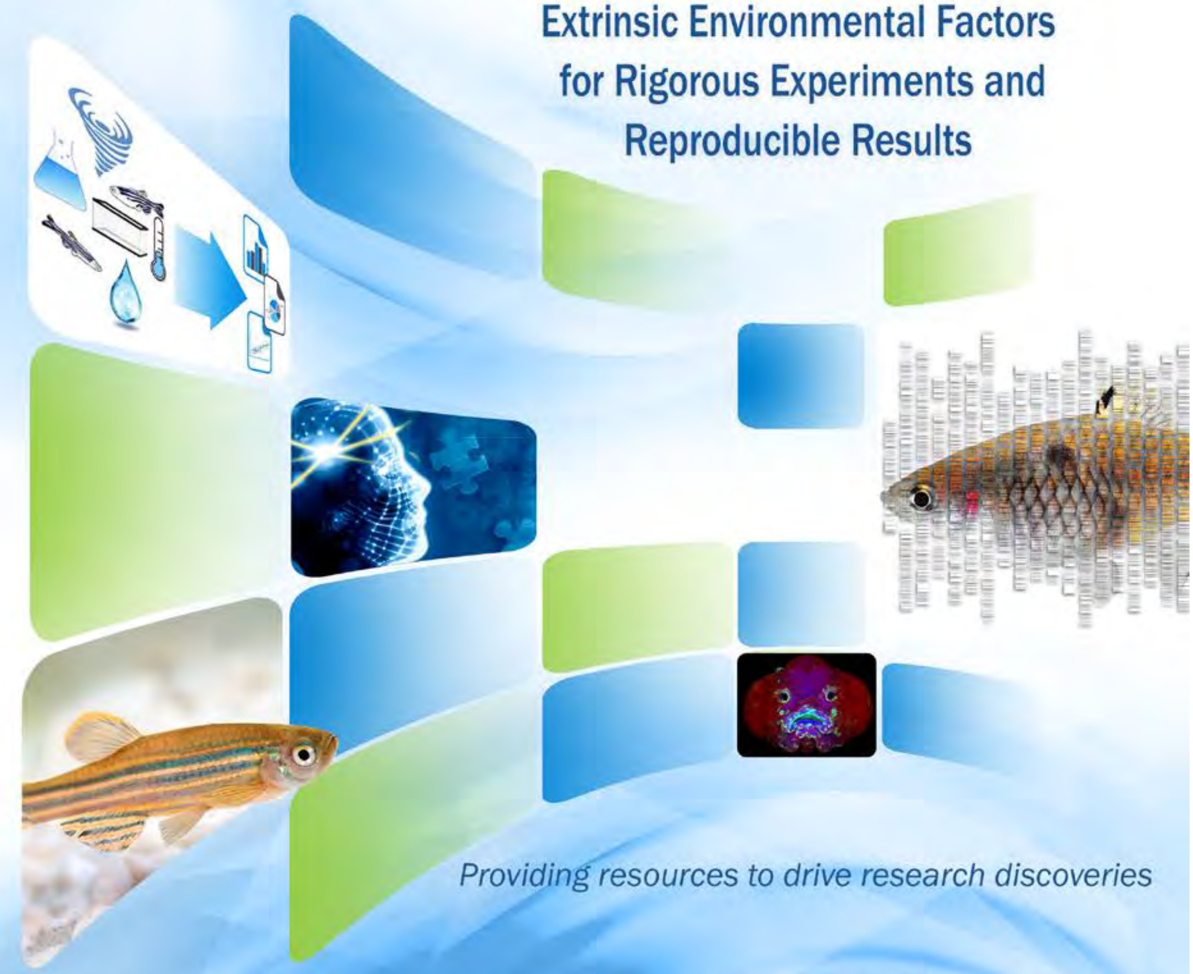
ORIP
OFFICE OF RESEARCH INFRASTRUCTURE PROGRAMS

# Zebrafish & Other Fish Models

## Extrinsic Environmental Factors for Rigorous Experiments and Reproducible Results



*Providing resources to drive research discoveries*

NIH National Institutes of Health
*Office of Research Infrastructure Programs*

**Workshop held in Bethesda, MD**
**September 11 - 12, 2017**

**Department of Health and Human Services**
**National Institutes of Health (NIH)**
**Division of Program Coordination, Planning, and Strategic Initiatives**
**Office of Research Infrastructure Programs (ORIP)**

# Zebrafish and Other Fish Models: Extrinsic Environmental Factors for Rigorous Experiments and Reproducible Results

**Bethesda Marriott Suites**
**Bethesda, MD**
**September 11–12, 2017**

**Workshop Report**

## <u>MONDAY, SEPTEMBER 11, 2017</u>

## INTRODUCTION TO THE WORKSHOP

### Welcome

*Malgorzata M. Klosek, Ph.D., Director, Division of Construction and Instruments (DCI), ORIP, NIH*

Dr. Malgorzata Klosek welcomed the participants to the workshop and introduced Drs. Franziska Grieder and Stephanie Murphy to provide the meeting introductions.

### ORIP's Support for Animal Models and Animal Facilities—Infrastructure for Innovation

*Franziska Grieder, D.V.M., Ph.D., Director, ORIP, NIH*

Dr. Franziska Grieder presented ORIP's mission of supporting research infrastructures—animal models and animal facilities—that help facilitating rigorous and reproducible experimental results. Dr. Grieder encouraged the workshop attendees to examine ORIP's Strategic Plan for Fiscal Year (FY) 2016–2020 for further insight to the Office's objectives of providing research resources and supporting infrastructure for the benefit of researchers funded across NIH's Institutes and Centers (ICs). ORIP is structured into two divisions: the Division of Comparative Medicine (DCM) and DCI, which work closely together to implement ORIP's mission. This workshop focuses on zebrafish and other fish research models and how to account for extrinsic environmental factors within aquatic research facilities. This is imperative because environmental factors greatly affect the rigor and reproducibility of experimental results. This objective aligns with ORIP's mission to support research infrastructure across NIH ICs.

### Rigor, Reproducibility, and Animal Model Research

*Stephanie Murphy, V.M.D., Ph.D., Diplomate of the American College of Laboratory Animal Medicine (DACLAM), Director, DCM, ORIP, NIH*

Dr. Stephanie Murphy provided an overview of the NIH's and ORIP's interest in the topic of rigor and reproducibility in animal model research. The challenge of achieving experimental reproducibility has generated much discussion within the biomedical community, particularly concerning preclinical research. The NIH has created measures for enhancing research reproducibility and transparency. In June 2016, the NIH published a Guide Notice on reproducibility through rigor and transparency and described four focus areas. The Notice informs scientists of changes and updates to application instructions and review criteria, related to scientific premise, rigorous experimental design, consideration of relevant biological variables, and authentication of key biological or chemical resources. Further highlighting NIH's efforts, Dr. Murphy summarized NIH's principles that are most relevant to the workshop's discussion: transparency in reporting, data and material sharing, and establishing best practice guidelines.

Dr. Murphy explained how improving research reproducibility using disease models is part of ORIP's Strategic Plan for FY 2016–2020. ORIP's website includes information and resources for the extramural community to address rigor and transparency in grant applications and progress reports. The Institute for Laboratory Animal Research has published a journal issue bridging the gap between reproducibility and translation and explaining NIH's policy on rigor and transparency.

**Meeting Logistics**
*Malgorzata M. Klosek, Ph.D., Director, DCI, ORIP, NIH*

Dr. Klosek introduced the workshop's organizers, members of the steering committee, and key ORIP staff. Dr. Michael Chang is the Deputy Director of ORIP. Within DCM, Drs. Miguel Contreras and Oleg Mirochnitchenko, and Sige Zou (Health Scientist Administrators) oversee animal model portfolios. Dr. Klosek introduced the workshop facilitators: Drs. Alena Horska and Willie McCullough and Mr. Esmail Torkashvan. The workshop is designed for stakeholders to address how to account for extrinsic environmental factors to improve rigor and reproducibility of experimental results using zebrafish and other fish models. It is important that if by the workshop participants can reach a consensus is on how to appropriately describe these factors, ORIP would like to work with the community on the next steps. Dr. Klosek invited Dr. Stephen Ekker to expound on the objectives of the workshop.

**Goals and Expected Outcomes of the Workshop**
*Stephen Ekker, Ph.D., Professor, Department of Biochemistry and Molecular Biology, Mayo Clinic Cancer Center*

Dr. Stephen Ekker thanked the participants for attending the workshop. Highlighting the importance of enhancing the use of the zebrafish model, Dr. Ekker informed the participants that zebrafish is the second most used animal model in research; the diversity of methods of growing these animals surpasses that of mice. Various environmental facets are important to consider for reproducible outcomes in zebrafish experiments across different laboratories. The intent of the workshop is to identify how to address the environmental variables that affect reproducibility. The expected outcome of the workshop is a white paper structuring the workshop's conversation, which will be distributed among the zebrafish community and other stakeholders, such as scientific journals and funding agencies. Dr. Ekker described the workshop's format and introduced the plenary session on water chemistry.

## PLENARY SESSION—WATER CHEMISTRY
*Moderator: George Sanders, D.V.M., M.S., Director, Department of Comparative Medicine, University of Washington, School of Medicine*

**Basic Information Regarding Water Chemistry from Zebrafish Facilities**
*George Sanders, D.V.M., M.S.*

Dr. George Sanders described the important parameters for tier 1 and tier 2 level water chemistries (i.e., pH, temperature, and conductivity/salinity) that affect the stages of zebrafish development and physiology. The goal of his talk is to discuss (1) Which parameters are measured and how are they measured at different facilities? (2) How frequent are the measurements? (3) How do the parameters vary over time? (4) What are the set points and ranges for these parameters? and (5) How are the ranges maintained?

To illustrate the diversity of water chemistry parameters between zebrafish facilities, Dr. Sanders outlined the parameters from the University of Washington, Duke University, and the University of Tennessee. The current parameter ranges at the University of Washington are a pH of 6.85–7.27, a conductivity of 1,488–1,537 microsiemens per meter (µS/m), a temperature of 25–29℃, a nitrate level of 0–40 milligram/liter (mg/L), an alkalinity of 30–51 mg/L, and a hardness of 154–188 mg/L. Regarding the  the facilities at the University of Washington, Dr. Sanders noted that this facility has four multi-rack and seven single-rack systems, full-time staff and student workers, seven laboratories and 40 researchers, reverse osmosis (RO) -treated municipal water, and several water additives.

Duke University's parameter ranges are a pH of 7.3–7.5, a conductivity of 1,100–1,200 µS/m, a temperature of 27–28℃, a nitrate level of 5–15 mg/L, an alkalinity of 40–85 mg/L, and a hardness of 80–120 mg/L. Municipal water is RO/electro-deionization (EDI)-treated and supplemented with several additives. The facility has seven multi-rack and 14 single-rack systems, full-time staff and student workers, and supports 11 laboratories and 70 researchers.

At the University of Tennessee, the parameters ranges are a pH of 6.5–8.0, a temperature of 27–29℃, and a nitrate level of 0–5 parts per million; conductivity, alkalinity, and hardness are not tested. Municipal wares RO/EDI-treated, and supplemented with several additives. The facility has a maximum capacity of 30 2.5-gallon static aquarium tanks, one staff member and several student workers, five laboratories and eight researchers.

**Effects of Water Chemistry on Zebrafish and Research Endeavors**
*Jim Burris, M.S., Facilities Manager, School of Medicine Zebrafish Core Facilities, Duke University*

Mr. Jim Burris presented on the effects of water chemistry on zebrafish research. The goal of facility managers is to ensure that facilities maintain optimal water quality that promotes the health of fish. However, sustaining a balance of factors within the recirculating aqua system is challenging. An imbalance of water parameter relationships (i.e., alkalinity, carbon dioxide, and pH) negatively affects the water quality and zebrafish viability. For example, as total ammonia nitrate levels increase, pH increases; conversely, as carbon dioxide increases, pH decreases.

Feeding protocols affect water chemistry. For example, high-fiber diets contribute to liquefied feces that cannot be filtered out of the water system. Also, feeds containing elevated levels of organic material promote heterotrophic bacteria production and an increased biological oxygen demand on the system. Although algae are a normal component of aquatic systems, excessive levels may contribute to the growth of biofilms harboring opportunistic pathogens, such as mycobacteria. Biological filtration is important for maintaining healthy water quality—ammonia toxicity is the most common cause of mortality in aquaculture systems. Filtration includes the removal of organic load and chemicals, including nitrate.

Changes to water parameter ranges affect aquatic species differently. Wide ranges may hinder fish homeostasis; rapid changes to this range may cause deleterious effects. Wide ranges of parameters can stress the biological filtration process. The stability of the fish environment and non-pathogenic microbial community depends on the maintenance of a narrow range, which promotes reproducible research.

Poor water quality can contribute to slow fish growth, slow maturation rate, and low survival. Low alkalinity and hardness can reduce egg hatchability and affect larval development. An elevated pH can lead to hypertrophy in fish gills and corneal damage. Consequently, poor water quality leading to poor fish health results in substandard research and data. Zebrafish can undergo genetic changes due to variations in water parameters, such as the activation of stress response genes. It is important to determine the boundaries of normal parameters beyond which genomic and behavioral alterations may become a concern. The aforementioned factors will determine short- and long-term economic investments. Another issue is that the equipment used to monitor water chemistry varies in cost and accuracy, and the device selection depends on the facility's size. These factors are significant considerations when maintaining water quality for zebrafish research.

**Discussion**

In response to a question from Dr. Michael Hucka, Mr. Burris replied that scientists at Duke record chemistry parameters during experiments via hard copy (written) and automated systems (data logs).

Dr. Allan Kalueff wondered what the effect of antidepressant drugs that feed into municipal water systems is on water chemistry. Mr. Burris replied that investigators can contact their local municipality to obtain an annual report of their region's water quality parameters; testing for prescription drugs is not done in the facilities. Dr. Sanders added that testing for individual drug compounds is not feasible.

Dr. Marnie Halpern remarked that some investigators use municipal water containing chlorine in their experiments. The most common cause of water system failure in research laboratories is disruption of the RO system, which causes bacterial contamination. Mr. Burris acknowledged that equipment failure is the major reason for non-protocol-induced mortality. He reiterated that ammonia is the major cause for water system failure and added that municipal water quality will fluctuate throughout the year. Dr. Halpern agreed that there is fluctuation; however, using water that humans consume is optimal for use in the zebrafish model. Dr. Sanders commented that the choice of water depends on the study. Dr. Halpern cautioned against standardization of water chemistry for zebrafish research. Mr. Burris reiterated that the water quality will depend in part on the size of the facility that will require the use of different water testing equipment.

Regarding parameter monitoring, Mr. Christian Lawrence pointed out that the parameters in husbandry systems can differ from those under experimental conditions.

Dr. Ekker emphasized that the purpose of the workshop is not to standardize scientific methods. The goal is to determine which parameters should be measured and which may restrict reproducibility in research.

Dr. Robert Gerlai cautioned against assuming that a narrow range of stable systems results in reproducibility. Allowing a certain degree of system flexibility mirrors the natural environment of zebrafish. He questioned why the conductivity/salinity (sodium chloride) in experimental systems surpasses the level found in nature. He recommended that in zebrafish research, measuring the speed of change, not the absolute value, is more relevant. Mr. Burris warned against comparing water conditions in nature versus in facilities because fish bred in laboratories may not thrive in natural conditions.

Dr. Lilianna Solnica-Krezel thanked the NIH for supporting the zebrafish community and noted that the most important point to consider is how alterations of the water conditions will affect studies. Recommendations from the Institutional Animal Care and Use Committee (IACUC) at each institution may influence the choice of study conditions. There is no scientific basis for developing strict guidelines for zebrafish research. Dr. Solnica-Krezel commented that she hopes the NIH will support the research addressing biological questions that are raised during the workshop. Dr. Klosek reiterated that the goal of ORIP is to support the development of animal models and improve reproducibility and robustness by providing access to important tools. Dr. Klosek acknowledged the myriad biological questions that may arise, but indicated that they do not align with the workshop's goal.

Dr. Ronald Walter agreed with Mr. Lawrence's earlier point that housing animals and using them in experiments are different concepts. Understanding these differences is important for experimental outcome. Dr. Sanders added that data reporting should be based on the specific conditions for each study. Dr. Ekker agreed with these assertions, but as a manuscript reviewer, he observed that authors and reviewers are provided little guidance on how to report experimental conditions. Knowing these reporting parameters for manuscripts is necessary for reproducibility. Dr. Sanders said that husbandry information is not included typically in an IACUC protocol. Dr. Solnica-Krezel responded that she has witnessed IACUC impose strict guidelines in certain instances. Dr. Gerlai added that the NIH should support scientists conducting research characterizing the environmental factors that are most optimal for zebrafish. Dr. Sanders replied that the immediate goal is reviewing what parameters different institutions are using.

Dr. Halpern commented that zebrafish researchers should leverage the large amount of knowledge of fish aquarists and husbandry experts who have already optimized environmental conditions. Certain participants cautioned against optimization, which they deemed is not feasible biologically.

Dr. Kalueff said that researchers conducting experiments should exploit the variable ecological niches (e.g., temperature) of zebrafish in their natural habitat. Genetic strain and sex differences also are important when formulating potential experimental recommendations.

In response to a participant's inquiry, Dr. Sanders said that investigators from the institutions he mentioned use the same methodologies for measuring environmental conditions irrespective if they move to another institution. Certain participants indicated that the conditions are underreported when laboratories change physical locations; it is difficult to account for the changes in conditions when animals are moved to different rooms within a single institution. Identifying what minimal set of parameters should be reported is important. Dr. Halpern replied that bringing to the conversation experimentalists such as experts on imaging or drug screening, may assist in identifying what environmental conditions are important to report. Dr. Ekker clarified the discussion by saying that identifying variables that prevent reproducibility between laboratories is most important.

Dr. Gina Alvino agreed with Mr. Lawrence's comments and mentioned that as the Senior Editor for the *Public Library of Science (PLOS) ONE* journal, she works with animal research and reporting issues. The majority of animal model submissions to *PLOS ONE* do not have sufficient information regarding environmental conditions. She recommended that participants report details of these conditions.

Dr. Gerlai added that there is a rise in "sophistication" in zebrafish research, and determining what causes stress on the animals is important.

## PLENARY SESSION—PHYSICAL ENVIRONMENT

*Moderator: Ronald Walter, Ph.D., Professor and Chair in Cancer Research, Department of Chemistry and Biochemistry, Texas State University*

### Zebrafish Husbandry and Breeding Require Careful Consideration of Environmental Conditions: The Growing Need for Systematic Studies on a Large Number of Factors

*Robert Gerlai, Ph.D., Professor, Department of Psychology, University of Toronto Mississauga*

Dr. Gerlai presented an overview of zebrafish husbandry and breeding. Achieving a consensus during the workshop is unlikely due to the differing scientific approaches and opinions from zebrafish researchers. The goal is broadening ideas and providing feedback to the zebrafish community. He expressed his appreciation to the NIH for organizing the workshop.

Replicability is a hard issue because of high experimental error / variation. This variation arises mainly from the lack of attention to and knowledge about important environmental factors. The ability to increase statistical power and to control for environmental factors that have deleterious effects on results is needed.

Several environmental factors that contribute to irreproducibility often are not reported. Because of the lack of knowledge of optimal parameter ranges, it is important for the NIH to support studies identifying these ranges. Dr. Gerlai reiterated previously discussed example parameters that include, but are not limited to, physiological and behavioral stressors, pH, light, temperature, and diet. Dr. Gerlai concluded that breeding methods should be also published.

### Physical Factors in Zebrafish Facilities: What Varies and Should We Care?

*Christian Lawrence, M.S., Zebrafish Facility Manager, Division of Hematology and Oncology, Boston Children's Hospital*

Mr. Lawrence said that several physical factors in zebrafish facilities warrant discussion. Zebrafish that have subclinical infections display altered behavioral symptoms. The variability in zebrafish behavior can arise because of factors that are not immediately evident to the researcher. He presented examples of variations of physical factors that affect zebrafish experiments. Extrinsic physical factors include light, temperature, and sound. To illustrate light variability, Mr. Lawrence presented light values of tanks from the Aquaneering and the Tecniplast™ at the facilities at Boston Children's Hospital. Tanks were positioned at high, medium, or low heights on the racks, and values were collected at the front and back of each tank. There were quantifiable differences in light values in the front versus the back of each rack; the brightest light was observed at the highest rack. Light variability at such levels is expected to affect the zebrafish. Regarding the temperature, the points of variation occurs with the incubators, fish rooms, and tanks. One must be aware that discrepancies often arise between set temperatures versus actual temperatures, and the laboratory bench versus the system's temperature. Vibration is another physical variable of importance here. Variability in temperature, sound, and light is easily measurable using widely available devices. He recommended that facilities normalize values of environmental parameters across their infrastructure. This could be accomplished using a sticker at each facility displaying the standardized parameters of each room, system, and water, as well as behavioral, nutritional, and pathogenic profiles. This process is helpful for identifying "gaps" in measured values.

**Physical Environmental Parameters Affecting Experiments: Neural/Behavioral Studies as an Example**
*Mary Halloran, Ph.D., Professor, Departments of Zoology and Neuroscience, University of Wisconsin, Madison*

Dr. Mary Halloran described the effects of environmental stimuli on neurological development, function, and behavior of zebrafish. Behavior is affected by experimental conditions and husbandry. The environmental parameters that should be reported are temperature, water chemistry, light levels during testing experiments, the description of testing arena (e.g., dimensions of chamber), fish handling methods, and a description of stimuli. She suggested that reporting should be via manuscripts and archived in a model organism database. Understanding experimental parameters related to live imaging (which is a common technique for zebrafish research,) is important. Outcomes are affected by the temperature of the imaging chamber, the methods of anesthesia, mounting or immobilization, and any noise or vibration. For behavioral studies especially, baseline reporting is important. The type of tank system, flow rate, density of fish, and the type of apparatus used for rearing embryos and larvae all affect animal health and, thus, the experimental results. The type of incubators (e.g., an incubator with a light cycle or a dark incubator) used to raise zebrafish is underreported and can affect circadian rhythm and visual development.

**Establishment and Maintenance of Genetic Homeostasis: Light Effects on Zebrafish Gene Expression**
*Ronald Walter, Ph.D., Chairperson, Department of Chemistry and Biochemistry, Texas State University*

Dr. Walter presented an overview of studies conducted at Texas State University showing the effect of light on zebrafish gene expression and overall genetic homeostasis. The effects of the type and intensity of light on zebrafish are largely ignored by the community. Light source wavebands alter selectively genetic states in vertebrate animals. Fluorescent light sources and waveband exposures of 10 kilojoule/meter squared alter the expression of genes found in the skin, liver, and intestine of platyfish, medaka, and zebrafish species, as observed by RNA sequencing and quantitative real-time polymerase chain reaction (PCR). The most prominent alterations occurred in important signaling pathways—insulin-like growth factor 1, oncostatin M, and acute phase (inflammatory) signaling pathways. Also, the platyfish oppositely regulates cellular proliferation and immune responses compared to the medaka and zebrafish species. These data led to the hypothesis that genetic responses to light wavebands are conserved and deeply embedded in the vertebrate genetic regulatory repertoire. The downregulation of necrosis-related genes occurred upon exposure to a 300- to 350-nanometer (nm) waveband, compared with an increase of expression at 450 nm. Dr. Walter showed additional examples of waveband-dependent differential transcriptional regulation of gene clusters (epidermal growth factor) in other bodily areas (brain and liver). Each tissue preferentially modulated expression at different wavebands. These data demonstrate that different wavebands are used by the fish for modulating the expression of various genetic pathways. The implication of these findings is that gene expression can be manipulated experimentally by exposure to various wavebands in the laboratory. As light will differ between facilities, the type of waveband must be considered when conducting experiments.

**Discussion**

Dr. Ekker recommended that participants use a smartphone application for measuring light at facilities, as an effective and less costly alternative to other instruments. Drs. Walter and Ekker agreed that the effect of light measurements is underreported. Dr. Gerlai added that the use of a light cycle in incubators that house larvae has myriad effects on immunological and behavioral responses. Dr. Maurine Hobbs commented that the University of Utah fish facility uses bright lights, affecting behavior in its zebrafish and algae growth. She is unaware of what the optimal light range should be. Dr. Walter replied that the algae is not a problem and is a normal part of the zebrafish ecosystem; the type of light bulb is important to report.

Dr. Kalueff added that the size and color of the housing tank/racks affects experimental results. Dr. Walter responded that monitoring the genetic alterations is important for determining the normal light range. Dr. Gerlai added that layering different types of rack materials (e.g., wood and plastic) reduces vibration frequencies.

Dr. Brant Weinstein said that a researcher must consider the reasonability and practicality of recommendations for reporting; a cost-benefit analysis is needed.

## PLENARY SESSION—NUTRITION

*Moderator: Liliana Solnica-Krezel, Ph.D., Alan A. and Edith L. Wolff Distinguished Professor, Department of Developmental Biology, Washington University School of Medicine*

### Zebrafish Nutrition and Nutrition Research

*Stephen Watts, Ph.D., Professor, Department of Biology, The University of Alabama at Birmingham*

Dr. Stephen Watts stated that nutrition is a complex phenomenon about which much remains to be learned. Historically, nutrition was studied as early as 400 B.C. with an understanding that it is important for human health. By the early 1800s, it was recognized that energy is dependent largely on food consumption. Significant nutrition-related findings occurred by the 1940s (e.g., recommended daily allowance) and the 1960s (the NIH's National Health and Nutrition Examination Survey). Additional information can be found on myplate.gov (U.S. Department of Agriculture). In the laboratory, animal nutrition has been studied using animal models. The development of dietary standards for rodents addressed an observed lack of experimental reproducibility. The first major standardization of laboratory animal diets was the American Institute of Nutrition (AIN)-76A rodent chow. This diet was updated in 1994 (AIN-93 Growth and AIN-93 Maturation). The AIN-93 represents the current dietary standard for rodent research. Dr. Watts encouraged the workshop participants to review the National Animal Nutrition Program to understand the nutrient composition of diet for fish. Dr. Watts highlighted the negative effects of not standardizing nutrition for research animals. He alluded to a 30-year study conducted in Wisconsin comprising two experiments in which caloric restriction and aging were assessed in two groups of animals fed different diets. These experiments could not be compared because the diets altered the phenotype of the animals. The inability to compare experiments resulted in a major financial loss.

A "holy grail" zebrafish diet is needed to promote healthy growth, viability, egg production, and consistent quality. A purified standard reference diet with defined ingredients that will support all life stages of zebrafish also is required. Ultimately, it is important to add a practical chow diet that is necessary for basic colony maintenance. Determining which diet is appropriate will rely on several markers that include behavior, growth, and reproductive success. Moving forward, the zebrafish community must identify nutrition research as a priority, partner with the NIH, and obtain intellectual support from other animal nutrition specialists (e.g., American Society of Nutrition, National Animal

Nutrition Program). Researchers should consider how to feed transgenic animals, manage feed appropriately, increase education (knowing the difference between dietetics and nutrition), and try novel approaches.

**Variability in Adult Zebrafish Feeding Standard Operating Procedures (SOPs)**
*Maurine Hobbs, Ph.D., Director, Centralized Zebrafish Animal Resource, University of Utah*

Dr. Hobbs presented data from a survey across 17 different facilities describing the variability in zebrafish feeding SOPs. The survey covered various aspects of feeding, which included how often animals are fed and how much food is given. Most facilities feed zebrafish twice daily and provide options for users to deliver snacks to the fish. The frequency of feed is significant because it correlates with breeding success. Most respondents indicated that the amount fed is based on percent body weight. Other facilities feed according to the number of animals per tank, size, etc. Different facilities have established diverse average target weights for adult fish. Most of the users stated that larger fish do not breed well, therefore, target weight requirements were established. Dr. Hobbs discovered that the majority of respondents use dry feed, whereas the second most common method is dry feed mixed with water. Several facilities use more than one food type; the majority use two different feeds, mainly the Gemma Micro 300 (Skretting Zebrafish). To minimize the likelihood of under- or over-feeding, the University of Utah implemented a sentinel program for monitoring weight progression among the fish across animal rooms. Most facilities monitor the health and mating success as the goal is to maintain healthy reproducing fish. Based on the results of the survey, Dr. Hobbs recommended that when reporting on husbandry conditions investigators describe their feeding regime and the average target weight, age for breeding, etc. Also, it is important for users to specify variations of individual fish lines.

**Assessing Nutrition in Zebrafish**
*Lauren Pandolfo, M.S., Animal Aquatics Facilities Manager, Research Animal Management Branch, Division of Intramural Research,* Eunice Kennedy Shriver *National Institute of Child Health and Human Development (NICHD)*

Ms. Lauren Pandolfo described the complexity of assessing nutrition in zebrafish. Because of the vastness of NIH's animal facility and the diversity of research conducted, NIH staff, not individual investigators, are responsible for husbandry. Ms. Pandolfo outlined the general system conditions at NIH's facility, which has approximately 100,000 liters of water capacity and a high number of water changes. The typical conductivity value is high (1,000 µS/m). In an effort to improve husbandry practices and protocols, her staff considered developing a new feeding regimen and defined diet for juvenile zebrafish. These changes included the use of a feed gun to better control food amounts, rather than the traditional "squirt bottle" or pressure pump method. Also, instead of the flake commercial diet mixed with krill and *Artemia* (FKA), processed commercial diet with *Artemia* (PA) was used, and the rate of fish development and reproduction were monitored. The FKA diet increased the percent fertilization and viable embryos when compared to the PA approach. These findings support the need for further nutrition studies and funding. Use of *Artemia* presents several challenges. Because of *Artemia*'s limited commercial availability and high cost, alternative feed regimens should be explored. Possibilities include replacing *Artemia* with a rotifer regime, changing commercial brands, or replacing *Artemia* with Gemma Micro. Another challenge is that no dietary nutritional control exists for experiments because *Artemia* is grown in the wild. The current diet scheme at NIH's central zebrafish facility includes feeding with rotifers in green water culture at early larval stages, with flakes during the juvenile stage, and with

Gemma Micro during the adult stage.

**What Data Is or Should Be Collected?**
*Liliana Solnica-Krezel, Ph.D., Alan A. and Edith L. Wolff Distinguished Professor, Department of Developmental Biology, Washington University School of Medicine*

Dr. Solnica-Krezel presented on various aspects of the Washington University fish facility. The facility contains a nursery, purified RO-treated water, 7,000 tanks, and mechanical equipment separated from each other to minimize vibration; is staffed by four full-time employees; and is utilized by 23 users in 12 departments. Highlighting the importance of monitoring zebrafish feeding, Dr. Solnica-Krezel said that the type of feeding approach determines the rate of fish development and productivity. Feeding SOPs outline the feeding schedules for zebrafish at various developmental stages; rotifers are used for both young and adult fish. Multiple feedings with small amounts of food increased the time to fertility from 6 to 8 weeks of age. Reporting includes monitoring of sickness, productivity (e.g., genetic crossing success), and survival for wild-type and genetically altered fish. Another reported parameter is the purity status of the water. Dr. Solnica-Krezel concluded with a historical perspective of the type of feed for larvae and adult fish across facilities throughout her academic career. She mentioned that at her current institution, desired experimental results are observed when using rotifer live feed with algae and adult brine shrimp.

**Chromium-Contaminated Adult Zebrafish Diet Negatively Impacts the Viability of Larval Progeny**
*Mark Masino, Ph.D., Associate Professor, Department of Neuroscience, University of Minnesota*

Dr. Mark Masino presented data illustrating the negative effect of chromium contamination in non-hatchable, decapsulated brine shrimp feed for adult zebrafish. He recommended not using this type of food source because of the high levels of chromium. Dr. Masino described various effects of chromium contamination at his facility, including embryos' becoming orange-red in color, severe deformations resulting in low survival rates, perturbed posture, and light sensitivity. He speculated on the potential sources of chromium—carotenoids, pathogens, water, or food. Testing revealed that chromium and other heavy metal levels in the decapsulated food source were elevated compared to other types of feed. The chromium levels were higher in embryos from clutches with orange-colored yolks. These data suggested that the chromium levels in the food were responsible for the observed symptoms.

Because of the chromium contamination, decapsulated food was replaced with commercially produced pellet food (Gemma Micro); the tank lids and floors were cleaned. The University of Minnesota Department of Health and Safety was contacted. As predicted, the orange color of the yolks and morphological abnormalities diminished after these measures were implemented; however, clutches of embryos with orange-colored yolks, developmental delays, and behavioral defects occasionally were found in spawning tanks up to 10 months after the diet change. These results suggest that chromium accumulated in the ova of adult females fed the contaminated food, and the effects of the heavy metal contamination could persist for several months following its elimination from the diet regimen. Dr. Masino stressed the importance of performing quality control of food.

**Discussion**

Dr. Gerlai asked Dr. Masino if he plans to perform a follow-up dose-response analysis on chromium effects. Dr. Masino replied that the risk to the facility is too great to perform such studies.

Dr. Halpern wondered whether the chromium is from the decapsulation process in brine shrimp. Dr. Hobbs replied that the chromium does come from the process of decapsulation. Dr. Masino added that the decapsulation process is not controlled by vendors.

Clarifying Dr. Watts' earlier comment, Dr. Cory Brayton said that the AIN-93 diet is a maintenance diet and is not grain-based; only a minority of rodent research facilities use this feed type.

Dr. Hobbs said that in response to her institution's discovery of chromium, 8-week diet studies were performed using a single food source. Fish became ill after changing to a particular type of flake diet (TetraMin). Her facility has adopted a protocol to perform a 2-month food study each time a new type of food source is introduced into the facility.

Dr. Kalueff wondered about the usefulness of feeding zebrafish fish-based food, which may elevate pheromone hormone stress levels. Ms. Pandolfo described her facility's experience of discovering metal shavings in the *Artemia* feed. She added that that the NIH regularly tests for heavy metals in new feed batches, but this may be cost prohibitive at other institutions. She added that the goal is to determine how to periodically evaluate the actions facilities are taking to make healthy choices while avoiding perturbing the progress of research. Dissemination of information across institutions and partnering with vendors is significant. Ms. Priscilla Shirley remarked that having facilities provide feedback to vendors is important for addressing the issue of contamination. Dr. Solnica-Krezel added that it should not be the responsibility of the user to test for heavy metals in food.

## PLENARY SESSION—HEALTH MONITORING AND BIOSAFETY

*Moderator: Susan Farmer, D.V.M., Ph.D., Co-Director, Zebrafish Research Facility, Assistant Director, Animal Resources Program, The University of Alabama at Birmingham*

**Biosecurity, Importation, and Exportation**

*Katrina Murray, D.V.M., Ph.D., Veterinarian, Zebrafish International Resource Center (ZIRC), University of Oregon*

Dr. Katrina Murray detailed aspects of biosecurity, importation, and exportation of zebrafish. ZIRC's mission is to acquire and distribute mutant, transgenic, and wild-type fish lines. She highlighted the large biosecurity risk of importation of pathogens into facilities. The large number of lines available for importation at ZIRC increases the risk of asymptomatic infections, pathogens surviving cryopreservation, and contamination of sperm samples. Because of these various risks, Dr. Murray described how ZIRC analyzed its importation strategy, which included quarantine of submitted adults and cryopreservation. ZIRC has implemented three approaches regarding importation of fish: requesting the health history of imported adult male zebrafish, requiring that all mutant and transgenic lines undergo cryopreservation of sperm, and minimizing of the number of live lines at the facility. For wild-type lines, the quarantine room is emptied and decontaminated before importation of 20 or more adult pairs. Obtaining cryopreserved sperm is the best opportunity to knock back pathogens, and the importation requires a health history and fixed moribund or pre-filtration sentinel fish for histopathology. Assignment of a health status requires a diagnostic evaluation process for live males, live wild-type pairs, and cryopreserved sperm. A three-tiered level of risk exists—none, low-risk, or high-risk pathogens.

Dr. Murray mentioned that the exportation process involves an online animal health report accessible to users that describes the water system, diagnostic, and sentinel results. ZIRC recommends the strict use of quarantine practices for all imported fish.

**Health Monitoring and Diagnostic Testing in Zebrafish Facilities**
*Susan Farmer, D.V.M., Ph.D., Co-Director, Zebrafish Research Facility, Assistant Director, Animal Resources Program, University of Alabama at Birmingham*

Dr. Susan Farmer presented the importance of health monitoring for protecting zebrafish health and preventing loss of unique animal models. Diseases found in fish can be acute or chronic and, in many cases, subclinical. The immune responses (e.g., inflammation) can alter experimental results. The most common pathogen types are viral, fungal, bacterial, and parasitic, with the most common threats to zebrafish research being microsporidiosis and capillariasis, as well as mycobacteria and myxozoan species. Many of these pathogens are resistant to common disinfection techniques and their presence of is often detected through necropsy, PCR, histology, and bacteriology-based methods. PCR be performed on the whole animal or on individual organs or tissues can provide specific and accurate pathogen identification. Histological approaches include staining techniques using hematoxylin and eosin or gram stain. Other testing methods include bacterial cultures (in select media) or matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. Little is known about viruses in zebrafish; however, importation of spring viremia of carp (caused by a rhabdovirus) is a concern in several countries.

Important aspects of health surveillance in a zebrafish facility include the frequency of testing, facility size, sources of pathogens, and types of research being conducted. Reporting these factors in connection to the types of research being conducted is important. Also important is the proper documentation on the trends in spawning and larviculture t. Dr. Farmer mentioned several online pathogens testing resources, including Charles River Laboratories and IDEXX BioResearch. She added that the type of information that should be shared with other investigators/publications is a general description of the surveillance programs and testing parameters (which agents, methods, sample size).

**Finding Meaning in Zebrafish Sick Reports**
*Joseph Schech, D.V.M., DACLAM, Animal Program Director, Research Animal Management Branch, Division of Intramural Research, NICHD, NIH*

Dr. Joseph Schech presented on the types of morbidity ("sick") reports for zebrafish. Sick or mortality animal reports typically include numbers and trends that indicate system health or growing problems. These reports may be compiled into a database, which allows tracking facility trends over time. The reports for individual zebrafish are not useful in monitoring the entire system's health. Establishing and disseminating to investigators baseline morbidity/morality rates helps them identify problems with animal systems. For example, an investigator can determine the abnormal/normal ranges of dead versus sick fish. Reporting the variability between facilities or systems provides a method for evaluating and sharing husbandry methods between facilities and investigators.

Dr. Schech illustrated NICHD's zebrafish health monitoring or "stop light" system, which incorporates general appearance, activity, body morphology, bone formation, and overall condition for helping investigators evaluate their fish. Visual assessment techniques based on head and body sizes are performed for evaluations. Based on these assessments, feeding and housing methods can be adjusted. Dr. Schech concluded by presenting data that showed percent mortality between various systems increasing over time, demonstrating the need for assessing the health of animals housed within each system.

**Physical Appearance and Lesions: Identifying Sick Zebrafish**

*Heather Shive, D.V.M., Ph.D., Diplomate of the American College of Veterinary Pathologists (DACVP)**,** Assistant Professor**,** Department of Population and Pathology**,** North Carolina State University*

Dr. Heather Shive described the methods of identifying sick zebrafish, including assessing their physical appearance antemortem and postmortem. Fish require regular inspection to prevent the spread of illnesses. She explained the basic process for performing an antemortem examination—the physical appearance of the skin and gills may present with ulceration, hyperemia or discoloration, and the body may show mass lesions or changes in body conformation. Behavior is another aspect of antemortem evaluation; swimming behaviors, activity level, interest, and food are common metrics that require monitoring. Regarding postmortem evaluation, an investigator should consider histological examination of any zebrafish with abnormalities identified by antemortem evaluation. She suggested performing gross or stereomicroscopic dissection prior to fixation. Formaldehyde-based fixation is important for optimal identification of disease processes, whereas decalcification is required for any bone tissues prior to processing and preparation of histological sections. One must use the appropriate ratio of fixative to tissue. Because of the small size of zebrafish, the preparation of tissues for sectioning must be precise. Histological assessment must be done by an experienced evaluator, such as an animal pathologist. Dr. Shive emphasized the importance of incorporating ancillary testing, such as PCR and microbial culture, when possible.

Dr. Shive reviewed several diseases, their associated lesions, and how to identify them histologically. Mycobacterial species and *Edwardsiella ictaluri* are identifiable using specialized staining of granulomas or granulomatous inflammation. Certain cofounding factors that may affect histological identification include the discovery of dead fish, inadequate sampling, or fixation. The appropriate protocols for tissue collection, processing, and histological assessment are essential for optimal analysis; however, ancillary testing may be necessary for achieving a definitive diagnosis.

**Impacts of Infectious Diseases: Should Pathogens Be Reported?**

*Christopher Whipps, Ph.D., Associate Professor, Center for Applied Microbiology, State University of New York College of Environmental Science and Forestry*

Dr. Christopher Whipps described bacterial and parasitic diseases of zebrafish and their reporting. He presented data showing that the most common zebrafish pathogens in facilities, reported by ZIRC, are *Pseudoloma* and *Mycobacterium.* However, in *Pseudoloma*-negative fish, the presence of mycobacteria is difficult to eliminate. This highlights the difficulty in removing mycobacteria from zebrafish and the importance of assessing the effect of this pathogen on fish. Pathogens that could most affect experiments involve those associated with tumors, lesions, and abnormal kidney and nervous system function. Such pathogens include *Pseudocapillaria tomentosa* and *Mycobacterium*. In some cases where a pathogen is discovered, there are no deleterious effects on the zebrafish. For example, *Mycobacterium* infection does not affect egg production. Understanding that there are unknown effects of pathogen presence and infections is important. For instance, the protozoa *Myxidium* is found commonly in zebrafish, but does not cause disease sequelae. The level of sensitivity to the presence of pathogens and their effects on experimental outcomes depend on the type of research being conducted. Investigators should consider whether the health status of fish should be evaluated before or after each experiment to maintain the uniform disease/health baseline. More research on these common disease agents is needed for defining the baseline information that would suggest a concern.

**Discussion**

Dr. Ekker wondered how many microbial species discovered in zebrafish are pathogenic versus non-pathogenic (normally found). It is unclear when facilities should be concerned with the presence of a bacterium or parasite. Dr. Whipps replied that *Mycobacterium chelonae*, which is commonly found in zebrafish, causes disease only under certain circumstances. Conversely, *M. haemophilum* is pathogenic and will cause mortality.

Dr. Michael Kent reiterated Dr. Whipps' comment that infection with *M. chelonae* is asymptomatic, but does cause multiple granulomas. Dr. Kent said that these infected fish have elevated levels of cytokines, which will affect immunological studies.

A participant noted that a growing number of facilities are moving toward centralized housing systems. Such arrangements may affect experiments belonging to many investigators. Dr. Marcus Crim said that there is a trade-off between biosecurity versus operating efficiency. Greater redundancy in the system allows greater flexibility when addressing a suspected pathogenic infection; recirculation of air and water in a centralized system may support pathogen persistence.

Dr. Gerlai asked what is known about the bacterial flora in zebrafish. Dr. Murray replied that Washington State University and the University of Oregon are conducting research to identify the composition of this flora. She cautioned against unnecessary sanitizing, which may disrupt the normal flora. Dr. Kalueff asserted that the presence of high amounts of algae in zebrafish facilities may affect health.

Dr. Yuk Fai Leung said that measuring genetic variations is important for health monitoring and biosecurity.

## PLENARY SESSION—WELFARE AND BEHAVIOR

*Moderator: Michael Kent, Ph.D., Professor, Department of Microbiology, Oregon State University*

### Behavioral Assays: Reproducibility and Rigor

*Marnie Halpern, Ph.D., Staff Member, Department of Embryology, Carnegie Institution for Science*

Dr. Halpern said that behavior is an important aspect of reporting. Various types of behavior (e.g., locomotor activity, swimming, learning) have been examined in zebrafish for several decades. The current scientific trend is towards understanding the underlying neural circuitry that mediates behavior.

She indicated that behavior is affected by many factors, such as age, sex, water quality, and light. She presented unexpected results from her laboratory demonstrating that light exposure affects gene expression and thus behavior in zebrafish. Selecting the appropriate genetic background is important for rigor and reproducibility.

Additional factors that may influence behavioral results and are important for reporting are the numbers of experimental subjects and trials, transgene expression, image analysis, and immobilization of the zebrafish during *in vivo* imaging. Certain opportunities and challenges exist when assessing zebrafish behavior. Permitting repeatability of conditions across experiments and laboratories makes comparisons between experiments feasible and provides statistical power for relating behavior to neural function. A negative aspect of this approach is that the brain has evolved to generate behavior in complex natural environments, which may not be captured under certain conditions. An experimental system where we can study naturalistic behaviors combined with a tight control of experimental parameters and comprehensive measurements of behavioral output and physiology is ideal. Investigators must consider the artificiality of laboratory conditions when studying zebrafish.

**Reproductive Performance**

*Brant Weinstein, Ph.D., Senior Investigator, Section on Vertebrate Organogenesis, Division of Developmental Biology, NICHD*

Dr. Weinstein expressed that reproductive performance is possibly the most important husbandry parameter for zebrafish. Reproductive performance is widely measured in all laboratories. Reproduction is critical because stressed fish do not spawn; therefore, reproductive performance can be used as a proxy for husbandry conditions and animal welfare. Reproductive performance is influenced by myriad factors—water quality, diet, light, etc.—and is measured by the rates of fertilization and spawning success. Unfortunately, this performance is highly variable within and across facilities. Dr. Weinstein pointed out data showing that reproductive performance is not greatly influenced by stocking density (i.e., the number of fish housed per liter). He recommended that reproductive performance should be assessed across multiple facilities. The current assessment of the effects of various husbandry parameters may not be correct and rigorously controlled studies should be undertaken to test such effects. Genetics likely has a great influence on reproductive performance. Dr. Weinstein noted the need for funding multicenter studies on the effects of husbandry parameters and develop healthy fecund "real" inbred strains for wide use.

**In Search of Parameters Affecting Variation and Reproducibility When Using Zebrafish to Model Disease**

*Brian Link, Ph.D., Professor, Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin*

Dr. Brian Link presented parameters affecting variation and reproducibility of experimental results in zebrafish. He discussed two important goals in zebrafish research: (1) to identify sources of variation and reproducibility and (2) to provide best practices guidance on experimental design, analysis, and reporting procedures. Examples of source variation include non-controlled genetic background heterogeneity, underpowered sample sizes, and undefined qualitative assessment. Certain sources are not as apparent, but still require more research; these include variations in aquatic composition, feeding parameters, and rearing density. Stress induces a range of genetic changes affecting phenotypic results.

Highlighting the environment's role in affecting phenotype, Dr. Link discussed various studies demonstrating the effects of temperature-dependent genetic changes on phenotypes. Temperature-induced mutations in the gene *puma* changed the pigment pattern of skin and the growth rates of zebrafish. This phenotype plasticity also is observed in studies that measure sex as a variable affecting the formation of the zebrafish jaw. He alluded to previous findings that heat-shock protein 90 is a capacitor of phenotypic variation. Reducing function of this protein produces an array of morphological phenotypes. Manipulating this protein offers a tool for harnessing unknown genetic variations and for elucidating the interplay between genotypes and environments in the determination of phenotypes. Phenotypes can be selected against (or for) using genetic manipulations. Direct experimentation is needed to rigorously identify key reportable variables that affect reproducibility.

**Effects of Chronic Infections on Behavior Endpoints**
*Michael Kent, Ph.D., Professor, Department of Microbiology, Oregon State University*

Dr. Kent described the effects of chronic infections on behavioral endpoints. He alluded to data from his laboratory demonstrating the effect of subclinical disease in zebrafish. Infection with *Pseudoloma neurophilia* is asymptomatic and extremely common in zebrafish. Pathologically, the parasite infects the central nervous system tissue and causes diffuse chronic myositis. Dr. Kent referred to studies led by veterinary pathologist Dr. Sean Spagnoli (Oregon State University) showing that this parasite clusters in the ventral white/gray matter and the nerve roots. *P. neurophilia*-infected fish had a lowered rate of habituation to a startle response than control fish. Also, infected fish avoided net capture and maintained reduced mean inter-fish distances, demonstrating that they have elevated escape responses and higher rates of shoaling. These findings raise the question of whether studies should be conducted in zebrafish without first verifying the absence of brain parasites. Dr. Kent and investigators from Oregon State University and Champalimaud Centre for the Unknown (Lisbon, Portugal) are researching the effects of *Pseudoloma* infection on shoaling behavior. From this collaborative effort, it also was demonstrated that sub-clinically infected fish have reduced inter-fish distance compared with those that have clinical symptoms.

**Behavior: Accepting and Implementing Reporting Procedures**
*Rebecca Van Beneden, Ph.D., Professor, Department of Molecular and Biomedical Sciences, University of Maine*

Dr. Rebecca Van Beneden reiterated the importance of studying and reporting behavior in zebrafish. Recommendations to promote good practices in research are linked closely to research reports. These reports support the need for adhering to guidelines for planning animal research and testing, as well as for improving methods of research reporting. The two guidelines are Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE). PREPARE includes guidance for formulating studies, dialoguing with scientists and the animal facility staff, and quality control. The ARRIVE reporting guidelines include a description of the introduction, methods, results, and discussion of experiments.

Systematically assessing the effects of housing on the stress response in zebrafish is important. Knowing the housing conditions that fish prefer is crucial for experiments. Preference is measurable using tank diving assays in which the anxiety response to novel environments is assessed. In one diving assay

(Parker et al., 2012), zebrafish introduced to a novel tank exhibited freezing movement and erratic swimming patters that correlated with anxiety and stress reactivity. Also important is understanding the suitable number of animals, behavioral preferences (solitary versus schooling), stock density, and sex ratios. In some cases, greater stocking density leads to increased aggression in adults and imposes stress in larvae or embryos. Dr. Van Beneden mentioned that behavior also may depend on sex differences.

The lack of reporting of such details as housing conditions, stocking density, and male-to-female ratio contributes to also omitting their effects on behavior and an overall behavioral assessment. Because of the rapidity of the developmental process, the window of opportunity to measure many of these relevant variables of interest is short. Proper reporting guidelines should involve defining the responsibility of the animal facility versus that of the researcher, understanding the requirements of funding agencies, and stricter enforcement of reporting guidelines by publishers and reviewers.

**Discussion**

Dr. Farmer wondered if infected zebrafish release alarm substances from their body. Dr. Gerlai replied that it is unclear whether these substances are released, but they can be located in a necropsied body or observed as bruising of the body. Dr. Kalueff added that stress can be communicated in a variety of ways, such as secretion of cortisol in urine; fish may have a stress response analogous to that in rodents (pheromones). Dr. Kalueff said that shoaling behavior is difficult to interpret; proper interpretation requires multiple confirmatory experiments. Dr. Gerlai said that many zebrafish communication methods are not chemical based; infection in a single zebrafish can be sensed non-chemically by neighboring fish. A participant referred to the occurrence of mycobacteriosis infection in his facility; the behavior of non-infected zebrafish alerted investigators to the presence of a pathogen in the system.

Dr. Gerlai remarked that non-behavioral scientists who study behavior deem that behavioral analysis in fish is unreliable. Behavioral analysis permits researchers to detect what other forms of phenotypic testing would be missed. It is important to discuss how to study behavior because it is an excellent discovery tool.

In support of Dr. Weinstein’s earlier comment, Dr. Dustin Graham stated that clearly defining experimental versus observational units is important.

Dr. Halpern asserted that the experimental variability when using transgenic models to study zebrafish behavior is underreported. Researchers must know the expression level of transgenes in experiments.

## PLENARY SESSION—COMMERCIAL EQUIPMENT AND DATA FORMATS

*Moderator: Stephen Ekker, Ph.D., Professor, Department of Biochemistry and Molecular Biology, Mayo Clinic Cancer Center*

**Design for Success: “Smart” Data Collection and Analysis**
*Mark Francis, President, Aquaneering, Inc.*

Mr. Mark Francis presented on technologically advanced or “smart” methods of data collection and analysis applicable to zebrafish research. Mr. Francis reviewed a few of the current smart advances, including real-time data collection using smartphones, novel transmitters that provide graphical representation of data trends, and devices that measure parameters of lumen/light cycles and vibration levels. In the future, artificial intelligence will be used to warn researchers of water quality trends. Parameters that will be easily measurable using future technology are fish size, density, sex, feed portion, and fish growth. Mr. Francis noted that several laboratories have requested chlorine measuring in their water systems as a result of the water crisis in Flint, Michigan. Chlorine measuring is difficult and requires pH and oxidation-reduction potential electrodes; however, new transmitters have the capacity for this type of measuring.

The results of a survey of 27 research articles published in the journal *Zebrafish* indicate an increasing interest in measuring light cycles, water temperature, feeding schedule, diet, fish type, and sex. Device manufacturers can work toward supporting the community's testing needs by providing the technology that makes data collection possible.

**Aquatic Systems: Technical Feasibility and Practicality**
*Massimo Ferrari, Aquatic Product Specialist, Tecniplast USA, Inc.*

Mr. Massimo Ferari discussed the feasibility and practicality of implementing changes (e.g., new standards and technologies) to aquatic systems. He mentioned that the point of his talk is to establish an open discussion between Tecniplast™ and zebrafish investigators to determine what technology is most suitable for implementation. Mr. Ferrari reviewed the various causes of failure in zebrafish systems and recommended approaches for monitoring system parameters. Polyvinyl chloride (PVC) ultraviolet light sterilizers often break, causing leakage of PVC material into the water and catastrophic failure. Stainless steel or titanium housing UV sterilizers are a viable replacement option for PVC because of the savings in labor and increased safety for housed fish. Mr. Ferrari highlighted the importance of monitoring and controlling system water parameters (e.g., pH, conductivity, temperature) with alarming and system shutdown features. Because some systems are prone to leaks and do not have working floor drains, water sensors should be installed to shut the system down and alarm when water is detected on the floors. Another underestimated parameter is total dissolved gas (nitrogen) pressure rate, which has caused major fish kills. A single probe used for monitoring parameters is insufficient because of false readings, lack of calibrations, and possible computer failures. The development of smarter probes will presumably address these limitations.

Installation of an uninterrupted power supply system is a suitable option for resolving the power failure issues seen with the use of programmable logic controllers (PLC). Safety is the most important aspect of water system monitoring; all components should comply with local standards and laws. Underwriters Laboratories is a company approved to perform safety testing. Component certification of meeting minimum equipment requirements also is necessary. Additional significant monitoring parameters include sentinel systems and food intake or diet regimen.

**Aquatic Feeds in the Research Industry**
*Priscilla Shirley, Sales Specialist, Pet and Lab Feeds, Zeigler Bros., Inc.*

Ms. Shirley outlined the various aquatic feeds used in the research industry. She highlighted Ziegler's core capacity of providing aquatic feed to researchers and mentioned several of the company's milestones. She outlined the various aspects needed for good quality aquatic feeds. Good nutrition relies on feed formulation and manufacture, as well as on appropriate feeding methods. In addition to the variety of factors that are required for manufacturing aquatic feeds (e.g., desired ingredients and palatability), a suitable quality assurance (QA) program is necessary. QA is built into every step of the process including understanding customer specifications, analyzing the ingredients, producing the feed, analyzing the product, and collecting customer feedback. Ingredient specifications include more than 1,500 different ingredients that are monitored for several characteristics.

Ziegler sells standardized aquatic diets that have fixed formulations and are irradiated. Ziegler currently supports "open" formula diets allowing scientists to control nutrient and contaminant concentrations that are suitable for experiments. This type of manufactured specialized aquatic diet is managed by Zeigler's research and development technical center. Zeigler adheres to the 2011 National Research Council's Nutritional Requirements of Fish and Shrimp Guidelines for aquatic feeds.

Ms. Shirley presented on precise feeding methods; the difficulty in feeding zebrafish is caused by the volume of fish in any given facility. Precision feeding, which provides each animal with exact quantities and nutrition, is crucial for obtaining consistent results. She solicited participants for their feedback and invited them to visit the Zeigler facility.

**Creating a Health Management Program for Aquatic Establishments**

*Nick King, M.S., Operations Director, Fish Vet Group, Inc.*

Mr. Nick King described how to create a health management program for aquatic facilities that have a recirculation aquaculture system (RAS) for zebrafish housing. The benefits of RAS are consistency, reduced pathogen exposure, and the ability to raise fish anywhere in the world with water access, irrespective of climate or geography. Recommended components of a successful facility health management program (FHMP) are sourcing of seed stock, quarantine, biosecurity, cleaning and disinfection, pathogen screening and surveillance, intervention thresholds, and management practices and staff training. An FHMP reduces the risk of catastrophic loss due to diseases, provides an occupational health program, and ensures evidence-based decisions regarding the transfer of fish between laboratories.

Pathogen screening and surveillance monitoring are used to establish periodic baselines for facilities; no facility is ever pathogen free during operation. Disease surveillance (targeted investigation for a specific pathogen) and disease screening (casting a wide net to survey the population for underlying disease) are two common methods for pathogen screening and surveillance. The Fish Vet Group uses a signature health inspection method incorporating bacteriology-, virology-, parasitology-, and histopathology-based assays. Also, important for fish screening is determining the statistically significant number of samples to test; determination should adhere to the guidelines from the American Fisheries Society and the World Organization for Animal Health. Determining what constitutes a bio-secure unit requires knowing whether water or equipment is shared between holding units. Mr. King concluded by summarizing example results of a fish health inspection conducted by the Fish Vet Group.

**Health Monitoring and Health Status Reporting for Zebrafish Colonies**
*Marcus Crim, D.V.M., M.B.A., M.S., Head of Microbiology Labs and Aquatic Diagnostics, IDEXX BioResearch*

Dr. Crim described the objectives for monitoring zebrafish health. These include, but are not limited to, improving zebrafish health and husbandry practices, ensuring the validity of experimental data, determining appropriate measures when introducing new animals into a colony, and preventing the spread of pathogens within or between facilities. Protecting human health is also an important objective; cases of *Mycobacterium marinum* infection in zebrafish handlers have been reported.

To illustrate the importance of health monitoring, Dr. Crim described the evolution of health monitoring in rodent laboratories. Previously, diagnostics were based on observed clinical signs ("sick" era) or the presence or absence of death ("dead" era). The currently adopted "positive" era employs the use of approved vendor lists, comprehensive health surveillance, and systematic diagnostic examination. The approach for zebrafish health monitoring is changing globally. Maintaining zebrafish health involves monitoring a spectrum of diseases, which requires a risk assessment in each facility. Using the method of exclusion (using animals from selected sources, such as ZIRC) will minimize the likelihood of pathogen introduction into a facility or a catastrophic event. If exclusion is not possible, having more than one quarantine protocol is useful. Biosecurity and operating efficiency is a tradeoff, which is illustrated by the use of rack-level biocontainment (stand-alone racks) that provides increased flexibility versus system-level biocontainment (modular system with central filtration), which may be more efficient. Monitoring should be a comprehensive system of multiple diagnostic platforms (e.g., molecular tests, microbial culture, simple direct examinations, and histopathology). IDEXX provides a range of health monitoring at both the facility level and the level of the individual zebrafish. By request, investigators can obtain exportable custom health reports from IDEXX.

**Efficient and Effective Data Collection to Support and Connect the Zebrafish Research Community**
*David Weintraub, President, Fulcrum Automation and Control Technologies, Inc.*

Mr. David Weintraub described the efforts of Fulcrum Automation and Control Technologies to provide efficient and effective data collection method systems to the zebrafish research community. The company discovered that environmental data are affected by facility processes, which in turn affect the research conducted and published by the investigator. Mr. Weintraub discussed the amount of data that can be collected and used to help investigators' experiments. An important factor when determining this amount is the cost-benefit ratio.

A necessity for most zebrafish scientists is efficient data collection in facility, veterinary, and investigative processes. Efficient data collection is defined as information gathering without interruption of workflow while using multiple parameters. Inefficient automated processes of data collection occur, for example, as a result of having multiple water quality systems from various manufactures. Automation in a more uniform and efficient manner would involve combining a facility's different water quality systems into one. This one system has a common communication protocol supporting data access using higher-level computers.

**Discussion**

Dr. Ekker commented on the great talks covering multiple topics that he believed encouraged investigator partnerships. He wondered about the technical challenge of installing a cost-effective probe into every system. Mr. Francis replied that the electrodes are complicated and delicate and have two different pressure systems. Dr. Ekker said installing probes is an aspirational goal, but an absolute need for facilities.

Dr. Halpern mentioned that incorporating technological advances discussed during the workshop into fish facilities may be cost prohibitive. Dr. Ekker replied that commercial partners may assist with creating accessibility to these technologies. Dr. Halpern added that the price of zebrafish feed from certain vendors also may be cost prohibitive, thus limiting food choices. Dr. Solnica-Krezel remarked that the introduction of artificial intelligence technology will require additional layers of human inspection.

Dr. Solnica-Krezel asked the companies providing pathology services about their experimental controls for false positives. Dr. Crim replied that at IDEXX all positive samples are run twice in a high-throughput real-time PCR assay prior to reporting to the client. Mr. King added that at the Fish Vet Group all positive samples are rechecked.

A participant commented on the importance of addressing zebrafish nomenclature, which should be included in the workshop’s ongoing discussion.

Ms. Pandolfo asked what additional information vendors can provide to facilities. It is important for investigators to know what they are receiving. Dr. Crim replied that different types of health information can be requested; investigators should clearly define their questions for vendors.

Dr. Hobbs remarked on the need for a more reliable method of feeding that can accommodate food dosing, under various physical limitations of the facility staff such as small hands. Dr. Halpern mentioned that her laboratory is using a modified version of Dr. Ekker’s feeding method; the difficulty is not with performing manual dispensation, but with the food that gets clogged in the system. Mr. Rory Francis said that his company, Danio Lab, has feeders that accommodate small hands. Dr. Halpern encouraged the companies attending the workshop to address the challenge of food clogging when using hand-held feeders. Dr. Kalueff asked how the color of the instruments in zebrafish facilities is selected; the color (among other variables) should be researcher driven, not determined by the companies. Mr. Francis agreed with the need for more input, but said that it is helpful to receive feedback from investigators who are willing to try new products.

Dr. Hucka said that open-source solutions can address the issue of the high cost of novel software. The development of software can be incentivized by the NIH. Regarding monitoring systems, he cautioned against the use of artificial intelligence/machine learning systems that introduce errors into machine learning algorithms when analyzing very complex parameters.

Ms. Pandolfo agreed with Ms. Shirley’s earlier comment that customer feedback on what researchers need is important.

In an effort to reduce feed costs in her laboratory, Dr. Hobbs had reviewed studies that compared Zeigler's adult diet and Gemma Micro; these studies demonstrated the same growth rates in zebrafish. She recommended that other laboratories perform similar comparisons. Ms. Shirley indicated that there is a reasonable cost associated with buying feed, irrespective of the type of formulation. Zeigler recently changed one of its feed formulations to reduce system clogging.

## CONCLUSIONS

### Questions and Issues for the Breakout Groups, Part I

Dr. Ekker explained that day 2 of the workshop will focus on "packaging the group's wisdom" to establish a starting point of conversation about the workshop conclusions. He recommended that the breakout sessions comprise two groups that can partner for discussion. Dr. Klosek described the agenda for day 2; the topics of the two plenary morning sessions will be (1) technical aspects and feasibility of data collection and sharing and (2) how journal editors and scientific societies can assist zebrafish researchers in disseminating data and provide input regarding specific recommendations.

Dr. Klosek reiterated her previous remark that ORIP's goal is neither to impose regulations on facilities regarding raising fish nor to support efforts toward standardization of husbandry practices. What is needed is for researchers to share their methods (data) of fish rearing. She noted that discussion will include more than two breakout groups; she recommended that participants formulate questions in advance.

## TUESDAY, SEPTEMBER 12, 2017

## PLENARY SESSION—DATA COLLECTION AND SHARING

*Moderator: Michael Hucka, Ph.D., Staff Scientist, Department of Computing and Mathematical Sciences, California Institute of Technology*

Dr. Hucka commented that the objectives of the plenary sessions are to determine how participants are storing and capturing data and to develop minimal information guidelines for data collection and sharing.

### Current Status of Guidelines for Minimal Information Reporting

*Cory Brayton, D.V.M., DACLAM, DACVP, Associate Professor, Department of Molecular and Comparative Pathobiology, Johns Hopkins University*

Dr. Brayton presented on the current status of guidelines for minimum information reporting. She explained that guidelines that focus on animal welfare (i.e., ARRIVE) are converging with those that emphasize science-related issues. Minimum information reporting guidelines are emerging, such as for patient-derived xenograft animal models. She offered two examples of mouse databases using minimum reporting guidelines—the Mouse Phenome Database (MPD) and the International Mouse Phenotyping Consortium (IMPC). Within the IMPC is a large-scale international initiative to discover gene functions that support human health. IMPC has created and characterized 20,000 knockout mouse lines and associated protocols (baseline phenotyping); this information is publicly available online. The IMPC protocol, International Mouse Phenotyping Resource of Standardised Screens, captures the husbandry and environmental factors influencing experimental phenotypes in rodents. Other websites exist wherein investigators can retrieve information regarding genetically altered mouse welfare assessments from IMPC, for example, the phenotypic variation between sub-strains of C57/BL6 mice depending on diet, bedding, and sex. The guidelines for zebrafish nomenclature can be modeled on rodent designations. Dr. Brayton concluded by referencing journal articles that support the need for microbiome reporting in zebrafish.

**A Neuroscientist's Perspective on Tools for Data Collection and Sharing**
*Allan V. Kalueff, Ph.D., Director, ZENEREI Research Center, Professor, St. Petersburg University, Professor Southwest University, Chongqing, China*

Highlighting the globalization of zebrafish research, Dr. Kalueff mentioned that the International Zebrafish Neuroscience Research Consortium (ZNRC), of which he is the chairperson, has laboratory branches in China and Russia. ZNRC and the International Stress and Behavior Society (ISBS) established a taskforce recommending procedures for handling the effects of stressors on the behavior and physiology of zebrafish. This joint effort between ZNRC and ISBS aligns with the goals of the workshop. Dr. Kalueff discussed the unfortunate discontinuation of the previously developed phenotype-based zebrafish database. In addition to a zebrafish phenome database, the following are needed: a large data cloud computing system collecting phenotypic data, crowd-based information collection and open-access analyses, "smart" algorithms to extract new phenotypic patters, and conceptual innovation (e.g., recognizing new phenotypes). Dr. Kalueff described the importance of having better descriptions of phenotypes based on three principles—recognize, report, and reproduce. The goal of zebrafish research is to translate animal models into human disorders (and the reverse). Understanding the limitations in zebrafish models is important.

**Husbandry Data Collection and Environmental Data Monitoring at the Zebrafish International Resource Center**
*Zoltan M. Varga, Ph.D., Director, ZIRC, University of Oregon*

Dr. Zoltan Varga presented on husbandry data collection, and the environmental data monitoring and alert system at ZIRC. On the ZIRC website, the resources and inventory section is a tool intended to help investigators maintain nursery and adult lines or strains. The inventory contains information on the fish line type, genotype, fish tanks, and cryopreservation status. ZIRC's alarm method is the Supervisory Control and Data Acquisition (SCADA) system, which connects to smart devices. Typical system monitoring consists of temperature readers for freezers, water quality, pH, and conductivity. The data from these measurements are collected and illustrated via graphs viewable by investigators. Data collection can be manual, semi-automated, or automated.

Despite generous support from ORIP, a database cannot provide all needed functions. Scalable methodology for data collection is necessary and should be reported. An important remaining question is how to collect continuous data. Language/terms and units (e.g., volume, concentration, and environmental terms) need to be standardized, and reporting approaches between facilities need harmonization.

**Data Collection at the University of Oregon Zebrafish Facility**
*Timothy Mason, Director, Aquatic Animal Care Services, University of Oregon*

Mr. Timothy Mason presented the process of data collection at the University of Oregon's aquatic animal care facility. Highlighting the need for a centralized database committee, Mr. Mason said that data generated from experiments are collected via notebooks, on clipboards, on tanks, in SCADA or PLC, by email, via calendars, in spreadsheets, and in individual investigator desktop databases. The format of this data is often text, image, or video and is in a column or row format. The University of Oregon has a database committee creating databases using FileMaker Pro software.

The University of Oregon has a number of stakeholders of the database project. Primary stakeholders are investigators, research staff, veterinary and husbandry staff, facility administrators, and others closely associated with the facility. Secondary stakeholders are the institution's research office and those involved with material transfer agreements and IACUC. Tertiary stakeholders are research colleagues and funding agencies. Some general requirements for the University's database project include accessibility via a web browser with secure passwords and data that are extensible and sharable. The University decided to use an open-source system for database access, wherein data can be shared, and software licensure restrictions are avoided. Some of the trackable information in the database's Animal Colony Tracker includes personnel, animal genetics, and fish. The tracker enables users to create a data trail, full reports, and reference databases. A future goal for the tracker project is ensuring that it interfaces with the University's top-level database.

**A Few Words About Minimal Information Guidelines**
*Michael Hucka, Ph.D., Staff Scientist, Department of Computing and Mathematical Sciences, California Institute of Technology*

Dr. Hucka elaborated on his earlier remarks regarding the guidelines for minimal information. Minimal information guidelines are focused on a subset of information to be communicated and are an intersection of what people need, can obtain, and can afford to gather and report. A common guideline format is a checklist or text description providing what people want to know. Desirable criteria for these guidelines are unambiguity, specificity, and conciseness. He outlined critical ways for developing guidelines, for example, gathering a small set of people to generate a draft checklist. He suggested not worrying about file formats, but focusing on the content. The simplest and easiest format that each researcher community can use is most suitable. At a later stage of development, other software needs must be addressed (i.e., specify a formal data model and define how to store data in other formats).

**Data, Data Collection, Data Sharing**
*Yuk Fai Leung, Ph.D., Associate Professor, Department of Biological Sciences, Purdue University*

Dr. Leung described various data formats and data sharing methods. The goal of zebrafish research is to conduct rigorous experiments that produce reproducible results. Such objectives are dependent in part on genetics. Zebrafish genetics is associated with husbandry data. Dr. Leung reviewed ZeBase at Purdue University, an open-source zebrafish inventory database. The database allows multiple users to keep track of genetic-based information, survival, and breeding. It is an automated reporting system based on user-defined queries; the addition of husbandry and environmental data has been partially implemented. An overlooked factor when using data is compliance. This relates to minimum information standards, which is defined as a set of reporting guidelines for data derived by relevant methods in biological sciences. An example minimum information standard is Minimum Information About a Microarray Experiment (MIAME). MIAME has software tools and data exchange formats that assist scientists in producing MIAME-compliant descriptions for their experiments. Regarding guidelines for zebrafish, ARRIVE is intended to improve the reporting of animal research. Several funding bodies were consulted, and more than 300 journals have endorsed ARRIVE. Two years after implementation, journals are recommending the ARRIVE guidelines for pre-clinical animal studies. Major lessons learned from MIAME and ARRIVE initiatives include (1) realizing that compliance will become an issue in the scientific community, (2) having a standard form pre-populated with common parameters may enhance compliance, and (3) understanding the incentive or motivation for laboratories to comply is important.

**Discussion**

Dr. Ekker commented that several laboratories have attempted building zebrafish databases, but only 10 percent of initiated database projects are being used. He suggested following up with those 10 percent of databases to determine which are most helpful and which can assist with knowing the data types used when collecting zebrafish from the wild. Dr. Varga agreed with the lack of database usage and added that ZIRC is interested in scaling back its support of unused databases. A participant added that the steering committee must consider metrics, especially when evaluating the implementation of recommendations from the workshop.

Dr. Solnica-Krezel said that it is important to use the same units and nomenclature across all suggested approaches. It also is imperative to gather all existing information to guide development of novel software and other approaches. Dr. Hucka added that efforts to standardize nomenclature for the zebrafish community have been made in the past.

Dr. Kalueff commented on the survival of databases by saying that they can be useful but are not sustainable. Resources and motivation drive the successful use of a database.

Dr. Solnica-Krezel remarked that the NIH is supporting integration of several animal model databases into one unit. She hopes that this effort involves creating uniformity of nomenclature.

Dr. Gerlai pointed out that the neuroscience community has struggled with implementing a database and determining how to collect complex data coming from imaging and genetic studies. Databases must capture the number of breeding generations, because different laboratories may observe divergent phenotypes caused by genetic drift and housing conditions.

Ms. Pandolfo mentioned that most NIH researchers who already use several databases do not want to maintain additional databases, which are perceived as laborious, for compliance purposes. Dr. Ekker stressed that one database will not solve all of the needs for researchers. Dr. Varga added that identifying standards for implementation is a feasible effort for database sharing.

## PLENARY SESSION—DISSEMINATION QUESTIONS

*Moderator: Stephen Ekker, Ph.D., Professor, Department of Biochemistry and Molecular Biology, Mayo Clinic Cancer Center*

Dr. Ekker posed two key questions to consider during the remainder of the workshop: (1) How can participants engage the broader research community and devise a strategy of working together toward improving issues of reproducibility? and (2) How can researchers identify novel scientific areas? As the Editor-in-Chief for the journal *Zebrafish*, Dr. Ekker provides guidance for reviewers when assessing data quality and reproducibility.

### *PLOS ONE*'s Efforts to Promote Reproducibility in Science Publishing

*Gina Alvino, Ph.D., Senior Editor,* PLOS ONE

Dr. Gina Alvino described *PLOS ONE*'s efforts to promote reproducibility in scientific publishing. As the Senior Editor for this journal, her primary interest is in animal research reporting. Dr. Alvino introduced three areas that *PLOS ONE* has implemented for promoting reproducibility. The mission of the journal is to accelerate progress in science and medicine by leading transformation in research communication — "openness inspires innovation." The online journal provides immediate open-access publishing without restrictions, which supports reproducibility.

Publication criteria are based on experimental rigor, methodology, and proper ethical standards. The journal has continued its efforts toward enhancing reproducibility in methodological reporting. Authors are encouraged to report basic experimental details, such as adherence to internationally accepted standards and IACUC protocols. Recent efforts focus on developing specific author guidelines pertaining to studies involving death as an endpoint and methods of euthanasia.

Regarding additional reporting guidelines that enhance reproducibility, *PLOS ONE* has implemented community-based reporting guidelines — Transparent Reporting of Systematic Reviews and Meta-Analyses and ARRIVE. Data must be rigorously reported as defined by these standards. Additional measures enhancing reproducibility involve partnering with protcols.io, which is an open-access repository of science methods. Further highlighting its efforts, the *PLOS ONE*'s recent data policy dictates that authors must make all the data in their manuscripts fully available without restriction when at all possible. *PLOS* supports the collaborative development of community guidelines for zebrafish research.

### Comments from an Editor/EB/ERB Member and Reviewer

*Cory Brayton, D.V.M., DACLAM, DACVP, Associate Professor, Department of Molecular and Comparative Pathobiology, Johns Hopkins University*

Dr. Brayton, Co-Editor-in-Chief for the journal *Institute for Laboratory Animal Research*, alluded to a goal of the workshop—creating a white paper for publication in *Zebrafish*. Some questions for discussion include the journal's policies regarding page limits, rules about supplementary material, and formatting, as well as how the zebrafish community can engage societies and journals in disseminating data. She reiterated that the two mouse reporting databases that should be reviewed are IMPC and MPD.

**Comments from a Director of the Society for Developmental Biology**
*Ida Chow, Ph.D., Executive Officer, Society for Developmental Biology*

Dr. Ida Chow provided an overview of how the Society for Developmental Biology supports dissemination of information between the Society, community, and the journals. The Society holds several activities and disseminates information via regional meetings. Instructing trainees in proper data collection and analysis is crucial and influences scientific reproducibility. Dr. Chow encouraged the participants to improve their training methods. She highlighted important activities of the Society — grants for fish-related research conferences and BioEYES, a partnership to advance scientific education in young students. The Society's journal, *Developmental Biology*, is a way to help promote these activities. Creating a special issue of the Society's journal can support the dissemination of best practices. Publishing in this journal carry several advantages (e.g., no page limits). She concluded her talk by highlighting the journal and Society's websites.

**Zebrafish Husbandry Association: A Learning Community Focused on Advancing Standardization of Zebrafish Research**
*Dante D'India, A.L.M., President, Zebrafish Husbandry Association, Research Assistant, Harvard Medical School*

Mr. Dante D'India described the Zebrafish Husbandry Association (ZHA), a nonprofit group consisting of facility managers, technicians, and veterinarians who share the mission of promoting and developing zebrafish husbandry standards through education, collaboration, and publication. ZHA accomplishes this mission through educational and training programs that are available online for ZHA members. ZHA's website posts funding announcements, working groups' information and community surveys, publications and contact information, quarterly newsletters, and webinars. ZHA has active social media platforms on Facebook, Twitter, and LinkedIn, where users can receive immediate feedback from senior ZHA members. ZHA has approximately 305 members from 32 institutions; 20 vendors also are members. Most of ZHA's activities are made possible by vendor support.

**Comments from an Editor of *Lab Animal*, Nature Research**
*Dustin Graham, Ph.D., Chief Editor of* Lab Animal*, Nature*

Dr. Graham described *Lab Animal*, a journal focused on *in vivo* science and technology using model systems for human disease. *Lab Animal* recently has enforced the ARRIVE guidelines as part of the peer-review process. The journal's mission is publishing work that improves the use of model organisms and bridging the gap between the animal vivarium and the laboratory.

Some of the journal's editorial practices relevant to the discussion of dissemination and reproducibility include (1) ensuring that editors review data blinded, (2) making sure the review process is based on the importance of the study, not the novelty, (3) promoting rigor and transparency by encouraging authors to report negative data in their publications, and (4) improving the generalizability and encouraging scientists to repeat studies in different settings. *Lab Animal* has mandated compliance of ARRIVE by submitting authors, which is intended to improve the peer-review process. Another effort to enhance transparency includes encouraging authors to submit their manuscripts to third-party sites (e.g., bioRxiv.org [Bioarchive]) so the data are accessible to the community during the review process. Lastly, authors are solicited to use *Nature* journal's protocol exchange.

***Discussion***

Dr. Ekker asked how the protocols on these various websites compare. Dr. Alvino responded that the protocols on protcols.io are open access and provide the ability to share methods. Dr. Graham added that he is unsure whether the for-profit status of these websites plays a role; a lot of researchers are unaware of these websites.

Because of the different formatting requirements for various journals, a participant recommended developing a template of items that journals will accept, which will make the submission process easier. Dr. Ekker replied that this is possible via consultation with the editorial board. Dr. Chow added that some publishers are reformatting submitted articles to fit their journal's format. Dr. Alvino said that *PLOS ONE* encourages the use of supplementary files. Dr. Graham supposed that the use of ARRIVE guidelines is widely accepted; quite a bit of overlap in checklist requirements exists across journals. Dr. Graham indicated the importance of performing meta-analysis with manuscripts.

## BREAKOUT GROUPS

The participants were organized into six breakout groups for discussing various issues, formulating recommendations, and suggesting future actions based on the topics presented during the workshop.

## PLENARY SESSION

### Report from Breakout Groups

Dr. Ekker welcomed the participants back from the breakout session. He indicated that the six breakout groups will present their top recommendations and future opportunities for zebrafish scientists. The written documents summarizing the breakout discussions will be emailed to the participants after the workshop.

*Group 1—Water Chemistry*

Dr. Sanders presented the following recommendations and opportunities for reporting requirements intended for water chemistry parameters:

- Report tier 1 and tier 2 water chemistry parameters for housing and experimental factors; report a range (or average) of parameters throughout the course and time of each study.
- Report water sources per facility and location; indicate what purification method is being used.
- Evaluate RO water systems.
- Validate water quality test methods (e.g., colorimetric versus test strips).
- Improve total gas pressure monitoring methods.
- Harmonize software capabilities for electronic probe systems.
- Improve communication through cloud-based systems; avoid firewalls.
- Evaluate the microbiome system in fish.
- Address the probiotic factors in fish.
- Consider the tank size, configuration, and color.

- Develop collaborations between the multicenter husbandry studies that ORIP sponsors and societal organizations, such as ZHA.
- Provide guidance to new principal investigators regarding purification methods and the use of different water sources.

*Group 2—Physical Environment*

A participant presented a Strengths, Weaknesses, Opportunities, and Threats (SWOT) analysis and described reporting opportunities intended for physical environments.

**Strengths**
- The zebrafish environment is controllable.
- Quantifications can be made.
- Outstanding genomic tools exist.
- The spaces in which fish are housed are easily modifiable; there are many ways to house fish.

**Weaknesses**
- The environment is an extremely complex ecosystem.
- Researchers are unaware what and where to quantify and do not know how the physical environment affects fish.
- There are many ways to house fish.

**Opportunities**
- Outstanding genomic tools can be used to inform management—couple biology to physical parameters; there are many ways to house fish.

**Threats**
- The parameters often are unreported or not quantified.

The participant reviewed a template of suggested basic reporting parameters for four physical conditions (temperature, light, sound/vibration, housing characteristics). Some of these suggestions will overlap with water chemistry; redundancy is acceptable. Specific reporting parameters are (1) the temperature of the system, ambient air, and sampled tanks; (2) the light level in a system and the light intensity in sampled tanks and incubators, the light source, and the light treatment on embryos/larvae; (3) the sound/vibration in the system and sampled tanks; and (4) the housing characteristics, such as the tank's size, shape, color, material composition, piping, pump type, and holding density. Knowing the environmental changes that affect fish biology is important. Creating a genetic profile/baseline of fish before studying the effects of the environment is recommended. The effect of environment on zebrafish biology can be accomplished by sampling genetic outputs (gene expression) while the physical parameters are measured.

*Group 3—Nutrition*

Dr. Solnica-Krezel presented SWOT analyses applicable to general and nutrition-related zebrafish reporting.

## General Reporting

**Strengths**

- Zebrafish is a mainstream vertebrate model for human development and disease that is constantly expanding in utility across research areas.
- The zebrafish research community is collaborative and well-integrated.
- Zebrafish has an established database (the Zebrafish Information Network) with clear genetic nomenclature rules and research information.

**Weaknesses**

- There is a lack of community-wide guidelines for reporting.
- There is a lack of rigorous studies investigating the effects of environmental factors in genotype-to-phenotype relationships.
- There is a lack of funding for systematic and rigorous studies investigating the role of nutrition and environmental factors in genotype-to-phenotype relationships.

**Opportunities**

- Develop community-wide standards for reporting husbandry and nutrition data.
- Work with industry to develop new products.
- Establish grant opportunities for multi-institutional studies.
- Create NIH Small Business Innovation Research (SBIR)-like collaborative grants between research institutions and businesses.
- Launch partnerships between commercial vendors and laboratories for developing new feeds.

**Threats**

- Scientific rigor of zebrafish publications may decrease because of inconsistent reporting of husbandry and nutrition.
- Innovative methods of zebrafish husbandry, nutrition, and research are lessening.

## Nutrition-Related Reporting

**Strengths**

- Zebrafish are robust and can grow using a variety of feeds and feeding regimens.
- Quality Assurance (QA) processes and ingredient profiles have been implemented by some fish feed manufacturers.

**Weaknesses**

- Nutritional requirements for fish models are unknown.
- There is a lack of standardized diets and chemically characterized feed.
- There is uneven and insufficient QA for commercial diets.
- The availability of commercial food sources and prices are shifting.
- Laboratories have insufficient purchasing power to provide market motivation and pressure.

**Opportunities**

- Develop new chemically defined diets.
- Establish standard QA procedures for food production and delivery.

- Partner between commercial vendors and research laboratories in developing new feeds.
- Encourage commercial vendors to develop food testing services.
- Educate the community and trainees on the significance of nutrition for their research and the available resources (e.g., ZHA).
- Develop a feed-related troubleshooting guide.
- Create a communication channel in the community.

**Threats**

- Processed diets can interfere with filtration systems.
- Contaminants in live and formulated diets can threaten fish and jeopardize experiments.
- There is reduced feed availability if a vendor leaves the commercial market.

Dr. Solnica-Krezel concluded by describing an example of how nutrition parameters can be reported in manuscripts.

*Group 4—Health Monitoring and Biosecurity*

Dr. Farmer presented a SWOT analysis on health monitoring and biosecurity reporting.

**Strengths**

- Pathogens are generally identifiable, and good testing resources are available.
- A small set of pathogens is responsible for most of diseases.
- Some pathogens can be detected antemortem in environmental specimens (e.g., feces).
- Published literature on health monitoring and biosafety is growing.

**Weaknesses**

- The levels of education, training, and awareness among researchers are variable.
- There is a reluctance to know, acknowledge, or report potential pathogens in a colony.
- There are possible unknown health monitoring and biosecurity-related effects on research results.
- Implementation of control and treatment measures is extremely variable.
- Testing, treatment, quarantine, disinfection, and labor can be expensive.

**Opportunities**

- Develop new ways to eliminate pathogens.
- Change the design of facilities to make the system modular to facilitate disinfection and treatment for subgroups.
- Facilitate or enhance collaboration and communication between researchers, veterinarians, and husbandry personnel.
- Create reviewer checklists that include the indications for health status testing.

**Threats**

- Infectious disease fish research in facilities may threaten unrelated nearby fish.
- Multiple vectors can introduce pathogens into live food, water, etc.
- Emerging diseases that have not been recognized in zebrafish exist.
- Mixing of species can introduce new pathogens.
- Pathogens cannot be eradicated because of disinfection constraints or treatment options.
- Genetic diversity can cause altered and undefined susceptibility to pathogens.
- Differences in susceptibility are observed in zebrafish of different age, sex, strain, and genotype.

- Testing services may not be budgeted in grants nor are supported by institutions.

The nutrition group developed the following reportable parameters for publications:

- Determine if testing is being done, what tests are performed for what agents, and how frequently.
- Define what pathogens are excluded from testing and how testing was performed in specific pathogen-free colonies.
- Include a short statement describing the protocol when using outside fish lines and on health testing of control and experimental populations.

The group discussed a few desired devices and testing methods that may enhance reporting:

- Obtain better methods for chorion disinfection to eliminate pathogens.
- Acquire different laboratory-ready rapid tests for the most common pathogens (e.g., tank-side test).
- Obtain equipment to isolate, shut down, and disinfect portions of a system.

*Group 5—Welfare and Complex Phenotype*

A participant presented a SWOT analysis of animal welfare and complex zebrafish phenotype reporting.

**Welfare Reporting**

**Strengths**

- Zebrafish are tolerant to a variety of conditions; colonies are easy to maintain via euthanasia.
- Welfare is addressed by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)/IACUC.
- Signs of stress are starting to be understood; perhaps novel stress markers are needed.

**Weaknesses**

- There is no standardized facility management throughout the zebrafish community (centralized versus satellite).
- The investment and training for staff varies.
- AAALAC/IACUC provides minimal criteria and guidance for zebrafish at certain institutes.
- It is difficult to recognize sick fish and diagnose specific ailments.

**Opportunities**

- Multiple disease diagnostic options are available, but they can be cumbersome and costly.

**Threats**

- Poor welfare management will affect experiments and reproducibility. There is a wide variety of scale and types of habitat systems (and water parameters).

The welfare and complex phenotype group created a set of reportable welfare parameters for publications:

- Reporting light cycles, stocking densities, and system design is important.

- The group was undecided on whether listing of common pathogens that likely to affect complex phenotypes (e.g., schooling behavior), is important for publications. This decision will presumably be study-specific.

**Complex Phenotype Reporting**

**Strengths**
- Zebrafish are complex and can be used to study complex phenotypes.
- The affordability of animals makes large sample sizes r possible.

**Weaknesses**
- There is a lack of understanding of complex phenotypes.
- Unknown effects of disease history on phenotype exist.

**Opportunities**
- Identify key parameters and markers (e.g., assays measuring stress and inflammation).

**Threats**
- The non-standardization of complex phenotype analyses inevitably will cause variability and affect reproducibility.

The group developed a set of reportable complex phenotype parameters for publications:

- Include detailed information on genetic backgrounds/strains.
- Test and report phenotype modifications.
- Report the growth stage, sex, temperature, time of day for the analysis, experimental design, and data selection.
- Emphasize experimental variability (e.g., transgenic, behavior and disease phenotypes).

To guide reporting, the group proposed the following question: What are the critical sources of variation and reproducibility?

*Group 6—Data Collection*

Dr. Hucka presented a list of topics that the data collection group discussed. These topics were related to the methodology of data collection, monitoring equipment and data output, and SOPs with associated data. Reportable data include data related to genotype information and are defined by "controlled" terminology. Perhaps Uniform Resource Locators to existing online protocols can be included in reporting.

Regarding principles of data collection and sharing, the group recommended developing a principles document, rather than a checklist. Some proposed guidelines in creating this document include:

- Focus on data that are typical and arise from common procedures.
- Provide a set of data groups.
- Define data characteristics that should be reported.
- Explain how to disclose methodologies used to acquire data and sources/resources, as well as how and where the data are shared.

Dr. Hucka posed the following question: How much data are required, and how should updates to data be handled? To address this, feedback from the community is necessary. An example method of receiving feedback is the Mouse Tumor Biology database, which has an open web forum for comments.

**White Paper**

Dr. Ekker reiterated an earlier question regarding how to engage the zebrafish community for comments**.** He suggested developing a workshop report to coalesce the group discussions. Drs. Ekker and Solnica-Krezel agreed that disseminating to the zebrafish community a draft white paper that summarizes the group's recommendations and soliciting feedback is a prerequisite for formalizing a white paper. Dr. Ekker wondered what would be necessary for developing a formal white paper for the NIH.

Dr. Kalueff said that to make an impact on the zebrafish field, a formal summary of the discussions in an elegant and concise manner is needed. This document should be published in *Lab Animal* or *Zebrafish*.

Dr. Chow suggested that the participants adhere to the workshop's original objective and identify NIH's expectations before formulating a white paper. A participant added that knowing the definitions and requirements for a white paper is compulsory.

**ORIP Recommendations**

ORIP bridges across all NIH ICs by supporting shared instrumentation and improvements of facility; the Office also supports the development of animal models and their preservation. ORIP's mission is addressing the research needs of scientists from all ICs and biomedical research disciplines. Dr. Klosek raised several points. The workshop should draft a white paper for about the minimal reporting requirements and their formats for for zebrafish (and other fish model) research and share it with ORIP. ORIP is interested in identifying better approaches and methods for describing animal models, to enhance rigor and reproducibility. It seems that the lack of ARRIVE enforcement is due, in part, to limited communication between research communities. Communication between facilities that collect environmental data and investigators whose research outcomes are affected by the extrinsic environment is needed. Better collection, management, and sharing of extrinsic environmental data are proposed ways to improve animal facilities.

**Dissemination of Outcomes, Feedback from the Research Community, and Future Actions**

Several participants proposed methods of disseminating the workshop's discussed points. Dr. Gerlai suggested that the groups develop a priority list of the most pressing needs, as well as a cost analysis for these needs, before requesting funding from the NIH. Dr. Ekker solicited the participants' comments regarding a workshop draft report for dissemination to scientific societies (i.e., International Zebrafish Society, Zebrafish Disease Models Society, ZHA, and yet-to-be-identified Chinese or European zebrafish societies). Dr. Ekker added that the draft report will outline a list of achievable opportunities (regarding environmental factors) for improving zebrafish science. Ms. Pandolfo wondered if a follow-up discussion is necessary after the draft is formulated and reviewed by the participants.

A participant recommended that the steering community create a document summarizing the workshop discussions. This document can be disseminated to the workshop subgroup leaders, who will seek comments from members of each group. The steering community can perform a final review of this document before it is submitted to the zebrafish community. Dr. Solnica-Krezel suggested that creating a workshop report (without recommendations) that is submitted to the community allows dissemination to move quickly. Dr. Solnica-Krezel advised developing two documents—a white paper sent to funding agencies describing research areas that are deemed important for moving the field forward and a document detailing general reporting recommendations for the community. These recommended guidelines will be better directed by emerging science.

Dr. Ekker indicated that the workshop report is a clear outcome of the meeting and will be used for engaging the broader community; the ultimate goal is creating a white paper for the NIH. Guidance on what to include in the white paper is necessary. He announced that IACUC must receive a report.

A participant suggested that each group leader create a document summarizing the group's conclusions, which can be forwarded to the committee. Several participants agreed that 2 weeks suffices to provide their documents to the committee. Dr. Ekker reiterated that the workshop report will be sent to the committee for review, editing, and ultimately publishing. The published report then will be disseminated to all of the communities; this document will serve as the basis for creating a white paper for the NIH. A second document of general reporting recommendations for the zebrafish community will be developed. A meeting participant recommended that the report be used as a basis to solicit feedback from the entire community (e.g., a survey at scientific conferences).

Dr. Ekker thanked the participants for their attendance at the workshop and commented that he looks forward to seeing the written documents. In response to Dr. Sanders' question, Dr. Ekker indicated that participants can submit supplementary information (e.g., research recommendations) while each group's document is in circulation among the committee.

## ADJOURNMENT

Dr. Klosek announced two ORIP's program announcements for SBIR and STTR Programs. They support the development of tools and devices that can improve experiments using animal models.

She thanked the participants for attending and adjourned the workshop.

## APPENDIX A: Workshop Agenda

**MONDAY, SEPTEMBER 11, 2017**

7:30 – 8:30: Registration

**INTRODUCTION TO THE WORKSHOP**

8:30 – 8:35: Welcome – Malgorzata M Klosek, Director DCI/ORIP/DPCPSI/OD/NIH

8:35 – 8:45: ORIP's Support for Animal Models and Animal Facilities - Infrastructure for Innovation – Franziska Grieder, Director ORIP/DPCPSI/OD/NIH

8:45 – 8:55: Rigor, Reproducibility, and Animal Model Research – Stephanie Murphy, Director DCM/ ORIP/DPCPSI/OD/NIH

8:55 – 9:05: Meeting Logistics – Malgorzata M Klosek

9:05 – 9:15: Goals and Expected Outcomes of the Workshop – Steering Committee: Stephen Ekker, Christian Lawrence, Zoltan Varga

**PLENARY SESSION**

**9:15 – 10:05: Water Chemistry** – Discussion Leader: George Sanders

Jim Burris, Stephen Ekker, Steven Ripp, & George Sanders:

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| 9:15 – 9:35: | | *Basic Information from Zebrafish Facilities Represented by the Water Chemistry Group* |
| 9:35 – 9:50: | | *Effects of Water Chemistry on Zebrafish and Research Endeavors* |
| 9:50 – 10:05: | Q&As | |

**10:05 – 10:20: Break**

**10:20 – 11:10: Physical Environment** – Discussion Leader: Ronald Walter

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|---|---|---|
| 10:20 – 10:30: | Robert Gerlai: | *Zebrafish Husbandry and Breeding Require Careful Consideration of Environmental Conditions: The Growing Need for Systematic Studies on a Large Number of Factors* |
| 10:30 – 10:40: | Christian Lawrence: | *Physical Factors Impacting Zebrafish Experiments: What Are They and How Do You Measure Them?* |
| 10:40 - 10:50: | Mary Halloran: | *Physical Environmental Parameters Affecting Experiments: Neural/Behavioral Studies as an Example* |
| 10:50 – 11:00: | Ronald Walter: | *Establishment and Maintenance of Genetic Homeostasis: Light effects on Zebrafish Gene Expression* |
| 11:00 – 11:10: | Q&As | |

**11:10 – 12:10: Nutrition** – Discussion Leader - Lilliana Solnica-Krezel

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| 11:10 – 11:20: | Maurine Hobbs: | *Standardization Feed SOPs - Food Delivery Methods and Feed Types Across Different Institutions* |

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|---|---|---|
| 11:20 – 11:30: | Lauren Pandolfo: | *Bcs and Larvae vs Adults Needs and Diets* |
| 11:30 – 11:40: | Stephen Watts: | *Nutrition and What Is Done in Other Model Organisms* |
| 11:40 – 11:50: | Lilliana Solnica-Krezel: | *What Data is or Should Be Collected?* |
| 11:50 – 12:00: | Mark Masino: | *Chromium-contaminated Adult Zebrafish Diet Negatively Impacts the Viability of Larval Progeny* |
| 12:00 – 12:10: | Q&As | |

**12:10 – 1:10: Lunch Break**

**1:10 – 2:10: Health Monitoring and Biosafety** – Discussion Leader: Susan Farmer

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|---|---|---|
| 1:10 – 1:20: | Katy Murray: | *Biosecurity, Importation, and Exportation* |
| 1:20 – 1:30: | Susan Farmer: | *Health Surveillance: How Often and What Is Available* |
| 1:30 – 1:40: | Joseph Schech: | *Finding Meaning in Zebrafish Sick Reports* |
| 1:40 – 1:50: | Heather Shive: | *Physical Appearance / Lesions: Identifying Sick Fish and Diseases* |
| 1:50 – 2:00: | Christopher Whipps: | *Impacts of Diseases (such as Pseudoloma and Mycobacterial Diseases): Which Pathogens Should Be Reported?* |
| 2:00 – 2:10: | Q&As | |

**2:10 – 3:10: Welfare and Behavior** – Discussion Leader: Michael Kent

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| 2:10 – 2:20: | Marnie Halpern: | *Behavioral Assays: Reproducibility and Rigor* |
| 2:20 – 2:30: | Brant Weinstein: | *Egg Production and Fertility* |
| 2:30 – 2:40: | Brian Link: | *Parameters Affecting Variation and Reproducibility when Using Zebrafish to Model Disease: Implications for Disease Modeling* |
| 2:40 – 2:50: | Michael Kent: | *Effects of Sub-Clinical Infections* |
| 2:50 – 3:00: | Rebecca Van Beneden: | *Accepting and Implementing Reporting Procedures* |
| 3:00 – 3:10: | Q&As | |

**3:10 – 3:30: Break & Group Photo**

**3:30 – 5:30: Commercial Equipment and Data Formats** – Discussion Leader: Stephen Ekker

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| 3:30 – 3:50: | Mark Francis: | *Design for Success: 'Smart' Data Collection and Analysis* |
| 3:50 – 4:10: | Massimo Ferrari: | *Aquatic Systems: Technical Feasibility & Practicality* |
| 4:10 – 4:30: | Priscilla Shirley: | *Lab Feeds* |
| 4:30 – 4:50: | Nick King: | *Creating a Health Management Program for Aquatic Establishments* |
| 4:50 – 5:10: | Marcus Crim: | *Health Monitoring and Health Status Reporting for Zebrafish Colonies* |
| 5:10 – 5:30: | David Weintraub: | *Efficient and Effective Data Collection to Support and Connect the Zebrafish Research Community* |

**CONCLUSIONS AND SET-UP FOR THE NEXT DAY**

5:30 – 5:45: **Questions and Issues for the Break-out Groups, Part I:** Steering Committee, Discussion Leaders, and all other participants

## TUESDAY, SEPTEMBER 12, 2017

**PLENARY SESSION**

8:30 – 8:35: Plans for the Day: MM Klosek

**8:35 – 9:45:** **Data Collection and Sharin**g – Discussion Leader: Mike Hucka

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| 8:35 – 8:45: | Cory Brayton: | *Current Status of Guidelines for Minimal Information Reporting* |
| 8:45 – 8:55: | Allan Kalueff: | *A Neuroscientist's Perspective on Tools for Data Collection and Sharing: The Researchers Need* |
| 8:55 – 9:05: | Zoltan M Varga: | *Husbandry Data Collection and Environmental Data Monitoring at the Zebrafish International Resource Center* |
| 9:05 – 9:15: | Timothy Mason: | *Data Collection at the University of Oregon Zebrafish Facility* |
| 9:15 – 9:35: | Yuk Fai Leung & Michael Hucka: | *Data Formats and Data Sharing: Minimal Information Model and its Potential for Growth and Integration* |
| 9:35 – 9:45: | Q&As | |

**9:45 – 10:15:** **Dissemination Questions:** How to Engage Professional Organizations / Journals – Input for the Break-out Group Sessions – Discussion Leader: Stephen Ekker

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|---|---|
| Gina Alvino: | *Comments from an Editor of PLOS ONE* |
| Cory Brayton: | *Comments from an Editor/EB/ERB member and reviewer* |
| Ida Chow: | *Comments from a Director of the Society for Developmental Biology* |
| Dante D'India: | *Zebrafish Husbandry Association: A Learning Community Focused on Advancing Standardization of Zebrafish Research* |
| Dustin Graham: | *Comments from an editor of LabAnimal, Nature Research* |

**10:15 – 10:30: Break**

**10:30 – 10:45: Questions and Issues for the Break-out Groups, Part II:** Steering Committee, Discussion Leaders, and all other participants

**10:45 – 10:50: Break-out Groups Set-up**

**BREAK-OUT GROUPS**

**10:50 – 12:30: Break-out Groups Discussions:** Open Issues, Recommendations and Further Actions

**PLENARY SESSION**

**12:30 – 1:30:** **Lunch Break**
**1:30 – 2:00:** **Reports from Break-out Groups:** Discussion Leaders
**2:00 – 2:30:** **White Paper:** Steering Committee
**2:30 – 3:00:** **Recommendations for ORIP:** Steering Committee and Discussion Leaders

**3:00 – 3:15: Dissemination of Outcomes, Feedback from the Research Community, and Future Actions:** All Participants

**3:15 – 3:30: Wrap-up**

**3:30: Workshop adjourns**

## APPENDIX B: Workshop Roster

### Participants

**Gina Alvino, PhD**
Senior Editor
PLOS ONE
San Francisco, California

**Cory Brayton, DVM, DACLAM, DACVP**
Associate Professor
Department of Molecular & Comparative Pathobiology
Johns Hopkins University
Baltimore, Maryland

**Jim Burris, MS**
Facilities Manager
School of Medicine Zebrafish Core Facilities
Duke University
Durham, North Carolina

**Stephan Canters**
Director of Business Operations
Fulcrum Automation & Control Technologies, Inc.
Beechwood, Ohio

**Daniel Castranova, MS, LATG**
Zebrafish Technician
Section on Vertebrate Organogenesis, Division of Developmental Biology
*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
Bethesda, Maryland

**Ida Chow, PhD**
Executive Officer
Society for Developmental Biology
Bethesda, Maryland

**Marcus Crim, MBA, DVM, MS**
Head of Microbiology Labs & Aquatic Diagnostics
IDEXX BioResearch
Columbia, Missouri

**Andrew Davis, LATG**
Research Technician
Section on Vertebrate Organogenesis, Division of Developmental Biology
*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
Bethesda, Maryland

**Dante D'India, ALM**
President, Zebrafish Husbandry Association
Research Assistant, Harvard Medical School
Boston, Massachusetts

**Stephen Ekker, PhD**
Professor
Department of Biochemistry & Molecular Biology
Mayo Clinic Cancer Center
Rochester, Minnesota

**Susan Farmer, DVM, PhD**
Co-Director, Zebrafish Research Facility
Assistant Director, Animal Resources Program
University of Alabama at Birmingham
Birmingham, Alabama

**Massimo Ferrari**
International Sales Area Manager
Tecniplast, Inc.
Milan, Italy

**Mark Francis**
President
Aquaneering, Inc.
San Diego, California

**Rory Francis**
Founder
Danio Lab
Boston, Massachusetts

**Robert Gerlai, PhD**
Professor
Department of Psychology
University of Toronto Mississauga
Mississauga, Ontario

**Dustin Graham, PhD**
Chief Editor of Lab Animal
Nature
New York, New York

**Mary Halloran, PhD**
Professor
Departments of Zoology & Neuroscience
University of Wisconsin–Madison
Madison, Wisconsin

**Marnie Halpern, PhD**
Staff Member
Department of Embryology
Carnegie Institution for Science
Baltimore, Maryland

**Maurine Hobbs, PhD**
Director
Centralized Zebrafish Animal Resource
University of Utah Health
Salt Lake City, Utah

**Michael Hucka, PhD**
Staff Scientist
Department of Computing & Mathematical Sciences
California Institute of Technology
Pasadena, California

**Allan V. Kalueff, PhD**
Director
ZENEREI Research Center
Slidell, Louisiana
Professor
St. Petersburg University, Russia
Southwest University, Chongqing, China

**Michael Kent, PhD**
Professor
Department of Microbiology
Oregon State University
Corvallis, Oregon

**Nick King, MS**
Operations Director
Fish Vet Group, Inc.
Portland, Maine

**Chris Knox**
Vice President and Director of Technical Services
Tecniplast USA, Inc.
West Chester, Pennsylvania

**Christian Lawrence, MS**
Zebrafish Facility Manager
Division of Hematology & Oncology
Boston Children's Hospital
Boston, Massachusetts

**Yuk Fai Leung, PhD**
Associate Professor
Department of Biological Sciences
Purdue University
West Lafayette, Indiana

**Brian Link, PhD**
Professor
Department of Cell Biology, Neurobiology, & Anatomy
Medical College of Wisconsin
Milwaukee, Wisconsin

**Mark Masino, PhD**
Associate Professor
Department of Neuroscience
University of Minnesota
Minneapolis, Minnesota

**Timothy Mason**
Director
Aquatic Animal Care Services
University of Oregon
Eugene, Oregon

**Katrina Murray, DVM, PhD**
Veterinarian
Zebrafish International Resource Center
University of Oregon
Eugene, Oregon

**Lauren Pandolfo, MS**
Animal Aquatics Facilities Manager
Research Animal Management Branch, Division of Intramural Research
*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
Bethesda, Maryland

**Steven Ripp, PhD**
Assistant Director
The Center for Environmental Biotechnology
University of Tennessee
Knoxville, Tennessee

**George Sanders, DVM, MS, Fish Pathologist (AFS/FHS)**
Senior Lecturer & Aquatic Animal Program Director
Department of Comparative Medicine
University of Washington, School of Medicine
Seattle, Washington

**Joseph Schech, DVM, DACLAM**
Animal Program Director
Research Animal Management Branch, Division of Intramural Research
*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
Bethesda, Maryland

**Priscilla Shirley**
Sales Specialist
Pet & Lab Feeds
Zeigler Bros., Inc.
Gardners, Pennsylvania

**Heather Shive, DVM, PhD, DACVP**
Assistant Professor
Department of Population and Pathology
North Carolina State University
Raleigh, North Carolina

**Massimo Ferrari**
Aquatic Product Specialist
Tecniplast USA, Inc.
West Chester, Pennsylvania

**Lilianna Solnica-Krezel, PhD**
Professor and Department Head
Department of Biology
Washington University in St. Louis
St. Louis, Missouri

**Rebecca Van Beneden, PhD**
Professor
Department of Molecular and Biomedical Sciences
University of Maine
Orono, Maine

**Zoltan M. Varga, PhD**
Director
Zebrafish International Resource Center
University of Oregon
Eugene, Oregon

**Ronald Walter, PhD**
Chair in Cancer Research
Department of Chemistry and Biochemistry
Texas State University
San Marcos, Texas

**Stephen Watts, PhD**
Professor
Department of Biology
University of Alabama at Birmingham
Birmingham, Alabama

**Christopher Whipps, PhD**
Associate Professor
Center for Applied Microbiology
State University of New York College of Environmental Science and Forestry
Syracuse, New York

**Brant Weinstein, PhD**
Senior Investigator
Section on Vertebrate Organogenesis, Division of Developmental Biology
*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
Bethesda, Maryland

**David Weintraub**
President
Fulcrum Automation & Control Technologies, Inc.
Beechwood, Ohio

**ORIP Staff**

**Alena Horska, PhD**
Program Officer
Division of Construction and Instruments
ORIP/DPCPSI/OD/NIH

**Malgorzata Klosek, PhD**
Director
Division of Construction and Instruments
ORIP/DPCPSI/OD/NIH

**Willie McCullough, PhD**
Program Officer
Division of Construction and Instruments
ORIP/DPCPSI/OD/NIH

**Oleg Mirochnitchenko, PhD**
Program Officer
Division of Comparative Medicine
ORIP/DPCPSI/OD/NIH

**Stephanie Murphy, VMD, PhD, DACLAM**
Director
Division of Comparative Medicine
ORIP/DPCPSI/OD/NIH

**with Assistance:**

**Miguel Contreras, PhD**
Program Officer
Division of Comparative Medicine
ORIP/DPCPSI/OD/NIH

**Sige Zou, PhD**
Program Officer
Division of Comparative Medicine
ORIP/DPCPSI/OD/NIH

**Support Staff:**

**Terry Clark**
Staff Assistant
ORIP/DPCPSI/OD/NIH

**TJ Smith**
Staff Assistant
ORIP/DPCPSI/OD/NIH

**Desiree Von Kollmar**
Health Science Policy Analyst
ORIP/DPCPSI/OD/NIH

**NIH Extramural Attendees**

**Doreen Bartlett, RVT, LATG**
Senior Assurance Officer
Division of Assurances
OLAW/OER/OD/NIH