Published in final edited form as:

Comp Biochem Physiol C Toxicol Pharmacol. 2009 March ; 149(2): 121–128. doi:10.1016/j.cbpc.2008.12.006.

Aquatic Animal Models of Human Disease: Selected Papers and Recommendations from the 4th Conference

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Background

Historically, the motivation for studies with aquatic invertebrates and fishes was due in large part to the ease with which natural processes could be observed and relevant tissues harvested. Highly successful, work of this nature led to Nobel Prizes for: Ilya Ilich (Elie) Metchnikov (Metschnikoff), Hodgkin and Huxley, Christianne Nüsslein-Volhard, Jens Skou and Eric Kandel who respectively examined: starfish larvae and adults describing phagocytosis and ushering in the cellular phase of immunology; principles of signal propagation using giant squid axon; identification of genes controlling development in drosophila and zebrafish; leg nerves of the shore crab or rectal gland of dogfish shark identifying an enzyme that works with ATPase to regulate cellular levels of potassium and sodium, cellular basis of memory and learning using sea hares. However, these widely acclaimed studies are only part of what is possible given the fact that fishes are the most numerous, the most diverse, and the oldest group of vertebrates (Beyenbach 2004). Furthermore, following a review of the phylum, chordata, Hickman et al., (1984) concluded that chordate evolution continues today to “unfold a colorful spectrum of functional and structural diversity that has no equal among vertebrates”.

Less recognized, but increasingly important, were early studies of the renal physiology of fishes (see review by Beyenbach, 2004) that led to diagnostic methods remaining in use in human medicine today. Also of increasing importance are contributions of aquatic invertebrates and fishes to the detection of toxic responses from: metal mining and smelting impacts (Luoma et al., 2008); polycyclic aromatic hydrocarbons and persistent organic pollutants and their adverse effects in harbors and coastal estuaries (Myers et al., 1998; Johnson et al., 2008; Tillitt et al., 2008; Meyer et al., 2003; Hahn, 2002), including the verification of remediation processes (Johnson et al., 2008); off-site transport and biological effects of agricultural control agents (Bailey et al., 1994); personal care products and pharmaceuticals (Richardson et al., 2005); and, to epizootics of tumors in wild populations (Myers et al., 1998; Vogelbein et al., 1990). The importance of this capacity for detection was witnessed with the emergence of a number of different endocrine disrupting chemicals during the 1980's and 1990's (Sweeting 1981 as reviewed in Tyler et al., 2008). The responses to low concentrations of these compounds were first detected in birds, reptiles, fish and invertebrates, and possibly influence sperm counts and development of testicular malignancy in humans (European Environment Agency, 2001). Taken together these illustrate the important roles of aquatic organisms as environmental sentinels warning of potential harm to man. Both field observations and laboratory experiments are essential to unravel the complexities of these risks (Luoma et al., 2008). Examples of laboratory experiments addressing mechanisms and processes modulating responses after previous studies of natural field populations include: selenium (Ohlendorf et al., 1986), diseases developing after stress of aquaculture restraints (Groff et al., 1992; Marty et al., 1995) or the international movement of aquaculture organisms (Cole et al., 2008) or feeding upon contaminated foodstuffs (Lee et al., 1991) or residing at contaminated sites and feeding upon contaminated food organisms (Malins et al., 1985).

In laboratory experiments, disease states are induced under controlled circumstances, often in an attempt to determine the cause(s) and mechanisms of a specific disease agent or to evaluate the role of xenobiotic chemicals in the etiology or modulation of the process. *It is clear that much insight into human health and safety can be gained through research involving various aquatic species.* Much effort has been put into using certain fish species to address specific questions. Zebrafish are being used to model cancer, aging, anemia, tissue regeneration and stem cell biology and many additional genetic diseases and disorders (Feitsma and Cuppen, 2008; Gerhard, 2007; Meeker and Trede, 2008; North and Zon, 2003; Poss, 2007; White and Zon, 2008). However, certain species possess novel traits making them strong model species for specific diseases; for example *Xiphophorus* serves as a model for melanoma. And, recently,

expanded attention has been directed toward the emerging impact of zebrafish technology on preclinical safety decision making in relation to development of pharmaceuticals.

As the genomes of multiple fish species have been sequenced, many examples of ancient and recent genome duplications and very compact and very large genome sizes as well as individual gene losses and duplications have emerged. These findings emphasize the importance of taking a comparative approach to understanding genomics in these groups as well as the power of examining the functional implications of these different genome structures in fishes. Thus there is not likely to be one prototype fish species for studies of genomics and some caution should be applied when extrapolating observations made in single fish species to other species. Similarly, while most cellular and molecular processes are well conserved across vertebrate groups, bony fish and mammals diverged ~400 million years ago. As with translation of research results from any animal model system to humans, careful analysis of similarities and differences from molecular to organismal levels must be conducted before proposing the use of a particular model system. Given these limitations there are a wide variety of examples of opportunities for translational research such as high throughput chemical screens with embryonic fish to provide a rapid and affordable means for discovery drugs which may ultimately be applicable in human medicine. The broad spectrum of disease research involving aquatic animals integrates a wide variety of disciplines such as molecular biology, analytical- and biochemistry, physiology, embryology, immunology, microbiology, and genetics. This research has historically focused not just on a limited number of laboratory species but has constantly expanded to include species encountered in wild populations. These species are typically selected for study based on their unusual physiological adaptations, diseases or exposure/response to toxicants in polluted habitats. This diversity of research disciplines and species involved in development of aquatic animal models of human disease has led to a diverse community of researchers who may not interact on a regular basis at the more established, discipline-oriented, scientific meeting. The need to bring this diverse group of scientists together has led to establishment of cross-disciplinary meetings focused on all aspects of the use of aquatic organisms as models of human disease, begun in 2000.

Contributions of this issue- this special issue of Comparative Biochemistry and Physiology contains 17 manuscripts from a conference entitled “The Fourth Aquatic Animal Models of Human Disease Conference” held from January 31, 2008 to February 3, 2008 at Duke University, Durham, North Carolina. This issue joins similar special issues developed from the three previous meetings in this series held in 2000, 2003 and 2005 (Nairn et al., 2001; Schmale, 2004; Schmale et al., 2007). The studies presented in the present issue again demonstrate the wide range of disease-related questions that can be addressed by these unique models for human disease processes.

1) Models of carcinogenesis/mutagenesis

Background- we are witnessing the dawn of perhaps a new era in safety assessment. As new modalities emerge for testing the potential mutagenicity and/or carcinogenicity of industrial chemicals, consumer products, and environmentally discharged compounds, aquatic animal models are playing increasing roles in comparative risk assessment. Validation of new cell and tissue arrays will require animal models that can be used in large numbers and with high through-put, yet maintain convincing comparisons to human responses. Basic research with aquatic models will continue to play a vital role in the discovery of common cancer mechanisms that can be applied to such testing as well as to cancer therapeutics. In addition, the aquatic environment is a natural “final sink” for numerous cancer-causing chemicals that may contaminate drinking water sources. In this section leading scientists in comparative cancer research present work with several aquatic animal models that will figure prominently in this new era.

Aquarium fish models such as genetic hybrids of the genus *Xiphophorus* have provided useful tools to assess the role of specific genes in tumor formation. For example exposure to ultraviolet light (UV) or to a direct acting carcinogen, N-methyl-N-nitrosourea (MNU), resulted in melanoma formation. In the study by Rahn et al., (2009 - this issue) crosses between *X. maculatus* and *X. helleri* were exposed to MNU with resultant tumor formation enabling analysis of genes putatively involved in melanoma formation. After sequencing the tumor suppressor gene, *CDKN2AB*, and finding no mutations, they concluded that melanoma may result from mutation of other critical genes.

Detection of mutations within key fish genes may form the basis for a population-based approach of use to regulatory agencies responsible for assessment of long-term population health consequences. Rotchell et al., (2009- this issue) analyzed tumors in livers of marine flatfishes for mutations in the retinoblastoma (*Rb*) gene, a tumor suppressor. *Rb* gene alterations were localized to adenomas and carcinomas but not to normal adjacent tissue. Furthermore, they report a profile similar to that observed in human tumors where mutations are spread across the gene, particularly in the functionally important region involving exons 8–23.

Hobbie et al., (2009- this issue) made use of recent advances in the medaka (*Oryzias latipes*) model to compare test results for carcinogenic potential of a given compound, dimethyl nitrosamine (DMN). Using a molecular dosimetry approach, quantitative adduct tests compared and integrated factors such as chemical exposure, uptake, distribution, metabolism, that tend to vary widely between different phyletic levels. Next, mutation frequencies were measured in medaka and rat using transgenics of each organism. Interestingly, DNA adduct concentrations were similar between species but mutation frequencies of medaka were up to 20 fold over that of the Fisher 344 rat. It is thought that future work with other compounds using this same approach will provide a comparative dose response between various model organisms, honing our ability to guide risk assessors as to appropriate “alternative models”.

Using Japanese medaka infected by *M. marinum* and exposed to the mutagen and carcinogen, benzo – e,a,f,-pyrene, Broussard et al., (2009- this issue) examined the role that chronic mycobacterial infections play in overall cancer risk. *M. marinum* infections in medaka resemble the pattern seen with the closely related disease, tuberculosis. The finding of increased hepatocellular neoplasms in carcinogen-exposed fish infected with *M. marinum* is evidence that the infectious disease can act as a tumor promoter. These findings suggest that increased risk for cancer promotion may be seen in human populations with chronic tuberculosis infections.

Telomeres and telomerase, the cellular activity that synthesizes them, are ends on chromosomes formed by a special chromatin preventing their degradation and recombination. They are of critical importance in aging and cancer. Au et al., (2009- this issue) compare genomic and protein information in various lower vertebrates and demonstrate how telomerase reverse transcriptase (TERT) of the Japanese medaka (*Oryzias latipes*) shares the highest similarity to the human among all small aquarium fishes including pufferfish and zebrafish. In addition, medaka telomere length is closest to that of the human. These authors present previous and current findings supporting the medaka as a model for studies of aging, tissue regeneration, and carcinogenesis.

The study by Teutschbein et al., (2009- this issue) established and used signaling pathways in *Xiphophorus* fish to demonstrate that the Src family kinase/Focal Adhesion Kinase (FAK) complex plays an essential role in transformation downstream of oncogenic growth factor receptors. In melanomas of *Xiphophorus* fish, an oncogenic EGF receptor orthologue, *Xiphophorus* melanoma receptor kinase (*Xmrk*), causes continuous activation of the Src family kinase Fyn but not in other members of the family. Also, in *Xiphophorus* melanomas, Fyn is

responsible for promoting tumorigenic events. The workers showed that the prominent role of *Xiphophorus* Fyn also was seen in mouse and in human melanoma cell lines. This illustrates how findings in fish can be used to find hitherto unrecognized signaling in tumorigenesis of human melanomas.

The study by Williams et al., (2009- this issue) is a well – presented and brief review of the very interesting work using the rainbow trout model for cancer studies. Summarizing several decades of important research in hepato-carcinogenesis, the paper presents major findings of the completed ultra low dose, dibenzo[a,l]pyrene, study and the ongoing aflatoxin study. A major thrust of the report is the value of the trout model in estimating a virtually safe dose with less uncertainty than can be achieved using rodent models. In these studies the major, unique contribution of the trout model is the clear demonstration that a linear extrapolation of predicted tumor incidence to ultra low doses is likely to produce significant errors in estimating a virtually safe dose.

2) Toxicology models

Background- the recent review of toxicokinetics in fishes by Kleinow et al (2008) denotes special features of aquatic organisms and the unique medium in which they reside; and, serves as useful background information to this section. For example, fishes are intimately linked to their aqueous habitats; eating, obtaining sensory information, reproducing and spatially orienting within this single surrounding and contiguous medium. Many of the structural and physiological adaptations permitting survival in aqueous environment also strongly impact upon interactions of these vertebrates with xenobiotic substances. While the epidermis of mammals is dead and keratinized and does not possess a circulatory component, fishes have a living epidermis perfused by an underlying secondary circulation. In very small fish or bottom-dwelling (benthic) species, constant contact with sediments and their anthropogenic contaminants is maintained and the skin thereby becomes an important exchange surface for potentially toxic compounds, *i.e.*, these organisms bioaccumulate compounds of interest in human health.

We know that large differences often exist among environmental toxicant concentrations and the basis for certain differences are environmental factors while other factors are associated with aquatic organisms themselves (Kleinow et al. 2008). Water dissolves more substances and dissolves them more completely than does any other liquid. Nevertheless some non-polar substances are relatively insoluble in water. Water of natural systems, being a composite of H₂O and materials that are dissolved and suspended in it, is known to affect complexation, binding, precipitation and chemical form with ultimate effect on bioavailability of these. For this reason water may not be viewed as a uniform or generic medium and chemical detection of levels of compounds in water may not reflect actual risk to aquatic biota or their wildlife and human consumers (*i.e.*, biomarker responses and bioassays are needed). Information of this nature must be applied in studies comparing fish and mammalian responses.

From the above it is obvious that aquatic species have numerous properties that are similar to their mammalian counterparts, yet there are significant differences and the medium of aquatic organisms must be taken into consideration. If for no other reason, toxicity bioassay using fish in specific waters with varying concentrations of compounds are necessary and a portion of the papers at the 4th Aquatic Animal Models of Human Disease Conference covered this interesting aspect.

Not only do aquatic organisms *bioaccumulate* substances but they *biotransform* them as well. Biotransformation is a two-phase process involving enzymatic reactions to convert non-polar lipophilic chemicals to polar water-soluble metabolites (Schlenk et al., 2008). It is an unfortunate reality that the alteration of chemistry required for enhanced polarity often creates

reactive intermediates through bioactivation with the result that these intermediates can be more biologically hazardous than are the parent compounds. Biotransformation of specific compounds by fishes has received extensive attention in a variety of freshwater and marine species (Schlenk et al., 2008) and we now have much information concerning the occurrence, regulation, catalytic activities, genes involved and specific proteins related to biotransformation in various fish species (Schlenk et al., 2008). We recognize that alterations in enzyme expression can dramatically affect the sensitivity of an organism to the toxic insult of a xenobiotic. We now know that alteration may occur as a result of genetics, diet, gender, or history of exposure to other xenobiotics.

The study of mechanisms of toxicity in fish was recently reviewed (Hahn and Hestermann, 2008) and two distinct rationales that follow from the coverage above are of fundamental importance. *Fish may serve as the target and as the model.* One major theme in earlier proceedings of Aquatic Models meetings and the current issue is aquatic organisms as *animal models* in toxicological research. Close evolutionary relationships to humans (Hahn and Hestermann, 2008) are evidenced by shared genes and biochemical pathways that have become even more apparent as a result of whole-genome analyses (Aparicio et al., 2002; Jaillon et al., 2004). As is seen with certain of the fish species reported herein advantages include: small size, rapid development and short generation time, and transparent, externally developing embryos that facilitate experiments in developmental toxicology and mixture evaluation. Transgenic species (Linney and Udvardi, 2004a; Kurauchi et al., 2005; Scholz et al., 2005; Chen et al., 2007) and gene knockdown (Nasevicius and Ekker, 2000) are well-developed approaches serving as powerful tools for mechanistic research. These and other advantages of small fish models have been described in detail elsewhere for zebrafish (Carvan et al., 2005; Hill et al., 2005; Linney et al., 2004b,) medaka (Oxendine et al., 2006), Atlantic killifish (*Fundulus heteroclitus*) (Burnett et al., 2007) and rainbow trout (*Oncorhynchus mykiss*) Bailey et al., 1996). New contributions to the aquatic toxicology literature reflect in part the strategic use of available resources and chronicle the advantages inherent when these model- and sentinel species are coupled to modern experimental approaches with robust toolkits.

DNA damage is an important endpoint of toxicity for a variety of pollutants. However, widespread use of this approach has been hampered by the fact that extensive nuclear DNA sequence data are available in a limited but growing number of laboratory model fishes. Many of the more than 20,000 species of bony fishes lack this data but remain environmentally relevant species. Jung et al., (2009- this issue) take an alternate approach centered upon analysis of mitochondrial DNA, since mitochondrial genomes are frequently sequenced in full. First, they conducted laboratory exposure of Atlantic killifish to benzo a,c pyrene in liver, brain, and muscle. Next, they measured DNA damage in liver and muscle tissues from killifish residing at a site highly contaminated with polycyclic aromatic hydrocarbons. In both cases DNA damage extent proved comparable in both nuclear and mitochondrial DNA. Jung et al., (2009 - this issue) adapted a PCR-based large amplicon quantitative assay (LA-QPCR) for this work and argue that assessment of mitochondrial DNA damage will be a valuable approach in ecotoxicological studies and human disease related laboratory experimentation.

The paper by Segner (2009- this issue) is as much about future needs in research with aquatic animal models such as the well-established zebrafish (*Danio rerio*) model as it is a review of a body of work related to endocrine disruptive compounds (EDCs) in the aquatic environment. Widespread, EDCs require special tests to detect endpoints; and, until recently these compounds were not detected by the battery of existing bioassays (Tyler et al. 2008). As Segner maintains, the zebrafish has a preponderance of reasons favoring its use to investigate EDCs and their associated alterations in development, physiological homeostasis and health of vertebrates. Not the least of these reasons are a rich history in developmental biology, abundant genomics resources and abundant use in toxicity testing. Segner stresses the need for a good

knowledge of the biological traits of any species as a pre-requisite for rational design of test protocols and endpoints as well as accurate interpretation and extrapolation of associated toxicological findings. To illustrate the situation with this organism, he reviews the mode of sexual differentiation of zebrafish that impacts EDCs testing. Finally, using EDCs as an example, Segner (2009- this issue) stresses the need for more basic understanding of how molecular alterations translate into animal disease.

The paper by Lammer et al., (2009-this issue) is a thorough report that examines data derived from the Fish Embryo Toxicity test and compares it with that of the conventional, fish acute toxicity test in Germany derived from high quality literature. The driving force for production of such a report has been the multinational efforts that seek to constrain the number of vertebrates used for the purpose of research in the context of global chemical management programs. Policy makers, scientists, and other stakeholders in Europe anticipate unprecedented levels of animal tests to provide needed safety information under the REACH legislation (Registration, Evaluation, Authorization and Restriction of Chemicals) that went into force in 2008. Efforts to replace, reduce, and refine the use of animals in toxicity testing are being demanded. Embryos as well as eleutheroembryos (free swimming forms with yolk sac unresorbed) may prove viable alternatives. North American investigators, perhaps, have not been made so aware of these developments as have our colleagues in the UK and the other member nations of the EU. An emerging topic that is rising in the Discussion is the potential expanded role for a more robust usage of embryos and eleutheroembryos in future testing, risk assessment, and research. The stage is set for this debate to begin. Given the massive amount of data derived recently by the prolific use of zebrafish, and to some extent other species as mentioned herein, in gene identification, drug formulation evaluation, safety testing with personal care products, and in nanomaterials research, the argument will be made. Clearly these models have strong support and potential integration across biological levels of organization. This paper serves to show that refined aquatic models have strengths that are becoming increasingly more difficult to ignore.

The paper by Wise et al., (2009- this issue) is an excellent example of the use of resources from aquatic model species to establish genotoxic responses and to compare these to various species of interest, in this case a marine mammal, the North Atlantic right whale. Cultures from an established medaka fin cell line were exposed to an emerging pollutant of concern for marine waters, hexavalent chromium (Cr(VI)) and concentration-dependent genotoxicity (chromosomal aberrations) characterized and quantified. Next, lung and testicular cell lines from the North Atlantic right whale were exposed to the same form of the metal and genotoxicity established as above. Genotoxic responses of medaka were comparable to those of the marine mammal cells. Results suggest medaka to be a useful model for other species some of which may be threatened or endangered.

3) Research Resources (needs)

From the first Aquatic Models Conference to the edition to which this issue is dedicated, biological and database resources supporting aquatic animal research have commanded considerable attention (Nairn et al., 2001; Schmale, 2004; Schmale et al., 2007). A few aquatic models, such as zebrafish, have become commonplace at many university and government laboratories such that well established protocols and shared facilities are available. However, the vast majority of scientists working with marine or freshwater animals represent small scientific communities. Such researchers find it necessary to personally collect, rear, and care for the animals used in their studies. Separation of labor for animal care and maintenance from that needed for the more technical tasks of experimentation is rare. Standardized protocols in animal care, such as those available for mouse or primate facilities at typical medical schools, are only now being established in aquatic animal science. This is not to say that varied, valuable

and important aquatic resource centers do not exist. Examples of national stock centers for aquatic organisms include the National Resource for Zebrafish, the Xiphophorous Genetic Stock Center, the National Resource Center for Cephalopods and the National Resource for Aplysia. These Centers, funded by the National Center for Research Resources of NIH, provide needed standardization of aquatic animals and other resources for use by scientists employing these particular aquatic models.

This issue provides an example of how one of these centers, the National Resource for Aplysia, has expanded to meet the growing demand for the California sea hare, *Aplysia californica*, as a model organism Capo et al., (2009-this issue). *A. californica* has played an increasingly important role as a model organism in the neurosciences. As for most marine animals, the pelagic larval life stage of *Aplysia* is the most fragile and difficult to raise. Thus, this developmental stage is typically a bottleneck in any culture operation. Capo et al (2009-this issue) demonstrate that optimization of algal diets and larval density can greatly increase the yield and consistency of laboratory reared *Aplysia*. These and other developments leading to optimized protocols for *Aplysia* culture have greatly improved the numbers and value of these organisms for research.

As aquatic resource centers develop they tend to carry more animal lines and/or strains with a concurrent increase in labor requirements to maintain them. One avenue to reduce this labor load that had been virtually unexplored until recently is research aimed at establishing cryopreservation capabilities for each of the extremely varied aquatic models. Past conferences have witnessed research reports that collectively frame a consistent effort to develop cryopreservation protocols for model fish species (Tiersch, 2001). A workshop was devoted to Cryopreservation in the 4th Aquatic Animal Models Human Disease Conference. And, in this issue, Yang and Tiersch (2009-this issue) describe the current state of affairs regarding development of effective cryopreservation protocols for fish sperm derived from zebrafish, medaka and *Xiphophorus*. As research with these aquatic organisms has increased, so have the needs to maintain pedigreed lines and various mutant strains. Cryopreservation has emerged as the most promising means to achieve these needs. Of importance to all who use marine and freshwater species is the basic biology and structure of the testis, sperm maturation morphology and basic physiology that are uncovered while considering cryopreservation methods. Coverage on protocol development for sperm cryopreservation is thorough given the demands of egg bearing and live-bearing species. Finally, evaluation procedures and criteria for success are presented along with the application of cryopreservation to select aquarium species. In a related paper (Yang et al., 2009- this issue) using cryopreserved sperm from *X. couchianus*, virgin females from *X. helleri* were inseminated and followed for 90 days. In two of three tanks, offspring were produced signifying the success of the procedure for the first time in this species. Paternity of offspring was verified both by phenotype (body color) and genotype using microsatellite genetic markers. And, a protocol is provided. This work is not only important for research but illustrates also how endangered species may be maintained.

One of the key areas in which aquatic animal resources have lagged behind the well-established mammalian systems is in routine disease monitoring. Disease monitoring in laboratory colonies of aquatic animals is not yet widely implemented. Thus, there is considerable interest in developing acceptable and standardized methods to assess disease prevalence in aquatic colonies. Accordingly, in past conferences and proceedings the susceptibility of experimental fish to various mycobacteria has been the subject of several reports (Kent et al., 2004, Watral et al., 2007; Burge et al., 2004; Broussard and Ennis, 2007).

In this issue, detection, management, and prevention of infectious diseases in research colonies also takes center stage. Recommendations by Kent et al, (2009 – this issue) emphasize the need for control of infectious disease in fish used for biomedical research. Not only are they

concerned that acute diseases will cause morbidity and mortality, but low grade or sub-clinical infections may confound research results as well. Strategies for control of such diseases, while not completely developed to date, deserve attention given source and quality of incoming water, the varied design of facilities used by individual investigators, and the need to transport such animals between laboratories. In addition to discussion of protocols that may be employed to maintain disease free fish colonies, an understanding of pathological states of infection are useful to diagnose the degree to which a colony may be compromised and is necessary to establish protocols for its return to a disease-free state. Accordingly, Spitsbergen et al., (2009-this volume) present a review of current and future finfish and aquatic invertebrate pathology resources. In this paper they underscore the need for sophisticated understanding of pathologic lesions in the various organ systems in these diverse species. Such pathological understanding is the first step toward development of databases that will allow evaluation of changes in organisms within an experimental colony. As pointed out by Spitsbergen et al., (2009-this volume) there is a current rapid global expansion in aquaculture coupled with increasing threats to natural populations of aquatic species largely due to diminished habitat and pollutants in the environment. Thus, if we are to realize all the important advances in genetic, genomic, proteomic, and metabolomic resources, it is imperative that we first understand the systemic pathology of these species. In addition, there is a need for aquatic animal models to “bridge the gap” between the new cell and tissue arrays (currently under study by the United States Environmental Protection Agency and the National Institute of Environmental Health Sciences) and human tissue/organ responses. There is a critical need to maintain *in vivo* models yet with high through-put capabilities. To reach these goals: corresponding research funding support as well as commitment of academic institutions to train these scientists. In short, there is a need for more pathologists to anchor new phenotypes (*i.e.*, produce morpho-functional correlates) as was mentioned in a recent letter to the Editor of Nature Biotech [“Do-it-yourself (DIY) Pathology” Vol. 26 No. 9, Sept. 2008 p. 978].

Development of database resources for aquatic models is also a highly represented topic in the history of the Aquatic Models conferences as has been reported the corresponding journal issues. Research resources related to genomics (Clark et al., 2001; Nonaka et al., 2001), development of microarrays (Oleksiak et al., 2001; Linney et al., 2004, b; Ju et al., 2007; 2007a; Page, et al., 2007; Hook et al., 2007), development of mutagenesis assays (Shima and Shimada, 2001; Winn et al., 2001), construction of BAC libraries (Miyake and Amemiya, 2004), adaptation of proteomics (Oehlers et al., 2007), and establishment of cell culture capabilities (Parton et. al., 2007; Butler et. al., 2007; Buck et. al., 2001) have mirrored state of the art application of these technologies in mammalian research studies. Although genomic sequencing is rapidly progressing for quite a few diverse animals, many of the useful aquatic models do not have the benefit of genome, large scale EST, or proteomic databases to support them. However, use of these models may still be informative via comparative genetic analyses using tools developed for closely related species.

The report by Boswell et al., (2009- this issue) documents the plausibility of gene expression profiling in one species, *Xiphophorus*, using a microarray developed from the closely related aquaria model, medaka. Herein, gene expression patterns were assessed after exposure to hypoxia and it was determined that the tissue source of RNA contributed more to observed response of gene targets than did the different species from which the RNA was isolated. Target organs and potential biomarkers of response that can be used to identify hypoxia exposure were also highlighted.

4) Future Directions

Scientists working with aquatic models require access to genomic, proteomic, metabolomic and/or functional genomic databases that are on par with those available for many mammalian

model systems. The slow development of such databases is a direct consequence of the relatively small numbers of scientists using any one of these varied aquatic model systems. However, a clear value of the aquatic systems is the comparative genomics insight they can provide. Among these various genera are represented a plethora of alternative and/or modulated biochemical processes that have evolved to bestow rare and unique abilities to eke out a living in extremely variable niches. The robust radiation of fishes, for example, extends from benign and temperate clines to seemingly uninhabitable regions and hostile environments and is illustrative of the role of gene and whole genome duplication in the evolution of the most species and diverse group of vertebrates. The acquisition of genomic sequences and other database resources for these varied and interesting models would not only allow scientists to perform novel comparative analyses that delve into the evolution of specific physiological mechanisms (*e.g.*, tumorigenesis, renal function, etc.), but also, and perhaps more importantly, serve as a set of reference genomes for new studies aimed at determination of the mechanics behind complex life history traits such as maturation, aging, and behavior. The investment of large scale resources in developing databases for genome and EST projects for mammalian species has paid great dividends. One of the goals of this conference was to demonstrate the usefulness of employing a diverse array of model systems to address specific research problems. As technologies for genome and proteome sequencing continue to improve rapidly, corresponding costs are reduced. The opportunity to apply these advancing technologies to realize the full potential of valuable aquatic animal models can only be realized through cooperation and collaboration of the type encouraged by these conferences on aquatic animal models.

We are gratified to see that genomes have now been or are being sequenced from: zebrafish (Jekosch 2004), medaka (Kasahara et al. 2007), stickleback (<http://www.genome.gov/12512292>), Takifugu (Aparicio et al, 2002), Tetraodon (Jaillon et al. 2004), elephant shark (Venkatesh et al. 2007), coelacanth (<http://www.broad.mit.edu/node/437>), sea urchin (Sodergren et al. 2006), Ciona (Dehal et al. 2002), amphioxus (Holland et al. 2008) and Aplysia (<http://genomics.biotech.ufl.edu/aplysia/>). And, we look forward to the inclusion of other aquatic species. As has been stated above, there are compelling reasons for continued use of Aquatic Animal Models. The value of comparative approaches to basic biological questions and the need to complement these investigations using more traditional animal models are two strong rationales. During the 4th Conference, the question “why are well-developed aquatic models not used in risk assessment?” was raised repeatedly.

The future directions of Aquatic Animal Models research will likely see continued use of gene knockdown technology and we note the recent development of gene knockout models involving medaka and zebrafish. The continual refinement of the zebrafish genome sequence and the completion of the first draft of the medaka genome sequence and subsequent releases of additional genome coverage (Kasahara et al. 2007) have further contributed to the placement of zebrafish and medaka among the leading model organisms for genomics and developmental genetics research. Most recently, by capitalizing on these expanding genomic databases, procedures for the disruption or knock-out of the function of specific genes has been demonstrated in medaka and zebrafish (Taniguchi et al., 2006; Moens et al., 2008). The approach, referred to as TILLING (Targeting Induced Local Lesions in Genomes), was first used in *Arabidopsis* and other plants, and is now used in large scale efforts to disrupt genes in zebrafish and medaka. TILLING entails the introduction of random mutations in the male fish genome using the chemical germ cell mutagen ethylnitrosourea (ENU) followed by breeding with wildtype females to establish a library of genomic DNA and sperm from the F1 generation male offspring. The genomic DNA from the library is sequenced to identify individuals carrying specific mutations for the gene of interest. Individual animals with mutations in the targeted genes are then retrieved using *in vitro* fertilization with the

cryopreserved sperm, and lineages are established by subsequent outcrossing. The generation of gene knockout models by target-selected mutagenesis represents a significant achievement by providing the means to develop new lineages carrying mutations for virtually any gene of interest to explore the mechanisms of gene function and related disease processes.

Although TILLING has been used to generate gene disruptions in zebrafish since 2002 (Wienholds et al., 2002)) additional gene knock-out technologies are being developed in zebrafish which utilize zinc-finger nuclease fusion proteins to generate targeted gene disruptions (see review by Ekker 2008). *In vitro* transcribed mRNAs encoding these proteins are injected into the 1-cell embryo with the result of the fusion protein transiently expressed during the early cell division. The zinc finger portion of the fusion protein targets the nuclease to a specific nucleotide sequence within the genome which then introduces double strand breaks. The double strand breaks are then repaired, however the repair mechanism is imprecise typically resulting in the insertion of additional random nucleotides which can generate frame shifts, destroy splice sites and even introduce the occasional premature stop codon. This strategy should be applicable to a variety of other fish species which lay eggs and can be mated in the laboratory. The expansion of genetic resources, including the development of specialized lineages such as targeted gene knockouts, promise to provide unprecedented research opportunities.

Thus, in this “genomic” era one may argue there seems no better use of limited resources than to stimulate the collective scientific ability to approach novel questions in evolutionarily distinct and varied organisms. It would be a mistake to lose experimental models that exhibit high speciation, expansive morphological variability, and environmental resiliency. Basic research models may be thought to represent volumes of a biological library, and each volume having its own special characteristics may be selected and used to access new understanding. Experience teaches us that when libraries lose volumes they become less valuable and at some point upon successive losses, the library will cease to be of value at all. Continued use and promotion of the varied aquatic models by those faculty and students attending and presenting their research results at these Aquatic Models of Human Disease conferences each serve as shining examples of the value such models have to the overall scientific community. The organizing committee would like to acknowledge their contributions and thank all who came and supported this meeting. We hope to see you again in Oregon at the 2010 meeting.

Acknowledgements

5. Acknowledgement of the supporters of the meeting

Lastly, we wish to acknowledge those who supported this conference that was hosted by Duke University and the Nicholas School of the Environment. The meeting would not have been possible without the generous support of the National Center for Research Resources, the Office of the Dean of the Nicholas School, the Nicholas Institute for Environmental Policy Solutions, the Duke University Comprehensive Cancer Center, Fred and Alice Stanback through their support for the Cancer and the Environment Program at Duke University, the Roy F. and Joann Cole Mitte Foundation, and the Duke University Integrated Toxicology and Environmental Health Graduate Training Program. We would especially like to thank the members of the organizing committee who, in addition to the authors of this preface were: Marjorie Oleksiak (University of Miami) and David E. Williams (Oregon State University).

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