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Rigor and Reproducibility of Animal Studies: Extrinsic Factors Workshop

Session 1. Aquatic Animals

September 23, 2022
Virtual Meeting

Final Report

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Executive Summary

The Extrinsic Factors Workshop was held in three sessions to better understand extrinsic factors and their effects on biomedical research. Session 1 was focused on extrinsic factors in the use of aquatic animals for biomedical research. Drs. Stephen Ekker and Robyn Tanguay served as the Session 1 co-chairs. Session 1 topics addressed shared challenges and needs of diverse aquatic animal models as well as extrinsic factors, aquatic housing, and monitoring that impact and enhance rigor and reproducibility in studies using aquatic animals. The speakers identified various extrinsic factors for consideration in research, including water quality, feeding regimens, pathogen exposures and non-pathogenic diseases, temperature, season, flow rates, tank size, population density, enrichment, strain, parental stock, incubator light cycles, stress, time of day, experimental technique, and movement and noises. As new model organisms are developed, the unique requirements of individual aquatic species must be considered. Additionally, lessons learned from monitoring extrinsic factors in other model organisms—such as rodents—can be applied to aquatic facilities. The participants discussed recent innovations (e.g., improved reverse osmosis systems, real-time pathogen monitoring, whole-genome sequencing, artificial intelligence) and their potential for implementation across facilities. Several participants emphasized that monitoring and reporting on extrinsic factors is the first step toward standardization; journals and funding agencies can play a role in this area. They also discussed the need to ensure that smaller facilities are provided access to affordable options for meeting new standards. The importance of fostering community engagement in discussions on this topic also was emphasized.

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Robyn Tanguay, Ph.D., Oregon State University

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Bobbi Baur, Aquaneering
Iain Drummond, Ph.D., Mount Desert Island Biological Laboratory
Gianpaolo Milite, D.V.M., M.Sc., Tecniplast
Katy Murray, D.V.M., Ph.D., Zebrafish International Resource Center (ZIRC)
Corbin Schuster, Ph.D., ZIRC
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Workshop Report

Opening Remarks

Robert W. Eisinger, Ph.D., Acting Director, Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director (OD), National Institutes of Health (NIH)
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Dr. Franziska Grieder, Director, ORIP, and Dr. Robert W. Eisinger, Acting Director, DPCPSI, welcomed the attendees to Session 1 of the workshop. In his opening remarks, Dr. Eisinger affirmed that the NIH emphasizes the importance of rigor and reproducibility in biomedical and biobehavioral research. The NIH upholds the highest standards of scientific integrity, bioethics, public accountability, and social responsibility in the science that it supports. In 2017, the Advisory Committee to the NIH Director (ACD) proposed an initiative on enhancing rigor, transparency, and translatability in animal research. In 2021, the ACD Working Group on Enhancing Rigor, Transparency, and Translatability in Animal Research recommended that the NIH encourage and support work to better understand, monitor, record, and report important extrinsic factors related to animal care that might affect research results. ORIP is modifying its infrastructure programs to address reproducibility in animal studies. The Extrinsic Factors Workshop seeks to better understand extrinsic factors and their effects on biomedical research.

Dr. Grieder noted that ORIP's mission is "Infrastructure for Innovation." ORIP awards grants to support research resources, which include animal models for human disease; state-of-the-art biomedical instrumentation; animal models that serve as a bridge between basic science and human medicine to enable scientists to better understand, diagnose, and treat human diseases; and research-related resources that play an important role in biomedical experiments. ORIP's 2021–2025 Strategic Plan commits that ORIP will assess the contribution of its resources to improving scientific rigor and reproducibility and will make strategic investments in methods and infrastructure tools to enhance the rigor and reproducibility of animal models and related biomaterials.

Dr. Grieder explained that this was the first of three sessions of the workshop. In the past, ORIP has devoted efforts in enhancing rigor and reproducibility through workshops (e.g., Zebrafish and Other Fish Models: Extrinsic Environmental Factors for Rigorous Experiments and Reproducible Results; Validation of Animal Models and Tools for Biomedical Research) and publication of future funding opportunity announcements (e.g., NOT-OD-22-039). This workshop is one of several steps going forward in fulfilling ORIP's Strategic Plan by addressing the important endeavor of enhancing animal study and reproducibility in NIH-supported research.

Dr. Jeff Wang, Workshop Coordinator, also welcomed the attendees. He provided examples of extrinsic factors related to animal research, which include temperature, humidity, noise, and lighting. Housing conditions—such as size and material of enclosure, number of animals per enclosure, bedding material and thickness, and cleanliness and cleaning schedules—also must be considered. Dr. Wang emphasized that the effects of extrinsic factors can be highly complex and often include multiple interactions. This issue has been understudied and under-documented. The goal of the workshop is to discuss the current status, needs, and strategies related to management, monitoring, and reporting of extrinsic factors to enhance the reproducibility and rigor of animal research. The focus is on the most widely and commonly used animal models, relevant extrinsic physical factors, and modern technologies. Dr. Wang expressed appreciation to the organizing committee members, speakers, and participants for their engagement.

Dr. James Fox, Workshop Chairperson, previewed Sessions 2 and 3. He emphasized that the topic of extrinsic factors is highly relevant to biomedical research, both for investigators and vivarium staff

members. He recalled his own experience of addressing extrinsic factors in zebrafish studies at the Massachusetts Institute of Technology. Drs. Stephen Ekker and Robyn Tanguay, Session 1 co-chairs, introduced themselves.

Keynote Presentation: Shared Challenges and Needs of Diverse Aquatic Animal Models
Iain Drummond, Ph.D., Mount Desert Island Biological Laboratory (MDIBL)

Dr. Iain Drummond presented on innovations related to reproducibility. MDIBL's mission is to improve human health by discovering novel mechanisms of tissue repair, aging, and regeneration. An educational program also is in place. The facility supports 11 laboratories that use non-mammalian species—most of which are aquatic organisms—using a comparative approach. These species have included dogfish, salmon, mummichog, bullfrog, cane toad, *Xenopus*, sea urchin, and are now principally zebrafish, axolotl, and African turquoise killifish. Dr. Drummond explained that zebrafish, axolotl, and African turquoise killifish are maintained in separate rooms so that their unique conditions for growth, fecundity, and husbandry are met.

Current scientific activities at the MDIBL include early-life stress impacts, vasculature, neural stem cell differentiation, immunology and cell lineage in limb regeneration, kidney regeneration, and aging. Dr. Drummond explained that as the science has evolved, endpoint measurements have become increasingly sensitive. Unexpected variables have been identified over time. Dr. Drummond noted that different aquatic animals have unique requirements related to conductivity, salinity, temperature, trace metals, and general water quality. Each room at the facility is regulated independently for these environmental variables. Aquatic facilities, however, often do not report these variables. Better communication across facilities is needed.

Most facilities are equipped with online reporting systems, but these systems might not be analyzed frequently. Dr. Drummond noted that these systems provide a valuable historical record that can be useful in assessing unexpected experimental results. He highlighted a case example of the African turquoise killifish, which is highly sensitive to changes in water quality. Stress is a potential confounder in behavioral and physiological assays, and low light is beneficial in managing stress. Dr. Drummond emphasized that reliable reporting is needed for reproducibility. Over time, standardization of conditions will be achieved within the field for this species. He noted that these issues relate to facility infrastructure and management, and water sustainability should be considered.

The effects of temperature variation on growth are not fully understood in the axolotl, and temperature is likely to affect expression of growth-related genes. In the zebrafish and African turquoise killifish, these effects are more standardized. Dr. Drummond also noted the importance of reporting feeding in publications, rather than simply citing standard references. The effects of feeding variables differ among the axolotl, zebrafish, and African turquoise killifish. The experimental questions also must be considered in this context. Potential solutions for standardizing feeding include 3D-printed feeders and iron magnetic artemia (cyst-free brine shrimp quality). Pathogens also represent a significant extrinsic factor, and vigilance in monitoring pathogens is critical. Normalized PCR assays and other in-house detection methods are needed. Costs in this area also must be reduced. Several variables related to housing—such as tank size, population density, enrichment, and enclosure tents—can affect growth rates, stress, and survival. Seasonable variables also must be considered across species. Dr. Drummond presented an example of unexpected variance in RNA sequencing experiments; the order of replicate sampling resulted in variance in expression. Other potential variables can include strain, parental stock, incubator light cycles, stress, time of day, and experimental technique.

Dr. Drummond noted that it may not be possible to understand the full dynamics of all extrinsic factors. He emphasized, therefore, the importance of recording all relevant metadata so that variance can be mapped onto both intended and unintended experimental variables. He highlighted opportunities for

improvements, which include newer reverse osmosis water purification systems, physical parameter reporting, pathogen detection and standardized health monitoring, and standardization of husbandry conditions (e.g., water, feeding, light) and housing. Opportunities for innovation include magnetic removal of brine shrimp cysts, standardized feeders, and racked axolotl cups in tub enclosures for efficient and reproducible offline water changes.

Discussion

- Dr. Emily Franklin asked for clarification on the temperature, growth, and regeneration data in the axolotl. Dr. Drummond explained that those data were collected in a pilot experiment. He clarified that the animals grown at warmer temperatures regenerated more quickly; the mechanisms are not fully understood. Dr. Prayag Murawala added that animal size also must be considered in regard to regeneration. Dr. Ekker added that the developmental stage is closely affected by temperature. In mice, this effect can lead to complications for researchers.

- Dr. Reid Landes commented that standardized variables have not been established in rodent models, despite a longer history of work in that area. Dr. Drummond noted that two elements of standardization exist: standardization of growth methods and standardization of reporting. The latter element might be easier to address. He noted that increased requirements for reporting in publications have been successful. The zebrafish field has coalesced in regard to several relevant factors.

- Dr. S. Randal Voss remarked that breeding patterns of the axolotl represent the species' ancestral vestige. He also emphasized the importance of backup water systems in the event of a failure.

- Dr. Fox asked whether the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines could be applied to studies of aquatic animals. Dr. Drummond agreed that more discussion in this area is needed. Dr. Landes added that the ARRIVE guidelines are applicable to all organism types, but more guidance is needed on organism-specific metadata reporting.

Presentations: Extrinsic Factors That Impact Rigor and Reproducibility in Studies Using Aquatic Animals

Lessons Learned from the Zebrafish International Resource Center (ZIRC)

Zoltan Varga, Ph.D., ZIRC

Dr. Zoltan Varga presented on animal environments developed through ZIRC, which serves as a central genetic repository for zebrafish, provides diagnostic zebrafish health services, and researches and disseminates zebrafish health and husbandry standards. He began by highlighting an example of how excessive movement and irregular noises (e.g., building renovations, fire) can lead to aggressive behaviors. The effects of these factors are complex and challenging to characterize.

Dr. Varga explained that responses to movement change over time following repeated exposures. He also presented data on the potential effects of the fire alarms in the building, noting that the alarms are insignificant compared to ambient noises in the tank. The alarms do not appear to affect behavior. He also presented data from a study on water quality across seven facilities. Results indicated significant variation in metals and metalloids across the facilities. Dr. Varga explained that various factors, including diet and equipment, contribute to these differences. He affirmed, however, that reverse osmosis filtration still is the best approach to standardize water composition.

Dr. Varga presented a schematic diagram of ZIRC's new recirculating systems. The systems and equipment are monitored for various factors, including pH and temperature. Data can be monitored in real

time and reviewed using supervisory control and data acquisition (SCADA) software. A paging system sends mobile alerts that allow users to respond in real time. He noted that the monitoring system is backed by an uninterruptable power supply. He presented an example of data outputs. Examples of SCADA applications include continuous monitoring and logging of systems and the animal environment, storage of logged data and back-tracing of environmental changes, environmental predictions based on previous data records, and an alert system when the environment exceeds set thresholds.

Areas for future work at ZIRC include consistent and standardized conditions, research into monitoring equipment, correlations between environmental changes and fish performance, and other correlations related to animal behavior. To this end, ZIRC has established a collaboration with Martineau & Associates, Inc., which has developed a commercial Remanent Imaging video recording system—CanaryTank—that can help researchers better integrate, log, and analyze sentinel fish position and behavior in the aquarium, and can thus track indicators of animal health continuously, remotely, and in real time. Dr. Varga shared a representative CanaryTank recording and explained that these data can provide insight into the correlations between environmental disturbances and fish behavior as an indicator of well-being. He remarked that behavioral, health, and husbandry research are needed in the context of environmental change, with a focus on both short- and long-term responses. Additionally, thorough, and well-defined reporting on the husbandry environment is needed.

Review of Diseases and Impact on Research
Katy Murray, D.V.M., Ph.D., ZIRC

Dr. Katy Murray discussed diseases that affect zebrafish in the context of their effects on scientific research. She explained that most of these diseases have been well characterized in terms of diagnosis and treatment. Bacterial, microsporidian, metazoan, protozoan, viral, and noninfectious diseases, as well as neoplasia, occur naturally in zebrafish.

Mycobacteriosis is caused by an acid-fast bacterial pathogen that exists in fish and biofilms and results in chronic inflammatory lesions in fish tissues. Different mycobacterial species vary in virulence. Impacts on research can include asymptomatic presentation, bacterial autofluorescence, human infections, granulomas to sheets of macrophages, acute versus chronic transcriptome signatures, and inflammatory cytokine upregulation.

Psuedoloma neurophilia, a microsporidium, is an obligate intracellular pathogen with high infectivity. Both horizontal and vertical transmission can occur. Impacts on research can include weight loss and skeletal deformities, mortality in immunosuppressed fish, reduced fecundity, and downregulation of immune response genes. Behavioral impacts can include responses to stress and startling stimuli, altered shoaling, capture avoidance, increased stress and anxiety, reduced locomotor activity, and sex-specific changes in exploration.

Pseudocapillaria tomentosa is a capillarid nematode that infects the intestinal tract and is spread via direct fecal–oral transmission. Impacts on research can include moderate to high morbidity and mortality, inflammatory response, and microbiome disruption. This pathogen is a tumor promoter, and infection is associated with the development of intestinal neoplasms.

Supersaturation and gas bubble disease occur when the total pressure of dissolved gases exceeds atmospheric pressure. This disease represents a population-level problem, but not all fish develop clinical disease. Impacts on research can include mortality, formation of bubbles in tissues, and occlusion necrosis and death. Behavioral impacts associated with gas bubble disease can include lethargy, altered buoyancy, disequilibrium, reduced feeding, exophthalmia, rapid respiration and hovering at the bottom of the tank, decreased growth rate, and secondary infections.

Dr. Murray noted the importance of strain-specific considerations in regard to behavior, disease susceptibility, and presentation. She also spoke on the effects of disease on the sharing of animals among facilities and receiving new lines for research. Factors for consideration include zoonotic risk and liability, shipping conditions, and national regulations.

Dr. Murray concluded by emphasizing that zebrafish are susceptible to a range of infectious and noninfectious diseases. Some of the most common diseases have the potential to affect research in both subtle and dramatic ways, including morbidity and mortality; organ- and tissue-specific pathologies; cytokine, transcriptome, and microbiome profiles; behavioral alterations; tumor incidence; decreased growth and secondary infections; strain-specific effects; and shipping challenges.

Developing Real-Time Pathogenic Testing in Aquatic Systems
Corbin Schuster, Ph.D., ZIRC

Dr. Corbin Schuster discussed monitoring of zebrafish pathogens in tank water at ZIRC. He explained that as a researcher, he must consider the extent to which subclinical diseases affect the zebrafish model (e.g., physiologically, immunologically). His research focus is on *P. neurophilia* in the context of altered startle response, increased stress and anxiety, reduced activity, and changes to the brain transcript profile. He is studying the application of diagnostic tools (e.g., histopathological, wet-mount, molecular) to eliminate *P. neurophilia* from research facilities.

In his experiments, most infected animals were asymptomatic, making diagnosis difficult. For small populations of valuable fish, testing of almost all animals is needed. One proposed solution is to use nonlethal and relatively noninvasive assays, such as skin scraping and water and sediment assays. This approach is broadly applicable to zebrafish and other biomedical models used in research and is particularly useful for small populations and small water volume.

Dr. Schuster presented a case for digital PCR (dPCR) to detect pathogens in the environment. This platform helps address inconsistency issues in quantitative PCR assays. Primers and probes could be transitioned across the two approaches. dPCR limits the potential for PCR inhibition from environmental factors. Dr. Schuster noted that this platform is available through several vendors.

dPCR results in absolute quantification, limiting inhibition. This approach enables detection of rare events. Development of a nonlethal assay, however, has presented a challenge, because different facilities face different dynamics within their systems. Standard practices have not been established. Dr. Schuster explained that the process involves collection, filtration, sonification, environmental DNA extraction, and dPCR amplification. Detection of the pathogen in water was sporadic for the first 11 weeks after infection, indicating a key point in infection.

Dr. Schuster hypothesized that detection of *P. neurophilia* in the water corresponds to its life cycle. This approach enables early detection of pathogens. The nonlethal assay now is being integrated at ZIRC, where screening is being performed in different populations. The limit of detection was found to be lower than the minimum infection in larval fish. A multisite occupancy model was developed to evaluate relationships between habitat, sampling method, distribution, abundance, and overall detection. The group's findings suggest that spores are present frequently and sporadically, but often in low numbers.

The dynamics of detection were correlated with days post-exposure, suggesting that the detection of the parasite is dependent both on the system dynamics and life cycle. Diagnostic application results were consistent with those generated by the model. Dr. Schuster detailed current data collection efforts at ZIRC. His group has sampled 20-gallon, 5-gallon, and 1-gallon (sentinel) tanks. Only one positive *P. neurophilia* case was determined and confirmed by histopathology. Using dPCR, the group determined that an infection had been established in this tank.

The group now is expanding assays beyond *P. neurophilia* and plans to make the assays available to the research community. These efforts would promote in-house screening and lower costs of surveillance efforts. The dPCR system is more costly than other approaches but offers an avenue for environmental sampling. An assay for *P. tomentosa* has been published. In the future, ZIRC will move toward the development of multiplex assays. Dr. Schuster highlighted current data related to efforts in this area. The group also is interested in developing nonlethal assays to help in the facility's efforts toward specific-pathogen-free (SPF) animals.

Dr. Schuster also noted that automation of water sampling is being pursued to reduce sampling bias and time requirements. To accomplish this effort, an understanding of facility requirements and dynamics is needed. Increased throughput, ease of application, and various dynamics (e.g., flow rates, stocking density, racks) must be considered.

Discussion

- Dr. Michael Britt Williams asked about the water flow and availability of space for swimming. He also asked whether the observed swimming patterns were sustained over long periods. Dr. Varga affirmed that the patterns were sustained over 16 hours, and the pattern was established within 1 hour. He estimated that the tank had a volume of 4 liters and contained six fish.

- In response to a question about countermeasures, Dr. Murray explained that specific treatments have been investigated for *Pseudocapillaria*. For most other diseases, researchers focus on biosecurity and exclusion of pathogens. An understanding of the prevalence of different strains and their impacts on research are important. Decreasing stress also is beneficial. Dr. Tanguay added that multiple pathogen-related factors interact with one another.

Presentations: Aquatic Housing and Monitoring That Enhance Rigor and Reproducibility in Studies Using Aquatic Animals

An Attempt to Standardize the Approach to Microbiological Monitoring in Zebrafish Research Units

Gianpaolo Milite, D.V.M., M.Sc., Tecniplast

Dr. Gianpaolo Milite discussed approaches to monitoring the health and microbiological status of aquatic animals. He first outlined factors related to infections in aquatic animals, which increased susceptibility to subclinical infections, zoonotic diseases, and other infections, as well as to altered immune response, altered physiological response, altered research parameters, and increased contamination of transplantable tumors. By eliminating pathogens from a colony, researchers can understand the full scope of the effects of pathogens.

Dr. Milite clarified that infection is not synonymous with disease. Researchers no longer are concerned only with the health of the animal, but also with the organisms that infect animals with no clinical—or even pathological—effects. These organisms can still interfere with research. Additionally, the distinction between health monitoring and microbiological monitoring must be considered.

A working group within the Federation of European Laboratory Animal Science Associations (FELASA) completed a survey on species of fish used for research, methods of euthanasia, health monitoring, and biosecurity in Europe, North America, and Oceania. One-fourth of the responding facilities did not have a health monitoring system in place, and only a small fraction reported quarantine routines to ensure reliable biological barriers. Additionally, little consensus was observed among facilities in regard to biosecurity measures.

FELASA also published guidelines on the monitoring and reporting of diseases and health status in laboratory fish, as well as biosecurity in aquatic facilities. Approaches to health and microbiological monitoring of zebrafish include pre- or post-filtration sentinels, sump swabs, sludge analysis via microscopy or PCR, and direct investigation of sick fish (e.g., gross pathology, histopathology, PCR). Dr. Milite explained that to date, these analyses have not been standardized. Dr. Milite described his efforts to combine these methodologies. The aim of this work was to develop a standard device for key carriers (e.g., sludge, biofilm) of environmental microorganisms.

The InterZebTEC is a self-contained device capable of collecting debris from a large number of fish tanks while allowing the simultaneous development of biofilm. The InterZebTEC can perform over prolonged periods (i.e., weeks) to record a "video" of the microbiological condition. It is sensitive to the point of representing a true, reliable environmental monitoring device and is easy to install and remove. The overall goal of this effort was to develop a standardized sampling method.

Dr. Milite described PCR testing on InterZebTEC exposed over variable periods of 5 to 7 weeks over 12 months of screening. The main unit indicated strong modifications in terms of animal population, tank occupancy, gender, and age. The washing procedure of stock tanks was carried out routinely and occasionally was followed by autoclaving. PCR testing revealed detection of multiple microorganisms. Dr. Milite explained that the InterZebTEC also can be used for standardized bacteriological procedures.

The InterZebTEC can be used conveniently to simplify and standardize the environmental microbiological monitoring of aquatic units. This methodology, in combination with sampling of sick animals, leads to stronger monitoring results. Dr. Milite noted that the device could be used to determine reproducibility of experiments across different facilities, including among SPF versus non-SPF animals.

Monitoring and Recording Water Quality Parameters
Bobbi Baur, Aquaneering

Ms. Bobbi Baur discussed the use of water quality and environmental sensors in the support of rigor and reproducibility in animal research. She listed common parameters for water monitoring, which include temperature, pH, conductivity, nitrates, nitrites, and ammonia. Less common parameters include dissolved oxygen, total gas pressure, water hardness, alkalinity, and water flow. Ms. Bauer briefly outlined techniques for monitoring these parameters. She also noted the importance of considering what is actually being reported within facilities (e.g., location within the system of tanks).

Currently, no standard has been established for reporting water quality parameters. Ms. Bauer completed a review of recent publications. All publications reported the strain used, and most publications reported water temperature, light cycle, and pH. Some publications shared information related to conductivity, diet, density, tank size, water exchange rate, nitrates, nitrites, and ammonia.

Ms. Bauer explained that some parameters must be reported in studies, whereas others might be unnecessary. Water temperature affects activity and immunity, and pH and conductivity affect metabolism and osmoregulation. She emphasized the importance of a top-down reporting requirement (e.g., journals, funding agencies) for investigators.

Discussion

- Dr. Ekker responded to Ms. Bauer's comments about reporting requirements. He stated that needs for statistical reporting often differ among types of species and emphasized the importance of community input on these topics. Additionally, journals' instructions to authors should include a checklist of minimum guidelines in this area. He also noted the need to maintain a balance between fostering innovation and following core standards.

- In response to a comment from Ms. Alissa Hatfield, Dr. Milite stated that a joint working group of veterinarians in the United States and Europe representing the American Association for Laboratory Animal Science and FELASA recently published recommendations for health monitoring, reporting, biosecurity, and quarantine of aquatic laboratory species.

Group Discussion and Summary

- Dr. Varga commented on ongoing discussions related to standardization of feeding. He stated that a reference diet could help investigators better understand the nutritional requirements of zebrafish and other aquatic species. Dr. Tanguay noted that this topic might be outside the scope of the workshop but agreed that diet is an important factor in research. Dr. Varga pointed out that feeding can affect water quality. Dr. Tanguay agreed and noted that this effect is challenging but important to address. Dr. Wang added that automatic feeders can be helpful for standardization.

- Dr. Drummond remarked that some investigators are moving toward whole-genome analysis, which has become more affordable in recent years. He wondered whether the filters fitted onto the InterZebTEC could provide new insight into water quality. Dr. Milite stated that some laboratories are moving in this direction.

- Dr. Varga commented that genetic background is an important factor for variation. He wondered about ways to address the needs of investigators who do not have access to certain strains.

- Dr. Ekker pointed out that some microbial organisms are pathogenic to humans only in clinical settings; the same principle applies to animal facilities. He emphasized the importance of clearly defining pathogens in research. Calibration curves for facilities are needed. Dr. Drummond agreed, noting that all pathogens cannot reasonably be eliminated from a facility. The immune system is the most important factor. Metrics for determining innate immunity are needed.

- Dr. Allison Neely asked about approaches for a pilot study on tank density (e.g., length of time, number of fish, performance outcomes). Dr. Ekker explained that he was involved in the publication of a health and husbandry issue in *Zebrafish*. A survey of laboratories' current density practices was conducted, and a baseline was established. He noted that current practices could be determined in a follow-up survey. Additionally, scoring criteria are needed.

- Dr. Varga pointed out that facilities should report set points, as well as the actual range, because the fluctuations can differ among facilities.

- Dr. Ekker underscored the importance of measuring calcium levels and noted that new measurement tools are needed. Dr. Varga remarked that calcium hardness can be estimated based on pH and temperature.

- Dr. Tanguay commented that many common practices have been set arbitrarily within facilities, and setting new standards can be challenging. The NIH can play an important role in this effort. Reporting is the first step, but the ultimate goal must be standardization. Dr. Varga added that the Zebrafish Information Network has established committees for various topics and suggested that the workshop participants pursue a similar effort.

- Dr. Ekker highlighted opportunities through other workshops and relevant tools. The University of Minnesota has maintained a real-time sewer-monitoring system that detects SARS-CoV-2 and other pathogens. He was unaware of similar systems within animal research facilities. Opportunities for deployment of such technologies in research settings could be pursued.

- Dr. Varga noted that technologies for reverse osmosis filtration also have advanced in recent years. Engagement with vendors would be beneficial, and the group could provide guidance in this area to the scientific community.

- Dr. Landes asked about ways that journals can help investigators adhere to reporting guidelines (e.g., templates for metadata). Dr. Ekker noted that engagement with journal editors and scientific societies would be beneficial. A centralized approach, however, is needed.

- Dr. Williams pointed out that fish experience temperature fluctuations in natural environments. Dr. Varga agreed, noting that multiple parameters must be considered. He added that insight into variation is relevant from both scientific and health perspectives.

- Dr. Ekker noted that standardization of lighting (e.g., wavelengths) is challenging at his facility. He can monitor and report this parameter, but he has minimal control as an investigator. Dr. Tanguay noted that a better understanding of lighting could enable the development of standards in this area, and facilities would need to adapt to the new requirements. Dr. Ekker pointed out that smartphones can function as light meters. Dr. Williams added that most laboratories now use green exit lighting for dark cycles, rather than red lighting; this change was carried out in response to a study on the topic. Awareness of such findings is needed across the research community.

- Dr. Xiang-Ning Li asked about deficiencies in the monitoring, recording, and reporting process that should be addressed. Dr. Drummond reiterated the potential of whole-genome sequencing in gaining new insights (e.g., the cause of embryo deaths). Dr. Murray emphasized the importance of histopathology, particularly for diagnostic modalities. Dr. Ekker noted that artificial intelligence (AI), which is used for diagnosis of altered morphology in humans, could be applied in this area.

- Dr. Oleg Mirochnitchenko asked about approaches for monitoring physiological parameters. Dr. Ekker noted that behavioral changes often are the first sign of a physiological problem. Dr. Varga added that another marker is increased cortisol in the water. Dr. Tanguay noted that mass spectrometry can enable detection of water changes at high sensitivity. Dr. Mirochnitchenko suggested developing publications that focus on physiological outcomes.

- Dr. Alexander Wisner wondered about a video database of common behavioral phenotypes. He noted that these phenotypes are challenging to monitor in smaller laboratories. Dr. Varga was unaware of such a database but noted that standard operating procedures for physical and behavioral monitoring are available through ZIRC.

- Dr. Mirochnitchenko wondered about studies to characterize and monitor abnormal behavior. He noted that the Knockout Mouse Project (KOMP) is following individual mice to detect phenotype and uses some elements of AI. Dr. Ekker agreed that such an approach would be beneficial, but a validated algorithm must be developed. He added that a video tracking system also could be applied for monitoring density and codifying feeding regimens.

- Dr. Ekker spoke on the need for a repository dedicated to computer-aided design drawings associated with publications. Dr. Drummond suggested developing a subsection within an established resource. Dr. Varga noted that the NIH 3D Print Exchange has been established for this purpose. Ms. Bauer added that Aquaneering also provides a platform for 3D-printing resources. Dr. Ekker suggested conveying information about these resources to the scientific community.

- Dr. Varga emphasized the importance of considering the needs and physiological profiles of aquatic species beyond zebrafish. Dr. Ekker agreed, noting that husbandry requirements differ among species. Dr. Drummond noted that this topic can be discussed at organism-specific meetings. Dr. Ekker added that one of his colleagues is engaged in biomedical research using cephalopods, and the field is emerging.

- Dr. Ekker remarked that with the development of CRISPR genome editing, nearly any organism theoretically can become a model organism. For this reason, new model organisms likely will emerge, and various factors must be considered.

- Dr. Ekker wondered about topics of discussion among non-aquatic communities. Dr. Drummond highlighted efforts at The Jackson Laboratory (JAX) to monitor animals in the dark. Dr. Fox agreed, noting that this topic will be addressed in Session 2. Dr. Mirochnitchenko added that JAX is offering a short course on the application of machine learning to automated quantification of rodent behavior. This information could be applicable to other animals.

- Dr. Ekker also noted that air quality represents an additional consideration. Dr. Fox added that many laboratories and standard operating procedures have been designed primarily for mammalian use. Individual species' needs must be considered.

- Dr. Ekker encouraged the attendees to consider opportunities related to the monitoring of mutant animals. He suggested the development of a relevant toolbox for investigators.

- Dr. Wang asked whether ZIRC's data acquisition and monitoring systems can be applied to other facilities. Dr. Varga explained that the system is largely self-made, but most of the components were sourced through Aquaneering. All data, tools, and equipment can be made available to other facilities. The facilities would need to maintain their own software, however.

- In response to a follow-up question from Dr. Wang, Dr. Tanguay explained that her facility has a dedicated space for behavioral monitoring—from an experimental perspective—using custom algorithms. These systems are not yet in place at a tank level. She noted that tank-level monitoring is challenging in aquatic species, compared to other animals. Dr. Varga noted that his imaging system can capture 3D data to account for these differences. Dr. Tanguay responded that this system is difficult to apply at a large scale.

- Dr. Ekker added that commercialization of monitoring technologies is needed for standardization. Additionally, cloud-based solutions are needed. Dr. Fox also noted that filter devices, similar to those in murine systems, could provide monitoring service on a routine basis and at a reasonable cost to investigators. He added that not all investigators have access to the same resources for analysis.

- Dr. Ekker asked the participants to identify recommendations and next steps. He commented on the importance of community engagement and feedback. Dr. Fox noted that a new edition of the *Guide for the Care and Use of Laboratory Animals* is being developed. Standards for the design of aquatic facilities have been a topic of discussion in this effort. He agreed to provide contact information for the committee responsible for updating the guide. Dr. Ekker also emphasized the importance of considering accessibility of new technologies.

Session Wrap-Up and Adjournment

Dr. Wang thanked the speakers, organizers, and participants for their engagement during the meeting. He noted that the discussions encompassed multiple species and extrinsic factors, and further considerations will be needed. Dr. Wang encouraged the participants to register for Sessions 2 and 3. Dr. Li also thanked the participants and underscored the importance of monitoring and reporting extrinsic factors. Additionally, Dr. Li stated that managing extrinsic factors presents many challenges. He emphasized the importance of fostering community engagement and collaboration in this effort. Dr. Li adjourned the meeting.

Appendix A: Meeting Agenda

Session 1. Aquatic Animals
Virtual Meeting
September 23, 2022

12:10–12:30 p.m.	**Opening Remarks** *Robert W. Eisinger, Ph.D., Acting Director, Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director (OD), National Institutes of Health (NIH)* *James Fox, D.V.M., M.S., DACLAM, Workshop Chairperson, Massachusetts Institute of Technology* *Franziska Grieder, D.V.M., Ph.D., Director, Office of Research Infrastructure Programs (ORIP), DPCPSI, OD, NIH* *Guanghu (Jeff) Wang, Ph.D., M.B.A., Workshop Coordinator, ORIP, DPCPSI, OD, NIH*
12:30–1:20 p.m.	**Keynote Presentation: Shared Challenges and Needs of Diverse Aquatic Animal Models** *Iain Drummond, Ph.D., Mount Desert Island Biological Laboratory*
1:20–2:20 p.m.	**Presentations: Extrinsic Factors That Impact Rigor and Reproducibility in Studies Using Aquatic Animals** Lessons Learned from the Zebrafish International Resource Center (ZIRC) *Zoltan Varga, Ph.D., ZIRC* Review of Diseases and Impact on Research *Katy Murray, D.V.M., Ph.D., ZIRC* Developing Real-Time Pathogenic Testing in Aquatic Systems *Corbin Schuster, Ph.D., ZIRC*
2:20–2:40 p.m.	**Break**
2:40–3:40 p.m.	**Presentations: Aquatic Housing and Monitoring That Enhance Rigor and Reproducibility in Studies Using Aquatic Animals** An Attempt to Standardize the Approach to Microbiological Monitoring in Zebrafish Research Units *Gianpaolo Milite, D.V.M., M.Sc., Tecniplast* Monitoring and Recording Water Quality Parameters *Bobbi Baur, Aquaneering*
3:40–4:30 p.m.	**Group Discussion and Summary**
4:30–4:40 p.m.	**Session Wrap-up**
4:40 p.m.	**Adjournment**

Appendix B: Participants List

Session 1. Aquatic Animals
Virtual Meeting
September 23, 2022

Stephanie Achilles, The University of Alabama at Birmingham
Stefani Albrecht, The University of Kansas Medical Center
Ashrifa Ali, The University of Texas at Austin
Matthew Arnegard, Office of Research Infrastructure Programs (ORIP), Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director (OD), National Institutes of Health (NIH)
Tracie Baker, University of Florida
Ashley Barnes, ORIP, DPCPSI, OD, NIH
Bobbi Baur, Aquaneering
Christopher Bohince, ORIP, DPCPSI, OD, NIH
Samantha Brims, University of Southern California
Patricia Brown, Office of Laboratory Animal Welfare (OLAW), Office of Extramural Research (OER), OD, NIH
Sandra Buhl, Max Planck Institute for the Biology of Ageing
Angelica Cabrera, Bristol Myers Squibb
Jessie Carder, U.S. Department of Agriculture
Alexandra Ceurvorst, Mount Desert Island Biological Laboratory (MDIBL)
Susan Chandran, ORIP, DPCPSI, OD, NIH
Michael Chang, ORIP, DPCPSI, OD, NIH
Chris Chao, National Institute of General Medical Sciences (NIGMS), NIH
Megan Clark, OLAW, OER, OD, NIH
Mackenzie Connell, University of Florida
Miguel Contreras, ORIP, DPCPSI, OD, NIH
Devon Crawford, National Institute of Neurological Disorders and Stroke (NINDS), NIH
Janice Cui, University of Missouri
Subham Dasgupta, Clemson University
Erin Daugherity, Cornell University
Jami de Jesus, The University of Alabama at Birmingham
Jillian Dietrich, The University of Toledo
Iain Drummond, MDIBL
Samantha Earlywine, Nationwide Children's Hospital
Mark Eichelberg, American Physiological Society
Michael Eichner, Office of Research Services, Office of Management, OD, NIH
Robert W. Eisinger, DPCPSI, OD, NIH
Stephen Ekker, Mayo Clinic
Laverne Estanol, University of California, Santa Cruz
Jeetendra Eswaraka, Rutgers University
Ted Evans, Georgia Institute of Technology
Jeffrey Everitt, Duke University
Cynthia Faulk, The University of Texas at Austin
Logan Fehrenbach, Nationwide Children's Hospital
Cameron Fili, U.S. Food and Drug Administration
Aspen Foote, Boise State University
James Fox, Massachusetts Institute of Technology

Jennifer Fox, National Institute on Aging (NIA), NIH
Olga Franco, National Institute of Allergy and Infectious Diseases, NIH
Emily Franklin, Massachusetts Institute of Technology
Maria Fe Lanfranco Gallofre, NIA, NIH
Michael Garcia, Texas A&M University–Corpus Christi
Chelsea Garrison, Boise State University
Neera Gopee, OLAW, OER, OD, NIH
Vijay Kanth Govindharajan, Qatar University
Franziska Grieder, ORIP, DPCPSI, OD, NIH
Susan Harper, Inwood Animal Center
Alissa Hatfield, American Physiological Society
Nancy Hitt, NINDS, NIH
Tuan Hoang, Fluid Synchrony, LLC
Logan Holfelder, The University of Alabama at Birmingham
Marko Horb, Marine Biological Laboratory, The University of Chicago
Sandra Jablonski, Georgetown University
Glenn Jackson, Cornell University
Crystal Johnson, Georgetown University
Katherine Johnson, Boise State University
Cheol-Hee Kim, Chungnam National University
Kim Klukas, The Hormel Institute, University of Minnesota
Sailaja Koduri, NIGMS, NIH
Donna Kupniewski, Monell Chemical Senses Center
Erica Lachenauer, Rutgers University
Reid Landes, University of Arkansas for Medical Sciences
Chelsea Landon, Duke University
Kang-Han Lee, Chungnam National University
Karen Lencioni, California Institute of Technology
Xiang-Ning Li, ORIP, DPCPSI, OD, NIH
Sarah Long, Duke University
John Manker, Turner Scientific
Pierre Martineau, Martineau & Associates, Inc.
Maura McGrail, Iowa State University
Danel Medelbekova, Max Planck Institute for Biology of Ageing
Ana Melero, University of Valencia
Anne Merley, Brown University
Gianpaolo Milite, Tecniplast
Reginald Miller, Icahn School of Medicine at Mount Sinai
Yang Ming, University of Missouri
Dvir Mintz, Technion
Oleg Mirochnitchenko, ORIP, DPCPSI, OD, NIH
Jennifer Mitchell, MD Anderson Cancer Center
Elizabeth Moore, Cornell University
Rafael Moreno Gómez-Toledano, Universidad de Alcalá
Prayag Murawala, MDIBL
Stephanie Murphy, ORIP, DPCPSI, OD, NIH
Katy Murray, Zebrafish International Resource Center (ZIRC)
B. Natterson-Horowitz, Harvard University
Allison Neely, The University of Kansas Medical Center
Richard Noel, Georgia Institute of Technology
John Norton, Duke University

Albert Gris Oliver, August Pi i Sunyer Biomedical Research Institute
Payton Oswalt, University of Missouri
Annette Parks, Bloomington *Drosophila* Stock Center, Indiana University
Wuhong Pei, National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH
Mahesh Pillai, The University of Toledo
Gessica Piras, Università degli Studi di Cagliari
Larisa Poluektova, University of Nebraska Medical Center
Cate Pritchard, OLAW, OER, OD, NIH
Michael Pryor, Vanderbilt University Medical Center
Reza Raeisossadati, The University Texas at Austin
Gregory Reinhard, University of Pennsylvania
Charles Sassine, Texas A&M University–Corpus Christi
John Scarpa, Texas A&M University–Corpus Christi
Caroline Schomer, The University of Texas at Austin
Corbin Schuster, ZIRC
Anna Skorupski, University of Pittsburgh
Heather Smith, Office of Animal Care and Use, Office of Intramural Research, OD, NIH
Jeff Stanton, Oregon National Primate Research Center
Christine Steinke, Scripps Institution of Oceanography
Eric Stone, Aquatic Enterprises, Inc.
Xiaoping Sun, NIA, NIH
Debra Szczepanski, The University of Texas at Austin
Robyn Tanguay, Oregon State University
Ginger Tansey, National Eye Institute, NIH
Nick Tataryn, Vanderbilt University Medical Center
Biao Tian, ORIP, DPCPSI, OD, NIH
Elizabeth Tobey, National Agricultural Library
Drew Townsend, National Institute on Drug Abuse, NIH
Hung-Chi Tu, University of California, San Diego
Jacquelyn Tubbs, OLAW, OER, OD, NIH
Zoltan Varga, ZIRC
Tyara Vazquez, The University of Toledo
Daniel Vinci, Aquatic Enterprises, Inc.
Jayalakshmi Viswanathan, NIA, NIH
David Volz, University of California, Riverside
S. Randal Voss, *Ambystoma* Genetic Stock Center, University of Kentucky
Guanghu (Jeff) Wang, ORIP, DPCPSI, OD, NIH
Stephen Watts, The University of Alabama at Birmingham
David Wiest, Fox Chase Cancer Center
Michael Britt Williams, The University of Alabama at Birmingham
Alexander Wisner, The University of Toledo
Michael Wisnieski, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH
Kim Woodard, Georgia Institute of Technology
Dan Xi, National Cancer Institute, NIH
Jianhua Xu, NIGMS, NIH
Phil Zerofski, Scripps Institution of Oceanography
Sige Zou, ORIP, DPCPSI, OD, NIH