NIEHS Workshop: Unique Marine/Freshwater Models for Environmental Health Research

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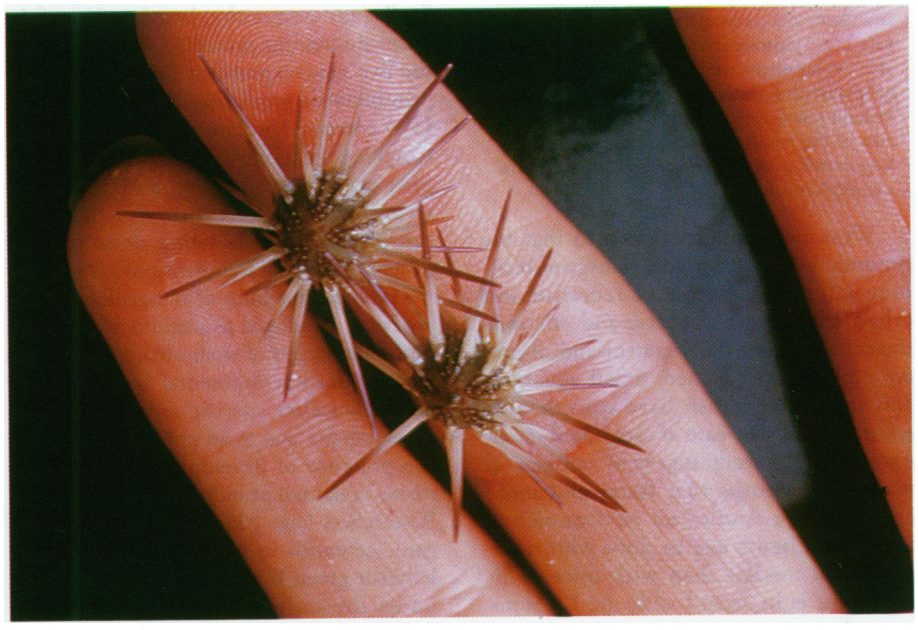
This NIEHS-sponsored workshop highlighted the advances in environmental health research being made by using marine and freshwater model systems to add greater definition to the roles of genetic and environmental factors in disease. Workshop participants considered human health problems, features of marine and freshwater models that facilitate studies of these problems, and obstacles to taking full advantage of these models. Cancer, developmental defects, and neurological disorders affecting humankind are being clarified by studies that use these model systems to probe the genetic and environmental components of disease. The features of the models that facilitate these studies include simplicity, relatively low cost to generate statistically valid results, wide range of sensitivities, short development times, and ease of generating and maintaining useful mutant or genetically engineered strains. The primary obstacles to be overcome are lack of institutional infrastructure and lack of appreciation among the scientific community of the advantages inherent in use of these models. Workshop participants recommended the creation of localized supportive infrastructure and regional centers of expertise to train and educate researchers in the use of these models, and the communication of these results.

Organized by the five NIEHS Marine/Freshwater Biomedical Sciences Centers, the workshop convened at the NIEHS Conference Center in Research Triangle Park, North Carolina, on 20–21 April 1998. Educators, researchers, students, physicians, and administrators gathered to discuss the challenges and opportunities involved in using marine and freshwater organisms in environmental health research. Overviews of agency perspectives and research needs set the stage for presentations, posters, and discussions. A keynote address by John Stegeman put forward a cautionary note to the effect that investigators and reviewers often have differences of opinion about what a model is, how well a particular model is justified for a particular research question, and what attributes characterize a good model. He emphasized that the suitability of a model depends on the question being asked and the attributes of the system that lend themselves to answering that question. With this caveat in mind, three groups focusing on zebrafish, other vertebrates, and invertebrates then met to consider the needs and strategies possible with these models for environmental health advancement. The focus groups summarized their conclusions at the end of the meeting.



Studies with marine and freshwater organisms are being transformed by the application of modern genetic methods and protein-engineering techniques. These techniques make it possible to design and implement studies that provide greater insight into the mechanisms that underlie adverse environmental effects and the interplay between genetic and environmental factors in disease. This understanding can revolutionize approaches to the diagnosis, treatment, and prevention of disease. Notably, "engineered" organisms and mutant strains that have amplified or otherwise distinctive responses to environmental challenges are being generated. This approach can reveal unexpected connections between disease and environmental conditions or confirm the role of a gene or gene product in variable disease susceptibility.

The workshop was characterized by a sense of excitement derived from the fact that new and powerful ways of using marine and freshwater models are being discovered. Presentations and posters at the workshop encompassed a broad range of studies that illustrated how environmental effects on health are being addressed with marine and freshwater models. Notable among these was a poster that showed that fish scales, like tree rings, can provide a history of exposure to environmental pollutants. Both presentations and posters illustrated ways that nerve systems of molluscs such as squid, octopus, and sea hare (*Aplysia*) are being used by researchers to clarify the molecular basis of nerve functions such as tactile sensations, learning, and memory. Modern genetic methods are being used with sea urchins, enhancing their use as models for understanding adverse environmental effects on early development. Similar progress characterized reports of ongoing studies with spiny dogfish, little skate, winter flounder, the killifish *Fundulus*, zebrafish, and marine polychaetes such as *Glycera*.

Hope was expressed that future transgenic studies would allow human metabolic pathways to be "knocked-in" and explored in fish models. Also, attention was drawn to a recent program announcement (PA) on an

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NIH initiative to increase support of the zebrafish as an animal model for research. As indicated in the PA,

> . . . mutational analysis in vertebrates has lagged behind such investigations in invertebrates . . . . When combined with genetic combinatorial analyses, mutational analyses can identify specific genes that act during embryonic development, provide insight into how they function, and clarify the pathways in which they participate . . . . [Studies show] that there is remarkable evolutionary conservation in the genetic programs that determine embryo formation, including such early patterning events as formation of the embryonic axes, but also including later events such as development of eye, heart, and other organs.

Workshop attendees affirmed that the genomic information and transgene techniques for effective use of zebrafish and other fish to probe genetic and environmental effects on development and disease are rapidly being gained. Small aquarium fish such as zebrafish have considerable advantages of size, ease of care, rapid development, and a ready supply of embryos. It was the consensus that faster and more cost-effective studies on environmental impacts on health can be done with transgenic fish models than with mice. This consensus is in accord with the PA statement that

> While reverse genetics (e.g., knock-outs) have been useful in the mouse model, the substantial costs of maintaining large mouse colonies has limited the applicability of forward genetic approaches, which will have a profound impact on our understanding of development.

Pharmaceutical companies are closely monitoring these developments because it would be of great benefit to reduce the cost of testing new drugs and medicines.

Zebrafish, characterized by their small size and transparent embryos, are leading the way in attracting interest to marine and freshwater model systems. These fish are rapidly becoming recognized as premiere organisms for studies of vertebrate development and genetics. Many features of this fish model, including patterns of early cardiac and nervous system development and aspects of cell fate and lineage determination, have been characterized. Researchers have developed techniques for removing or transplanting individual cells to explore questions about induction and cell fate. Researchers have generated and recovered zebrafish mutations that affect a wide range of genes including ones that regulate patterns of development, organogenesis, physiology, and behavior. Positional cloning of genes identified by mutation has recently been accomplished. It is important that the functions of many of these genes appear to be conserved among different vertebrate groups. The analysis of zebrafish mutations and their responses to environmental factors can thus clarify genetic and environmental linkages to health in other vertebrates, including humans.

Progress is being made on ways to enhance the production of developmentally regulated transgenics in zebrafish to provide for live gene-expression imaging. Use of the green fluorescent protein (GFP) may allow for live gene-expression imaging plus the possibility of producing three-dimensional (3D) computer archives of gene expression. Coupling technologies for transgenesis, live gene-expression imaging, and 3D reconstruction of gene expression with the zebrafish system is being done to gain insight into signal transduction questions.

Bioindicator lines of trangenic zebrafish are being developed as identifiers of specific molecular targets of environmental toxins, as a screen for environmental agents and for genes that alter specific aspects of neural development; and as an online biosensor for the presence of developmentally relevant toxins in natural or industrial water supplies. Researchers have generated a line of transgenic zebrafish in which expression of the jellyfish green fluorescent protein GFP is under the control of a defined promoter from the mammalian *GAP-43* gene. This promoter is activated when differentiating neurons acquire the ability to extend an axon and is then silenced when those neurons successfully complete axonal outgrowth and establish functional connections. GFP expression in live zebrafish embryos and hatchling fish shows that activation of this GAP-43 promoter construct is tightly restricted to neurons and is temporally regulated at appropriate stages of development. Application of this approach to promoters for other genes activated at specific stages of neuronal and glial differentiation would make it possible to use a panel of bioindicator fish for broad and rapid screening or monitoring of environmental toxins affecting central nervous system development.

The zebrafish has features that make it an attractive model system in which to study cardiovascular development. Congenital cardiac defects occur in about 8–12 in 1,000 live births in humans as the result of the interaction between genetic susceptibility and environmental factors, resulting in an enormous social and economic burden. To understand the complex etiology of congenital cardiac defects, model systems that are amenable to simultaneous genetic and environmental manipulation need to be developed. The utility of the zebrafish as a model of mammalian vascular development clearly depends on the conservation of molecular and morphogenetic mechanisms of cardiovascular development. Zebrafish homologues of Tie1 and Tie2, receptor tyrosine kinases that are known to be involved in mammalian cardiovascular development, have been recently cloned and found to be strikingly similar to their mammalian counterparts. These findings suggest that the molecular mechanisms of cardiovascular development are highly conserved across vertebrate species and that the zebrafish should be considered as an alternative to more standard model systems to study the complex etiology of congenital heart disease. Along similar lines, a genetic screen using zebrafish was performed to identify the important steps and pathways of vertebrate heart development. Mutations affecting discrete steps of the morphogenesis of zebrafish heart, including individual components, patterns, and asymmetry, have been identified. These mutations provide further insights to vertebrate heart development and aid in understanding the molecular basis of heart morphogenesis.

Results on the integrative mapping of the zebrafish and trout genomes were presented. These results are relevant to the objectives of the Environmental Genome Project, which seeks to examine variation in 200 or more genes to identify alleles that determine environmental disease susceptibility. Zebrafish can be used in the identification of genes involved with resistance or susceptibility to environmental chemicals by using the saturation mutagenesis approach, an approach that has been very successful in isolating developmental mutations. Knowledge of phenotypes of zebrafish mutations can aid in the determination of the functional significance of mutations in human genes. With increasing knowledge of the interplay between environmental and genomic factors in disease, we can generate transgenic fish to serve as environmental sentinels, the water-dwelling equivalent of the miner's canary. Activation of transcription factors to three classes of environmental contaminants, aromatic hydrocarbons, electrophiles, and heavy metals, was examined in normal zebrafish cells. These xenobiotics were found to alter gene expression through transcription-factor interactions with defined response elements in the enhancer regions of specific genes. Constructs have been designed to use xenobiotic-inducible promoters or synthetic response elements or both to induce dose-dependent expression of reporter genes in zebrafish cells upon exposure to specific xenobiotics. To determine the molecular basis for species differences in dioxin (TCDD)-induced developmental toxicity and to better understand the roles for aryl hydrocarbon receptor (AhR) and AhR nuclear translocator

(ARNT) in normal development, it is essential to characterize the AhR signaling pathway in fish. Researchers have cloned AhR and ARNT from both zebrafish and rainbow trout and found several differences between the fish and mammalian AhR and ARNT proteins. First, there are multiple forms of both AhR and ARNT in fish. The C-terminal domain of fish AhRs are approximately 200 amino acids longer and do not contain a Q-rich transactivation domain found in mammalian AhRs. The trout and zebrafish AhRs are expressed and functional in the early embryo suggesting a potentially important role for AhR in normal development. Interestingly, unlike the case in mammals, TCDD exposure was found to increase AhR mRNA in both zebrafish and rainbow trout.

Related studies reported by other researchers concerned early life-stage toxicity of TCDD and related AhR agonists in trout and zebrafish. Signs of toxicity in lake trout and rainbow trout early life stages, exposed as fertilized eggs to TCDD, were manifested during the sac fry stage as cardiovascular toxicity and arrested development of skeletal and soft tissues culminating in mortality. While similar signs of early life-stage toxicity occur in other ecologically relevant species and experimentally advantageous species such as zebrafish, there are significant differences in the susceptibility of these different fish species to early life-stage mortality. Lake trout and rainbow trout are among the most sensitive species, and zebrafish is the most resistant, requiring a 40-fold higher dose of TCDD to cause embryotoxicity. Ongoing studies are designed to clarify the cause of the species difference in embryotoxic potency of TCDD and ultimately to understand the physiological functions of the AhR signaling pathway in vertebrate development. In this regard, presenters suggested that generation of a pool of mutant zebrafish, differentially insensitive to certain signs of TCDD toxicity, would be useful in elucidating the molecular pathways leading to adverse developmental effects of TCDD.

Fish models in addition to zebrafish are claiming their share of the attention. The *Xiphophorus* fish system has been used for over 60 years for studies of spontaneous and induced carcinogenesis. Application of modern approaches and techniques for the molecular dissection of genetic components contributing to tumor susceptibility in the numerous *Xiphophorus* hybrid tumor models has not yet been fully exploited. Various and genetically diverse *Xiphophorus* interspecies hybrids, which exhibit both spontaneous and induced tumors of several types, have been developed for research use. There is notable conservation of gene maps in *Xiphophorus* and other teleost fishes. Comparisons of gene maps of fishes, amphibians, and mammals have provided support for hypotheses that some chromosome segments have been conserved through over 400 million years of vertebrate evolution. What is learned with one fish may thus have relevance to other fish and to other vertebrates as well.

The small killifish species medaka and *Fundulus* are among the fish species that meet the combined requirements for ease of gene transfer and continuous culture. Both of these hardy fish are well known to toxicologists. *Fundulus* advocates noted that for studies of pharmacological and environmental effects, it is significantly easier to obtain sufficient quantities of liver tissue for assays from *Fundulus* than from either zebrafish or medaka. One illustration of how these fish models can be used to address important environmental questions was the use of medaka to clarify the organismal significance of exposing male fish to (xeno)estrogens such as octylphenol (OP). It had been previously shown that such exposure typically results in elevated levels of vitellogenin (VTG) in blood. It has now been demonstrated that vitellogenin in male fish can be used as a predictive indicator of impaired reproduction. The observed OP-induced decrease in egg production, combined with fewer eggs fertilized and fewer embryos surviving, may have serious ecological implications.

Researchers using the trout as a model system claimed advantages for this rather large fish that offset its longer developmental time. Compared to zebrafish, trout exhibit greater sensitivity and utility for dose response, molecular dosimetry, and chemoprevention cancer studies. Remarkable studies of 42,000 trout with the environmental carcinogen dibenzo(*a*,*l*)pyrene (DBP) and a chemoprevention study with 10,000 trout were reported. The molecular dosimetry studies showed that dietary chlorophyllin (CHL) strongly reduces aflatoxin B_1 (AFB_1)–DNA adduction and strongly inhibits hepatic tumors 9 months later, and that DNA adducts are quantitatively predictable biomarkers of reduced carcinogenesis at lower CHL doses. Based on these results and the demonstration that the simple protective mechanism was directly translatable from trout to F344 rats, a double-blind, placebo-controlled CHL intervention trial has been initiated for AFB_1-exposed participants in Qidong, China. This is believed to be the first direct translation of fish cancer research to a human health intervention trial.

Animals without backbones were represented by enthusiasts who are quick to point out that the simpler organ systems of the invertebrates can lead to more definite interpretations and often more elegant experiments. Their position is inarguable, given the long history of scientific advances made with the nerve systems of squid and other molluscs such as *Aplysia*. Recognizing the need for cultured molluscs for experimental use, primarily in the disciplines of neuroscience and behavior, the National Center for Research Resources established National Resource Centers for *Aplysia* and for Cephalopods. These invertebrates have great potential for use in the study of the mechanisms of developmental toxicology in that large and individual neurons, linked to specific simple behaviors, can be tracked throughout development and can be studied electrophysiologically. Although advances are being made in this area with vertebrate models (e.g., zebrafish), nearly all research in vertebrate models involves the use of biochemical proxies for nerve function and development rather than direct observation of nerve function as is possible in many invertebrates.

Invertebrates are also being used as environmental sentinels or biomarkers. Success with this approach was reported for a study that deployed hatchery-reared juvenile oysters at 15 sites that were classified as reference, agricultural, suburban, or urban/industrial. Researchers analyzed a suite of cellular responses and found that environmental pollutants associated with anthropogenic activities led to adverse effects on lysosomal destabilization, depletion of glutathione, increases in lipid peroxidation, and induction of metallothioneins and heat-shock proteins. These fundamental cellular responses reflect the kinds of responses of mammalian as well as marine systems to environmental chemicals, and can be used to elucidate the subtle effects of toxins on disease susceptibility and chronic disease processes. These types of studies will also facilitate our ability to identify linkages between ecological health and human health. Aquatic organisms may thus function as important "canaries" of potential environmental hazards associated with urban and industrial activities.

Despite having favorite model organisms, the workshop participants were unanimous in their accord that a broad range of environmental health topics can be successfully addressed when one draws on the widely varying properties of current and emerging marine and freshwater models. A major conclusion of the roundtable discussion was that environmental effects on reproduction and development, neural function, and patterns of gene

expression in organisms and ecosystems are areas that are poised for advances coupled to increased use of marine/freshwater models. Important questions of whole animal biology and whole ecosystem responses can also be uniquely addressed with these models, where individual susceptibility to environmental parameters can be selected and/or manipulated.

The marine and freshwater models have, in many cases, been shown to offer distinct advantages for many studies in terms of simplicity, fecundity, rapid development times, broadly varying sensitivities, range of adaptive responses, and greatly reduced maintenance and handling costs. Such models often provide for relative structural simplicity, ease of observation, and simplicity at cellular or genetic levels while revealing processes that are closely related to those found in human health and disease. More widespread use of these models would reduce the number of higher vertebrates used in environmental health research. This advantage is, in some cases, coupled with the opportunity to discover novel protective or medicinal products of human health significance.

The presentations at the workshop covered uses of marine/freshwater models that represent organisms closely related to humans as well as organisms with distinctive biochemical pathways. With this broad diversity of form and function, it was agreed that appropriate models can potentially be chosen for a wide range of environmental health questions. It was concluded that more cellular level research, particularly mechanistic studies and toxicological investigations, will help researchers select the marine or freshwater model most appropriate to provide answers to their questions.

In light of the advances being made in use of these models in environmental heath research, it was recommended that an advisory group be formed to interact with the National Institutes of Health (NIH). The responsibility of the group would be to keep agency leaders informed about research advances in the use of marine and freshwater models, suggest relevant programmatic initiatives, and aid in setting up separate study sections for targeted funding opportunities. The objective would be to increase the level of funding for investigations using these models, with the rationale that this would have a high payoff in advancing our understanding of the environmental and genetic contributions to disease. It was stressed that the uses of marine and freshwater models should be integrated into research with mammalian models and that toxicology training programs should include training in the selection of appropriate models from both classes.

Other recommendations made for more effective use of marine and freshwater models in environmental health research centered on ways to enhance development and distribution of genetically manipulated organisms and to enhance the infrastructure for their use at research institutions. Users of fish models are often handicapped by being the principal animal care providers. This differs from the situation with users of rodent models, where multiple users benefit from university facilities and the support services of veterinarians. Additionally, the long-term culture of transgenic lineages can pose significant challenges. Infrastructure at research institutions appropriate to the increasing use of normal and genetically manipulated forms of marine and freshwater models is clearly needed.

Research to develop well-characterized, disease-free strains and ways to distribute these are also needed. Once generated, transgenic organisms can be best used by researchers trained in their use and in avoidance of diseases. These needs could be met by creating regional training and distribution centers and by agency initiatives that encourage collaborative studies. This concept was amplified by each of the focus groups, with features specific to zebrafish, other vertebrates, and invertebrate models. The regional centers could be set up partly by the regional user groups, but some support from the research institutions and state and federal agencies would be advantageous in aiding the exchange of models and training researchers to use them.

Individual researchers were urged to work with collaborators and with centers to achieve better communication about the advantages inherent in the use of marine and freshwater models. Better communication was among the primary ways mentioned that would aid in putting these models on an equal footing with traditional rodent models (when appropriate). Other ways were debated. Agency-supported initiatives, such as the recent NIH-wide Program Announcement that invites researchers to submit proposals that make use of the zebrafish model, were regarded very favorably.

It was recommended that marine/freshwater centers and specialized resource centers for this kind of research and training be restructured to have broader scope or increased in number or both. These centers would become more effective focal points for generation and distribution of needed research tools, such as isolated proteins, gene libraries, genetically enhanced models, and cell lines, and for training in their use. Great value was ascribed to the existing National Resources and Centers of the NIH that provide organisms, materials, and information for work with *Caenorhabditis elegans*, *Drosophila melanogaster*, *Aplysia*, cephalopods, zebrafish, yeast, cultured cells, and molecular biology tools. These centers and the NIEHS-sponsored Marine/Freshwater Biomedical Science Centers have Web pages and annual reports that provide in-depth information of current research. Reports and summaries that communicate advances being made with marine and freshwater models were also endorsed.