-1 (ET-1) acting through an ET-B receptor, nitric oxide synthase (NOS), cyclic GMP and protein kinase C. A number of tubular nephrotoxicants, including radiotracers, aminoglycoside antibiotics and heavy metal salts, activate the same sequence of events. In contrast, luminal Mrp2 activity and protein expression are increased 24 hours after transient exposure to ET-1 or nephrotoxic agents. This may result from an induced de novo synthesis of Mrp2, or post-transcriptional regulation involving receptors and signaling pathways to affect Mrp2 function. Unlike mammalian liver, Mrp2 expression in killifish kidney is not regulated through activation of nuclear receptors, viz. the pregnane xenobiotic receptor (PXR), constitutive androstane receptor (CAR), or farnesoid xenobiotic receptor (FXR). After screening of a number of compounds known to be activators of mammalian FXR and PXR, only dexamethasone induced Mrp2-mediated transport. In the present study we investigated dexamethasone signaling to Mrp2.

For transport experiments, freshly isolated killifish tubules were exposed to dexamethasone without and with inhibitors of signaling. After a predetermined exposure time, 2 μM fluorescein-methotrexate (FL-MTX) was added to the medium and Mrp2-mediated transport was measured by confocal microscopy and quantitative image analysis using ImageJ 1.34s (NIH, MD, USA). For immunostaining, incubated tubules were fixed in 2% formaldehyde/0.1% glutaraldehyde and after permeabilization incubated with an Mrp2 specific antibody. Mrp2 staining was visualized using a fluorescent secondary antibody.

Exposure of killifish tubules to concentrations ranging from 0.25 – 10 μM dexamethasone for 3 h increased FL-MTX transport at all concentrations tested (p < 0.01; n = 48-231 tubules). Highest increases were found with 1 μM dexamethasone (218 ± 10%); this concentration was used for subsequent experiments. Figure 1 shows that the induction in Mrp2-mediated transport was rapid and significant even after 15 min of treatment. Increasing incubation time for up to 3 h did not further enhance Mrp2 induction (data not shown).

![Figure 1. Rapid stimulation of FL-MTX transport by dexamethasone.](image)

Tubules were incubated with or without (control) 1μM dexamethasone. The fluorescence intensity of the lumen is depicted as measured arbitrary units at indicated time points, mean values ± S.E.M. are shown for 9-16 tubules. Significantly different from control, * p<0.05; ***p<0.001
To determine whether dexamethasone signaled through the ET-1 pathway, we tested the effect of N-G-methyl-L-arginine (iNOS inhibitor) and bis-indolylmaleimide (PKC blocker). However, neither altered dexamethasone-induced stimulation of Mrp2 activity. Dexamethasone is a potent synthetic glucocorticoid. Other GR-ligands, cortisol and triamcinolone acetonide (TA; both at 1.0 µM) also stimulated Mrp2-mediated transport. The order of effectiveness of the GR ligands was TA>cortisol>dexamethasone. Cortisone, an inactive metabolite of the native fish GR-ligand, was without effect. The GR-antagonist, RU-486 (0.5 µM), abolished the effects of dexamethasone, TA and cortisol on Mrp2-mediated transport.

Consistent with action through a non-genomic mechanism, dexamethasone up-regulation of Mrp2-mediated transport was insensitive to cycloheximide (100 µg/ml). Immunohistochemistry revealed that dexamethasone did not alter Mrp2 expression in the luminal membrane. Dexamethasone-enhanced Mrp2 activity may be mediated by phosphorylation of a peptide domain of the transporter by kinases. Figure 2 shows that K252a, an inhibitor of the tyrosine kinase TRK subfamily, reduced the effect of dexamethasone, as did the specific c-Met kinase inhibitor, PHA-665752.

In conclusion, we have found a novel signaling pathway for the rapid induction of Mrp2-mediated transport in renal proximal tubules. Signaling involved glucocorticoids acting though GR and tyrosine kinases, but did not involve increases in protein synthesis or Mrp2 expression. This work was supported by a travel grant from SNUF, B090148, to B. Prevoo and by the NIEHS Intramural Research Program.

Genomic regulation of ABC transporters by the aryl hydrocarbon receptor (AhR) in killifish (Fundulus heteroclitus) kidney tubules

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Chemical pollutants like polycyclic aromatic hydrocarbons are classical ligands of the AhR that plays a pivotal role in the regulation of detoxifying mechanisms. Here, we show that transport activity and protein expression of P-glycoprotein, Mrp2 and BCRP, three ATP-driven, xenobiotic efflux pumps, are increased by the AhR in isolated renal proximal tubules. Using specific inhibitors of transcription and translation revealed a genomic signaling pathway.

In renal proximal tubules, ABC transporters remove metabolic wastes and xenobiotics from blood and secrete them into urine. Recent studies in hepatocytes show that several ABC transporters are transcriptionally regulated by AhR²,¹⁰. This cytoplasmic receptor binds environmental pollutants such as polychlorinated biphenyls and polycyclic aromatic hydrocarbons, and translocates to the nucleus where the receptor binds to a “dioxin responsive element” (DRE) in the promoter regions of the target genes. AhR induction of xenobiotic metabolizing enzymes and transporters is a major protective mechanism. We recently provided evidence for AhR-mediated upregulation of transport on three ABC transporters in killifish renal tubules: P-glycoprotein (P-gp), Breast cancer resistance protein (BCRP) and Multidrug resistance-associated protein 2 (Mrp2)⁸. The present report extends those findings, demonstrating for each of those transporters that exposure of tubules to AhR ligands increases transcription and translation.

Transporter activity in killifish tubules was determined using confocal microscopy to measure specific accumulation of fluorescent substrates (NBD-CSA for P-glycoprotein, fluorescein-methotrexate for Mrp2 and mitoxantrone for BCRP) in lumens of intact killifish renal tubules⁶,⁹. Transporter protein expression was measured with quantitative immunostaining. Incubation of renal tubules with the selective AhR agonists, β-naphthoflavone (BNF, 0.1-1.0 μM, established by previous dose response experiments⁵) or 2,3,7,8-tetrachlorodibenzodioxine (TCDD, 0.1-1.0 nM, Kᵰ=0.56nM⁵) for 3h caused concentration-dependent increases in transport activity and protein expression for all three transporters. This upregulation was abolished by the selective AhR antagonists, resveratrol (1.0 μM) and α-naphthoflavone (10.0 μM, determined by dose response experiments). No increase in activity was observed for renal organic anion transport (measured using fluorescein). Importantly, for all three ABC transporters, BNF induction of transport was blocked when tubules were pretreated with inhibitors of transcription (actinomycin D, 100μg/ml³) and translation (cycloheximide, 2.5μM⁷).

The present study shows for the first time that AhR-dependent upregulation of the P-gp, Mrp2 and BCRP in killifish renal tubules is mediated via a genomic mechanism comprising of changes on the transcriptional and translational level. Future investigations will focus on the effects of in vivo TCDD exposure on killifish ABC transporters and will test the involvement MAPK-signaling in the action of AhR. Funded by: DFG grants GF1211/12-1 and GF1211/13-1, NIH grant MDIBL-CMTS (ES03838) and the NIEHD Division of Intramural Research.


Development of a permanent cell line derived from fathead minnow (*Pimephales promelas*) testis and their applications in environmental toxicology

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Fathead minnow (*Pimephales promelas*) is a widely used aquatic vertebrate model organism used extensively in toxicology both for regulatory testing and research. Fathead minnows are especially important in the study of environmental endocrine disruptors (EED’s) and cell lines derived from these organisms are being sought for ecotoxicological studies. In this study, we report on a newly established epithelial cell line from fathead minnow testis (FHMT-W1) and on their applicability for cytotoxicological studies. These cells could be useful for understanding mechanisms of testicular cell growth and differentiation in fathead minnow species as well as in toxicity response in aquatic animals.

Fathead minnows are important model organisms in aquatic toxicology¹. As their use increases in ecotoxicological testing, simpler, faster, cheaper alternatives are being sought. Cell lines derived from fathead minnow are few and although a cell line, FHM, has been available since the 1960’s⁴ and has been used in cytotoxicity testing of aquatic pollutants², there is a need for target-tissue derived cell lines, such as the liver as the main target for toxicants, or gonad-derived cell lines that could be used for testing endocrine disruptors. In this study, we report on the establishment of a continuous cell line from fathead minnow testis: FHMT-W1 and compare their usefulness in environmental toxicity testing alongside a newly established fathead minnow liver derived cell line: FHML2-6⁸.

Cell cultures were initiated from the testis of a mature fathead minnow (approximately 9 months old that had reached sexual maturity). The dissected testis was placed in a sterile petri dish with Hanks’ buffered salt solution (HBSS) with antibiotics, and cleaned of connective tissue covering and blood vessels. The soft tissue was minced by mixing up and down in a sterile centrifuge test tube with a Pasteur pipette in Leibovitz’s L-15 media with 10% fetal bovine serum (FBS) supplement. The cells were allowed to grow out of tiny explants then passaged into new culture flasks using TryplE, a recombinant form of trypsin trademarked by InVitrogen. Growth assays were performed by hemocytometer cell counts or using fluorescent viability assays³ (Figure 1).

![Figure 1. Alamar Blue (AB) fluorescence assay for measuring cell viability.](image)

Figure 1. Alamar Blue (AB) fluorescence assay for measuring cell viability. Standard curves were generated with increasing cell numbers that provided a linear correlation between relative fluorescence units (RFU) and FHMT-W1 cell numbers. Points represent mean RFU of 6 replicate wells per cell number.

Initially, cells were kept at 24°C and passaged within 3 weeks of culture, and temperature preference experiments showed similar growth at 28°C (Figure 2) in agreement with FHML2-6⁸. For ease of maintenance, cells have been kept at an average 26°C, although they can also be maintained at room temperature. Cells were
passaged using TrypLE for over 25 generations and various passages have been successfully cryopreserved and thawed with high viability (>80%).

Figure 2. Effect of incubation temperature on FHMT-W1 growth. Cells were plated in 96-well plates and incubated at the indicated temperatures for up to 9 days. Mean relative fluorescence units (RFU’s) as % of control (day 1) is presented (n=6).

FHMT-W1 cells are pleomorphic (Figure 3) with various shapes and sizes of cells although the epithelial morphology is predominant. Cells are also rapidly adherent to tissue culture plates and most cells attach to growth surfaces within 2 h. However, in non-tissue culture plates, cells aggregate and form spheroid-like bodies reminiscent of embryoid-bodies characteristic of stem cells. The aggregates formed by FHMT-W1 cells were not as compact as FHML2-6 spheroid bodies, which could form readily in hanging drops with cells at various densities (Figure 4). Cells at high densities formed spermatocyte-like cells similar to those reported for zebrafish testis primary cell cultures (Figure 5). These cells had condensed nuclei that stained darkly with Giemsa stain.

Figure 3. Morphology of FHMT-W1 under phase contrast microscopy. Cells exhibiting various morphologies can be found within a monolayer. Panels A-D show various areas within a flask of confluent FHMT-W1 cells at passage 14 grown at 26°C. A- epithelial cells, B- highly proliferative stem cell-like cells, C- pleomorphic cells with islet of differentiating cells, D- fibroblastic cells. All pictures were taken at same magnification (10x). Bar = 100 μm
Figure 4. FHML2-6 and FHMT-W1 Spheroid Bodies (SB) after 3d of culture in hanging drops at different seeding densities. A-C phase contrast images of FHML cells forming tightly packed round SB’s. D-F FHMT-W1 cells displayed irregular shaped loosely packed SB’s. A,D ~10^4 cells; B,E ~5 x 10^4 cells; C,F ~10^5 cells. Bar=100 µm

Figure 5. Differentiation potential of FHMT-W1. Giemsa stained confluent FHMT-W1 cells show cells at various stages of nuclear condensation which are depictive of spermatogenic process. The small dense nuclei visible in all frames (these were from passage 23) at various densities are reminiscent of spermatocyte-like cells Bar =100 µm

FHMT-W1 cells responded in a dose-dependent manner to a selection of chemicals including hormones (not shown) and toxicants (Figure 6), and the calculated EC50 values (effective concentration to kill 50% of cells)
were compared with reported 96h LC50 values (lethal concentration to kill 50% of organisms) for fathead minnows (http://www.pesticideinfo.org).

Figure 6. Effect of various chemicals on FHMT-W1 viability. Cells were exposed in replicates of 6 to 12 wells to various dosages of chemicals (SDS=sodium dodecyl sulfate, CuSO4 = copper sulfate) for 24h at 26°C. Alamar blue &/or CFDA viability assays were performed and data was expressed as percent of vehicle controls. EC50 values were calculated using Prism (GraphPad).

FHMT-W1 joins a growing list of poikilothermic cell lines, and appears to be the only immortal fathead minnow testis derived cell line. These cells grow well within the physiological temperature range of fathead minnows and have been conclusively identified using molecular probes as derived from the fathead minnow, Pimephales promelas. Derivation of testis cell lines with stem cell characteristics has been reported with mammalian cells, thus the adult fish testis may also be able to give rise to stem cells as has been shown with adult mouse testis. These cells could be very useful for toxicology and endocrinological studies and further molecular characterization is in progress.

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Establishment of a myofibroblast cell line from the gastrointestinal tract of Atlantic salmon, *Salmo salar*

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Cell lines are cultures of animal cells that can be propagated repeatedly and used as convenient alternatives to whole animals in research. The aquaculture of Atlantic salmon is an economically important industry that is looking to soybean meal as a cheap and readily available component for feeds of the future, but soybean meal has been found to cause gut inflammation, which restricts growth. Myofibroblasts regulate inflammation so the availability of a cell line could allow the soybean components responsible for inflammation to be identified and studied more quickly than with fish. Towards this goal, we describe herein a cell line, with characteristics of myofibroblasts, derived from the gut of Atlantic salmon.

The subject of intestinal inflammation recently has become important in fish biology because of efforts to develop new feeds for aquaculture. The rapid growth of aquaculture is likely unsustainable if only fishmeal continues to be used in feeds². Fishmeal is made from wild-caught species whose populations fluctuate and could decline in the future. Therefore, plant meals are being investigated as alternatives. Soybean meal is the most attractive plant substitute but causes enteritis or intestinal inflammation, which impairs fish growth⁵,²⁰. It is unclear how soybean meal causes enteritis and what the responsible soybean components may be.

Normal and abnormal intestinal inflammation has long been an interest in human medicine, and the cells involved have been studied both *in vivo* and *in vitro*. These studies have been pursued in order to understand and cure conditions such as inflammatory bowel disease (IBD). The first cells to be intensively studied were immune cells, such as macrophages, dendritic cells and lymphocytes, but increasingly the importance of non-immune cells in intestinal inflammation is being recognized⁶. Examples of non-immune cells include enterocytes and subepithelial fibroblasts. The subepithelial fibroblasts or intestinal myofibroblasts are found just below the enterocytes at the interface with lamina propria and contribute to the normal physiology of the gut mucosa by integrating a variety of processes³,⁷. They participate in inflammation by affecting the recruitment and retention of immune cells and by acting as non-professional antigen-presenting cells⁷,¹⁸.

For mammals, the use of cell culture models has been vital to study at the cellular level, physiological processes of the gastrointestinal tract (GIT), such as inflammation. These include primary cultures and cell lines. A variety of human intestinal epithelial cell lines are available, with perhaps Caco-2 being most commonly used, including those for studies of soybean components¹⁴,¹⁵. Conversely, fewer human intestinal myofibroblast cell lines have been developed, but a common one is 18Co¹⁷,¹⁹. A characteristic feature of intestinal myofibroblasts is their capacity to rapidly and reversibly change shape in response to changes in intracellular cAMP levels⁷. This is sometimes known as stellate transformation (ST) and is a property maintained by 18Co¹⁹. For fish, the GI tract has been an infrequent source of cell cultures⁵. Although, we recently described the first cell line, RTgutGC, which is epithelial-like and were derived from the rainbow trout GI tract¹¹, and the preparation of primary cultures from the Atlantic salmon GIT¹³. Here we describe how one of these primary cultures was developed into a cell line.

As described previously¹³, primary cell cultures were prepared from the intestine of healthy female Atlantic salmon (*Salmo salar*) that were obtained from the National Cold Water Marine Aquaculture Center at Franklin, ME. The fish were 2 to 3 yrs old and weighed 1.1-3.8 kg. The gut contents were removed by running cold tap water through the lumen for approximately 5 min to empty the gut content and remove most of the bacterial flora before rinsing several times in 15 ppt seawater. Intestines were cut into anterior and posterior segments. These were rinsed 3x in cold sterile Hank’s Buffered Salt Solution (HBSS) with Penicillin/Streptomycin/Ampicillin, everted with sterile plastic transfer pipettes, and further rinsed in HBSS with antibiotics/antimycotic. All subsequent solutions and incubations were at room temperature. For the cultures that led to ASimf20, posterior segments were used. They were briefly incubated in Ca/Mg free HBSS and cut with fine scissors into 2-4 mm cubes that were placed lumen side down into 12.5 and 25 cm² tissue culture flasks (Falcon). Enough growth medium was added to cover but not float them. The growth medium was Leibovitz’s 15 (L-15) supplemented with 10 % fetal bovine serum (FBS) and Penicillin/Streptomycin. The tissue pieces were incubated this way for
a few hours to overnight in which time many of them anchored to the flask surface. This was followed by addition of normal volumes (5-10 ml) of growth medium. Over time cells migrated out of the tissue pieces and eventually covered the flask surface. Confluent cultures were subcultivated or passaged one to one with TryplE (Invitrogen).

One such culture underwent the cycle of growth to confluency and have been passaged 17 times, and is referred to as the cell line Atlantic salmon intestinal myofibroblast 20 (ASimf20). ASimf20 required FBS in order to grow but cell viability was maintained for a time in L-15 alone (Fig 1). In cultures without FBS cell number remained unchanged for at least 3 days and even after 15 days had only declined by approximately 50%. The initial cultures had epithelial-like and fibroblast-like cells, but after approximately 3 passages the cultures contained nearly all fibroblast-like cells (Fig. 1). The cells have been successfully cryopreserved at passages 8 to 10. The experiments described below use ASimf20 at passages between 9 and 16.

![Figure 1: Growth of ASimf20 in L-15 alone or L-15 with 10% fetal bovine serum (FBS).](image)

Cultures were initiated at approximately 50,000 cells per well of a 12 well culture plate in L-15 with 10% FBS. The next day the cell number in each of 3 wells was counted with a Coulter Counter to give the initial cell number. The other wells received either L-15 alone or L-15 with 10% FBS. Three wells were counted for each time point and the values expressed as a percentage of the starting cell number.

Treatment of ASimf20 cultures in L-15 with db-cAMP (dibutyryl-cAMP, 500 µg/ml) caused the normally flat bipolar cells to change shape (Fig. 2). The cells acquired a stellate morphology; this shape consisted of a central refractile body from which extend thin cell processes (Fig. 2B). The stellate transformation was obvious by 4 h, and by 24 h nearly all cells in a culture had adopted this new morphology. When the medium with db-cAMP was removed from cultures after 24 h, and replaced with just L-15 containing FBS, all the cells recovered their normal morphology by 4 h (Fig. 2C). Little or no stellation was observed upon the addition of cAMP (not shown). For cells in general, intracellular cAMP levels are raised by exogenous db-cAMP and not exogenous cAMP because db-cAMP better resists degradation and enters cells. The db-cAMP did not cause a change in shape when the culture was supplemented with serum, which might be partially attributed to db-cAMP degradation in serum. In contrast to ASimf20 cells in L-15, cell of the rainbow trout intestinal epithelial cell line, RTgutGC, in the same growth medium showed no change in shape upon the addition of db-cAMP (not shown).

ASimf20 appear to be myofibroblasts because the rapid and reversible shape change that the cells underwent in response to db-cAMP is a characteristic feature of mammalian intestinal myofibroblasts upon treatment with agents that raise intracellular cAMP levels. Primary cultures and cell lines from rodent and human intestinal cells underwent the stellate transformation to myofibroblasts after treatment with a variety of agents, including db-cAMP, that elevate internal cAMP levels. Higher intracellular cAMP levels appear to cause stellation by promoting the depolymerization of actin. The shape changes of intestinal myofibroblasts in vivo are thought to mediate several important actions that contribute to the functions of villi. These include
regulating the passive and active movements of villi and acting as a sieve for nutrients, ions, water and immune cells.

Figure 2: Differential effects of db-cAMP and endothelin-1 alone or together on the morphology of salmonid intestinal fibroblasts. Phase contrast images of ASimf20 cultures are presented with the scale bar denoting 100 µm. Panel A: control culture. Panel B: culture after 24h with db-cAMP (500 µg/ml). Panel C: culture 24 h after the termination of a 24 h exposure to db-cAMP (500 µg/ml). Panel D: culture treated with endothelin-1 (ET-1) (5 µg/ml). Panel E: culture 24 h after the addition of db-cAMP (500 µg/ml) and ET-1 (5 µg/ml) together. Panel F: culture 48 h after the addition of db-cAMP (500 µg/ml) and 24 h after the addition of ET-1 (5 µg/ml) so that the two agents were together for the last 24 h.

The stellate transformation of ASimf20 by db-cAMP was modulated by endothelin-1 (ET-1). On its own, ET-1 at 5 µg/ml caused no change in the shapes of cells (Fig. 2D). However, the addition of ET-1 (5 µg/ml) concurrently with db-cAMP (500 µg/ml) prevented cells from developing the stellate shape characteristic of db-cAMP-treated cultures (Fig. 2E). Likewise, the addition of ET-1 to cultures in which the cells had been exposed to db-cAMP for 24 h, and undergone the stellate transformation, caused the cells to rapidly lose their stellate form and acquire their normal shape, despite the continued presence of db-cAMP (Fig. 2F). The intestinal myofibroblasts of mammals, including the human cell line 18Co, reverse the stellate transformation in response to endothelins\textsuperscript{7,19}. Although not specifically associated with the GIT, the endothelin system, endothelin ligands and their receptors, has been demonstrated in several teleosts\textsuperscript{5}. The responsiveness of ASimf20 to ET-1 further supports their identity as myofibroblasts, implies roles for the endothelin system in the fish GIT, and provides an in vitro tool for studying myofibroblast migration and contraction\textsuperscript{12}.

Myofibroblast cell lines, or in some cases just early passage myofibroblast cultures, have been developed from the intestines of humans, rats and mice and have been used for a variety of experimental purposes. For example, the human intestinal myofibroblast cell line, 18Co, has been used to study how myofibroblasts regulate the growth and differentiation of intestinal epithelial cells\textsuperscript{8}, participate in innate immune responses\textsuperscript{16}, and act as antigen presenting cells\textsuperscript{18}. The development of ASimf20 and other early passage myofibroblast cultures from Atlantic salmon GIT\textsuperscript{13}, could allow similar studies to be carried out in a fish species of tremendous aquaculture value. These cells could aid in the development of inexpensive plant-based feed formulations and help understand for example how soybean meal causes enteritis.
We thank Dr. William R. Wolters from the USDA, ARS National Cold Water Marine Aquaculture Center, Franklin, ME, for supplying the fish used in this research study. This research was supported by a New Investigator Award from MDIBL to LEJL and by the Natural Sciences and Engineering Research Council (NSERC) of Canada with Discovery and Strategic grants to LEJL, BD and NCB.

Influence of pH and egg quality on the yield of viable zebrafish embryos (*Danio rerio*)

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Many experiments in zebrafish addressing genetic or developmental issues rely on obtaining a sufficient number of viable embryos. We found that the pH in the embryo media plays a crucial role in the survival and development of the embryos especially when the egg quality is questionable. In this project we found that zebrafish embryos prefer a more acidic environment with a pH of 6.5 in the embryo media in contrast to the guidelines of the prevailing literature that propose a pH of 7.2. According to our results we believe that this recommendation may have to be revised.

Zebrafish are a well-established model organism in environmental and genetic studies mainly because of the great number of offspring with about 300 embryos per female and week. Despite standardized procedures and a high number of embryos with each spawning attempt, we noticed that the first 24 hours post fertilization is the most delicate time period. We attempted to optimize the yield of viable embryos by comparing different embryo media and measuring the effects on embryo survival. Furthermore we developed a scoring system through which we were able to preselect embryos of “good” and “bad” quality.

We found that the most widely used embryo media is the henceforth-called Embryo Medium 1 according to *The Zebrafish Book* by Westerfield. Commonly used embryo media are made up from similar basic salt solutions in varying concentrations, mostly consisted of NaCl, KCl, Na₂HPO₄, KH₂PO₄, CaCl₂, MgSO₄, and NaHCO₃ to buffer the media. However, the set point for the pH is not clearly defined. In fact, the only clear guideline to buffer the embryo media to a pH of 7.2 is recommended by Westerfield for the Embryo Medium 1. Westerfield also suggests another fairly quick protocol for so-called egg water consisting of 60 g/ml “Instant Ocean®” Sea Salts which should be used for raising very young embryos and for *in vitro* fertilization. In addition we tested the so-called E3 medium, which is described in *Zebrafish: a practical approach* by Nüsslein-Volhard. We found that the unadjusted pH of the simple egg water was ranging around 6.5, whereas the E3 had a final pH around 6.8 to 7.0.

In this study we compared the survival of embryos that were raised in egg water versus the Embryo Medium 1, and E3. We found that the survival rates of embryos in egg water (mean percent survival of 98% after 24hrs, n=98) were higher in contrast to those kept in Embryo Medium 1 (85%, n=70), and E3 (91%, n=35) (Fig.1A) in two consecutive experiments with embryos pooled from different clutches. To elucidate the possibility that a lower pH in the conventional media as described by Westerfield could improve the yield of viable embryos we looked at the survival of zebrafish embryos in Embryo Medium 1 with different pH ranging from 6.2 to 8.0 (n=70, in each group). Interestingly we could document improved survival rates for zebrafish embryos in Embryo Medium 1 with a lower pH as compared to embryos that were left in media with the recommended pH of 7.2 and higher (Fig. 1B).

Regarding the morphology of the zebrafish embryos we found that that clutches from various tanks that were spawned were rather inhomogeneous in terms of phenotype and development. We separately developed a highly reproducible system to separate “good” from “bad” embryos. Figure 2 shows some critical characteristics of these quality characteristics in early-stage embryos.
Fig. 1: Comparison of survival of zebrafish embryos in different embryo media and different pH settings. A) Survival of zebrafish embryos is higher in E3 and simple egg water than in Embryo Media 1 (ERM 1). B) Survival of zebrafish embryos in Embryo Media 1 by Westerfield set to different pH from 6.2 to 8.0.

Our results indicate, that zebrafish embryos prefer an acidic environment, a finding that has previously shown to apply to mammalian ovarian eggs in the setting of reproductive medicine as well1. We conclude that the pH setting is a very important physical property of the embryo media and that it critically influences the yield of viable zebrafish embryos.

Funding: Prof. Dr. Hermann Haller

Temporal sensitivity of oral-aboral axis specification to hypoxia in embryos of the sea urchin, *Strongylocentrotus purpuratus*

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Axis specification is the initial symmetry breaking event that establishes the spatial coordinates for the body plan during ontogeny. The body plans of Bilaterian animals are organized along two orthogonal axes that define the plane of bilateral symmetry. In sea urchin embryos the secondary axis is specified during embryogenesis through the activity of *nodal*, which is amplified and maintained via a positive feedback loop following its initial zygotic activation. Specification of the oral-aboral axis is disrupted by hypoxia, which inhibits *nodal* expression, producing malformed embryos. We show that the developmental timeframe for susceptibility to hypoxia corresponds to the stage at which *nodal* is initially activated.

In the first few days after fertilization, sea urchin embryos develop into pluteus larvae, which are bilaterally symmetric with respect to the plane created by the animal-vegetal axis and the oral-aboral axis. The animal-vegetal axis is determined by the maternally specified cyto-architecture of the egg, but the oral-aboral axis is determined by the zygotic development of oral and aboral ectoderm. Two extracellular signaling ligands, Nodal and Lefty, and the genes which code for them, are essential for the differentiation of oral and aboral ectoderm. Nodal promotes the establishment of oral ectoderm, while Lefty competitively interferes with Nodal. After its initial activation at late cleavage stage, Nodal expression is up-regulated through a positive feedback loop in which the signaling pathway activated by the Nodal protein induces more expression of the *nodal* gene. Nodal signaling also activates expression of Lefty, which inhibits Nodal signaling at a distance. The interactions between Nodal and Lefty thus constitute a reaction-diffusion system that confines *nodal* expression to the prospective oral ectoderm. For this system to work, only a shallow initial source gradient of Nodal in the early embryo is required.

As a member of the TGFβ family of signaling ligands, Nodal regulates gene expression by binding to a transmembrane receptor-kinase on the cell surface, which then phosphorylates and thereby activates members of the Smad2/3 family of transcription factors within the cytoplasm. These move to the nucleus, associate with various cofactors, and activate the expression of genes that have Smad target sites. In sea urchin embryos this pathway can be disrupted by environmental exposures to specific heavy metals (nickel and zinc) or hypoxia, resulting in abnormal expression of *nodal* and hence larvae with a radialized phenotype wherein ectoderm is specified as either all oral or all aboral. Such radialization produces severe malformations of the skeleton and nervous system. In the case of hypoxia *nodal* is underexpressed, for reasons currently unknown. The purpose of this study is to determine the developmental timeframe for this susceptibility.

Approximately 1000 sea urchin eggs were arrayed onto protamine sulfate coated glass bottoms of MatTek dishes and fertilized with dilute sperm using standard methods. To create the hypoxic environment, a glass coverslip was placed over the recessed area containing the embryos. This way, as the embryos developed, they would use oxygen from the water, which would not be replenished because of the cover glass. Control experiments showed that this treatment did not cause a significant drop in pH in the culture medium (data not shown).

In order to discover the period during which oral-aboral axis specification is sensitive to hypoxia, the coverslip was placed and removed in 6 and 12 hour intervals during the first 24 hours of development (Fig. 1A). After 24 hrs, when embryos were hatched blastulae, they were transferred to open culture dishes and developed an additional 24 hrs to late gastrula stage, when the oral-aboral axis is normally well-developed morphologically. At this point, the phenotype of fifty embryos from each dish were examined and scored as being normal, intermediate, or radialized. In normal embryos the gut was displaced toward the oral ectoderm and the two skeletal rods connected at the aboral pole, resulting in a somewhat triangular shape. Radialized embryos were completely round, with a straight centrally located gut and short, radially arrayed skeletal rudiments. Intermediate embryos were in between these two phenotypes, with a gut that was slightly displaced...
toward one side and bilateral skeletal rods which did not connect and were more parallel than normal. The results of several experiments are summarized in Fig. 1.

Figure 1. Temporal effects of hypoxia on oral-aboral axis development in the sea urchin embryo. (A) Experimental design and summary of results. The period during which embryos were under cover glass is indicated by black bars. The phenotypes that were scored (described in the text) are shown schematically, with percentages of each. The number of experiments for each condition is indicated in parentheses. (B) Graphical representation of the results from the three experiments that compared the effects of hypoxia over 0-12 vs. 12-24 hrs post-fertilization (hpf).

As shown in Fig. 1B, the percentage of radialization was much greater for embryos developed under cover glass during the first twelve hours of development compared to the second twelve hours. This suggests that oxygen plays a crucial role during the period when nodal expression is initiated, and is less important for its subsequent maintenance. This conclusion is further supported by the observation that embryos were effectively radialized by exposure to hypoxia limited to the first six hours of development, which is the interval leading up to the initial activation of nodal (Fig. 1A).

In sum, these data indicate that hypoxia affects the initial phase of nodal activation but not its subsequent auto-activation by positive feedback. Although the underlying mechanism for this effect remains unknown, mitochondrial H$_2$O$_2$ was recently shown to be required for the initial activation of nodal, but not its subsequent auto-activation. Thus, one hypothesis for the effects described here is that hypoxia suppresses mitochondrial H$_2$O$_2$ production.

This work was supported by NIEHS STEER program (R25ES016254) awarded to T.L., and by a grant from the NIH (R01 ES016722) to J.A.C.


Cis-regulatory analysis of the cyclinD gene in the sea urchin, Strongylocentrotus purpuratus

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Cyclin D promotes the G1-to-S phase transition of the cell cycle in response to mitogenic signaling during development, and is commonly overexpressed in cancer cells. To elucidate how cyclin D expression is controlled during normal development we have begun to analyze the cis-regulatory DNA sequences of the sea urchin cyclinD gene. Here we describe its temporal pattern of expression during embryogenesis and a computational analysis that identifies candidate cis-regulatory sequences.

Genes of the cyclin D (cycD) family are important in regulating G1 to S phase of the cell cycle during animal development⁹, and also play a role in cell differentiation⁴. Genes of this family have been shown to be mis-expressed in various cancers². To elucidate how cyclin D expression is normally controlled during development, we have carried out an initial characterization of the cyclin D gene (cycD) in the sea urchin S. purpuratus (Sp), measuring its expression level over time during embryogenesis, and computationally identifying candidate cis-regulatory sequences responsible for its control.

To determine the expression profile of cycD during embryogenesis, real-time reverse-transcription coupled PCR analysis was conducted on total RNA isolated from staged S. purpuratus embryos. The RNA was extracted with the RNasy Plus Mini Kit (Qiagen, Valencia, CA), quantitated by spectrometry, and equal amounts were used for cDNA synthesis with random hexamers using the FirstStrand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative PCR was conducted using Perfecta SYBR Green Fast Mix (Quanta Biosciences, Gaithersburg, MD) using a SmartCycler II PCR machine (Cepheid, Sunnyvale, CA). Primers for cycD and ubiquitin (ubi) were the same as reported previously¹⁰. Relative cycD expression at each time point was calculated as 2⁻⁰°Ct, where Ct is the number of PCR cycles required to achieve threshold fluorescence for a given primer pair, and ΔCt is the difference between the Ct values obtained with a given primer pair for egg RNA and RNA from a given time point, i.e.: ΔCt = Ct⁰ - Cttime point. ΔΔCt is then the ΔCt obtained for cycD normalized to that obtained for ubi (which does not change developmentally), i.e.: ΔΔCt = ΔCt⁰cycD - ΔCt⁰ubi.

The analysis showed that cycD expression is first expressed at the early blastula larva stage, peaking initially at 12 h post-fertilization then gradually increasing through the pluteus stage (Fig. 1). It should be noted that this increase occurs on a per-embryo basis over an interval when cell number is also increasing due to continued proliferation, albeit in a subset of tissues. These results are comparable to previously reported findings⁶.

Figure 1. Results of Real-Time PCR analysis to determine cycD expression in S. purpuratus embryos through the pluteus stage. The expression level for each time point is the mean for three experiments, each from a separate culture. Expression levels were calculated as described in the text. Error bars represent standard deviations.

Developmentally regulated genes contain cis-regulatory sequences that generally are non-coding (i.e., lie outside of exons). These sequences, usually several hundred basepairs in length, regulate a gene’s temporal and spatial expression profile by binding to specific transcription factors⁶. In cycD of S. purpuratus, candidate cis-regulatory regions were identified computationally based on meeting the following criteria, which we deduced
based on multiple findings from the literature. Such regions will (1) contain blocks of non-coding sequence that are conserved between *S. purpuratus* and other animals\(^4,6\); (2) have clusters of potential transcription factor binding sites\(^3,7\); and (3) have consensus binding sites for Runx and/or TCF, transcription factors that play key roles in coordinating cell proliferation and differentiation during development\(^10\).

To identify conserved regions, we first obtained the sequences of *cycD* from *S. purpuratus* (*Sp-cycD*, genbank NW_784111.1). For sequence comparisons, we used available sequences from *Lytechinus variegatus*, another sea urchin whose evolutionary line diverged from that leading to *S. purpuratus* approximately 50 million years ago\(^6\), and mouse, a more distantly related deuterostome. The available *L. variegatus* sequence (*Lv-cycD*) was from a BAC that only contained sequence upstream of exon 1. Mouse sequences of complete *cycD1*, *D2* and *D3* were obtained from mouse genome informatics (http://www.informatics.jax.org/). We next performed sequence comparisons using the program FamilyRelationsII\(^4\) (available at http://family.caltech.edu/). This program uses a “sliding window” to identify conserved regions between two or three sequences irrespective of where or how many times each region occurs in each sequence\(^4\). A sample output from a FamilyRelationsII analysis between *Sp-cycD* and mouse *cycD2* is shown in Fig. 2A. Regions with \( \geq 90\% \) sequence similarity between the two sequences are united by lines. Fig. 2B shows the identity of one of these conserved regions (highlighted by rectangles in Fig. 2A). It should be noted that the locations of exons and introns are not shown in the output from a FamilyRelationsII analysis. To determine whether a conserved sequence falls outside of an exon, further mapping is required.

![Figure 2](image.png)

**Figure 2.** Example of FamilyRelationsII output from a comparison between the *cycD* sequence of *S. purpuratus* (*Sp-cycD*) and *cycD2* of mouse (*Mm-cycD2*). (A) Comparison of 50 kb of sequence encompassing the entire transcription unit of *Sp-cycD*, plus several kilobases upstream and downstream, and 20.8 kb of sequence encompassing the *cycD2* locus of mouse. (B) Close up of an identified non-coding sequence conserved between sea urchin and mouse, shown by rectangles in (A).

For our analysis, we performed a series of comparisons. When comparing the *S. purpuratus* and *L. variegatus* sequences, only the region upstream of exon 1 was used due to the lack of downstream *L. variegatus* sequence. We performed pairwise analyses between *S. purpuratus* *cycD* and each of the other genes (*Lv-cycD* and each of the three mouse paralogues), as well as 3-way analyses between *Sp-cycD*, *Lv-cycD*, and each mouse parologue. We first searched for sequences that were greater than or equal to 20 nucleotides and that were greater than or equal to 75% identical. In many cases, when the 75% similarity threshold was used, FamilyRelationsII would show too many sequences to count. In these cases, the threshold of similarity was increased, generally to 90% similarity, to yield a more manageable number of sequences. We mapped the location of each conserved sequence to the *cycD* sequence of *S. purpuratus*. From this, we could determine which conserved regions fell outside of exons. In addition, we searched for regions in the *cycD* sequence where clusters of transcription factors might bind. Sequence sites where transcription factors cluster may be regulatory\(^3\). We used the program Cluster-Buster, a freely available program (http://zlab.bu.edu/cluster-buster/) that searches for clusters of motifs known to bind specific transcription factors\(^3\) to discover such potential sites in the *cycD* sequence of *S. purpuratus*. Four such clusters were identified and mapped to the *Sp-cycD* sequence. Likewise, we searched for and mapped consensus TCF and Runx binding sites to *Sp-cycD*. From a number of lines of evidence, Runx and TCF transcription factors have been proposed to cooperate to regulate *cycD* expression\(^10\).
Based on where the above regions of interest mapped to Sp-cycD, we identified 14 regions, located outside of exons that may either be single cis-regulatory modules, or regions containing multiple modules. Figure 3 shows schematically the locations of these regions within Sp-cycD, along within the location of the five exons. The criteria met by each region are summarized below the region in Fig. 3. In addition to the elements already mentioned, some regions contained G strings, which have been shown to be important in controlling transcription of developmentally regulated genes in sea urchins\(^\text{11}\).

Figure 3. Schematic of Sp-cycD (50 kb), showing locations of the 5 exons (blue, labeled with roman numerals I-V), and 14 conserved noncoding sequence elements (red) to be subcloned into EpGFPII. Key: Conservation between species and other criteria indicated as: Sp- S. purpuratus; Lv- L. variegatus; M: mouse; R: putative Runx binding site(s); T: putative TCF/Lef binding site(s); C: putative transcription factor cluster sites; Rep: highly repetitive DNA; G: G string.

Future work will determine the unique role of each of the above sequence regions in regulating the expression profile of cycD during the embryonic development of S. purpuratus. Toward that end, primers specific for each region have been designed and used to amplify each region of interest. Each amplicon was incorporated individually into the GFP expression vector EpGFPII\(^5\), then micro-injected into fertilized S. purpuratus embryos. This analysis will involve first determining the temporal and spatial expression profile of each construct. Next, we will use mutagenesis to explore the functionality of subregions, such as Runx and TCF binding sites, within each region.

This project was funded by the NIH (R01-GM070840 to J.A.C. and INBRE P20-RR-016463).

A green fluorescent protein reporter plasmid for identifying redox-sensitive cis-regulatory elements that mediate nodal activation in embryos of the sea urchin, Strongylocentrotus purpuratus

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In sea urchin embryos, oral ectoderm is specified by localized expression of nodal, which correlates spatially with the high end of a mitochondrial gradient in the early embryo. We have shown previously that initial activation of nodal requires mitochondrial H$_2$O$_2$. Here, a Green Fluorescent Protein (GFP) reporter plasmid was constructed as a reagent for identifying nodal cis-regulatory sequence elements that mediate this redox sensitivity. Preliminary experiments using a mitochondrially-targeted catalase suggest that like nodal, expression of this reporter may be responsive to mitochondrial H$_2$O$_2$.

The gene nodal encodes a TGFβ family signaling ligand that controls specification of the oral-aboral axis of the sea urchin embryo. Following its initial zygotic expression, Nodal activates its own production and that of its more quickly diffusible antagonist, Lefty, thereby localizing nodal expression to the prospective oral ectoderm of the embryo. The spatially asymmetric activation of nodal correlates with an anisotropic distribution of mitochondria in the early embryo and was recently shown to require mitochondrial H$_2$O$_2$.

Approximately one kilobase of 5' sequence flanking the first exon of nodal (nodal-5P) mediates its initial transcriptional activation in late cleavage stage embryos. Within nodal-5P, canonical target sequences for basic leucine-zipper (bZIP) transcription factors have been identified that are hypothesized to mediate the initial asymmetric activation of nodal in response to the redox asymmetry present in the early embryo. While the bZIP sites have been shown to be required for early nodal activation, their sensitivity to redox signaling has not been directly tested. We have constructed a reporter plasmid that encodes green fluorescent protein (GFP) linked to the nodal-5P cis-regulatory element, which will serve as a reagent for studies aimed at testing the redox-sensitivity of the bZip sites as well as other transcription factor target sequences within the nodal-5P region.

A bacterial artificial chromosome (BAC) containing the coding sequence of GFP inserted in place of the first exon of the nodal gene from S. purpuratus was used as a PCR template to amplify the 5P element together with the GFP coding sequence and associated downstream polyadenylation sequence. The PCR product was ligated into pGEM-T-Easy and transformed into E. coli. Qiagen mini-preps of plasmid DNA from twelve transformed bacterial clones were prepared and digested with EcoRI to check for the insert, and the nine containing the insert were then digested with NcoI to determine the insert orientation. Two of these sequences were then submitted for sequencing and aligned to genomic sequences generated by the sea urchin genome project stored in the NCBI database, confirming that the plasmid contained the correct sequences. One of the plasmids was linearized using SalI for injection into sea urchin embryos. In addition, a mitochondrially-targeted catalase (Mt-Cat) mRNA was prepared from a previously described plasmid construct, using the T7 mRNA Machine from Ambion.

Zygotes affixed to protamine sulfate-coated dishes were microinjected using standard methods of timed pressure injection. Linearized nodal-5P-GFP plasmid DNA was injected at a concentration of 2.5 ng/μl, in a solution containing 10 ng/μl restriction-digested sea urchin genomic DNA as carrier and 120 mM KCl. This amount of nodal-5P-GFP was empirically determined to be the minimum required to produce GFP fluorescence in the early embryo. After the injected zygotes had developed to the 2-cell stage, one of the two blastomeres was again injected with a solution containing 2 mg/ml fluorescent dextran in 120 mM KCl, either with or without Mt-Cat mRNA at a concentration of 162 ng/μl (Fig. 1). The embryos were then developed to the blastula stage, whereupon confocal fluorescence imaging was used to determine the spatial relationship between nodal-5P-GFP expression (green fluorescence) and dextran (red fluorescence) (Fig. 2). The prediction was that in embryos injected with dextran plus Mt-Cat mRNA, the frequency of co-localization between green and red fluorescence would be significantly lower than that in embryos injected with dextran alone, owing to reduced H$_2$O$_2$ levels (and hence lower rate of nodal activation) within the cells expressing Mt-Cat.
The results of three different experiments are shown in Table 1. Although Mt-Cat expression did produce the expected spatial bias in the pattern of nodal-5P-GFP expression in one experiment (Table 1, asterisk), this result was not reproduced in subsequent experiments. The reason for this variability is not known, although it may relate to the technical difficulty of injecting quantities of DNA and RNA that are within an optimal range. Additional experiments are therefore required.

Table 1. Quantitation of fluorescence co-localization results from three experiments in which nodal-5P-GFP-injected zygotes were injected at the two cell stage with fluorescent dextran alone (control) or dextran plus 162 ng/µl Mt-Cat mRNA, as illustrated in Fig. 1. Co-localization of dextran and GFP was then assessed as shown in Fig. 2. Only one of three experiments produced the expected effect (*).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fluorescence co-localized (# embryos)</th>
<th>Fluorescence not co-localized (# embryos)</th>
<th>Co-localization frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>4</td>
<td>6</td>
<td>0.4</td>
</tr>
<tr>
<td>Control 2</td>
<td>4</td>
<td>8</td>
<td>0.36</td>
</tr>
<tr>
<td>Control 3</td>
<td>2</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Control Total</strong></td>
<td><strong>10</strong></td>
<td><strong>15</strong></td>
<td><strong>0.4</strong></td>
</tr>
<tr>
<td>Mt-Cat 1</td>
<td>1</td>
<td>18</td>
<td>.05*</td>
</tr>
<tr>
<td>Mt-Cat 2</td>
<td>3</td>
<td>4</td>
<td>.43</td>
</tr>
<tr>
<td>Mt-Cat 3</td>
<td>12</td>
<td>7</td>
<td>.63</td>
</tr>
<tr>
<td><strong>Mt-Cat Total</strong></td>
<td><strong>16</strong></td>
<td><strong>29</strong></td>
<td><strong>0.36</strong></td>
</tr>
</tbody>
</table>

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Population genetics of eelgrass (Zostera marina) from the Jordan River

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\textsuperscript{2}Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672

Populations with high levels of genetic diversity are better able to adapt to change in their environment. Eelgrass from the Jordan River is being used to re-establish eelgrass beds at Hadley Point. Individual eelgrass plants from the Jordan River donor site were genetically characterized. This preliminary investigation found high levels of genetic diversity within the Jordan River.

Zostera marina collected from the Jordan River, Hancock County, Maine is being used to restore eelgrass beds at Hadley Point, Bar Harbor Maine. In 2007, 2008, and 2009 eelgrass was collected from the Jordan River and transplanted to protected areas to re-establish eelgrass habitat. Eelgrass communities across all regions of the world are being lost at a rapid rate\textsuperscript{8}. This preliminary study investigated the population genetics of the Jordan River eelgrass donor site. Manipulative field experiments\textsuperscript{1,4} demonstrate that the greater the genetic diversity in a population of Z. marina, the more resistant beds are to changes and disturbances. The success of MDIBL transplant efforts is therefore dependent on the genetic diversity of the Jordan River population. The purpose of this study was to evaluate genetic diversity at the donor site.

In June 2009, 80 samples of Z. marina were collected from a sampling grid (grid and sampling method published previously\textsuperscript{7}) set up parallel to the shore of the Jordan River. The grid consisted of 5 quadrants, each with an area of 10m\textsuperscript{2}. Sixteen samples were taken from each quadrant, with a minimum of 1m between samples to reduce the likelihood of collecting more than one sample from a single ramet\textsuperscript{7}. The northeast corner (bottom right corner in figure 1) of the grid was located at 44°28’18.12 N, 68°21’20.20 W.

Following standard CTAB DNA extraction, six polymorphic microsatellite loci\textsuperscript{6}, that exhibit high levels of polymorphism between genetic individuals, were individually PCR amplified using fluorescently labeled forward primers and unlabeled reverse primers. Amplified samples were multiplexed on the ABI 3130XL sequencers to determine size in base pairs. All 6 loci were polymorphic, and the proportion of distinct genotypes (non-clones) from the completely genotyped samples is G = 0.951 (Fig. 1). The Z. marina population in the Jordan River has relatively high levels of genetic diversity as compared to published data from North American sites, and the Mediterranean and Baltic Seas\textsuperscript{2,3,5,9}. The value of H\textsubscript{o} (observed heterozygosity) for the Jordan River is the 21st highest out of 83 sites, and 0.77 standard deviations above the mean (0.4199). The Jordan River donor site also exhibits a high level of intra-site genetic diversity. Most samples (58 of 61 completely genotyped) had unique genotypes. The sampling method was modeled from Reusch\textsuperscript{7} who found 12 shared clonal genotypes within 80 samples collected in a similar fashion. For the specific grid site used in this study, the Jordan River appears to have relatively low levels of clonal propagation. The Jordan River displays a low level of asexual reproduction, a high level of genetic diversity relative to other sites, and a higher H\textsubscript{o} than H\textsubscript{e}; this initial analysis suggests that the Jordan River is an appropriate donor site for transplants.

![Figure 1](image)

Figure 1. Locations of genotypes within the sampling grid. An open circle represents a genotype that occurs only once. A x represents a sample for which all six microsatellite loci have not been scored and could therefore be either a unique genotype or a clone of another sample. C1, C2 and C3 represent identical genotypes at all 6 loci; these are likely examples of clonal individuals.
This work was supported by a 2009 NSF REU (0453391) award to E. Correa.

Temporal Molecular Variation among *Squalus acanthias* from the Gulf of Maine

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The number spiny dogfish in the Northwest Atlantic is declining. For individual dogfish samples from 2004, 2007 and 2008, two types of genetic data were collected. Both genetic data sets exhibit declining levels of genetic diversity from 2004 to 2008.

The spiny dogfish *Squalus acanthias* L. is a highly migratory, small demersal shark species found globally in temperate shelf seas. In the north-west Atlantic, spiny dogfish are found from Labrador to the Florida Keys, migrating to the Gulf of Maine and Canadian waters in the summer and returning southward in the winter¹. Historically abundant worldwide, many stocks from around the globe are currently in significant decline prompting the species to be listed as vulnerable by the IUCN Red List of Threatened Species (http://iucnredlist.org/details/39326). In the north-west Atlantic, a 75% decline in the biomass of mature females recently occurred over a period of approximately a decade³. Proper management of the spiny dogfish is especially critical because its unique life history makes it particularly vulnerable to overexploitation. An understanding of a species’ genetic stock structure is a fundamental requirement for any long-term, effective management strategy and was highlighted as a research priority by the most recent stock assessment on spiny dogfish in US territorial waters³.

Genomic DNA was extracted from dogfish caught in 2004, 2007, and 2008 from the Gulf of Maine. Seven microsatellite loci and the mitochondrial D-loop were amplified via PCR and subsequently genotyped and sequenced, respectively²,⁴. Diversity statistics were calculated for all microsatellite loci combined (Table 1) and at the D-loop (Table 2).

### Table 1. Total number of individuals analyzed (*n*), mean observed number of alleles (*N₀*), mean observed heterozygosity (*H₀*), and mean Nei's diversity (*Dₙ*).

<table>
<thead>
<tr>
<th>Year</th>
<th><em>n</em></th>
<th><em>N₀</em></th>
<th><em>H₀</em></th>
<th><em>Dₙ</em></th>
</tr>
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<tbody>
<tr>
<td>2004</td>
<td>58</td>
<td>12.86</td>
<td>0.708</td>
<td>0.752</td>
</tr>
<tr>
<td>2007</td>
<td>53</td>
<td>13.00</td>
<td>0.693</td>
<td>0.725</td>
</tr>
<tr>
<td>2008</td>
<td>48</td>
<td>9.57</td>
<td>0.633</td>
<td>0.679</td>
</tr>
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</table>

### Table 2. Total number of individuals analyzed (*n*), the number of haplotypes revealed (*h₀*), haplotype diversity (*h*), and percent nucleotide sequence diversity (% π).

<table>
<thead>
<tr>
<th>Year</th>
<th><em>n</em></th>
<th><em>h₀</em></th>
<th><em>h</em></th>
<th>%π</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>44</td>
<td>32</td>
<td>0.982</td>
<td>0.5</td>
</tr>
<tr>
<td>2007</td>
<td>50</td>
<td>31</td>
<td>0.964</td>
<td>0.3</td>
</tr>
<tr>
<td>2008</td>
<td>49</td>
<td>23</td>
<td>0.953</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Gulf of Maine dogfish show a high degree of molecular variation at microsatellite loci and at the mitochondrial D-loop. Measures of observed numbers of alleles (*N₀*), observed heterozygosity (*H₀*), Nei's diversity (*Dₙ*), number of haplotypes (*h₀*), haplotype diversity (*h*), and nucleotide sequence diversity (% π) suggest reductions in molecular variation from 2004-2008 potentially due to mechanisms of genetic drift. Further analysis of additional temporal samples from 2009 may elucidate whether this trend continues. One of the foundations of evolutionary biology is that genetic diversity is required for populations to evolve in response to environmental change. If natural selection is, indeed, structuring this population and if measures of neutral molecular variation are, in fact, indicative of adaptive genetic variation, then this data suggests the possibility that dogfish from the Gulf of Maine may be adaptively constrained by an historical loss of heritable variance.

This work was supported by a Maine IDEA Network of Biomedical Research Excellence grant (Maine INBRE P20 RR-016463), a University of Maine System Maine Economic Improvement Fund grant, and by 2008 and 2009 MDIBL New Investigator Awards.

Characterization of pH and total alkalinity in waters along a north to south transect in Frenchman Bay, ME

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Carbon dioxide in the atmosphere is thought to be at the highest levels on earth in at least the last 800,000 years. Ocean acidification and effects on marine life are serious concerns. Here, we have begun baseline measurements of pH and CO₂ effects on the marine environment in Frenchman Bay.

Carbon dioxide absorption in the oceans liberates H⁺ ions from carbonic acid (H₂CO₃) and bicarbonate (HCO₃⁻) in a reversible and balanced reaction:\[ CO₂ + H₂O ⇌ H₂CO₃ ⇌ HCO₃⁻ + H⁺ ⇌ CO₃²⁻ + 2H⁺. \]

However, due to the increased amount of carbon dioxide in the atmosphere from anthropogenic activity, 20%-30% of the gas is absorbed by ocean surface, which amounts to more H⁺ in the oceans, thus increasing ocean acidity. Recent data indicates that this increased acidity is especially prevalent in the surface waters of the Atlantic Ocean. CO₂ first enters the ocean cycle from Southern oceans and is carried North by deep ocean currents, where high latitudes and cold waters enhance CO₂ solubility conditions. CO₂ saturated fog from New England industrial areas also often lingers in the areas of Maine and Nova Scotia. The current average ocean pH is around 8.1, and is predicted to decrease by 0.3-0.4 pH units by the end of the century. Currently there is little published data of the water pH in Frenchman Bay, ME near the MDIBL. In this preliminary study we have begun to characterize the pH and associated water quality parameters, including temperature, salinity, dissolved inorganic carbon (DIC), and total alkalinity (TA) of a small transect in Frenchman’s Bay, ME. In future experiments, ambient pH values will be used to test if gill sodium hydrogen exchangers (NHEs) of marine longhorn sculpin, *Myoxocephalus octodecemspinosus*, are modified to maintain homeostasis.

Twenty-one surface and bottom water samples were collected on three separate days (during the months of June and July) by lowering a Niskin bottle on a line at three coordinates along a South to North transect. The sampling sites began near the moorings off of the MDIBL (N44°26’02.3”, W068°17’25.5”; 6m depth), through pelagic waters (N44°26’21.8”, W068°17’21.8”; 20m depth) to Lamoine Beach (N44°27’00.4”, W068°17’10.2”; 11m depth). After lowering the bottle to the target depth, a weighted messenger was released down the line to stopper both ends of the bottle. After retrieval of the bottle, the water was immediately analyzed at the surface for salinity and temperature (YSI-85 handheld dissolved oxygen/ conductivity meter) and water was quickly transferred from the Niskin bottle through Silicone tubing to rinse a borosilicate glass bottle that was then filled with the water sample, from bottom to top and without any headspace or the introduction of air bubbles. Three replicates were collected from each Niskin bottle sample. To each bottle a drop of mercuric chloride (HgCl₂) was added to stop any metabolic activity. The samples where stored at 4° C prior to shipping on ice to the Ocean Process Analysis Lab (OPAL) at the University of New Hampshire in Durham, for analysis of DIC, TA, and pH (using salinity and temperature measurements). DIC was measured in quadruplicate while TA and pH were measured in duplicate.

Water samples from the bottom at each location in Frenchman Bay were found to have lower pH and higher TA than water taken from the surface at the corresponding locations (Table 1; p<0.001). Mean salinity and temperature taken from the bottom and surface were not significantly different. pH and TA between the three different sampling locations (Table 2) were also significantly different between sites (Mann-Whitney-U test and corrections for p-values was performed between pairs). Water pH was significantly lower mid-bay than at the MDIBL mooring area (p<0.01).

pH may be significantly lower at the bottom of Frenchman Bay because of ocean currents that mix the top layer of the seawater, which absorbs CO₂ from the atmosphere, with the bottom layer. It could also be due to near-bottom organismal metabolism, in which the total concentration of carbon outweighs TA. In other words the buffering capacity of CO₃²⁻ and HCO₃⁻ is outweighed by CO₂, making the water more acidic. The TA may
be higher at the bottom because CO$_3^{-2}$ release in the deep ocean increases TA$^{15}$. We had speculated that salinity would be greater and the temperature would be lower at the bottom (cooler, higher density water sinking) but there were no differences when all three sites are pooled and compared between surface and bottom though the bottom water temperatures ranged from 10.6° C off of Lamoine to 16.6° C at the MDIBL. Additional data will be needed to support the small sample size in this preliminary study. Nevertheless, our data does correspond to other salinity measurements taken at N44° latitude and W68° longitude$^1$. CO$_2$ has local fluctuation patterns in four dimensions: spatially (coastal vs. pelagic waters), diurnally (respiration is highest at night), vertically (height in the water column), and seasonally (cold waters in the winter naturally hold more CO$_3^{-2}$) $^{35}$. So although this study does not provide a complete picture of pH water quality parameters in Frenchman Bay, this sample of pH values are already at the projected pH values that are expected to occur by the end of the century $^{10}$. Funded by NSF IOB-0616187 to JBC

Table 1: Mean water quality parameters measured in Frenchman Bay, ME, taken within 1m of the surface and at the bottom of the bay at three different locations. pH U=1109, df = 1, p<0.001. TA U=1035, df = 1, p<0.001. Salinity U=42, df=1, p=0.2841. Temperature U=48, df=1, p=0.0899. Mann-Whitney-U test, mean ± SD. 

<table>
<thead>
<tr>
<th>Depth</th>
<th>pH</th>
<th>TA (µmol/kg)</th>
<th>Salinity (ppt)</th>
<th>Temperature (*°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>Mean</td>
<td>7.91 ±0.41</td>
<td>2536 ±79.3</td>
<td>31.0 ±0.24</td>
</tr>
<tr>
<td>Bottom</td>
<td>Mean</td>
<td>7.75 ±0.08</td>
<td>2086 ±32.5</td>
<td>31.3 ±0.56</td>
</tr>
</tbody>
</table>

Table 2: Water quality parameters measured in Frenchman Bay, ME. pH H=7.9764, df=2, p=0.019. TA H=6.6155, df=2, p=0.0366. Kruskal-Wallis test, mean ± SD. 

<table>
<thead>
<tr>
<th>Location</th>
<th>pH</th>
<th>TA (µmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDIBL: N 44°26'21.8'' W068°17'25.5''</td>
<td>Mean</td>
<td>7.93 ±0.45</td>
</tr>
<tr>
<td>Mid-bay: N44°26'21.8'' W068°17'21.8''</td>
<td>Mean</td>
<td>7.74 ±0.17</td>
</tr>
<tr>
<td>Lamoine: N44°27'00.4'' W068°17'10.2''</td>
<td>Mean</td>
<td>7.81 ±0.06</td>
</tr>
</tbody>
</table>

Eelgrass restoration over two summers at Hadley Point has resulted in a measurable increase in eelgrass coverage in upper Frenchman Bay, as determined by video monitoring and area estimation. In the process, community involvement has resulted in a greatly increased appreciation for the value of this species.

Eelgrass (Zostera marina L.) is a flowering plant formerly occurring in widespread meadows in sub-tidal areas along the coast of Maine. Eelgrass beds are home for a diversity of marine organisms, and serve as a nursery for a variety of larval forms including winter flounder, hake, pollock, and cod, as well as larval lobsters, mussels, and crabs. As a primary producer, eelgrass helps fuel the food chain, feeding organisms like bacteria, worms, and a host of marine invertebrates. Eelgrass also curbs erosion by stabilizing bottom sediments and may improve water quality by filtering excess nutrients from runoff. In Frenchman Bay, eelgrass has experienced significant declines, apparently related to dragging for mussels and other sessile species. At Hadley Point the bottom coverage was estimated at 60 - 80% coverage in 1996. By 2007, our pre-project underwater videography revealed that the coverage was down to <1%.

Pre-restoration water quality analyses indicated that Hadley Point was a suitable site for transplanting eelgrass, with excellent values for all parameters measured. We used the Preliminary Transplant Suitability Index (PTSI) model of Short and Burdick to confirm the suitability of the Hadley Point site.

In 2007 and 2008, multiple community groups and individual volunteers contributed to the restoration of eelgrass at Hadley Point. Plants were harvested from the Jordan River (located across the bay from Hadley Point) and tied to wire grids weighted with bricks in an adaptation of the TERF (Transplanting Eelgrass with Remote Frames) method. The grids were placed in the shallow sub-tidal area, and removed ~10 months later after the eelgrass plants had taken root. Fifty grids, each 2' by 2' (~0.4 m²), were transplanted each year (Figure 1). The restoration site was monitored by underwater video transects; coverage being determined by the ratio of the time eelgrass was seen to the total transect time. Using this method, we have determined that eelgrass coverage has increased from <1% to 8.4% in the restoration area at Hadley Point with two years of restoration effort (Figure 2).
Eelgrass area expansion was also determined by using a measuring stick to roughly estimate the size of all eelgrass patches in the restoration area at low tide. GPS coordinates were recorded for eelgrass patches over 1 m$^2$ (Figure 1). We transplanted a total of 36.4 m$^2$ of eelgrass (100 grids) in 2007 and 2008; and measured >500 m$^2$ of coverage in 2009, nearly a 15-fold increase. In addition, new eelgrass beds have developed, apparently by seeding from the transplanted plants, in nearby sites, including the east side of Hadley Point and the Lamoine shoreline.

Most important in the long run may be the results of our public outreach and education programs. Local students have contributed to restoration and monitoring, learning that it requires a lot of work to restore habitat once it has been disrupted. In addition, we have educated hundreds of visitors at the Myers Marine Aquarium at MDIBL about the importance of eelgrass as habitat in subtidal areas of Frenchman Bay.

We thank undergraduate interns Omar Aquino, Kavita Balkaran, Gar Gilchrist, Molly Miller and Casie Reed, (supported by NSF REU DBI-0453391), and Sarah Colletti (supported by EPA 97169501-0), high school interns Ellen Daily (supported by NIEHS STEER R25-E5016254). We also thank Eliza Childs, AmeriCorps Volunteer Leader Ariel Durrant, and Vista Volunteer Sarah Joy. Partners include the Town of Bar Harbor, Bar Harbor Marine Resources Committee, College of the Atlantic, Maine Mussel Harvesters Association, Acadia Aqua Farms, and the Bagaduce Watershed Association. The project was funded by a Restoration Grant from Gulf of Maine Council, a National Fish and Wildlife Foundation Habitat Restoration Grant and the Davis Conservation Foundation.

1. Barker, Seth. Maine Dept. of Marine Resources, Boothbay Harbor, personal communication
Drift buoys monitor surface currents driving dispersal of eelgrass (*Zostera marina*) seeds

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Eelgrass beds, which are important nurseries for many aquatic organisms, have been nearly removed from upper Frenchman Bay during the last decade. Our work efforts have shown that seed dispersal is an important part of restoration, for which water flow data are needed. This report summarizes one summer’s results with a radiotelemetry technique to track surface currents.

Eelgrass (*Zostera marina* L.) reproduces both by rhizomes and by seeds. The latter, with their capability for wide and rapid spreading, are probably very important in the reestablishment of normal eelgrass beds in denuded areas. Seeds are produced along the margins of the leaves, and are dispersed when the blade breaks off. Since the blades float, the attached seeds can be carried for long distances until they finally disintegrate, dropping the seeds to the bottom\(^6\). For maximum efficiency, we desire to transplant flowering shoots into areas where the currents will carry the seeds to suitable substrate.

Only a rough outline of current patterns in upper Frenchman Bay is available, mostly from anecdotal information. We therefore constructed “drift buoys” as shown in Figure 1 and equipped them with a combination GPS-controller-radio transmitter (Byonics MT-AIO)\(^3\) that is set to report its position every 2 minutes. The buoy floats as low as possible to minimize the direct effect of wind on the buoy. We use a frequency in the amateur 2-meter band (GWK is a licensed radio amateur) which is received by three recording/relay stations on shore, two of which were connected to computers running the WinAPRS program\(^7\) to log the data.

At 2-minute intervals, the unit activates its GPS receiver. When a valid position has been acquired, the controller transmits a NMEA\(^5\) GPRMC\(^4\) sentence containing date, time of day, latitude, longitude, and velocity, along with identification information. The position reporting resolution is 0.0001 minutes of arc or ~18 cm, which is greater than the accuracy of the GPS unit. The GPS resolution was checked with a series of 127 recordings over 63.5 min while the buoy was kept stationary. The maximum latitude error was 4.09 x 10\(^{-2}\) minutes of arc, which at 6000 ft (one nautical mile) per minute of arc is 245 ft (103 meters), while the standard error of these data was 6.73 x 10\(^{-4}\) minutes of arc, or 1.33 meters (4.38 ft). The longitude errors were similar, but since a minute of longitude is only 4500 ft at our latitude, the errors are smaller when expressed in distance units. To increase the accuracy of the velocity measurements, we calculated speed and direction from pairs of readings separated by at least 2 minutes in time.

One or two buoys were deployed at a time during parts of 37 days between July 1 and October 9, 2009. Variation in the time of high tide provides a range of tide conditions during daylight hours. We collected nearly 18,000 individual records; after rejecting duplicate and spurious data (recorded while the buoy was on the dock, washed ashore or in transit) the remaining 8522 records are the valid data set.

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Figure 1. Drift buoy made of PVC pipe and Styrofoam\(^*\), sitting on cement block. The 4” white pipe contains the GPS/transmitter; the 3/4” spike houses the radio antenna.
The sorting and plotting programs were written in JustBASIC®. For each running pair of points, we calculated the differences in latitude and in longitude (minutes of arc), converting these to distances in nautical miles. We then calculated the distance and direction between each pair of points, expressing the result as a velocity vector (speed and direction).

We plotted the data on a nautical chart, using UIView2 which is designed for real-time plotting of amateur APRS data and transferring the data to ArcGIS1 for presentation. Figure 2 shows an example of a single track, translated to GIS format and plotted on a nautical chart of the area. The buoy was released near our restoration area, which has had an increasing (now 4-8%) eelgrass coverage over the past two summers. It started east on the ebb tide, changed direction during the height of the flood, but subsequently resumed its easterly movement, running parallel to the Lamoine Beach shore. Many similar observations show a tendency for currents to flow from the restoration area onto the Lamoine shore, accounting for the incipient eelgrass beds recorded there in the summer of 2009.

It is difficult to summarize the 53 valid drift runs obtained, since current speed and direction must be correlated with latitude, longitude and time of tide cycle, at a minimum. Figures 3, 4, and 5 are our attempts at this process. Some parameters are relatively easy to present. For instance, the starting positions ideally should be evenly distributed both in space (latitude and longitude) and time (relative to the tide cycles). Spatial distribution is tested by
plotting the initial positions of each day's record, as shown in Figure 3. There are some obvious gaps (notably Berry Cove and west of Thomas Island) which are targets for future work, and there are some areas of concentration, such as the region around the Hadley Point restoration reserve, which was obviously of interest to this study. Practical considerations of distance from the laboratory and likelihood of the buoy's running aground were also factors, but this does not seem to have severely biased our conclusions.

To check the distribution within the tide cycle, we broke the data into 30-minute "bins" indexed to the previous high tide, calculated the velocity and direction of movement between each pair of points, and plotted these against location as a set of 25 charts covering the 12 hour 26 min tide cycle. The 25 bins contain 337 ± 6 records each, correcting for the short-time 25th bin, showing the expected uniform distribution of records across the tide cycle. The distribution of velocities varies greatly across the tide cycle, as might be expected. Figure 4 shows the average direction and speed for the 25 bins. During the ebb tide there is an easterly trend to the current, and the speed is also maximum at these times. However, the flood tide (bin 12 and above) does not show a corresponding westerly current. Two factors could account for this observation. First, the prevailing winds are from the west, and tend to produce surface currents which add to the tidal currents. The water would then be returned in a sub-surface current with the opposite orientation. Secondly, there is a considerable fresh water inflow from Northeast Creek, which varies in magnitude but is always present. This would establish a net outflow from Eastern Bay toward the east (flow through the narrow to the west is minor) and would particularly intensify the current at the surface. In the absence of complete hydrographic data, these remain speculations, but it is interesting that Eastern Bay is one of the few portions of the Maine coast which has never been closed to mussel harvesting due to red tide (Alexandrium sp.); this prevailing outflow might prevent the organism from entering the bay.

In an attempt to represent location, time and current flow, we laid down a rectangle 3.75 min (nm) in latitude by 7.5 min (5.6 nm) in longitude, with its southeast corner at 44°25.25' N by 68°14.25' W, which covers the recorded region. This rectangle was divided into a grid of 450 blocks, each 0.25 min on a side, and the data from within this block was averaged and plotted on the center of this block. Clearly some of these blocks will be unoccupied, as they fall on land, and some blocks will have a mid-point on land although there is valid data from that portion which falls on water. For each 30-minute tide bin, each data point was assigned to its position block, and the average velocity and direction for all points in the block was calculated. Figure 5 shows the results for the sum of bins 15 through 21, 7.7 to 11 hours after high tide. This covers the times for which the maximum flood currents should be observed. If the currents were exclusively tide-driven, one would expect this period to produce currents headed into Eastern Bay, that is, a westerly trend. Clearly this is not the case, and the velocity vectors are found in a range of orientations and magnitudes.
It is clear that even this large number of data points is not sufficient to give a complete picture of the currents in Eastern bay. We can, however, form some preliminary conclusions. One is that there are often currents which could carry propagules from our transplanted area west of Hadley point toward the Lamoine shore, which seem to be the reason for the recent identification of young eelgrass beds at the latter sites. We need to identify other trends as a guide to future transplantation efforts. Secondly, averaged over the tide cycles, there seems to be a general eastward trend to the currents, as might be expected from the fresh water input to Eastern Bay (Northeast Creek, primarily) and the prevailing westerly winds. We noticed many records in which the buoy movement is circular or contrary to the assumed tidal current flow, which would lead to leaf fragments "stalling" in eddies. This may be very important for determining the locations of seeding in *Zostera marina*.

Figure 5. A plot of the data from 7.5 to 11 hours after high tide (during the flood portion of the cycle), broken into spatial blocks 0.5 minutes on a side. (Not rectangular, since minutes of longitude are smaller than minutes of latitude.) The arrowhead in the center of each block shows the average current direction and speed for these data. While one might expect a westerly trend on a flood tide, the average currents are seen to flow in various directions depending on position.

MM supported by NSF DBI-0453391; the project funded by Gulf of Maine Council, the Davis Conservation Foundation, the National Fish and Wildlife Foundation and an anonymous donor. Many volunteers assisted in various portions of this work, especially in buoy recovery.

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Tidal height and herbivore density affect grazing intensity on *Ascophyllum nodosum*

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The rocky coastline of Maine is commonly inhabited by the knotted wrack seaweed (*Ascophyllum nodosum*). A frequently occurring periwinkle snail (*Littorina obtusata*) lives on and consumes *Ascophyllum*, forming grazing scars on the algae surface that may harm plant structural integrity and infection defense ability. To examine the impact of grazing activity on *Ascophyllum* success and how this impact varies across increasing tidal height (a proxy for changing stress), we manipulated grazer density at two different tidal heights and measured resultant grazing intensity. Our results show that *L. obtusata* generate wounds on the algae surface and that the intensity of this grazing increases with increasing tidal height, suggesting that snails may elicit top down control on these seaweeds and that this effect varies as a function of grazer density.

Stressors (grazing, desiccation, disturbance) affect seaweeds on rocky shores differently than terrestrial plants, as there is no potential for biomass allocation plasticity to root mass\(^1\). On the Maine shore, the macroalgae *Ascophyllum nodosum* (knotted wrack) is grazed by the periwinkle snail *Littorina obtusata*. At low tide, snails can be found at high densities grazing on exposed wrack in higher tidal strata. Given these observations, we asked the question: how does tidal height and grazer density affect grazing intensity on *Ascophyllum*? Based on initial observations, we hypothesized that surface wounds would increase with increasing snail density and tidal elevation due to increasing combined stressors (desiccation, disturbance, and grazing) at higher elevations.

To test this hypothesis, we conducted a 2-factor experiment from July to September 2009 on the rocky shore of Salisbury Cove at the Mount Desert Island Biological Laboratory. We examined the combined and separate effects of tidal height and grazing on algal growth and snail grazing intensity. The experiment consisted of 2 zones of intertidal elevation (high and low) and 4 levels of snail density (0,20,40,80). Initially, 64 rocks hosting wrack of equal heights, grazing intensity and plant girth were collected from intermediate tidal level. These were cleared of snails and divided randomly into sets of 8, half of which were placed at each tidal elevation. Locally collected snails were placed onto the seaweed sets at test densities (0,20,40,80) for both high and low intertidal zones. The experiment ran for 3 months and intermediate data were collected after 28 days and final data on 9/26/09. Intermediate data collection entailed recording snail density for 4 randomly selected replicates from each of the 8 sets and measuring grazing scars on 5-10 blades per sample rock (calculated as a percentage of total blade length grazed).

Results at 28 days suggest that grazing intensity increases with increasing snail density. Eighty-snail samples (mean=23.7%) had significantly more wounds than 0-snail samples (mean=8.74%). Grazing intensities for high (mean=15.36%) and low tide (mean=16.8%) were not statistically different (T-test P=0.75), but average snail count for low tide plants (mean=15.7) was higher than that for high tide (mean=12.9) suggesting a higher wound per snail ratio at higher tidal elevation. This result may be a function of higher physical stress which often makes plant more susceptible to grazing\(^3\). Desiccation, in particular, increases fragility of macroalgae which may decrease resistance to disturbance. Snails likely also graze more heavily on high elevation wrack because they are sheltered inside stationary seaweed patches and may feed for much of the day. These results show that snail grazing increases with increasing snail density and tidal height. This research was funded by a grant from the Andrew Mellon Foundation.

Gene expression and biochemical studies of the marine copepod *Calanus finmarchicus*

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The copepod *Calanus finmarchicus*, a small planktonic crustacean, is a key part of the food web in the North Atlantic Ocean, but scientists have been unable to determine what controls a critical part of its life cycle, the winter dormancy period. We are exploring the use of a functional genomics approach to investigate the role of lipid reserves in the seasonal cycle of the copepod. Here we present preliminary results indicating that gene expression patterns of animals sorted by the size of their lipid reserves parallels differences seen in the field between deep- and shallow-dwelling animals during overwintering.

*Calanus finmarchicus*, a dominant component of the North Atlantic zooplankton, undergoes a winter dormancy in the juvenile C5 copepodite stage, resembling in many respects the diapause stage of insects\(^3\). These C5s display distinct physiological characteristics including torpor, large lipid stores, and reduced metabolic and digestive enzyme activities\(^1,2,5\). However, individuals displaying normal activity patterns are often observed at the same time, generally segregated by depth. Active C5s tend to be smaller, with greatly reduced lipid sacs and elevated respiratory and digestive enzyme activities\(^1,2,5\). In shallow waters of the Gulf of Maine (*i.e.*, <100 m) the dormant individuals are unable to separate from the active and are found mixed together\(^4\). The factors that control the initiation and termination of this dormancy remain unknown, and thus limit our understanding of *Calanus*’ seasonal cycle. An important step towards identifying these factors includes the characterization of the physiological changes that precede dormancy and thus, initiate the transition to the diapause stage.

To elucidate the factors that regulate diapause in *Calanus* we have been developing a multi-level approach that incorporates biochemical analysis of individual copepods and gene expression studies using a microarray. A species-specific microarray was constructed for *Calanus finmarchicus* with 1000 probes. Fifty base-pair long unique oligomer probes were designed from a subset of EST sequences available for this species. The ESTs represented in the microarray included genes involved in growth and reproduction, respiration, digestion, lipid metabolism, stress response and biological rhythms. In the experiment reported here we tested the microarray with samples of *C. finmarchicus* that were sorted according to the status of their lipid reserves and compared these results to field samples collected during winter dormancy (October) at which time a portion of the population was present in surface waters. Our objective was to test whether patterns that were produced in a laboratory manipulation could reproduce patterns observed in the field.

*Calanus finmarchicus* were collected near Mount Desert Rock, Maine in 2008 and 2009. Animals were sorted upon capture into 10-liter buckets filled with cooled seawater. Upon return to the lab the most lipid-rich and lipid-poor stage C5 copepodites were sorted by microscope into two groups. Animals were preserved in RNAlater (Sigma-Aldrich, St. Louis MO) until extraction. Specimens were also photographed, and images were analyzed for lipid sac dimensions with ImageJ v1.38 (http://rsb.info.nih.gov/ij/). Conversion to percent lipid was done following Miller et al.\(^5\). Lipid-rich C5s were 21.6±2.9% lipid by volume, whereas the lipid-poor C5s were 5.8±1.4% lipid.

Additional samples of *Calanus finmarchicus* C5 were sorted from frozen samples collected by MOCNESS opening/closing net in Georges Basin in 1999 and stored in liquid nitrogen. Two deep (200 m) and one shallow sample (20-40 m) were sorted for C5 copepodites without distinguishing the size of lipid reserves.

![Figure 1. Electropherogram of total RNA extracted from a sample of *C. finmarchicus* collected in 1999 and stored in liquid nitrogen.](image-url)
Approximately 16-20 copepods from each sample were individually analyzed for protein content and the activities of citrate synthase (CS), hydroxyacyl CoA dehydrogenase (HOAD), and β-1,3-glucanase (laminarinase, a digestive enzyme). A second set of 20 to 35 individuals from each sample was used for the microarray experiment. High quality total RNA was extracted from these samples using the Qiashredder and RNeasy kits (Qiagen) (Fig. 1).

Deep and shallow C5s from Georges Basin could clearly be distinguished by differences in enzyme activities (Fig. 2). Surface animals had high laminarinase and CS activity, indicative of actively feeding animals, whereas deep-dwelling animals had very low digestive laminarinase activity, as well as lower CS activity, characteristics expected during dormancy. Higher HOAD activity in deep animals may indicate a greater need for catabolism of lipid reserves during dormancy.

Microarray comparisons were made between nearshore C5 copepodites sorted according to the status of their lipid reserves and the October Georges Basin C5s sorted by depth (shallow vs. deep). Deep-dwelling copepods are presumably in dormancy and would contain abundant lipid reserves. A non-parametric approach was used to ask whether gene expression patterns observed as a function of lipid reserves would be reflected in the field population of C5s that had behaviorally sorted themselves by depth. There were 4 replicate comparisons of fat vs. thin C5s (2 each in 2008 and 2009) and 2 comparisons between shallow and deep populations (Table 1).

The log ratio of fluorescence for each comparison was ranked, averaging the two replicate measurements for each microarray spot (n=992). The top and bottom 5% were then selected and from these 100 probes those that agreed consistently among replicates were chosen. Selecting for mRNA probes that were above the 5% cutoff among all replicates reduced the initial 100 to 46 genes for lipid-rich vs. lipid-poor and 34 for shallow vs. deep. The last step was to select those genes that were regulated in both sets of comparisons. Note that there were 4 replicate comparisons of lipid-rich vs. lipid-poor C5s (2008 and 2009) but only a single comparison between shallow and deep populations. This final step yielded a set of 15 genes that were similarly expressed in the two sets of comparisons, with the most common pattern being upregulation in the shallow; presumably active population, downregulation in the deep, presumably dormant animals (13 of 15 probes, Table 2). Thus, 15/34 or 44% of the genes that appear to be most strongly differentially expressed between the deep and shallow populations of Calanus in October are also differentially expressed in a different population that was sorted strictly by the size of the lipid sac.

Due to the lack of replication of the field microarray we cannot definitively identify ecologically-relevant genes that were differentially expressed. However, there were a number of probes that were below the cutoff for the above analysis but may provide insight into the differences between surface and

**Figure 2.** Protein content and enzyme activities (± st. dev.) of individual *Calanus finmarchicus* C5 from deep and shallow October populations in the Gulf of Maine. * denotes significant difference between deep and shallow samples (t-test, \( p<0.001 \), n=24 for each sample).

**Table 1.** Microarray comparisons (number of replicates).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Replicates</th>
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</thead>
<tbody>
<tr>
<td>Lipid-rich vs. Lipid-poor (MDI Rock 2008)</td>
<td>2</td>
</tr>
<tr>
<td>Lipid-rich vs. Lipid-poor (MDI Rock 2009)</td>
<td>2</td>
</tr>
<tr>
<td>Georges Basin shallow vs. Georges Basin deep</td>
<td>1</td>
</tr>
<tr>
<td>Lipid-poor (2009) vs. Georges Basin deep</td>
<td>1</td>
</tr>
</tbody>
</table>
deep animals in October. For instance, while neither CS nor HOAD met the cutoff, the trends in the microarray data are consistent with those from the biochemical analysis, with HOAD being relatively highly expressed in deep copepods and CS more highly expressed in the surface animals. Also of note was the high level of expression of the Δ9 fatty acid desaturase, involved in fatty acid synthesis, in shallow animals as compared to deep, suggesting the shallow population is actively synthesizing lipids. Similarly, a number of probes for digestive enzymes demonstrated relatively low expression in the deep copepods compared to shallow, as would be expected. Several other genes of potential interest include timeless (a clock-regulating gene) and prepro-type A-allatostatin, a peptide that regulates juvenile hormone production in insects, both of which appear to be downregulated in deep copepods. The differences observed between deep and shallow Calanus were paralleled by differences between Calanus sorted according to lipid reserves, with thin C5s resembling the shallow October population. The lipid-rich and lipid-poor C5s were collected in the same June tow, and were differentiated only on the basis of the level of lipid reserves, yet showed similarities in differential gene expression to October C5s that had behaviorally sorted themselves (and would be expected to show similar differences in lipid reserves). This suggests that the accumulation of lipid in the summer C5s is triggering physiological changes similar to those exhibited in dormancy. Further replication of the field microarrays, comparing deep with shallow copepods, may help clarify these patterns.

Supported by an Ohio University Research Council Award and a MDIBL New Investigator Award to R.P. Hassett and NSF OCE-0451376 to P.H. Lenz


<table>
<thead>
<tr>
<th>Downregulated in thin; upregulated in deep</th>
<th>copper resistance protein A</th>
<th>CFX00284_1</th>
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</thead>
<tbody>
<tr>
<td>Upregulated in thin; downregulated in deep</td>
<td>cytochrome P450 enzyme</td>
<td>CFX00331_1</td>
</tr>
<tr>
<td></td>
<td>urea active transporter</td>
<td>CFX00742_1</td>
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<tr>
<td></td>
<td>alpha 5 type IV collagen isoform 1, precursor</td>
<td>CFX00814_1</td>
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<td></td>
<td>Eukaryotic peptide chain release factor</td>
<td>CFX00977_1</td>
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<td></td>
<td>Methylenetetrahydrofolate dehydrogenase</td>
<td>CFX01008_1</td>
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<td></td>
<td>UDP glycosyltransferase 1 family</td>
<td>CFX01060_1</td>
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<tr>
<td></td>
<td>Lysosomal aspartic protease precursor</td>
<td>CFX03555_1</td>
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<tr>
<td></td>
<td>cytochrome P450 6a14</td>
<td>CFX03613_1</td>
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<tr>
<td></td>
<td>peptidase S10, serine carboxypeptidase</td>
<td>CFX01188_1</td>
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<td></td>
<td>LanC lantibiotic synthetase component</td>
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<td>transport protein Sec61 alpha subunit</td>
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<td>DNA polymerase theta</td>
<td>CFX01358_1</td>
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<td></td>
<td>ATP-sensitive inward rectifier K+ channel</td>
<td>CFX01880_1</td>
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</tbody>
</table>

| Upregulated in thin; upregulated in deep | phytanoyl-CoA 2-hydroxylase isoform a | CFX02697_1 |
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Robert Wheeler, Ph.D., The University of Maine
2009 SEMINARS

Monday Morning Science Seminars

July 6  “Moving parts of the CFTR chloride channel: Cysteine scanning, state-dependent reactivity and molecular dynamics simulation.” **David C. Dawson, Ph.D.**, Professor and Chair of Physiology and Pharmacology, Oregon Health and Science University

July 13 “How does C-type natriuretic hormone, acting through guanylate cyclase, stimulate CFTR-mediated chloride secretion in the shark rectal gland?” **John N. Forrest, Jr., Ph.D.**, Director Mount Desert Island Biological Laboratory

July 20 “An evo-devo approach to understanding vertebrate appendage development and regeneration” **Randall Dahn, Ph.D.**, Mount Desert Island Biological Laboratory

July 27 “CTD: A database resource to explore chemical-gene-disease interactions “, **Carolyn Mattingly, Ph.D.** and **Allan Davis, Ph.D.**, Mount Desert Island Biological Laboratory

August 3 “Protecting your proteosome: The perils of osmotic stress”, **Kevin Strange, Ph.D.**, Director, Mount Desert Island Biological Laboratory

August 10 “P-Glycoprotein, the Eight-Hundred Pound Gorilla of the Bloo-Brain Barrier”, **David Miller, Ph.D.**, Senior Investigator and Acting Chief, Laboratory of Pharmacology, Acting Chief, Laboratory of Molecular Toxicology, Acting Director, Environmental Toxicology Program, NIH/NIEHS

August 17 “Calcitonin-like peptides in crustacea: Identification, distribution and physiological actions”, **Andy Christie, Ph.D.**, Investigator/Director, Imaging Core Center for Marine Functional Genomics, Mount Desert Island Biological Laboratory

August 24 "CFTR@20", **John Riordan, PhD.**, Distinguished Professor, Biochemistry and Biophysics, University of North Carolina School of Medicine.

August 31 “Toxicants as Breakthrough Auxiliaries for understanding developmental networks “, **Antonio Planchart, Ph.D.**, Mount Desert Island Biological Laboratory

Friday Noon Brown Bag Seminars

June 12  5 minute “introductory” scientific talks.

June 19 **Andy Christie, Ph.D.; James Boyer, M.D.; Glenn Collier, Ph.D.; David Evans, Ph.D.; George Kidder, Ph.D.; Denry Sato, Ph.D.; Joe Shaw, Ph.D.**

June 26 “Salmon aquaculture project”, **Andy Christie, Ph.D.**, Mount Desert Island Biological Laboratory.

July 17  “Authorship: Why not just toss a coin?”, Kevin Strange, Ph.D., Professor of Anesthesiology and Pharmacology, Vanderbilt University and Director-elect of MOUNT DESERT ISLAND BIOLOGICAL LABORATORY

July 31  “Biogeochemistry of trace organic contaminants”, Jon Chorover, Ph.D., Professor of Environmental Chemistry, University of Arizona

August 14  Short Presentations by Nancy Berliner, M.D., Brigham & Women’s Hospital, Rebeka Merson, Ph.D., Assistant Professor, Rhode Island College, Larry Renfro, Ph.D., Professor, University of Connecticut, Hugo de Jong, Ph.D., Professor, Erasmus University Medical Center and Patricio Silva, M.D., Professor, Temple University Health Science Center

Wednesday Evening Seminars
July 1  15TH ANNUAL HELEN F. CSERR MEMORIAL LECTURE – “Lobster teeth and crab hearts: The role of neuropeptides in generating flexibility of rhythmic movements.” Patsy Dickinson, Ph.D., Josiah Little Professor of Natural Sciences; Director, Neuroscience Program, Bowdoin College.

July 8  27TH ANNUAL KINTER MEMORIAL LECTURE: “Dioxin, clocks and oxygen: prototype signals for a nuclear sensor.” Christopher Bradfield, Ph.D., Professor of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health

July 15  19TH ANNUAL THOMAS H. MAREN LECTURE: “The Ubiquitin Proteolytic System: From the Bench Through Human Diseases and onto Drug Targeting”, Aaron Ciechanover, M.D., Technion Distinguished Research Professor in the Ruth and Bruce Rappaport Faculty of Medicine and Research Institute at the Technion-Israel Institute of Technology, Haifa, Israel

July 22  “Crystal Structure of P-glycoprotein Reveals Strategies for Treating Multidrug Resistance in Cancer and Medicine”, Stephen G. Aller, Ph.D., Assistant Professor, Department of Pharmacology and Toxicology, University of Alabama at Birmingham

August 12  3RD ANNUAL RICHARD K. ORKAND MEMORIAL LECTURE IN NEUROSCIENCE AND CELL SIGNALING: Mark A. Sussman, Ph.D., Professor of Biology at San Diego State University.

Special Seminars and Presentations
January 15  Seminar: J. Sook Chung, PhD
February 13  Seminar: David Baldwin, PhD
February 25  "Next Generation DNA sequencing: Technologies and Applications."
March 10  INBRE seminar: “An inverted pendulum model for underwater walking”
June 20  Acadia Science Seminar Series, “Shipwrecks of Acadia: Acadia Maritime Cultural Resources Inventory”, Franklin Price, North Carolina Department of Natural Resources and Adjunct Instructor, East Carolina University.

June 25  “Family Roots and Icefishes: A perspective from Antarctica”, Lisa Crockett, Ph.D., Associate Professor, Dept. of Biological Sciences, Ohio University.

July 2  “One Biology, One Science: Biology for the 21st Century.” James Collins, Ph.D., Associate Director, National Science Foundation.

July 9  UNIQUE ATTRIBUTES OF MARINE AND FRESHWATER MODELS FOR TOXICOLOGY RESEARCH

Kevin Strange, Ph.D., Vanderbilt University, “Vision for Mount Desert Island Biological Laboratory Science and Environmental Toxicology.”

Bruce Stanton, Ph.D., Dartmouth College, “Environmental arsenic and salt adaptation in killifish.”

Carolyn Mattingly, Ph.D., Mount Desert Island Biological Laboratory, “Exploring the etiology of environmental diseases using the Comparative Toxicogenomics Database.”

James L. Boyer, M.D., Yale University School of Medicine, “The Discovery of Ost alpha-Ost beta from skate liver.”

Randall D. Dahn, Ph.D., Mount Desert Island Biological Laboratory, “A comparative approach to understanding vertebrate limb regeneration”

David Barnes, Ph.D., Mount Desert Island Biological Laboratory, “Arachidonic acid-induced expression of the organic solute and steroid transporter in a cartilaginous fish cell line.”

James Coffman, Ph.D., Mount Desert Island Biological Laboratory, “Sea urchin embryogenesis as a model for studying nodal-dependent pattern formation and its environmental susceptibility.”

Andrew Christie, Ph.D., Mount Desert Island Biological Laboratory, “Peptidurgic control of behavior in crustaceans.”

Antonio Planchart, Ph.D., Mount Desert Island Biological Laboratory, “Forkhead box transcription factors as mediators of chemically-induced craniofacial dysmorphology.”

Rebeka Merson, Ph.D., Rhode Island College, “Spiny dogfish shark aryl hydrocarbon receptors: Conservation and divergence of structure and function.”

Joseph Shaw, Ph.D., Indiana University, “Daphnia Genetics Consortium.”

Weiming Li, Ph.D., Michigan State University, “The biology of the lamprey.”
July 13  Special retirement lecture by **David Towle, Ph.D.**, "Crustaceans Aren't Just For Breakfast Anymore"

July 14  Frenchman Bay Crustacean Symposium:
**Tom Shafer**, University of North Carolina, Wilmington, *Co-Author: Julia Jenkins*, “Regulating calcification of the blue crab exoskeleton: From sequences to hypotheses and beyond. “

**Nora Terwilliger**, University of Oregon, “Crustacean molting: change is good. “


**Markus Frederich**, University of New England, “AMPK, a novel cellular marker for acute stress in marine invertebrates.”

**Don Lovett**, The College of New Jersey, *Co-authors: Michael Kaufer and Daniel Markowski* “Returning to the sea: A time-course study of gene expression in gills of blue crabs *Callinectes sapidus* transferred from low to high salinity.”

**Ray Henry**, Auburn University, “Neuroendocrine regulation of salinity-stimulated carbonic anhydrase induction in the gills of the euryhaline green crab, *Carcinus maenas.*”

**Sook Chung**, University of Maryland Biotechnology Institute, “Pleiotropic actions of crustacean hyperglycemic hormone in blue crab.”

**Andrea Tilden**, Colby College, “Melatonin and neurite outgrowth in crustacean X-organ cells.”

**Glen Collier**, University of Tulsa, “Evolutionary implications of crustacean arginine kinase gene structure.”


**Mary Kate Worden**, University of Virginia, “Lobsters in cyberspace: Neuroethology inspires educational gaming for math and science students.”


Caroline Wilson & Dan Hartline, Denison University & University of Hawaii at Manoa, “Calanoid copepod myelin and node development: structure and function.”

Pat Hassett & Petra Lenz, Ohio University & University of Hawaii at Manoa, Co-author: David Towle, “Functional genomics studies in Calanus finmarchicus: Preliminary microarray results.”

July 14 Mount Desert Island Biological Laboratory Developmental Biology interest group: Gary Conrad, Ph.D., “Extracellular Matrix (ECM) and Morphogenesis in the Cornea”

July 16 Chalk talk: Aaron Ciechanover, M.D., Technion Distinguished Research Professor in the Ruth and Bruce Rappaport Faculty of Medicine and Research Institute at the Technion-Israel Institute of Technology, Haifa, Israel

July 16 "How to get started on a new grant", David C. Dawson, Ph.D., Professor and Chair of Physiology and Pharmacology, Oregon Health and Science University, former Director of Mount Desert Island Biological Laboratory.

July 20 "Antarctic Fishes: Evolutionary Mutant Models for Human Anemia and Osteopenia", H. William Detrich, Ph.D., Professor of Biochemistry and Marine Biology, Director, Professional Science Masters Program, Bioinformatics, Northwestern University.

July 21 “Instrumentation Funding at NSF”, Nily Dan, Ph.D., Program Director, Instrument Development for Biological Research and The Major Research Instrumentation Program, BIO/DBI, National Science Foundation.

July 30 “Funding Opportunities at the National Science Foundations”, Mary Chamberlin, Ph.D., Program Director, The Physiological and Structural Systems Cluster, National Science Foundation.

August 5 15TH ANNUAL JOHN W. BOYLAN LECTURE presented A Sea Change, a new documentary directed by Barbara Ettinger and produced by Seven Huseby of Nijii Films.


August 18 Acadia Science Seminar, “Dramatic Shifts in shell size of Dogwhelks in Maine over the last century”, Peter Petraitis, Ph.D., Professor of Biology, University of Pennsylvania.
August 27  “A new chloride channel in cystic fibrosis”, Raymond A. Frizzell, PhD., Richard B. Mellon Professor and Chairman, Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine.

2009 CONFERENCES, SYMPOSIA, AND WORKSHOPS

January 26-27  Workshop in Biomedical Research Imaging
February 16-20  Molecular Biology Research Techniques
March 2-13  Functional Genomics of Membrane Transport
March 7-17  Evolutionary Molecular Genetics
March 7-17  Evolutionary Molecular Genetics
April 17-18  36th Maine Biological and Medical Sciences Symposium
May 10-23  Experimental Neurophysiology
May 30-June 6  11th Annual Course in Quantitative Flourescent Microscopy
June 6-13  Structure and Function of Polarized Epithelial Cells
June 19-20  Satellite Workshop to the Gordon Conference on Mycotoxins and Phycotoxins
July 8-9  16th Annual Environmental Health Sciences Symposium – “Comparative toxicogenomic strategies for unraveling mechanisms of environmentally-related diseases,” sponsored by the National Institute of Environmental Health Sciences (NIEHS) Center at Mount Desert Island Biological Laboratory, the Kinter Memorial Lectureship Fund, the Yale University Liver Center, the Dartmouth Environmental Health Sciences Center, and the Mount Desert Island Biological Laboratory.

Wednesday, July 8
27th ANNUAL KINTER MEMORIAL LECTURE

“Dioxin, Clocks and Oxygen: Prototype Signals for a Nuclear Sensor.” Christopher Bradfield, Ph.D., Professor of Oncology at the McArdle Laboratory for Cancer Research, University of Wisconsin School of Medicine and Public Health.

Thursday, July 9
Workshop Session I: The Power of the Comparative Approach

“Vision for Mount Desert Island Biological Laboratory Science and Environmental Toxicology”. Kevin Strange, Ph.D., Vanderbilt University

“Environmental arsenic and salt adaptation in killifish”, Bruce Stanton, Ph.D., Dartmouth College
“Exploring the etiology of environmental diseases using the Comparative Toxicogenomics Database”, Carolyn Mattingly, Ph.D., MDI Biological Laboratory

“The Discovery of Ost alpha-Ost beta from skate live”, James L. Boyer, M.D., Yale University School of Medicine

“A comparative approach to understanding vertebrate limb regeneration”, Randall D. Dahn, Ph.D., Mount Desert Island Biological Laboratory

Workshop Session II  Insights from Unique Animal Models

“Arachidonic acid-induced expression of the organic solute and steroid transporter in a cartilaginous fish cell line”, David Barnes, Ph.D., Mount Desert Island Biological Laboratory

“Sea urchin embryogenesis as a model for studying nodal-dependent pattern formation and its environmental susceptibility”, James Coffman, Ph.D., Mount Desert Island Biological Laboratory

“Peptidurgic control of behavior in crustaceans”, Andrew Christie, Ph.D., Mount Desert Island Biological Laboratory

“Forkhead box transcription factors as mediators of chemically-induced craniofacial dysmorphology”, Antonio Planchart, Ph.D., Mount Desert Island Biological Laboratory

“Spiny dogfish shark aryl hydrocarbon receptors: Conservation and divergence of structure and function “, Rebeka Merson, Ph.D., Rhode Island College

“Daphnia Genetics Consortium”, Joseph Shaw, Ph.D., Indiana University

“The biology of the lamprey”, Weiming Li, Ph.D., Michigan State University

July 14  2009 Frenchman Crustacean Association Research Symposium – Sponsored by the Mount Desert Island Biological Laboratory

July 16-17  Grant Writing Workshop, David C. Dawson, Ph.D. – Sponsored by the Mount Desert Island Biological Laboratory

July 28  2009 Student Symposium – Sponsored by Maine INBRE and the Mount Desert Island Biological Laboratory.

SESSION 1: COMPARATIVE FUNCTIONAL GENOMICS, CHAIR: CHARLES WRAY, PH.D., Mount Desert Island Biological Laboratory

Erica Brown, The University of Maine
Characterization of Ccr5 deficiency in the NOD-scid II2γKO Ccr5 KO mouse strain
Mentor: Leonard Schultz, Ph.D., The Jackson Laboratory
Yiftusira Gima Wondimu, College of the Atlantic
*The effect of the little mutation on the activation state of the mTOR pathway*
Mentor: David Harrison, Ph.D. and Kevin Flurkey, Ph.D., The Jackson Laboratory

Olivia Moody, Bates College
*Genomic instability in hematopoietic stem cells in B6 and BALB mice*
Mentor: David Harrison, Ph.D., The Jackson Laboratory

Emily Argo, College of the Atlantic
*Population genetics and phylogeography of Squalus acanthias*
Mentor: Charles Wray, Ph.D., Mount Desert Island Biological Laboratory

Stacy Littlechild, Kansas State University
*LASIK flap adhesion. Eye glue for you!*
Mentor: Gary Conrad, Ph.D., Kansas State University

Gage Brummer, Kansas State University
*Testing the effects of pyridoxal-5’pPhosphate on riboflavin/ultraviolet-A-crosslinking for the treatment of keratoconus*
Mentor: Gary Conrad, Ph.D., Kansas State University

Evelyn Dickinson, Mt. Ararat High School
*Allatostatin-C-type peptides in Homarus americanus*
Mentor: Andrew Christie, Ph.D., Mount Desert Island Biological Laboratory

Matthew Cronan, The University of Maine
Harrison Kilpatrick, University of Maine at Presque Isle
Jessica Shrader, The University of Maine
*Molecular and immunohistological characterization of nitric oxide synthase in the lobster stomatogastric nervous system*
Mentor: Andrew Christie, Ph.D., Mount Desert Island Biological Laboratory

Alexandra Pfister, Bowdoin College
*Analysis and quantification of anatomical characteristics of auditory neuron 2 and nerve 5 in Gryllus bimaculatus*
Mentor: Hadley Horch, Ph.D., Bowdoin College

Caitlin Stauder, Bowdoin College
*The role of Fgf10a in tooth morphogenesis of Danio rerio*
Mentor: William Jackman, Ph.D., Bowdoin College

**SESSION 2: PHYSIOLOGY, CHAIR: JIM STIDHAM, PH.D., Presbyterian College**

Jeremy Quail, High School for Math, Science and Engineering
*Functional analysis of the glomerulus filter in the larval zebrafish*
Mentor: Hermann Haller, M.D., Hannover Medical School
Christopher Wessner, Denison University
*Distribution of GYRKPPFNNGSIFamide (Gly1-SIFamide) in the stomatogastric nervous system (STNS) of the crabs Cancer borealis and Cancer irroratus.*
Mentor: Caroline Wilson, Ph.D., Denison University

Kentrell Burks
Will Epstein, Brown University
Brendan Vosburgh, Colorado College
*The world may never know: PDE-inhibitors, oocytes, and the rest*
Mentor: John N. Forrest, Jr., M.D., Yale University School of Medicine

Monica Orcine, University of Hawaii at Manoa
*Mapping a copepod nervous system*
Mentors: Daniel Hartline, Ph.D. and Petra Lenz, Ph.D., University of Hawaii at Manoa

Alyssa Simeone, Union College
*Characterizing water absorption in the spiral intestine of Leucoraja erinacea*
Mentor: Nicole Theodosiou, Ph.D., Union College

Henry Edelstein, Brookline High School
Megan Kelley, Fayetteville-Manlius High School
*Characterization of phosphodiesterase families in dogfish shark (Squalus acanthias) rectal gland*
Mentor: John N. Forrest, Jr., M.D., Yale University School of Medicine

Emily Miller, The University of Maine
*Cofilin 1 is an essential regulator of F-actin dynamics in podocytes*
Mentors: Sharon Ashworth, Ph.D., University of Maine, Orono and Mario Schiffer, M.D., Hannover Medical School

**SESSION 3: MARINE ECOLOGY, CHAIR: J.B. CLAIBORNE, PH.D.,** Georgia Southern University

Molly Miller, Vassar College
*Eelgrass restoration in Frenchman Bay: Past, present and future*
Mentor: George Kidder, Ph.D., Mount Desert Island Biological Laboratory

Kavita Balkaran, University of the Virgin Islands
*How does a 47-acre bottom mussel aquaculture operation influence mussel seed settlement on nearby restored eelgrass beds?*
Mentor: Jane Disney, Ph.D., Mount Desert Island Biological Laboratory

Elena Correa, Colorado College
*Population genetics of eelgrass in the Jordan River*
Mentor: Charles Wray, Ph.D., Mount Desert Island Biological Laboratory
Ellen Daily, Waterville Senior High School
Microbiological monitoring in the recreational waters of Mount Desert Island: an examination of the effect of incident light on Enterococcus faecalis levels in swimming areas
Mentor: Jane Disney, Ph.D., Mount Desert Island Biological Laboratory

SESSION 4: TOXICOGENOMICS, CHAIR: MICHAEL McKERNAN, Mount Desert Island Biological Laboratory

Benjamin Snowdon, Falmouth High School
Exploring the mechanisms underlying chemical-phenotype connections
Mentor: Carolyn Mattingly, Ph.D., Mount Desert Island Biological Laboratory

Chloe Taub, Ellsworth High School
Marisa Dzioba, University of New England
Salinity and arsenic regulate aquaporin 3 expression in Fundulus heteroclitus
Mentors: Bruce Stanton, Ph.D., Dartmouth Medical School and Joseph Shaw, Ph.D., Indiana University

SESSION 5: DEVELOPMENT, CHAIR: MICHAEL McKERNAN, Mount Desert Island Biological Laboratory

Taylor LaCasse, Mount Desert Island High School
Embryogenesis of Strongylocentrotus purpuratus: effects of hypoxia on oral-aboral polarity
Mentor: James Coffman, Ph.D., Mount Desert Island Biological Laboratory

Emilynne Buchanan, University of New England
Proximodistal patterning of vertebrate appendages
Mentor: Randall Dahn, Ph.D., Mount Desert Island Biological Laboratory

Poster Presentations:

Ethan Clement, Sheridan High School
Measurement of heart rate in Fundulus heteroclitus embryos after desiccation and subsequent rehydration
Mentor: Robert Preston, Ph.D., Illinois State University

Kaitlin Costello, Denison University
Sequencing the gene for the voltage-gated sodium channel in Calanus finmarchicus
Mentor: Caroline Wilson, Ph.D., Denison University

Ashley Dzioba, Dartmouth Medical School
Aquaporin 3 in Fundulus heteroclitus fails to show water channel properties
Mentors: Bruce A. Stanton, Ph.D., Dartmouth Medical School and Joseph R. Shaw, Ph.D., Indiana University
Sarah Ellis, Omaha High School
*Molecular cloning of neuropeptide hormone NPF from Helisoma trivolvis*
Mentor: Denry Sato, Ph.D., Mount Desert Island Biological Laboratory

Erin Flynn, Columbia University
*The molecular cloning of crustacean hyperglycemic hormone (CHH) neuropeptide family in Cancer borealis*
Mentor: J. Sook Chung, Ph.D., The University of Maryland

Edal Fontaine, Rockland District High School
*Compatible solutes and their role in dessication resistance of killifish embryo*
Mentor: Robert Preston, Ph.D., Illinois State University

Veronica Hernandez, University of Puerto Rico, Rio Piedras Campus
*Expression of Na+/H+- exchanger in cDNA from stickleback, Gasterosteus aculeatus*
Mentor: J.B. Claiborne, Ph.D., Georgia Southern University

Marina Karnofsky, Mount Desert Island High School
*Characterization of allatostatin-C-like immunolabeling in the nervous system of the copepod, Calanus finmarchicus*
Mentor: Caroline Wilson, Ph.D., Denison University

Erin Keim, The University of Maine
*Effects of arsenic and salinity on aquaporin 7 gene expression in killifish (Fundulus heteroclitus)*
Mentor: Bruce Stanton, Ph.D., Dartmouth Medical School

Anna Kufner, University of Vermont and
Gus Melita, University of Vermont
Mentor: John N. Forrest, Jr., M.D., Yale University School of Medicine

Max Mutter, Colby College
Mentor: Andrea Tilden, Ph.D., Colby College

Paul Ohno, Orono High School
*The search for orcokinin*
Mentor: Petra Lenz, Ph.D., University of Hawaii at Manoa

Michaela Petit, Arcadia University
*AQP3 mRNA expression in mid-stage killifish embryos.*
Mentor: Robert Preston, Ph.D., Illinois State University

Sarah Repasky, The Ohio State University
*Use of a nodal promoter element, gfp construct (p5gfp), to measure redox sensitive gene expression*
Mentor: James Coffman, Ph.D., Mount Desert Island Biological Laboratory
Brian St. Thomas, Bates College
*Transcription start site mapping of essential genes in diverse bacteria*
Mentor: Paula Schlax, Ph.D., Bates College

Caleb Swanberg, The University of Maine and
Christopher Preziosi, The University of Maine and
Catherine Morse, Amherst Regional High School
*Cofilin 1 is vital to normal zebrafish development*
Mentor: Sharon Ashworth, Ph.D., University of Maine, Orono

Bradley Wilbur, Georgia Southern University
*Rhesus proteins in carp teleosts*
Mentor: J.B. Claiborne, Ph.D., Georgia Southern University

August 7-8  
**8th Annual Mount Desert Island Stem Cell Symposium** – “Epigenetic Regulation of Stem Cells” Co-hosted by The Mount Desert Island Biological Laboratory, The Jackson Laboratory and Maine Medical Center Research Institute with support from the National Heart, Lung and Blood Institute.

**SESSION 1. REPROGRAMMING, CHAIR: JON EPSTEIN, M.D.**

Leonard Zon, M.D., HHMI / Children's Hospital, Boston
*Transcriptional elongation and hematopoiesis*

Alex Meissner, Ph.D., Harvard University
*Epigenomics and cellular states*

Bradley Cairns, Ph.D., HHMI / Univ. Utah School of Medicine
*Germline chromatin: concepts shared in germ and ES cells*

Konrad Hochedlinger, Ph.D., Massachusetts General Hospital
*Factors influencing nuclear reprogramming*

Nathan Lawson, Ph.D., Univ. Massachusetts Medical School
*Shaping the vascular system: two epigenetic fish tales*

**SESSION 2. GENERATION AND REGENERATION, CHAIR: PAUL COLLODI, PH.D.**

Alejandro Sánchez Alvarado, Ph.D., HHMI / Univ. Utah School of Medicine
*Stem Cells, regeneration and planarians*

Susan Mango, Ph.D., Harvard University
*Pluripotency and its loss during embryonic development*

Elaine Fuchs, Ph.D., HHMI / The Rockefeller University
*Wnt signaling and stem cell biology*

Harvey Lodish, Ph.D., Whitehead Institute
*Expansion of hematopoietic stem cells by multiple proteins coexpressed by a homogenous population of fetal liver stromal cells*
**Bruce Draper, Ph.D.**, University of California, Davis
*Germline stem cell fate in zebrafish is determined by the somatic microenvironment*

**Randall Dahn, Ph.D.**, MDI Biological Laboratory
*Re-armed: comparative approaches to understanding and modulating vertebrate limb regeneration*

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**SESSION 3. RETAINING, AND RELINQUISHING, STEM AND PROGENITOR CELL PLURIPOTENCY, CHAIR: DON WOJCHOWSKI, PH.D.**

**Rudolf Jaenisch, M.D.**, Whitehead Institute
*Stem cells, pluripotency and nuclear reprogramming*

**Jeanne Loring, Ph.D.**, The Scripps Research Institute
*Systems biology focus on pluripotence*

**Sheng Ding, Ph.D.**, The Scripps Research Institute
*A chemical approach to cell fate control*

**Hal Broxmeyer, Ph.D.**, Indiana University School of Medicine
*Influence of SIRT1, a member of the Sirtuin family of deacetylases, on maintenance and differentiation of mouse embryonic stem cells*

**Ren-He Xu, Ph.D.**, University of Connecticut Health Center
*New insights: how TGFb and FGF signaling act on human pluripotent stem cells*

**Kenneth Kosik, Ph.D.**, University of California, Santa Barbara
*microRNA-mediated exit from pluripotency*

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**SESSION 4. THERAPEUTICS, CHAIR: DON WOJCHOWSKI, PH.D.**

**Daniel G. Anderson, Ph.D.**, Koch Institute for Integrative Cancer Research, MIT
*Combinatorial development of biomaterials for stem cells*

**Leonard Shultz, Ph.D.**, The Jackson Laboratory
*Humanized SCID mouse models for stem cell research*

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**SESSION 5. TRANSCRIPTION AND EXPRESSION, CHAIR: KYUSON YUN, PH.D.**

**Richard Young, Ph.D.**, Whitehead Institute
*Programming ES cell state*

**Stuart Orkin, M.D.**, HHMI / Children's Hospital, Boston
*Polycomb function in embryonic stem cells and cancer*

**Mary Goll, Ph.D.**, The Carnegie Institute of Washington
*Exploring transcriptional silencing in zebrafish*
Kathrin Plath, Ph.D., UCLA
Reprogramming to pluripotency - insights into the mechanism

Alex Schier, Ph.D., Harvard University
The maternal-zygotic transition

Richard Gregory, Ph.D., Children's Hospital, Boston
Regulation of microRNA biogenesis in embryonic stem cells and cancer

Kyuson Yun, Ph.D., The Jackson Laboratory
Direct activation of stem cell genes by Notch in neural stem cells

August 28 Maine Microtechnology in Biology and Medicine Workshop – Sponsored by the Mount Desert Island Biological Laboratory

Session I: Microtechnology tool needs in biological research
Tony Robertson, Mount Desert Island Biological Laboratory
Molecular studies of sea urchin embryos

Rob Taft, The Jackson Laboratory
Microfluidics and Assisted Reproductive Technologies: Improving the state of the ART

Kevin Strange, Mount Desert Island Biological Laboratory
Physiological analysis and sorting of small tissues and model organisms

Paul Millard, The University of Maine
Tools for zebrafish research: What we have and what we need

Marie Hayes, The University of Maine
Physiological monitoring in high risk newborns

Sharon Ashworth, The University of Maine
Cultured kidney proximal tubule cells: A need for a new point of view

Session II: Microtechnology advances and biological applications
Rosemary Smith, The University of Maine
Principles of microfabrication

Scott Collins, The University of Maine
Microfabrication applications to biomedicine

Hang Lu, Georgia Institute of Technology
Microfluidic imaging, sorting and screening of C. elegans

Nikos Chronis, University of Michigan
Worm chips for neuronal functional imaging and axon regeneration studies

Ramunas Stepanauskas, Bigelow Laboratory for Ocean Sciences
Redefining microbial genomics: Sequencing individual cells

Ali Sarvestani, The University of Maine
Chemomechanics of tissue cells at engineered interfaces

Session III: Roundtable discussion
2009 COURSES

January 25 – 27  
**Workshop in Biomedical Imaging**  
Colby College INBRE workshop  
Andrew Tilden, Ph.D., Colby College

February 15 – 20  
**Molecular Biology Research Techniques**  
University of Maine – Farmington, University of Maine – Machias,  
University of Maine – Presque Isle INBRE course  
David Towle, Ph.D., MDIBL

February 28 – March 7  
**Functional Genomics of Membrane Transport: Toxicogenomics of Arsenic**  
University of Maine INBRE course  
Sharon Ashworth, Ph.D., The University of Maine  
J. Denry Sato, D.Phil., MDIBL  
Bruce Stanton, Ph.D., Dartmouth Medical School  
Jennifer Bomberger, Ph.D., Dartmouth Medical School  
Keith Hutchison, Ph.D., The University of Maine

March 6 – 17  
**Molecular Biology of Crustacean Neuropeptides**  
Bowdoin College INBRE course  
Andrew Christie, Ph.D., MDI Biological Laboratory  
Patsy Dickinson, Ph.D., Bowdoin College

March 16 – 27  
**Integrative Environmental Toxicogenomics**  
College of the Atlantic INBRE course  
Charles Wray, Ph.D., MDIBL

May 5 – 16  
**Experimental Neurophysiology**  
Bates College INBRE course  
Nancy Kleckner, Ph.D., Bates College  
J. Denry Sato, Ph.D., MDIBL

May 23 – 30  
**Structure and Function of Polarized Epithelial Cells**  
University of Pittsburgh School of Medicine  
Ray Frizzell, Ph.D., University of Pittsburgh School of Medicine

May 30 – June 6  
**Eleventh Annual Intensive Course in Quantitative Fluorescent Microscopy**  
Simon Watkins, Ph.D., University of Pittsburgh School of Medicine

June 6 – 13  
**Structure and Function of Polarized Epithelial Cells**  
Yale University School of Medicine  
John N. Forrest, Jr., M.D., Yale University School of Medicine
June 18 – 20  
**Satellite Workshop to the Gordon Research Conference: Early Warning and Integrated Management of Natural Toxin Events**
James J. Pestka, Ph.D., Michigan State University
Vera Trainer, Ph.D., NOAA Northwest Fisheries Science Center

August 24 – 28  
**Health and Colony Management of Laboratory Fish**
Paul Bowser, Ph.D., Cornell University
Michael Kent, Ph.D., Oregon State University
Jan Spitsbergen, Ph.D., Oregon State University

September 5 – 12  
**Origins of Renal Physiology**
Course for Renal Fellows
Mark Zeidel, M.D., Beth Israel Deaconess Medical Center

September 13 – 20  
**Course in Comparative Physiology**
Beth Israel Deaconess Medical Center, Harvard Medical School
William Aird, M.D., Beth Israel Deaconess Medical Center
Mark Zeidel, M.D., Beth Israel Deaconess Medical Center

October 11 – 16  
**Molecular Biology Research Techniques**
University of Maine – Fort Kent, University of Maine – Presque Isle
INBRE course
Randy Dahn, Ph.D.


Robertson AJ, Larroux C, Degnan BM, Coffman JA. The evolution of Runx genes II. The C-terminal Groucho recruitment motif is present in both eumetazoans and homoscleromorphs but absent in a haplosclerid demosponge. BMC Res Notes. 2009 Apr 17;2:59. PubMed PMID: 19374764; PubMed Central PMCID: PMC2674455.


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Aspirnaut Program

Cades Foundation Fellowship

Cleft Palate Foundation

Cystic Fibrosis Foundation

Davis Conservation Foundation

Denison University

DFG (Deutsche Forschungsgemeinschaft)

Environmental Protection Agency

Gulf of Maine Council

Kansas St. University  
*Terry C. Johnson Cancer Center*  

Maine Economic Improvement Fund

MDI Biological Laboratory  
*New Investigator Award*  

National Fish and Wildlife Foundation

National Institutes of Health (NIH)

NIH/National Institute of Environmental Health Sciences

NIH/National Center for Research Resources  
*Kansas INBRE*  

Maine INBRE

Oklahoma INBRE

National Science Foundation  

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