Report to SACATM

A collaborative workshop on aquatic models and 21st century toxicology: Leveraging small aquarium fishes to advance understanding of environmentally influenced human disorders and diseases

Held May 5 – 6, 2014, at North Carolina State University, Raleigh, NC

After a welcome by NCSU Dean of Biological Sciences, Daniel Solomon and Warren Casey of NICEATM, plus a brief overview by UNC Assistant Prof. of Biology, Antonio Planchart, Dan Villeneuve of the US EPA ORD (Mid-Atlantic Division) began the **Opening Session** by introducing the topic of the aquatic models (fish and amphibia) in regulatory programs, with example of EPA's Endocrine Disruptor Screening Program (EDSP) ("Aquatic animal models: not just for ecotox anymore"). He also introduced the concept of Adverse Outcome Pathways (AOPs) and described international efforts to formalize the concept, including OECD's guidance, handbook and shared knowledgebases. According to OECD's system, AOPs themselves are more-or-less linear; a description of one initiating event leading to one adverse outcome; however, it is a network of pathways that is the functional unit of prediction. He introduced Key Events as essential steps in AOP that can be shared between more than one pathway.   This is the context around the use of aquatic models that can be relevant for human health endpoints (e.g. for endocrine disruptors) as well as for ecotoxicity.

Jyotshnabala Kanungo from the US FDA described the use of zebrafish in drug safety assessment. She described a multi-pharmaceutical company consortium project evaluating the use of zebrafish to evaluate embryo toxicity. The results indicated several false negatives resulting from poor drug uptake as well as false positives caused by excessive bioaccumulation. They also discovered that 32 endpoints is excessive for a reasonable scoring protocol. Eventually the process will need to be automated. Also, she indicated that the presence of a liver is critical for screening. An FDA/ National Center for Toxicological Research zebrafish research program (beginning in 2010) is analyzing in vivo and in vitro endpoints with approved drugs, including ketamine, estradiol, and testosterone. Zebrafish showed good concordance with findings in mammals at physiological doses. It turns out that zebrafish share many of the mechanisms of ketamine activity with mammals; however, ketamine-treated zebrafish possessed fewer neurons, likely due to developmental delay. If zebrafish display similar phenotypes to humans or other mammals following treatment, they can be used to dissect drug mechanisms.

Matthew Winter from the University of Exeter also talked about the use of fish models in drug safety evaluation, from the pharmaceutical and European perspective. Only 11 percent of pharmacological compounds pass to registration, and this rate differs between therapeutic areas. The main reasons for this are efficacy, bioavailability, and some

toxicology. Companies have made great improvements in bioavailability, but not a lot of improvement in efficacy or toxicity, so better screening methods are needed, and zebrafish are seen as having potential. Very little compound is needed for testing, and animal protection in the EU starts at feeding (approximately 5 days post-fertilization in zebrafish) and are unregulated before that. Zebrafish can be useful for fulfilling unmet needs in the areas of drug development, including bridging gaps between mammalian and in vitro results (e.g. in cardiotoxicity), or looking at off-target effects (e.g. ototoxicity of analgesics). Zebrafish share much pharmacology with mammals, and the transparency is very helpful. Barriers to use include confidence in observations, uncharacterized bioavailability, and low throughput. A six-laboratory ring test was performed to evaluated zebrafish as a model for developmental toxicity found that discerning negatives was difficult and there is uncertainty around drug uptake. In order for zebrafish to become the standard, the confidence in zebrafish results needs to increase by more extensive validation. Zebrafish screening needs to be lower cost and throughput also needs to be improved.

Session 1 looked at the use of zebrafish for screening compounds for cardiovascular activity. Maria Bondesson from the University of Houston introduced the use of Kdr1:GFP transgenic fish (in whom vascular endothelial cells express GFP). In these fish, arsenic causes malformed vasculature along with other embryonic malformations at environmentally relevant concentrations. Arsenic interferes with vascular tubule formation, similar to what is seen in mammals. In a screen of 189 ToxCast compounds, 35 or so chemicals caused thin, short, non-parallel inter-segmental vessels. Zebrafish results were confirmed in vitro using C166 cells in a vascular tube formation assay. All of the zebrafish positive compounds were positive in the cell assay. Next, they need to test the negatives. They are dissecting the mechanisms of tube disruption and working with others to create a predictive AOP for zebrafish vascular development.

Warren Heideman of the University of Wisconsin describe his work on the effects of TCDD on the Aryl Hydrocarbon Receptor (AHR) in the zebrafish heart; looking at sublethal doses they were able to identify a development window during which the zebrafish heart is susceptible to TCDD exposure (between 48 and 96 hours post fertilization). They have looked at the mechanism of this activity (TCCD prevents epicardial cells from surrounding the nascent heart, and this activity involves the AHR. TCDD also affects brain development and caused trans-generational scoliosis n zebrafish.

Dave Volz from the University of South Carolina presented a high-through put screen for identification of chemicals impacting cardiovascular function in zebrafish embryos. The assay uses 384 well plates with 32 embryos per treatment. It takes 4 hrs to take 30,000 photographs per plate. The assay captures both morphological and functional endpoints including: body length, pericardial area, inter-segmental vessel area, heart rated, circulation (number of cells passing through a vessel over time). Body length and heart

rates are very consistent, ISV and circulation are more variable. They have designed a decision tree to decide whether an given experiment was good. Only two of the chemicals (one was TCDD) they tested had specific cardio effects.

Ken Poss from Duke University talked about heart regeneration. In general, the heart is poorly regenerating: heart attacks kill on average a billion cardiomyocytes in the left ventricle. Scar tissue forms and function is lost. Isolating cardiac stem cells and injecting back differentiated cardiomyocytes have yielded largely unremarkable results. Initial experiments using cardiomyocytes grown in vitro from human iHSCs injected into an infarct macaque model looks promising. Zebrafish experiments to ablate epicardium cells (using transgenic fish) implicate the myocardium in the regeneration process. However, using adult fish is too laborious so are now looking at explants of healthy zebrafish hearts. These experiments have shown that sonic-hedgehog signaling from outflow tract cells is critical for regeneration.

Session 2 focused on the role of developmental processes in cancer, toxicity and endocrine disruption. Shawn Burgess from NIH presented a rapid screening method for the identification of anti-metastatic compounds using zebrafish. Metastasis is what is lethal about cancer, and the deposition of primordium lateral line (pLL) cells from anterior to posterior during zebrafish development is a good imitation of metastasis. His lab found that expression of cell adhesion, guidance, polarization and other genes involved in cancer is enriched in pLL cells. So the screen involves looking for substances that block pLL migration. In the 1280 compound LOPAC library from Sigma, there were 66 "hits," a library of 800 natural products gave 63 hits, and an 880 compound library form GSK gave 9 hits. These hits were confirmed using an in vitro cell migration assay; the compound showed a large block but not complete inhibition. These chemicals included an estrogen receptor binder, matrix metalloprotease, PPAR inhibitor, neurotransmission inhibitor. A complementary approach they are taking is identifying genes involved in pLL migration by knocking out genes (using the CRISPR/Cas9 system) and looking for blocked migration. One such gene identified was SYK- a protein kinase. The proof of whether these results in zebrafish are relevant to humans will come when an identified drug is successful in humans; apparently drugs identified in zebrafish by Len Zon are currently in preclinical Phase III trials.

Mark Hahn from the Woods Hole Oceanographic Institution talked about the advantages of partial genome duplication: genome duplication occurred soon after divergence of bony fishes and many current fishes retain bits of this duplication, allowing them allelic diversity that mammals cant afford. For example, the humans have one Aryl Hydrocarbon Receptor (AHR) which is responsible for xenobiotic metabolism and is involved in dioxin toxicity. Fish have multiple AHRs at least two, and sometimes pairs of these two. Zebrafish have three AHRs, 1,2 and H: AHR 2 is involved in dioxin toxicity. AHR1b also binds dioxin, but is

expressed almost exclusively in the developing eye, and knock-down really messes up the eye (AhR also involved in eye development in drosophila). TCDD also causes eye malfunction in trout. Another example of divergent function is oxidative stress: there are 18 oxidative stress response genes in zebrafish that are known from mammals. These genes are differentially expressed in situations caused by different oxidative stressors, implying they have specialized.

Nancy Denslow from the University of Florida described molecular characterization of secondary sex effects in mosquito fish in response to exposure to androgens and progestins. In some contaminated rivers, female mosquito fish were found to have elongated gonopodia. This process involves the androgen receptor, and Bone Morphogenic Proteins (BMPs), which is also of fin regeneration of zebrafish. However, androgens actually inhibit fin regeneration – the opposite of anal fin elongation.

Session 3 focused on Emerging Technologies. *A highlight of the conference was Keith Cheng from Penn State describing his micron-scale synchrotron x-ray tomography as a tool for 3D assessment of cellular and tissue architecture.* This method is different from other x-ray methods in using a tube to direct the x-rays into a beam. This method gives high-resolution 3 dimensional images of the entire zebrafish embryo (that look somewhat similar to an MRI). The image can be moved or rotated to emphasize different structures, and is not subject to artifacts of other histological techniques that occur from fixation or sectioning (but the fish do look a bit dehydrated). The images were really quite remarkable.

Matthew Harris of Children's Hospital in Boston talked about large-scale genetic screens in zebrafish and medaka to identify genes involve in bone growth. They did two screens, one for recessive genes that have effects in adult fish (took about four years) and another that to look for genes that have dominant effects (took about 3 months). The genes were cloned by traditional systematic homozygocity. Is mad possible due to high variability in SNPs, allowing rather rapid mapping down to a few MB using only 100 or so SNPs. Then they were able to clone the genes using custom arrays and 60mers from coding sequences, but I am not sure exactly how – only that this process is getting faster. Currently they are comparing the identified zebrafish and Medaka genes. They are also looking at fish with more developmental plasticity, Krptolabias marmoratus, or mangrove swamp fish. This fish is a self-fertilizing hermaphrodite that can respond to environmental conditions by changing morphology. These fish like extreme conditions (low pH, low oxygen), can air breath through skin, can sit outside water for 3 – 6 months and the eggs can enter diapause – in other words they can exit development to resist dehydration. They are currently working on genetic markers and developmental screens, for example, for mutations that do not allow diapause.

David Reif from North Carolina State University presented a rapid motility assay for identification of neuromodulating chemicals in embryonic zebrafish. The screen involves measuring swimming activity affected by a light/dark transition in young zebrafish. They looked for hyper or hypo activity. They found one chemical that causes hyperactivity regardless of light stimulus, and one that decreases movement altogether. Behavioral screens provide a complement to other types of analysis, and are a useful integrating endpoint. A question for a large-scale screen using this method is whether chemical classes can be identified that can help inform the mechanism involved.

Rodolphe Barrangou also from North Carolina State University described CRISPR/Cas9 systems and genome editing. CRISPR/Cas systems evolved in bacteria as a defense against viruses (and other foreign DNA). This system requires genes that encode nucleases (Cas genes) as well as repetitive non-coding sequences (CRISPR arrays) that are transcribed and cleaved to form short RNA that guide the nuclease to a specific sequence. There are 3 types of CRISPR/Cas systems: Type II systems can be engineered to direct targeted double-stranded DNA breaks in vitro to specific sequences by using a single "guide RNA" with complementarity to the DNA target site and a Cas9 nuclease. The guide RNA is about 20 bases long and the nuclease will cut the DNA 3 bp away from the end. IN this way, a variety of surgical edits can be achieved: genome editing, inactivating genes, or you can add functions to a piece of DNA. In the past couple of years there has been a veritable CRISPR craze which has spread to zebrafish.

Session 4: Models of Neurobehavior and Neurotoxicology. I apologize for not covering some of these talks very well, including that of Marc Ekker from the University of Ottawa who talked about transgenic zebrafish models for the study of dopamine neuron development, loss, and regeneration. Dopaminergic neurons are tough to kill in whole fish – they are looking into the effects of the blood brain barrier by performing intracranial injections.

Stephanie Padilla from the US Environmental Protection Agency talked about using larval zebrafish in developmental neurotoxicity screening. With the current DNT guideline, only about 100 chemicals total have been tested since 1990. A high priority for EPA is DNT information. Bill Mundy and Tim Shafer have been working on an AOP and on collecting microarray data. She described a tracking assay similar to that described by David Reif. Light levels affect the results – it looks like lower light may actually be more sensitive. Generally saw concordance with mammalian data (e.g. lead caused a decreased activity in light with no difference in the dark as well as decreased neurons in the brain).

Andrew Rennekamp from Randy Peterson's lab at Massachusetts General Hospital described a rapid screen for small molecule effects on the vertebrate brain. Like Sean, Andrew emphasized that whole animal screening before targeted screening is critical for

improving success of lead compounds, especially for CNS (is not true for cancer). Zebrafish as a model has advantages over cell-based methods in that it has metabolism and a BBB (develops at day 3 pf) and uses the same neurotransmitters as humans. His assay tests 10 fish per well in a 96 well plate, one compound per well. In follow-up studies, 12 replicates are done. They use a setup like the one described by David Reif and Stephanie Padilla only it is also fitted with speakers and solenoids so it can deliver sound and electroshock as well as light. The assay takes only 7 minutes (420 seconds): no stimulus, then stimulus (fish freeze). Acetylcholine causes less activity during no stimulus plus hyper activity during stimulus. A muscarinic receptor agonist does a similar thing. They developed a semi-quantitative movement index: no movement (DMSO) = -10, constant movement = 0, therefore, hyperactivity during stimulus would be positive. They have created a similar coding for several characteristic behaviors and have formulated a system they call "behavioral barcoding." Then they look for chemicals that "phenocopy" known neuromodulators. For example, they administered a known ACHE and looked for chemicals that phenocopied the behavior and found a new one. Haloperidol mimics they found do bind the mammalian receptor, one binds as well as haloperidol. They can also look at safety, e.g. "anti-target" effects to avoid these. They can screen 10K compounds per day; the rate limiting step is loading embryos into plates.

Michael Carvan from the University of Wisconsin talked about neurological effects of environmentally-relevant methylmercury exposures in zebrafish. Methyl mercury decreases the visual startle in a dose-dependent way in zebrafish embryos; selenium protects the embryos from this effect, the same as in rodents. The effect is in the retinal electrophysiology; there is a big difference in potassium currents in bipolar cells in adult fish that had been exposed during development (first 24 hrs). In another assay based on spatial learning (they are taught where food will be), treatment with alcohol dur4ing the first 24 hpf increased the number of repeats required for learning the task. With methyl mercury exposure, at no concentration could the fish learn at all, demonstrating the dramatic effects of low levels of exposure. Methyl mercury causes effects 3 generations out at low levels of exposure in both zebrafish and perch. They are now looking at the effects of methyl mercury on global DNA methylation and beginning transcriptomics studies in brain and retina.

Jeff Bronstein from the University of California, Los Angeles: Studying Parkinson's disease-related environmental toxins using zebrafish. Several candidate disease pathways may start the PD process, including: protein degradation, synuclein oligomers, Lewy Body formation, mitochondrial dysfunction, and reactive oxygen species and pesticides that inhibit the ubiquitin-proteasome system (UPS). Behavioural experimetns in rats takes years, so are trying zebrafish. Exposing at 24 hpf can avoid the severe phenotypic effects seen by exposing earlier. Using VMAT2-GFP line marking Dopaminergic neurons (DA) they

can detect ziram-induced killing of DA neurons. Ziram also inhibits UPS in zebrafish. Zebrafish synuclein gene is truncated relative to mammals, but the protein (Syn) forms fibrils that have a beta amyloid structure. Like human Syn, injection of zebrafish Syn causes severe malformations and death, but this is not terribly convincing in terms of neurotoxicity. Syn knock-down results in better survival after ziram exposure implying that ziram functions through Syn. With regard to exposure to pesticides that inhibit UPS, similar effects are seen in zebrafish to those seen in mammals.

Session 5: Predicting Alterations to the Immune System

Carol Kim from the University of Maine talked about the effects of arsenic on the innate immune response. Apologies as I missed a bit of this talk. New England has high levels of naturally occurring arsenic due to the huge amount of granite. Arsenic exposure has been associated with high incidence of lung infection likely due to an effect on the innate immune response. She mentioned something about zebrafish not having lungs but perhaps being a model for cystic fibrosis, but I didn't understand how this was related to arsenic exposure. The Sod2 gene is up-regulated by arsenic. SOD2 knock-down morphants have decreased neutrophils. The Tnfaip8l1 gene family is implicated in apoptosis, cancer and immunity in mammals but their function is unknown in zebrafish. Knock-down increases mortality following P. aeruginosa infection. She would like to identify new things so would like to do a screen injecting different types of nucleic acids and look at their effect on neutrophils.

Jeff Yoder from North Carolina State University: strategies for in vivo immunotoxicology assays with zebrafish larvae. There have not been many functional studies of the zebrafish immune system, but the number of studies is increasing. There are many transgenic lines with markers related to the immune system that could be used as infection disease models. For example, microbial infection induces NF-kappaB and this could form the basis of a screen. Also, leucocyte chemotaxis could be a screen in zebrafish with neutrophils labeled with GFP with an induced tail wound. It would be better to be able to look at the immune reaction without inducing a wound. Another approach is to look at bacterial burden of a natural zebrafish pathogen, for example Edwarsiella tarda, labeled with CFSE-fluorescein. Percent mortality is a quantifiable effect, but the assay takes 9 days. They are now looking at the respiratory burst response which measures release of ROS in the presence of a fluorescent substrate. This assay can be high throughput. PMA stimulates the immune response; protein kinase C inhibitor decreases response. They are looking at 4 different exposure scenarios starting at 24 hpf to avoid early developmental effects. Percent mortality in this assay varies wildly in different zebrafish lines, perhaps due to gut flora?

Biobide lunch seminar: a representative of this company presented their multiplexing assays. Looking at markers for several toxicities at once can result in a 50% cost savings. They are currently multiplexing cardio-, neuro-, and hepatotoxicity. Developmental toxicity screen takes 7 days, due to final screening on day 5 pf. Zebrafish outperform cell-based assays for cardiotoxicity.

Session 6: Emerging Issues. Robert Tanguay from Oregon State University: In vivo behavioral and morphological screening of a 1078 chemical library using Zebrafish. He wished he would have met David Reif a lot earlier because he wasn't prepared for the data glut or analysis of a relatively high-throughput screen. They are taking a systems biology approach to early embryonic development. Most fundamental mechanisms are conserved amongst vertebrates. In zebrafish, diversity is an advantage. He talked about the elements that should be considered when designing a screen: exposure window, chorion or not, vehicle to use, amount of chemical to administer, as well as engineering parameters to consider: plate material, format, etc. there lab uses huge tanks (1,200 fish per tank)and eggs are collected through a valve on the side of the tank at intervals throughout the morning – they can get 40,000 eggs per tank per day. Dechorionation has also been automated; they can dechorionate 5000 embryos per 16 min cycles – using pronase and mild circular shaking. They screened ToxCast chemicals starting with nanomolar up to 64 micromolar. Every well is barcoded. They evaluate 32 developmental and neurotoxicity endpoints including mortality, developmental delay, spontaneous movement, notochord formation at 24 hpf, then at day 5 the do the motility assay, followed by euthanasia and several more endpoints. They built custom software to track inventory, record data, visualize results, and integrates the data. The software calculates LEL for all endpoints and the comparison is LELs. If the highest concentration shows no response, the LEL is 64 micromolar. The experiment was entirely blinded and all developmental neurotoxins were caught. Next they are working on refining the assays and translating the data as benchmarks for in vitro data. Phase I ToxCast chemicals were largely pesticides and gave many positives, phase II were largely inactive.

Jared Goldstone from the Woods Hole Oceanographic Institution: Cytochrome P450 in fish. Three-quarters of all drug metabolism is by p450's. CYPs in humans are encoded by 57 genes in 17 gene families. CYPs have many endogenous functions, e.g. hormone synthesis and metabolism, cholesterol and vitamin D production. CYP comparison in fish families: many CYP 2 s and 3's some CYP 11, CYP 27, but there is a huge expansion of CYP 2's. CYP16 is novel to fish but not in the main model species. There is a huge region of concordance between human and zebrafish CYP genes, but for many of the genes there is not too much. There is no CYP2E in fish that metabolizes alcohol in humans. But fish are susceptible to ethanol, and a CYP is induced by alcohol, but it isn't CYP2E. In other words there is the conservation of function but not the 1:1 conservation of orthologues. (This is a cautionary

note for AOP development: which orthologues is which?) There is only one CYP2J in humans; there are many in fish. In zebrafish they are called CYP2O, V, AD, N. They may be syntenic with cYP2J but there is not enough homology to define it as an orthologue. CYP2AA seems to be a zebrafish-specific gene. There are tandem duplications of about 10 genes with no known f(x). With so many genes, and so many differentially expressed, it will be difficult to unravel metabolism in zebrafish.

John Rawls from Duke University: Zebrafish models for investigating environmental regulation of adiposity. He uses Nile red staining to visualize adipose tissue in zebrafish and identified a mutation in growth hormone 1 (?); mutants are small but very fat and loaded with adipose tissue, obese actually. He is looking at the contribution of intestinal microbiota in zebrafish and mice on responses. Increased caloric intake will change the biota. If young mice are given antibiotics, they will get obese more often, and if you take the gut biota from obese mice and put it into a lean mouse it will become obese. Microbiota do metabolize complex carbohydrates and make them more absorbable. They also affect fatty acid and monoglyceride absorption.

John Colbourne from the University of Birmingham: Towards a science-driven solution for cooperative and effective management of chemical risks. He showed a photo of samples of water from Maywood California that are various shades of amber and brown and all are legally "safe" – there are so many different chemicals we don't even look for that are clearly present in drinking water. There are also a ridiculous number of species going extinct which will have a great impact on the environment. There is mounting frustration with current approaches which were mostly conceived in the 1930s. They are expensive, slow, use extremely high doses, not to mention the ethics of testing on mammals. After the recent chemical spill in West Virginia, a single test on 4-methylcyclohexane methanol showed an LD50 estimate was 825 ppm, so EPA added a 10X species factor + 10X humans being more sensitive, plus a random 8.25 X factor to account for non-lethal effects which magically resulted in a "safe" level of 1 ppm. In the EU, the Water Framework Directive declared that by 2013, all surface water be deemed safe. To do this, there will be a great need to generate a great deal of useful information. Water fleas (Daphnia) are environmentally sensitive, can reproduce sexually and asexually, generating genetically identical batches to experiment on. Comparison between species can lead to identification of conserved genes and processes, for example zebrafish and C. elegans. I think he mentioned a huge consortium involving China, Japan, Australia, and recently the JRC that is creating an enormous database of adverse outcome pathways.

Workshop Summary Discussion and Closing Remarks (I had to leave before this discussion was over). There was desire to include to include models other than zebrafish in future workshops. These big project need financing but it's not clear where the money is

going to come from. ADME is essential to making zebrafish (or any model) a proper toxicological model, so careful ADME measurements are necessary.