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Rigor and Reproducibility of Animal Studies:

Extrinsic Factors Workshop






September 23, 28, and 30, 2022

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U.S. Department of Health and Human Services
National Institutes of Health
Division of Program Coordination, Planning, and Strategic Initiatives
Office of Research Infrastructure Programs

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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Stephen Ekker, Ph.D., Mayo Clinic
Robyn Tanguay, Ph.D., Oregon State University

Presenters
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
Zoltan Varga, 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)
James Fox, D.V.M., M.S., DACLAM, Workshop Chairperson, Massachusetts Institute of Technology
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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
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John Scarpa, Texas A&M University–Corpus Christi
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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

U.S. Department of Health and Human Services
National Institutes of Health
Division of Program Coordination, Planning, and Strategic Initiatives
Office of Research Infrastructure Programs

Rigor and Reproducibility of Animal Studies: Extrinsic Factors Workshop

Session 2. Rodents

September 28, 2022
Virtual Meeting

Final Report

Table of Contents

Executive Summary

The Extrinsic Factors Workshop was held in three sessions to better understand extrinsic factors and their effects on biomedical research. Session 2 was focused on extrinsic factors in the use of rodent animals for biomedical research. Drs. Elizabeth Bryda, James Fox, and David Wiest served as the Session 2 co-chairs. Discussions in Session 2 addressed the effects of housing environment, equipment modernization, and new and emerging monitoring methods in addressing the need for rigor and reproducibility in rodent research. The speakers identified various extrinsic factors for consideration in research, including personnel, caging type, density, thermoregulation, food and water, bedding, enrichment, cage-change frequency, species-specific measures of behavior, the microbiome, housing density, lighting (e.g., quantity, spectral quality, duration), vibration, and air. The participants also discussed the need to balance energy-saving measures (e.g., retrofitting of light-emitting diode lighting) with scientific needs within facilities. In discussion, several participants noted that extrinsic factors in animal research never will be standardized fully across institutions, because some external variables always will be present. Additionally, it was proposed that variation within animal studies might better represent the biological systems of humans. The need for increased federal support on this topic, as well as for collaborations across both facilities and communities, was emphasized throughout the discussion.

Session Co-Chairs
Elizabeth Bryda, Ph.D., University of Missouri
James Fox, D.V.M., M.S., DACLAM, Massachusetts Institute of Technology
David Wiest, Ph.D., Fox Chase Cancer Center

Presenters
Brian Berridge, D.V.M., Ph.D., DACVP, National Institute of Environmental Health Sciences
George Brainard, Ph.D., Thomas Jefferson University
Jeffrey Everitt, D.V.M., Duke University
Mitchell Galanek, Radiation Protection Officer, Massachusetts Institute of Technology
F. Claire Hankenson, D.V.M., M.S., DACLAM, University of Pennsylvania
Ken Henderson, Ph.D., Charles River Laboratories
Vivek Kumar, Ph.D., The Jackson Laboratory (JAX)
Neil Lipman, V.M.D., Memorial Sloan Kettering Cancer Center
Steve Niemi, D.V.M., Boston University
Randall Reynolds, D.V.M., M.S., Duke University
Karen Svenson, Ph.D., JAX

Workshop Organizing Committee

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Guanghu (Jeff) Wang, Ph.D., M.B.A., Workshop Coordinator, 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)

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Workshop Report

Opening Remarks

James Fox, D.V.M., M.S., DACLAM, Workshop Chairperson, Massachusetts Institute of Technology
Xiang-Ning Li, M.D., Ph.D., 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)
Oleg Mirochnitchenko, Ph.D., ORIP, DPCPSI, OD, NIH

Dr. Xiang-Ning Li welcomed the attendees to Session 2 of the workshop. Dr. Li reminded the participants of NIH's dedication to rigor and reproducibility, which was emphasized by Dr. Robert W. Eisinger, Acting Director, DPCPSI, during Session 1. In 2021, the Advisory Committee to the NIH Director (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. Li also reminded the participants that ORIP has long devoted efforts to enhancing rigor and reproducibility, as emphasized by Dr. Franziska Grieder, Director, ORIP, during Session 1. ORIP has supported this effort through scientific research 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 publications of future funding opportunity announcements (e.g., NOT-OD-22-039). This workshop is one of several steps toward fulfilling ORIP's Strategic Plan by addressing the important endeavor of enhancing animal study rigor and reproducibility in NIH-supported research.

Dr. Oleg Mirochnitchenko 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. Mirochnitchenko 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. Mirochnitchenko expressed appreciation to the organizing committee members, speakers, and participants for their engagement.

Dr. James Fox, Workshop Chairperson and Session 2 Co-Chair, previewed Session 3, which will focus on large animals (i.e., nonhuman primates and swine). He emphasized that the topic of extrinsic factors is highly relevant to biomedical research, both for investigators and vivarium staff members. Dr. Fox also introduced Drs. Elizabeth Bryda and David Wiest, Session 2 Co-Chairs.

Keynote Presentation: Impact of Extrinsic Factors on Rigor and Reproducibility in Rodent Research

F. Claire Hankenson, D.V.M., M.S., University of Pennsylvania

Dr. F. Claire Hankenson presented on the ways extrinsic factors (as defined by the NIH Working Group) can affect rigor and reproducibility in rodent research. She emphasized the importance of explicit experimental planning to better control for these variables but noted that doing so has proven challenging. Many investigators have demonstrated improvement of reproducibility by enhancing external validity of

results. Dr. Hankenson also noted that the ACD Working Group was composed of various members of the research community, including research scientists, journal editors, statisticians and two veterinarians with expertise in a wide variety of animal models, as well as members of the internal NIH community.

Dr. Hankenson clarified the distinctions between reproducibility (i.e., getting consistent or duplicated results when starting from the same materials), replicability (i.e., getting consistent or duplicated results when using the same procedures or asking the same scientific question, but collecting new data), and generalizability (i.e., applying the results of a study in other contexts, situations, and populations). The Working Group was asked to consider various questions related to reproducibility, including what analyses can be performed to identify gaps and how the conditions in which animals are housed and bred affect experimental outcomes.

The Working Group identified the following extrinsic factors that can affect reproducibility: caging type, density, thermoregulation, food sources, bedding, water type, enrichment options, handling, cage-change frequency, species-specific measures of behavior, the microbiome, and refinements in care and well-being. Most of these factors are being tracked within animal facilities by animal care staff, but these data rarely are requested by scientific groups. Coordination between the veterinary and scientific communities therefore is needed. A review of relevant publications by members of the NIH Working Group also was performed and encompassed discussion on various species (rodents as well as larger animals and nonhuman primates), sex as a biological variable, neuroscience models, and statistical applications.

In its final report, the Working Group identified five themes in obtaining reproducible results for animal research: (1) improve study design and analytic rigor; (2) address bias, incomplete reporting, and questionable research practices; (3) improve relevance and use of animal models; (4) improve methodologic and results reporting; and (5) measure and evaluate effectiveness and costs. The Working Group expressed a shared foundational agreement, supported by the NIH Director, that animal studies contribute to significant findings and breakthroughs in both basic and translational research. Motivating problems that affect reproducibility were identified. First, transparent reporting of research methods is essential, yet frequent failures and shortfalls are present. Additionally, failure to record and report these factors degrades the reproducibility of findings. Furthermore, the completeness and granularity of reporting on animal husbandry factors is a quality issue and a topic for future research.

Dr. Hankenson highlighted several recommendations contained within the report's fourth theme. First, the NIH should expect that supporting data reported on animal research submitted in support of grant applications will include measures of quality and/or uncertainty for reported estimates and an interpretation of effect sizes within the context of the field. Additionally, the NIH should expect all vertebrate and cephalopod animal research to include the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) 2.0 Essential 10 Checklist at the publication stage. Furthermore, the NIH should work to understand, monitor, record, and report important extrinsic factors related to animal care that can impact research results. In the report, the Working Group emphasized the value of open-source methods for sharing findings and data. Methods reproducibility is dependent on transparency, and inferential reproducibility relates to the concept of generalizability.

Dr. Hankenson emphasized that more discussion on extrinsic factors is needed. Recent studies have demonstrated the complexity of this issue. Factors for consideration include age, feeding, replications, behavioral assessments, housing conditions, the 3Rs (i.e., replacement, reduction, and refinement) and 3Vs (i.e., construct validity, internal validity, and external validity) of animal research, and therioepistemology. She asserted that the limits of reproducibility are not violations of the 3Rs. Sound and reproducible science ultimately affects one or more of the 3Rs and might affect investigators' abilities to conduct appropriate cost–benefit analyses if work must be repeated with additional animals. Good study design and good data analyses, however, also are essential from an ethical standpoint.

Dr. Hankenson also remarked that limits of reproducibility permit scientific study. Investigators still are learning how extrinsic factors affect research results, and this topic has emerged as its own discipline in recent years. Animal housing, handling, and husbandry will never be standardized fully across institutions, because other external factors (e.g., personnel, building and facility age, HVAC, weather/seasonal changes) always will be present. Additionally, human conditions of disease are not standardized as they are studied; it is unrealistic to expect that animal conditions be identical.
Dr. Hankenson encouraged the participants to consider the concept of reproducibility of scientific ideas and conclusions, rather than reproducibility of research. An acceptable level of research variability—one that permits trust in experimental outcomes—must be determined. She emphasized that this topic requires engagement of both veterinary specialists and researchers.

Discussion

- Ms. Karli Gilbert highlighted a recent paper indicating that the sex of experimenters has significant and consistent effects on mouse behavior across different laboratories. She wondered about efforts to include this variable in reports. Dr. Hankenson responded that this variable can be reported but will never be controllable. Researchers will never be able to account for every extrinsic factor. In response to a follow-up comment from Dr. Fox, Dr. Hankenson recalled that the study accounted for the sex of the experimenters but not the animal handlers.

- Dr. Reid Landes asked about the value of a laboratory's purposefully increasing the variability of extrinsic factors in experiments so that the controlled factors can be more robust. Dr. Hankenson spoke on the value of repeating experiments and considering how the variability of extrinsic factors affects the application of outcomes.

- Dr. Brianna Gaskill remarked on the importance of NIH-funded work to examine experimental variables, rather than simply reporting them. Dr. Hankenson agreed and noted that investigators bring unique perspectives on these factors into the discussion.

- Dr. David Ashbrook raised the need for support of researchers to use multiple genetic strains, thereby increasing genetic diversity in their studies. Dr. Peter Nathanielsz reiterated the importance of considering these differences in human and animal studies, particularly in regard to pregnancy studies. Dr. Richard Nakamura suggested consulting experts on the ethology of the experimental animals, in the context of the species in the wild.

- Dr. William Gause highlighted recent studies suggesting laboratory housing conditions might not reflect the environment in which mice evolved to live. For certain experiments, researchers might consider housing mice under more natural conditions.

- A participant noted that incorporation of more variables increases the cost of performing research, and NIH grant budgets are limited. This limitation is likely to affect experimental design and reproducibility.

Presentations: Housing Environment That Impacts Rigor and Reproducibility in Studies Using Rodents

Effects of Increased Housing Density in Research Mice

Karen Svenson, Ph.D., The Jackson Laboratory (JAX)

Dr. Karen Svenson discussed the physiological effects of housing density in mice. She began by sharing a brief history of the *Guide for the Care and Use of Laboratory Animals*. The eighth edition of the *Guide*, published in 2011, included a recommendation to limit cages with breeding females to 51 square inches.

This guidance suggests culling litters to accommodate the recommendation in practice and appears to eliminate the possibility of using a trio breeding format, which is a common practice in research facilities. Dr. Svenson also noted that the seventh edition of the *Guide*, published in 1996, encouraged animal studies based on sound science to further define institute-specific guidelines.

Dr. Svenson presented a schematic of duplex cage setups at JAX, explaining that the layout ensures compliance with current *Guide* recommendations for housing density. A team of JAX scientists designed a study to characterize the changes in well-being that occur with increased housing density in mice. They also investigated ways to measure well-being in studies to assess such effects. They increased cage density using two approaches: (1) increasing group size and maintaining a single cage size or (2) maintaining group size and using smaller, variable caged. Mouse "clinics" were used to perform broad-based live animal phenotyping via an internally adopted strategy for assessing multisystem physiology.

Physiological effects of housing density on C57B/6J mice were assessed over a 9-month period. Mice were housed in groups of either five or nine animals per cage. The researchers concluded that in B6 mice, housing at twofold density had no measurable adverse effects; in fact, heart rate and adrenal weight were reduced in the higher-density group for both sexes. Cage air temperature and quality were measured in the study, and the frequency of cage changing (i.e., 1 week vs. 2 weeks) was assessed. The higher-density cages were about 3˚C warmer than the lower-density cages and were closer to the animals' thermoneutral zone. Additionally, the animals consumed less food. Humidity and carbon dioxide did not differ with density. Follow-up density studies at JAX did not detect measurable adverse effects at any density. The research also examined litter culling, which did not lead to improved health.

Studies performed by other groups have contributed to a growing body of evidence indicating that most mouse strains can be housed at higher densities than is recommended currently by the *Guide* and maintain good health. Dr. Svenson noted that several relevant literature reviews have been performed. She identified remaining gaps in this area, which include effects of lower densities (e.g., comparing one, two, three, or four animals per cage), additional studies in static cages, and use of outbred strains. Dr. Svenson concluded by emphasizing that housing density is an important extrinsic factor, and various components (e.g., number of cages, type of cage ventilation, range of densities, single-animal housing, management of cage attrition, use of enrichment) should be reported in research.

Minimizing the Impact of Habitat Lighting on Experimental Reproducibility for Rodent Studies
George Brainard, Ph.D., Thomas Jefferson University

Dr. George Brainard presented on the influence of lighting in rodent research, with a focus on wavelength. He first outlined physical parameters of photic input: quantity (e.g., light irradiance, illuminance), spectral quality (i.e., wavelength), timing, and duration. Systemic effects of wavelength include changes to circadian behavior, testicular weights, accessory sex organ weights, spleen and thymus weights, lymphocyte counts, pineal melatonin, pituitary prolactin, pituitary hormones, plasma triiodothyronine and thyroxine, and plasma testosterone.

A profound difference is present in the wavelengths that influence the visual system, compared to those that influence rodent behavior and physiology. Melanopsin, a protein found in 1–3% of ganglion cells in the retina, provides the cells direct photosensitivity, allowing them to influence a wide range of physiological processes. Dr. Brainard highlighted a study comparing fluorescence and solid-state lighting in animal facilities. Dr. Brainard noted that light-emitting diode (LED) lighting offers several benefits over fluorescent lighting. The two lighting types cover a similar spectrum but display differences in peak patterns, including at the wavelength of melanopsin sensitivity. In the study, the only variable was spectral characteristics of the two light sources.

Changes in lighting affected both melanopsin content and melatonin rhythm. C3H mice maintained in LED lighting showed reduced food and water consumption and grew at rates representative of a more youthful phenotype. Neurohormonal changes also were observed. These factors are associated with the promotion of animal health and well-being and therefore might influence scientific outcomes.
Dr. Brainard emphasized that numerous scientific opportunities exist in this area, and this topic must be considered by investigators as lighting systems are retrofitted in the future.

Vibration as an Extrinsic Variable for Research Outcomes
Randall Reynolds, D.V.M., M.S., Duke University

Dr. Randall Reynolds discussed the effects of vibration on experimental outcomes. He explained that vibration can serve as a general stressor for animals. Effects can include changes to reproductive parameters (e.g., nursing in mice); increased heart rate and mean arterial pressure in mice; increased stress hormones in mice, rats, and swine; startle response and fear-related behaviors in mice, swine, and poultry; and changes in brain neuroendocrine levels and vascular reactive oxygen species in rats.

Dr. Reynolds introduced the concepts of vibration in relation to waveform, directionality, and resonance frequency. He highlighted data reflecting the principle of resonance frequency in mice's startle response to vibration. The results provided insight into the range of vibration frequency in mice. Dr. Reynolds noted that secondary harmonic frequency ranges also must be considered. Other important principles of vibration include sound-induced vibration, periodicity, and habituation. Sounds produced in the environment can cause another object to vibrate if the frequency of sound matches the object's resonance frequency. Periodic vibration might be more problematic than constant vibration, and responses to repeated episodes may decrease over time.

Options for vibration control include cork, rubber, springs, and synthetic materials (e.g., polyvinyl chloride sheets, polyurethane foam). Methods for passive control include reducing the magnitude of vibration at the object's resonance frequency (i.e., damping) and changing the vibration frequency to which an object is exposed away from its resonance frequency (i.e., isolation). Dr. Reynolds outlined approaches to control vibration during construction-related activities and on a routine basis. He listed four elements (i.e., administrative, procedural, equipment, and engineering) of a construction-based sound- and vibration-control plan for construction and considerations for minimizing vibration that is inherent within a laboratory animal environment (e.g.,, equipment, housing location, husbandry procedures, transportation).

Administrative actions include developing a plan of action with the construction company and coordinating with research investigators. Procedural actions include premanufacturing ducting, pipes, and other materials in as large dimensions as possible off the job site and performing activities that produce more sound and vibration during non-business hours. Equipment-related actions include removing cinder block walls with power tools, rather than a sledgehammer, and removing vinyl tiles with power machines instead of scrapers and chisel bits. Engineering-related actions include using barriers and screens to block the direct path of sound and using rubber mats on the floor during demolition.

Equipment-related actions related to minimizing vibration that is inherent to facilities include employing low-vibration-producing equipment and ensuring continued maintenance of the equipment and physical plant. Actions related to housing location include housing larger species, which generate more noise, away from more sensitive species and housing breeding rodents away from the cage-wash area, autoclaves, and elevators. Husbandry-related actions include educating employees and addressing high-impact activities in the facility that can cause vibration. Transportation-related actions can include using towels on large carts or carrying by hand. Dr. Reynolds noted that vibration during transportation was found to be significant, even when using these minimizing approaches.

Dr. Reynolds spoke on the need for standardization of research and reporting of vibration. Frequencies should be tested near the animal's resonance frequency, and the magnitude of vibration used should be limited to what is within reason for the environment when studying the effects of vibration. Additionally, the effects of sound should be controlled when studying vibration. He also highlighted the *Reporting Guidelines for Whole-Body Vibration Studies in Humans, Animals, and Cell Cultures*, which lists 24 factors for consideration on this topic. Gaps in vibration research include more precise minimal magnitudes and frequencies that cause physiological and behavioral changes; magnitudes, frequencies, and periodicity for habituation; differential effects of vibration in *x, y,* and *z* directions; design criteria that prevent resonant and harmonic frequencies from affecting animals; additional studies on the magnitude and frequency of vibrations produced during construction and their associated effects on animals; and transportation methods to mitigate vibration.

Dr. Reynolds concluded by emphasizing that vibration is an important extrinsic variable in animal studies. Sensitivity to vibration differs among species and is dependent on the frequency of vibration. The resonance and harmonic frequencies both must be considered. A comprehensive vibration- and sound-mitigation plan is essential for construction activities. He also encouraged the participants to consult with knowledgeable engineers during facility planning and demolition/construction phases. The *NIH Design Requirements Manual* is an important resource, as are previous studies.

Environmentally Associated Lesions in Rodent Toxicology Studies
Jeffrey Everitt, D.V.M., Duke University

Dr. Jeffrey Everitt presented on lesions associated with environmental factors in rodent toxicology studies. He began by asserting that toxicology studies often serve as an exemplar for rigor and reproducibility in rodent studies. Studies often are repeated in the same facilities and with identical test systems. Additionally, the studies often employ relatively large groups of rodents with robust data-capture systems in place. Quality systems are employed for safety studies in the regulatory environment, and methods and endpoints are well established. Furthermore, standard nomenclature for lesions also has been established. Comparative pathologists are experts in working with animal models, and they spend much of their time distinguishing between treatment effects and extrinsic effects. The rigor and reproducibility of pathology data in academic studies, however, often are lacking.

Numerous extrinsic factors can lead to lesions in rodents. Major factors include air, housing, and diet. Rodents are obligate nasal breathers, and the nasal cavity is known to be affected by the environment. Additionally, the olfactory mucosa shows high metabolic activity. Effects of olfaction can extend to numerous endpoints, including neurobehavior. Dr. Everitt presented data suggesting the effects of cage changing on nasal lesion development. Volatile pollutants from soiled bedding can affect the development of lesions in the rat nasal cavity; these effects could not be attributed solely to the high presence of ammonia. He emphasized that further investigation in this area is needed.

Dr. Everitt briefly highlighted other examples of extrinsic factors that affect lesions. Obstructive genitourinary lesions in mice have been shown to be modulated by housing. These effects are also genotype dependent. Wire caging influences the development of dermal tumors in transgenic mouse models for carcinogenicity. Diet also represents a complex issue in this area that encompasses numerous factors, including chemical contaminants, nutrient content, form of diet, feeding methods, storage conditions, natural versus chemical ingredients, and open versus closed formula. He underscored the importance of using data from multiple laboratories to understand variables in experiments.

Historical pathology databases can contribute to understanding the robustness and reproducibility of rodent studies. Best practices have been established in this area in the toxicologic pathology community for sampling histopathology and rodent organs. Dr. Everitt emphasized that such standardized approaches should be established within the animal modeling community. Additionally, historical databases must be

treated as living documents with standard nomenclature and multi-facility data. He also noted that many investigators have written papers with less-than-optimal generation of pathology data in academic research.

Dr. Everitt encouraged ORIP to consider the question of quality and rigor of histopathology, and pathology in general, in NIH-funded studies and to foster best practices that can be better standardized across institutions. He listed challenges in this area, which include lack of best practices approaches and peer review in academia, cost, and limited infrastructure and access. He emphasized that the NIH could build pathology infrastructure similar to that within the toxicologic pathology community. This would include a robust community of animal model pathologists with common interest in rodent model best practices, necropsy, and pathology protocols; utilization of databases that incorporate digital pathology tools; and using digital imaging tools to move from qualitative to quantitative assessment of animal models pathology.

Discussion

- Dr. Amy Keller remarked that her laboratory has noted significant vascular physiological differences of rats housed at thermoneutrality, compared with those housed at human room temperature. Dr. Svenson noted research indicating that mice can mount an immune response to tumor invasion more readily at thermoneutrality. Dr. Svenson also emphasized that dedicated funding is needed for robust studies of extrinsic factors. She added that these efforts can provide insight when interpreting study results.

- In response to a question from Dr. Emily Franklin, Dr. Svenson confirmed that the density studies were performed in individually vented caging.

- Dr. Brainard clarified that cage light density was kept equivalent for each of the racks, with no housing on the top row. He emphasized the importance of considering rack design and location in studies.

- Ms. Kerith Coulson asked about ultrasound comparisons between lighting systems. She commented that fluorescent ballasts are thought to create more ultrasound and therefore might contribute to another extrinsic factor in addition to light wavelength. Dr. Brainard agreed to examine this question further. He added that flicker of light also should be considered, particularly in regard to variation among commercial products.

- Dr. Gaskill wondered how to account for ultraviolet wavelengths that can be seen by mice. Dr. Brainard explained that a rodent-based toolbox is used for calculating alpha-optic values. He agreed that this could be factor in experimental results but noted that the specified fluorescent lights emit little ultraviolet light.

- Dr. Vivek Kumar noted that most rodents live in amber boxes that are fitted with a filter, and the boxes tend to wear over time. He asked whether the filtration of light is being considered. Dr. Brainard responded that several experiments on this question have been performed.

- Dr. Landes noted that if all animals in a cage are part of the same experimental group, the cage inadvertently becomes the "experimental unit" and thus reduces the power of a study if any cage-to-cage variability is present.

- Dr. Miguel Contreras shared a publication demonstrating immune and inflammatory genetic responses to fluorescent light in vertebrate organs.

- Dr. Enrico Radaelli shared a publication on the reproducibility of histopathological findings in experimental pathology of the mouse.

Presentations: Equipment Modernization That Enhances Rigor and Reproducibility in Studies Using Rodents

New Methods for Performing Irradiation in Rodents

Mitchell Galanek, Radiation Protection Officer, Massachusetts Institute of Technology

Mr. Mitchell Galanek discussed the differences between cesium- and X-ray-based systems. He explained that isotope-based irradiators have been the workhorse of animal and cell irradiations for the past 50 years. Low-dose irradiators typically are based in cesium 137, which has a half-life of 30 years. Irradiators can function for decades with minimal maintenance. Advantages of the cesium-based irradiator include the mono-energetic gamma ray, reproducible dose rates, historical data on animal models, ease of use, low maintenance requirements, and safety. Disadvantages of the cesium-based irradiator include the non-collimated field, difficulty of shielding unwanted exposures to experimental animals, facility and researcher security requirements, and cost of final disposal.

In recent years, the U.S. government has encouraged laboratories to consider X-ray-based irradiators. Advantages of the X-ray-based irradiator include the monodirectional beam, collimated beam, lack of facility and researcher security requirements, safety, and capability for X-ray and bioluminescence imaging. Disadvantages of the X-ray-based irradiator include the lack of a monoenergetic beam; preventive maintenance requirements and costs; mechanical reliability; heat generation; and lifetime of X-ray tubes, which are expensive to replace.

Mr. Galanek shared several users' perspectives on the cesium- and X-ray-based systems. The users expressed that the cesium irradiator requires less training and minimal power consumption; the system works well for whole- and partial-body irradiation in rodents, as well as in vitro studies. Good dose homogeneity and dose rate were noted. The system mimics clinical radiation therapy and can allow reparable DNA damage. The cesium irradiator was perceived, however, to be less safe, and targeted irradiation is difficult to perform in animals. Additionally, expensive source exchanges may be required for older irradiators. units. Decommissioning and security requirements were a concern to users, as well as shielding requirements and continuing source decay.

The users also expressed that the X-ray irradiator is safe, with a tunable dose rate, and can be used easily to perform targeted irradiation in animals. The treatment area and platform height can be controlled, and cameras allow direct visualization. Additionally, energies are clinically relevant. The X-ray irradiator, however, requires more training, and the radiation energy is lower than clinical relevance. One user noted differences between moderate- and low-energy X-ray systems, remarking that a graded filter offers a reasonable option of whole- and partial-body irradiations. It was also noted, however, that extra filtration lowers the dose rate. Extra dosing works well for irradiating cells, but not for whole- or partial-body irradiations.

The U.S. Department of Energy Office of Radiological Security is sponsoring efforts to move toward X-ray-based irradiators. The Cesium Irradiator Replacement Program (CIRP) provides financial incentives to replace Cs-based irradiators with X-ray based systems. Mr. Galanek shared a case example of the removal of a cesium-based system at the University of Washington; the cesium source could not be removed from the shield plug, and the methods employed to remove the source led to widespread radioactive contamination in the immediate work area. Ten individuals were found to have skin contamination and were decontaminated by the first responders. All individuals were monitored for both external and internal radiation exposure. The highest internal dose was 70 millirems and the highest

external exposure was 55 millirems. He stated that the resulting decontamination project resulting from the source handling mistake was highly costly. Since then, the removal process has been changed so that the cesium source is not removed onsite, the irradiator is packaged and shipped as the entire unit.

Mr. Galanek concluded by posing the question of whether facilities should continue to use cesium-based irradiation systems. He answered that the Cs-based systems should be kept if the research warrants the use of these tools. A combination of cesium- and X-ray-based systems likely is the best solution.

Enhancing Animal Study Translation: Physiological Monitoring as a Key Contribution
Brian Berridge, D.V.M., Ph.D., DACVP, National Institute of Environmental Health Sciences

Dr. Brian Berridge discussed physiological monitoring in the context of understanding extrinsic factors as a key contribution to improving the translational relevance of animal studies. He began by remarking that animal studies are an important translational modeling platform used to support the full spectrum of exploratory to confirmatory biomedical research interests, where rodents are the predominant species used. These uses can include targeting and validation, hit and lead discovery and optimization, candidate selection, preclinical safety studies, and clinical assessment. He emphasized that the translational process presents multiple challenges, and success rates vary across therapeutic areas. Clinical experience can provide insight into translational weaknesses.

Reproducibility is an ongoing challenge in research. Three primary challenges in this area are reporting standards (e.g., insufficient experimental details to replicate study conditions), study design and conduct (e.g., bias, insufficient statistical power, technical consistency), and biology (e.g., natural validity, comparative relevance to humans). Dr. Berridge emphasized that more work is needed in the context of biological challenges (i.e., external validity). Based on these factors, the ACD Working Group recommended enhancing training in animal study design, improving access to statisticians, enhancing peer review of study plans, increasing expectations for describing animal study plans in grant applications, applying ARRIVE guidelines for reporting, improving rationalization for animal model selection, registering animal study plans, increasing funding for large-animal models, improving understanding and reporting of external factors, and assessing costs of these increased expectations.

Dr. Berridge remarked that animals can model important anatomic, functional, and mechanistic features of the human condition, but numerous differences between animal models and humans are present. These differences should be considered in model selection and study design. Environmental effects also must be considered. He noted that general health checks are standard in clinical care but typically are not monitored in the context of animal research. These technologies have been developed but often are not applied. He also highlighted the importance of monitoring behavior as a translational physiologic endpoint; new technologies are expanding capabilities in this area. Dr. Berridge briefly highlighted opportunities for monitoring physiologic and behavior endpoints in research.

In summary, Dr. Berridge encouraged investigators to think of their animals as the patients that they are intended to represent. He emphasized that animals will never be a perfect surrogate for patients, but translational relevance can be improved through more human context. Organ system function is a clinically important context for morphologic and molecular endpoints and measures. Additionally, technology solutions provide an opportunity to improve the human relevance of animal studies, as well as to optimize care and welfare.

Smart Cages Require Smart Management
Steve Niemi, D.V.M., Boston University

Dr. Steve Niemi spoke on the need for smart management of smart cages. He defined a smart cage as equipped to monitor various intra-cage parameters digitally and continuously, and inform personnel about

the status of those parameters remotely. Therefore, smart cages can provide researchers continuous information on the status of housed mice and represent a new generation of large-scale housing. Dr. Niemi began by explaining that mice must be observed at least once daily in accordance with regulatory requirements and good quality care. This practice can be challenging for institutions maintaining thousands of rodent cages daily. He presented data from an anonymous program indicating that most rodent health concerns were reported on weekdays, i.e., when the technical team was fully staffed, versus fewer health concerns reported on weekends and holidays when skeleton crews were used which indicated a need for more effective routine monitoring for better animal welfare especially during times when fewer personnel are on site. He hypothesized that similar effects might have occurred during the beginning of the COVID-19 pandemic when staff access to facilities was highly restricted.

Many options for housing are now available to researchers, including new platforms that can provide after-hours alerts of changes in intra-cage conditions, such as flooding, excess ammonia levels, unwanted temperature excursions, and animal activity. These capabilities address the need to inform staff and make these options more accessible to investigators. Rather than replace an institution's entire rodent caging inventory, Dr. Niemi proposed the use of smart caging on a limited scale for monitoring post-operative recovery and pain management, severe endpoints, difficult breeders, and hostile cage mates. Other opportunities include pilot studies to assess the effects of various factors (e.g., bedding, enrichment, room environment, housing density) on behavior and activity, staff and investigator training, and troubleshooting (e.g., suspect environmental controls, environmental disturbances).

Dr. Niemi also envisioned other "smart" cage accessories, such as food hoppers and water bottles that would monitor and broadcast if and how fast their contents were emptying. These enhancements could help researchers determine or confirm consumption of critical experimental components, such as medicated food or drinking water, and adequate agitations of chemical suspensions. He emphasized the importance of fostering collaborations between investigators and lab animal program managers to explore other benefits.

Highly Scalable and Reproducible Preclinical Rodent Behavioral Assays Using Machine Vision
Vivek Kumar, Ph.D., JAX

Dr. Kumar presented on the development of preclinical rodent behavioral assays using machine vision. He emphasized the critical need for new psychiatric treatments and better preclinical animal models, particularly rodent models. He explained that many current behavioral tests have low reproducibility and throughput. His work is focused on improving reproducibility by developing approaches that use novel instruments and equipment. Dr. Kumar is striving to achieve ethologically relevant monitoring of high-resolution outputs from neural circuits of multiple animals over long periods of time. He is working to manipulate the environment, nervous system, genetics, and pharmacology.

The field of computational ethology has expanded in recent years. Major advancements in statistical learning now are being applied to real-world problems. Dr. Kumar emphasized the need to democratize these new technologies. One opportunity in this area is automated annotation of animal behavior. Dr. Kumar briefly presented a representative recording using this method and explained how recordings can be used to detect behaviors in mice (e.g., grooming, gait, posture). He presented a readout of data annotation, explaining that multiple extrinsic factors (e.g., time, tester, light, noise, season, room of origin) can be considered.

Dr. Kumar proposed that highly reproducible and scalable motor assays could substitute for complex cognitive traits for screening purposes. He also spoke on the context of index-based phenotypes for generalizability in various contexts. For example, multiple behaviors contribute to developing these indices for biological age. He also presented an example of data monitoring to characterize social interaction; these data were found to be replicable.

The integrated mouse phenotyping platform involves steps of data acquisition, behavior annotation, classifier training, behavior characterization, and data integration. Dr. Kumar underscored the value of a standardized, high-quality data pipeline and a standardized analytic pipeline, with minimal human intervention. These systems would allow data comparisons across laboratories, location, and time. To achieve this outcome, sharing of data, hardware, software, and annotations will be needed. More infrastructure developments in this area are needed.

Discussion

- Dr. Ashbrook asked about factors related to genetic variation. Researchers tend to use single-genome rodents to model humans, which are genetically variable. Dr. Ashbrook noted that "translation" even between mouse strains sometimes is unsuccessful. Dr. Berridge responded that researchers are exploring ways to incorporate genetic variation into mouse studies. He suggested increasing the depth of evaluation in individual animals.

- Dr. Craig Franklin remarked that "pet shop" mice experience a higher antigen experience through exposure to a richer microbiome; this antigen experience correlates with immune system development that better replicates the adult human immune system. Moreover, a simpler microbiome might be more susceptible to change, which could impact reproducibility.

- A participant wondered whether any facilities are breeding "dirty" mice that still are classified for research. Dr. Wiest noted that investigators at the University of Minnesota are performing research using pet shop mice.

- Dr. James Burkett remarked that the ability to know immediately about adverse conditions does not necessarily imply that an immediate response is required for animal welfare. A reasonable response time could be defined. Dr. Niemi noted that the appropriate action is dependent on current circumstances, as well as the culture of the institution. He added that these practices now must be defined, rather than assumed.

- Dr. Wiest remarked that his facility is organized into different areas of health status. He noted that the smart caging technologies provide new opportunities but necessitate staffing considerations.

- Dr. Kumar commented that many of the smart sensors have been in place for decades but have not been implemented at scale in vivaria. He emphasized the need for scalable and affordable technologies. He proposed that a single sensor could be used for multiple modalities of monitoring. Dr. Niemi agreed, noting that specialized, premium equipment could be used for specialized situations. Scalable systems could be used in more general contexts.

- Dr. Wiest wondered about the use of machine learning to detect subtle behaviors, such as grooming versus scratching. Dr. Kumar replied that the algorithms are highly sensitive; for example, the breathing rate can be determined to distinguish sleep states. He added that frame rate and resolution must be considered.

- Dr. Wiest wondered whether the video systems could be implemented in cages. Dr. Kumar responded that this design would be feasible, but certain behaviors—such as strides—might be challenging to determine in cages. Other behaviors—such as sleep—might be easier to determine. He added that the algorithms are flexible and require only high-quality video and training data.

- In response to a question from Dr. Leah Villegas, Dr. Contreras noted that the ORIP small business programs could be applied for development of smart caging systems.

- Dr. Joseph Newsome remarked that the transition between isotope- and X-ray-based systems must be validated to ensure consistency in physiological effects.

Presentations: New and Emerging Monitoring Methods That Enhance Rigor and Reproducibility in Studies Using Rodents

Influence of Housing and Pathogen-Control Measures on Host Physiology and Reproducibility
Neil Lipman, V.M.D., Memorial Sloan Kettering Cancer Center and Weill Cornell Medicine

Dr. Neil Lipman spoke on the influence of housing and pathogen-control measures in the context of host physiology and reproducibility. He first presented a history of changes in rodent caging systems, which have evolved from wood to stainless steel and glass and, as currently utilized, thermoplastics. Several seminal events have shaped this industry. In the late 1950s, the first isolator cage and biological transfer hood was developed. In the 1980s, the design standard for most current isolator cages was developed.

Around this time, researchers began detecting new issues associated with these cages which affected mice health. The isolators led to changes in the microenvironment, particularly regarding ammonia levels. Bedding also plays a significant role in the accumulation of ammonia. As a result, researchers began exploring the concept of ventilated cages. This design helped to reduce ammonia accumulation in cages, it provided an additional level of protection for the cage occupants through intracage pressurization, and increased cage housing density. Dr. Lipman explained that this development occurred in parallel with the emergence of new approaches for genetic engineering in mice, which greatly increased the demand for their use in biomedical research.

Today, most facilitates use individually ventilated cages. Dr. Lipman noted that specific designs vary greatly across systems. Installation differences also should be considered. Dr. Lipman remarked that researchers often fail to adequately report the characteristics of the housing systems used in their studies, even when the studies are evaluating the systems. Often, researchers do not fully understand these systems. Dr. Lipman also noted that the ARRIVE guidelines provide inadequate details in this area. Information that should be reported could include airflow mechanics, rack ventilation, air-change rate, cage design, and intra-cage airflow dynamics. Dr. Lipman noted that reporting needs vary based on the type of study.

Dr. Lipman also discussed the use and processing of thermoplastics in animal research. These materials are used extensively in the production of rodent caging and water bottles. The plastics have been shown to degrade over time releasing bisphenols and other components. Bisphenols function as endocrine disrupters mimicking estrogen. This concern relates to the fundamental question of whether rodent caging should be routinely sterilized, because this process exacerbates the breakdown of caging materials. He presented data suggesting that cage-washing at industry standards might be sufficient to eradicate most murine pathogens. He concluded by underscoring the importance of understanding how practices and operations can introduce additional variables in animal studies.

Circumventing Challenges with Rodent Microbe Detection in Research Vivaria by Incorporating PCR-Based Environmental Screening Methods
Ken Henderson, Ph.D., Charles River Laboratories

Dr. Ken Henderson presented on the use of PCR-based methods for rodent microbe detection in animal facilities. He began by highlighting the work of Dr. Lisbeth Kraft, who described details of the microenvironment of research animals in 1957. Dr. Henderson briefly outlined examples of ways in which diseases can affect experimental outcomes in rodent research. Researchers must decide which pathogens in their rodent populations must be characterized and reported.

Methodology for PCR was first established in the 1980s, but this approach originally was viewed only as a good confirmation tool or as a secondary tool to traditional diagnostic methods. In 1998, researchers began using PCR to identify contact and air transmission risks for rat parvoviruses. They also detected externally released viruses from exhaust ducts. In 2004, the first proof-of-concept report of exhaust air duct collection on an individually ventilated cage rack was published.

At this time, many researchers were hesitant to change their current practices of using soiled bedding sentinels. Several years later, however, laboratory veterinarians became concerned about the use of these sentinels for quarantine. A fecal quarantine study was performed, and high-throughput PCR was incorporated. A larger-scale study was performed using pet shop mice, and researchers found that most infectious agents not detected in soiled bedding sentinels were detected via direct sampling for PCR.

Dr. Henderson explained that issues have occurred with the transition from open-top to microisolator caging. The effects on soiled bedding samples with regard to routine infectious agents were not considered. He highlighted additional advancements in biosecurity, which include cage-changing stations, use of surface decontaminants, decontamination of husbandry materials, and cell line and research biologics testing before use in animals.

Around 2009, researchers became aware of the prevalence of fur mites in research animals. Dr. Henderson was involved in efforts to better characterize these effects. In this process, the researchers developed the concept of routine environmental sampling for pathogen screening by PCR on individually ventilated cage racks. In recent years, several publications have supported the use of environmental PCR testing methods. Dr. Henderson began testing cage filters, which appeared to be more effective than the sentinels but still required a mouse for agitation. Additionally, in certain cage designs, the filters were difficult to remove.

A recent approach has involved manual agitation of soiled bedding with contact media. This approach does not require a mouse, and the data support good sensitivity for a small group of agents. Additionally, this method can be used with any cage type. Dr. Henderson collaborated with other groups to determine challenges within this system. They reported that cage shaking was cumbersome, and data for commonly excluded agents were limited. Standardized methods for agitation and evaluation of different contact media are needed. Furthermore, submissions have not been standardized.

Based on these challenges, the group standardized and optimized the agitation approach. They identified an optimal contact media treatment schedule and evaluated more than 20 contact media to select high-binding candidates. The cage was replaced with a collection box, which was used to agitate the collection media with the soiled bedding. This approach eliminates the need for soiled bedding sentinels.

Dr. Henderson concluded that environmental and exhaust dust sampling methods for PCR detection of rodent infectious agents are being used today by many institutions. These methods detect infectious agents typically found by traditional soiled bedding sentinel use, as well as a larger group of agents that are not. A better knowledge of which agents are present is important in understanding their potential effect on research outcomes. He emphasized the value of pursuing equivocal or superior methods that eliminate animal use in research.

Discussion

- No discussion occurred.

Group Discussion and Summary

- Dr. Wiest commented on the finding that LED lighting was associated with less feeding and drinking. He noted the link between caloric restriction and aging. He asked whether LED exposures lead to sustained reductions in feeding and whether this variable would be of interest to the aging research community. Dr. Brainard responded that the study was performed over 12 weeks, and he agreed on the importance of this effect. Continued investigation in this area is needed.

- Dr. Kumar noted that light pollution in facilities (e.g., from equipment) could be an area of concern. Dr. Brainard agreed, noting that darkness is relative and difficult to achieve in facilities. This is a particular issue for rodents, because they are nocturnal. In response to a follow-up comment from Dr. Fox, Dr. Brainard added that the effects of light cycles should be considered in this context. Light duration can prompt seasonal responses, triggering numerous physiological effects. The current edition of the *Guide* does not provide guidance in this area.

- Dr. Lipman asked whether LED lights can be tuned to address the observed effects in ganglia. Dr. Brainard commented that the International Space Station has been retrofitted with a tunable LED light source with a pre-sleep mode for astronauts. This tuning eliminates stimulation of the system. Similar strategies are being applied in other human studies. Dr. Brainard noted that tunable LED systems likely are not needed in all animal facilities at this point, but the engineering capacity has been established.

- Dr. Fox asked about the effects of environmental monitoring and energy-saving measures in the context of extrinsic factors. Dr. Lipman explained that air ventilation rates at his institution are controlled by various factors. The newly implemented system is designed to adjust air exchange as needed. Temperature and humidity are important factors for consideration. The new system is more cost-effective than previous systems, with a high return on investment. He noted that ventilation rates can be adjusted based on the presence of animals. Dr. Lipman added that the need for ventilation is driven primarily by the heat generated by the animals and equipment. He added, however, that such automated systems could introduce a new variable. Dr. Brainard emphasized that changes in facilities should be driven primarily by scientific needs.

- Dr. Lipman noted that the microbiome has emerged as an important topic in recent years. He added that as transgenic mice have been shipped across the globe, researchers do not truly know what new agents have been introduced to their facilities.

- Dr. Burkett asked about strategies to determine whether animal racks are being exposed to problematic vibrations. Dr. Reynolds noted that animals often exhibit stress responses, such as food grinding, reproductive issues, and cannibalization. Additionally, researchers can measure vibration directly within facilities, but problematic levels can be challenging to define.

- A participant asked about the duration of response to one-time significant vibration incidents. Dr. Reynolds replied that the response is dependent on numerous factors, and may be different for animals *in utero*. Direct testing would be needed to understand the effects fully.

- Dr. Gordon Lithgow shared information on the National Institute on Aging's Interventions Testing Program, which is designed to identify agents that extend life span and health span in mice.

- Dr. Lipman underscored the need for an NIH funding mechanism to study extrinsic factors in animal research. Dr. Li agreed on the importance of this issue. He noted that ORIP's recent concept clearance could provide some support in this area. Additionally, the outcomes of this workshop will be helpful in setting criteria for evaluation of grant applications that are addressing needs in this area. If NIH programs identify gaps in their funding portfolios, new funding opportunities can be developed.

- Dr. Everitt suggested that the NIH encourage inter-institutional studies to foster a better understanding of extrinsic factors. He reiterated the need for standardized methods. Dr. Fox noted that in private industry, many experiments are being performed by contracted laboratories. Dr. Everitt agreed that this practice can create challenges but noted that confirmatory studies, which are common practice within the pharmaceutical industry, have contributed to a stronger system of peer review.

- Dr. Marta Chesi wondered how the information discussed during the workshop could be incorporated into the *Guide*. Dr. Everitt remarked that numerous extrinsic factors are present, and researchers cannot account for every variable in research. He spoke on the need for tailoring controls to the type of research being performed. Dr. Wiest agreed, noting that researchers can take one of two approaches in addressing extrinsic factors: controlling for every variable or conducting studies on animals that are more representative of biological organisms in the real world (e.g., pet shop mice).

- Dr. Berridge emphasized that the issue of extrinsic factors will require large-scale collaborative efforts among the NIH, professional societies, and private industry entities. The NIH could help foster partnerships in this area. Dr. Lipman also suggested that ORIP convene a panel of experts to develop recommendations related to the use of LED lighting in facilities.

Session Wrap-Up and Adjournment

Drs. Fox and Mirochnitchenko thanked the speakers, organizers, and participants for their engagement during the meeting. Dr. Mirochnitchenko encouraged the participants to consider how the principles discussed during the meeting apply to other types of model organisms. Dr. Fox also encouraged the participants to register for Session 3. Dr. Mirochnitchenko adjourned the meeting.

Appendix A: Meeting Agenda

Session 2. Rodents
Virtual Meeting
September 28, 2022

12:00–12:10 p.m.	**Opening Remarks** *James Fox, D.V.M., M.S., DACLAM, Workshop Chairperson, Massachusetts Institute of Technology* *Xiang-Ning Li, M.D., Ph.D., 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)* *Oleg Mirochnitchenko, Ph.D., ORIP, DPCPSI, OD, NIH*
12:10–12:30 p.m.	**Keynote Presentation: Impact of Extrinsic Factors on Rigor and Reproducibility in Rodent Research** *F. Claire Hankenson, D.V.M., M.S., University of Pennsylvania*
12:30–1:50 p.m.	**Presentations: Housing Environment That Impacts Rigor and Reproducibility in Studies Using Rodents**
	Effects of Increased Housing Density in Research Mice *Karen Svenson, Ph.D., The Jackson Laboratory (JAX)*
	Minimizing the Impact of Habitat Lighting on Experimental Reproducibility for Rodent Studies *George Brainard, Ph.D., Thomas Jefferson University*
	Vibration as an Extrinsic Variable for Research Outcomes *Randall Reynolds, D.V.M., M.S., Duke University*
	Environmentally Associated Lesions in Rodent Toxicology Studies *Jeffrey Everitt, D.V.M., Duke University*
1:50–2:00 p.m.	**Break**
2:00–3:20 p.m.	**Presentations: Equipment Modernization That Enhances Rigor and Reproducibility in Studies Using Rodents**
	New Methods for Performing Irradiation in Rodents *Mitch Galanek, CHP, Massachusetts Institute of Technology*
	Enhancing Animal Study Translation: Physiological Monitoring as a Key Contribution *Brian Berridge, D.V.M., Ph.D., DACVP, National Institute of Environmental Health Sciences*
	Smart Cages Require Smart Management *Steve Niemi, D.V.M., Boston University*

	Highly Scalable and Reproducible Preclinical Rodent Behavioral Assays Using Machine Vision *Vivek Kumar, Ph.D., JAX*
3:20–3:30 p.m.	**Break**
3:30–4:10 p.m.	**Presentations: New and Emerging Monitoring Methods That Enhance Rigor and Reproducibility in Studies Using Rodents**
	Influence of Housing and Pathogen-Control Measures on Host Physiology and Reproducibility *Neil Lipman, V.M.D., Memorial Sloan Kettering Cancer Center*
	Circumventing Challenges with Rodent Microbe Detection in Research Vivaria by Incorporating PCR-Based Environmental Screening Methods *Ken Henderson, Ph.D., Charles River Laboratories*
4:10–4:40 p.m.	**Group Discussion and Summary**
4:40–4:50 p.m.	**Session Wrap-Up**
4:50 p.m.	**Adjournment**

Appendix B: Participants List

Session 2. Rodents
Virtual Meeting
September 28, 2022

Kristin Abraham, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH)
Stephanie Achilles, The University of Alabama at Birmingham
Yoko Ambrosini, Washington State University
James Amos-Landgraf, University of Missouri
Laura Anderson, The Jackson Laboratory (JAX)
Amanda Lee Armijo, Massachusetts Institute of Technology
Matthew Arnegard, Office of Research Infrastructure Programs (ORIP), Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director (OD), NIH
David Ashbrook, The University of Tennessee Health Science Center
Charles-Antoine Assenmacher, University of Pennsylvania
Julia Bachman, National Institute of Neurological Disorders and Stroke (NINDS), NIH
Alanna Backx, Massachusetts Institute of Technology
Amanda Barabas, Case Western Reserve University
Ashley Barnes, ORIP, DPCPSI, OD, NIH
Taylor Bennett, National Association for Biomedical Research
Zorana Berberovic, The Centre for Phenogenomics
Michelle Bernard, Oregon Health & Science University
Brian Berridge, National Institute of Environmental Health Sciences (NIEHS), NIH
Scott Birks, Boise State University
Himadri Biswas, The University of Toledo
Auke Boersma, University of Veterinary Medicine Vienna
Courtney Bouchet, Colorado State University
Aleaya Bowie, Vanderbilt University Medical Center
Felicia Boynton, Mayo Clinic
George Brainard, Thomas Jefferson University
Samantha Brims, University of Southern California
Mallory Brown, Johns Hopkins University School of Medicine
Patricia Brown, Office of Laboratory Animal Welfare (OLAW), Office of Extramural Research (OER), OD, NIH
Elizabeth Bryda, University of Missouri
James Burkett, The University of Toledo
Angelica Cabrera, Bristol Myers Squibb
Edgar Angelats Canals, August Pi i Sunyer Biomedical Research Institute
Jessie Carder, U.S. Department of Agriculture
Selen Catania, National Heart, Lung, and Blood Institute, NIH
David Cavazos, The University of Texas Health Science Center at San Antonio
Shreaya Chakroborty, National Institute on Aging (NIA), NIH
Susan Chandran, ORIP, DPCPSI, OD, NIH
Chris Chao, National Institute of General Medical Sciences (NIGMS), NIH
Sarah Chapman, University of Notre Dame
Naomi Charalambakis, Federation of American Societies for Experimental Biology
Julia Charles, Brigham and Women's Hospital
Lee Chaves, University of Kansas Medical Center

Amanda Chen, National Institute of Allergy and Infectious Diseases (NIAID), NIH
Marta Chesi, Mayo Clinic
Sangyong Choi, University of Connecticut
Debanik Choudhury, University at Buffalo
Eleanore Chuang, NIAID, NIH
Megan Clark, OLAW, OER, OD, NIH
Joyce Cohen, Emory University
Lesley Colby, University of Washington
Giancarlo Colombo, National Research Council of Italy, Istituto Di Neuroscienze
Karina Concha, Florida Atlantic University
Ronald Conlon, Case Western Reserve University
Miguel Contreras, ORIP, DPCPSI, OD, NIH
Kerith Coulson, University of Cape Town
Devon Crawford, NINDS, NIH
Joette Crews, Emory University
Lani Cupo, McGill University
Chi-Ping Day, National Cancer Institute (NCI), NIH
John Dennis, U.S. Food and Drug Administration (FDA)
CJ Doane, University of Arizona
Abigail D'Souza, Louisiana Tech Research Institute
Adrienne Duran, MD Anderson Cancer Center
Samantha Earlywine, Nationwide Children's Hospital
Catalina Echeverri, Rockefeller University
Samantha Edell, Cytokinetics
Mark Eichelberg, American Physiological Society
Michael Eichner, Office of Research Services, Office of Management, OD, NIH
Michael Ellis, JAX
Peter Ernst, University of California, San Diego, and University of California, Davis
Laverne Estanol, University of California, Santa Cruz
Rachel Sarabia Estrada, Mayo Clinic
Jeetendra Eswaraka, Rutgers University
Marissa Eudaley, University of Southern California
Jeffrey Everitt, Duke University
Angelika Fath-Goodin, ParaTechs Corporation
Chuhan Feng, McGill University
Steve Festin, Charles River Laboratories
Cameron Fili, FDA
Kelsey Finnie, The University of Tennessee, Knoxville
Ann Flenniken, The Centre for Phenogenomics
Craig Fletcher, The University of North Carolina at Chapel Hill
Loren Fong, University of California, Los Angeles
James Fox, Massachusetts Institute of Technology
Craig Franklin, University of Missouri
Emily Franklin, Massachusetts Institute of Technology
Julien Freeman, Massachusetts Institute of Technology
Ashley Gaffey, Georgetown University
Mitch Galanek, Massachusetts Institute of Technology
Judit Peix Gallofré, August Pi i Sunyer Biomedical Research Institute
Chelsea Garrison, Boise State University
Brianna Gaskill, Novartis
William Gause, Rutgers New Jersey Medical School

Karli Gilbert, Georgetown University
Sarah Gillis-Smith, Massachusetts Institute of Technology
Sylvia Gografe, Florida Atlantic University
Rafael Moreno Gómez-Toledano, Universidad de Alcalá
Neera Gopee, OLAW, OD, OER, NIH
Ernesto Gulin, Universidad de Buenos Aires
Bryan Hackfort, University of Nebraska Medical Center
Travis Hagedorn, Kansas University Medical Center
David Hamilton, The University of Tennessee Health Science Center
F. Claire Hankenson, University of Pennsylvania
Susan Harper, Inwood Animal Center
Melissa Harrington, Delaware State University
John Hasenau, Lab Animal Consultants
Alissa Hatfield, American Physiological Society
Hami Hemati, University of Kentucky
Ken Henderson, Charles River Laboratories
Beate Henschel, Indiana University
Renee Hernandez, GlaxoSmithKline
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Tuan Hoang, Fluid Synchrony, LLC
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Heather Holliday, Clemson University
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Crystal Johnson, Georgetown University
Katherine Johnson, Boise State University
Philip Jordan, Uniformed Services University of the Health Sciences
Vijay Kanth, Qatar University
Christopher Keator, Western Michigan University
Roseann Kehoe, Rutgers University
Amy Keller, Rocky Mountain Regional VA Medical Center
Lois Kelsey, The Centre for Phenogenomics
David Kennedy, The University of Toledo
Yong-Hwan Kim, Delaware State University
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Julia Kissling, National Aeronautics and Space Administration
Madison Klanke, Turner Scientific
Kim Klukas, The Hormel Institute, University of Minnesota
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Sailaja Koduri, NIGMS, NIH
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Vivek Kumar, JAX
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David Kurtz, NIEHS, NIH
Kelsey Lambert, Wake Forest University School of Medicine
Lorissa Lamoureux, University of Illinois Chicago
Reid Landes, University of Arkansas for Medical Sciences
Chelsea Landon, Duke University
Louise Lanoue, University of California, Davis
Elizabeth Lavin, Cornell University
Karen Lencioni, California Institute of Technology
Jori Leszczynski, University of Colorado Denver and University of Colorado Anschutz Medical Campus
Louis Leung, Martineau & Associates, Inc.
Denyse Levesque, Emory National Primate Research Center
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Meera Navaratnam, InterVivo Solutions
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John Norton, Duke University
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Alan Olzinski, GlaxoSmithKline
Carly O'Malley, Charles River Laboratories
Glen Otto, The University of Texas at Austin
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Scott Perkins, Tufts University
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Katy Phillip, The University of Arizona
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U.S. Department of Health and Human Services
National Institutes of Health
Division of Program Coordination, Planning, and Strategic Initiatives
Office of Research Infrastructure Programs

Rigor and Reproducibility of Animal Studies: Extrinsic Factors Workshop

Session 3. Large Animals (Nonhuman Primates/Swine)

September 30, 2022
Virtual Meeting

Final Report

Table of Contents

Executive Summary

The Extrinsic Factors Workshop was held in three sessions to better understand extrinsic factors and their effects on biomedical research. Session 3 was focused on extrinsic factors in the use of large animals, particularly nonhuman primates (NHPs) and swine, for biomedical research. Drs. Joyce Cohen and Kiho Lee served as the session co-chairs. Discussions in Session 3 addressed the use of precision medicine in NHP research; considerations for husbandry of swine; construction, housing, and caging factors; and equipment and technology factors. The speakers identified various extrinsic factors for consideration in research, including lifetime exposures, stress, social structure, sex and reproduction, diet, early biobehavioral organization, density, housing, enrichment, temperature, humidity, lighting, the microbiome, breed, disease status, and litter size. The participants also discussed monitoring and reporting extrinsic factors in research using large animals. They noted that these factors are important to monitor, but investigators often are discouraged from including extensive methodology sections in publications. Additionally, it was noted that many investigators are hesitant to modify their established systems. The participants discussed the need to maintain a balance between controlling extrinsic factors while ensuring that experiments remain generalizable and translatable. The need for additional funding to understand extrinsic factors was emphasized. Other considerations include the requirements for increased throughput and sample size. Investigators must remain flexible as technical and scientific opportunities present new considerations in biomedical research.

Session Co-Chairs
Joyce Cohen, V.M.D., DACLAM, Emory University
Kiho Lee, Ph.D., University of Missouri

Presenters
Timothy Allen, Ph.D., Florida International University
Kelly Ethun, D.V.M., Ph.D., DACLAM, Emory National Primate Research Center
Kathleen Grant, Ph.D., Oregon National Primate Research Center
Shannan Hall-Ursone, D.V.M., Southwest National Primate Research Center
Erin Kinnally, Ph.D., California National Primate Research Center (CNPRC)
Gota Morota, Ph.D., Virginia Polytechnic Institute and State University
Linda Saif, Ph.D., The Ohio State University
Gregory Timmel, D.V.M., M.S., DACLAM, CNPRC
Christopher Tuggle, Ph.D., Iowa State University
Kristin Whitworth, Ph.D., National Swine Resource and Research Center

Workshop Organizing Committee

James Fox, D.V.M., M.S., DACLAM, Workshop Chairperson, Massachusetts Institute of Technology
Guanghu (Jeff) Wang, Ph.D., M.B.A., Workshop Coordinator, 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)

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Workshop Report

Opening Remarks

James Fox, D.V.M., M.S., DACLAM, Massachusetts Institute of Technology
Xiang-Ning Li, M.D., Ph.D., 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)
Guanghu (Jeff) Wang, Ph.D., M.B.A., ORIP, DPCPSI, OD, NIH

Dr. Xiang-Ning Li welcomed the attendees to Session 3 of the workshop. Dr. Li reminded the participants of NIH's dedication to rigor and reproducibility, which was emphasized by Dr. Robert W. Eisinger, Acting Director, DPCPSI, during Session 1. In 2021, the NIH Advisory Committee to the Director 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.

Dr. Li also reminded the participants that ORIP has long devoted efforts to enhancing rigor and reproducibility, which was emphasized by Dr. Franziska Grieder, Director, ORIP, during Session 1. ORIP has supported this effort through scientific research 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 publications of future funding opportunity announcements (e.g., NOT-OD-22-039). This workshop is one of several steps toward fulfilling ORIP's Strategic Plan by addressing the important endeavor of enhancing animal study rigor and reproducibility in NIH-supported research. The Extrinsic Factors Workshop seeks to better understand extrinsic factors and their effects on biomedical research. ORIP is modifying its infrastructure programs to address reproducibility in animal studies.

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, briefly highlighted Sessions 1 and 2 of the workshop. He emphasized that the topic of extrinsic factors is highly relevant to biomedical research, both for investigators and vivarium staff members. Dr. Fox also introduced Drs. Joyce Cohen and Kiho Lee, Session 3 Co-Chairs.

Keynote Presentation: Precision Medicine—Targeting Individual Differences to Increase Rigor and Translatability

Erin Kinnally, Ph.D., California National Primate Research Center (CNPRC)

Dr. Erin Kinnally presented on rigor and reproducibility in the context of precision medicine. She explained that CPNRC's BioBehavioral Assessment (BBA) Program is focused on addressing the key questions of who gets sick and why. Dr. Kinnally briefly described precision medicine, which is based on the concept that humans differ in disease susceptibility, and treatments ideally will leverage these

individual differences to improve outcomes. Genetics, lifetime exposures, and biobehavioral organization might play a role. Biomedical models for disease should address these factors for optimal translatability.

The BBA Program has assessed 5,600 infant rhesus macaques to understand individual differences in temperament, stress, reactivity, cognition, and social motivation. Recently, the program was embedded into CPNRC's operating base grant. The program's assessments since have expanded to incorporate a lifespan approach; the team is developing an aging BBA for macaques older than 15 years of age. To date, 128 aging macaques have been assessed, and 50 aging animals will be assessed annually in the future. Dr. Kinnally highlighted discoveries resulting from BBA models, which relate to airway hyper-responsiveness, social motivation, and depressive behavior. Future directions of the program include studying long COVID-19, wildfire exposure, and retinal disease.

Dr. Kinnally highlighted work with the aging BBA. The goals of this initiative are to characterize healthy biobehavioral aging in monkeys and to determine the factors that promote healthy biobehavioral aging (e.g., social connection, lifetime exposures, stress, diet, genetics/epigenetics, early biobehavioral organization). The overarching objective is to increase use of individual differences in nonhuman primate (NHP) disease models to increase rigor and reproducibility. All data are made available to investigators.

The aging BBA was based on five major domains of macaque aging: cognitive, motor, physiological/cellular, emotional, and social. Various tests were developed based on metrics that allow investigators to determine how the monkeys are aging across these domains. The tests include vision (e.g., near, distance), gait (e.g., speed, cadence, number of steps), decision making (e.g., reaction time, complexity, predictability), depressive behavior, and social cognition (e.g., social stimulus response, human imitation).

Dr. Kinnally noted the importance of increasing throughput through technology. Efforts in this area include coding videos, automating behavioral observation software, and recording through eye-tracking tablets. She noted that automation software increases capacity for capturing certain metrics that previously were inaccessible to researchers.

The BBA programs can increase the use of NHP models for aging. Dr. Kinnally noted that stakeholders in this effort include NHP model developers, the National Primate Research Centers (NPRCs), and individual investigators. Engagement efforts include sharing standardized protocols, data, and video training resources. Dr. Kinnally noted that individual monkeys can be studied at investigators' request.

Discussion

- In response to a question from Dr. Amy Ryan, Dr. Kinnally affirmed that the program is assessing macaques across different housing conditions and rearing histories. The animals' health and research history can be accessed.

- Dr. Sarah Carratt wondered about differences in biobehavior between macaques housed in U.S. versus European Union facilities. Dr. Kinnally responded that this question would be interesting to explore.

- Dr. Cohen asked how often BBA information is leveraged before animals are assigned to projects. She asked whether this process occurs for all assignments at the CNPRC or whether it is implemented only upon request. Dr. Kinnally explained that BBA infant data have been available for years through the internal health record system, which is available to CNPRC investigators. This resource is used frequently for behavioral health studies. Data are being made available on the public website.

- In response to a question from Dr. Fox, Dr. Kinnally spoke on whole-genome sequencing efforts at the program. She noted that genotyping efforts in this area have expanded over the years. Several efforts related to whole-genome sequencing are being pursued, and many tissue samples are available for analysis.

- Dr. Fox asked whether the program is engaging with other NPRCs to implement the study in New World monkeys. Dr. Kinnally stated that she recently became involved in a multi-NPRC effort to develop BBAs for marmosets.

Keynote Presentation: Husbandry of Biomedical Swine Models
Kristin Whitworth, Ph.D., National Swine Resource and Research Center (NSRRC)

Dr. Kristin Whitworth highlighted the NSRRC and management of extrinsic factors in animal research. She first listed the objectives of the NSRRC, which are to (1) ensure that biomedical investigators have enhanced access to critically needed swine models involving human health and disease and (2) serve as a central repository for materials (e.g., germline and somatic cells, gene probes, markers) and information.

The NSRRC has generated more than 1,300 cloned pigs, of which 685 were cloned by zygote injection. More than 90 distinct models have been developed, 13 of which were developed in 2021. The NSRRC distributes pigs, pig tissues, pig cell lines, and protocols and facilitates cloning and embryo transfers across the United States. Five housing facilities have been established at the University of Missouri; the NSRRC is a high-biosecurity xenobiology building that can house about 100 pigs. Overall, the entire campus has the capacity to house between 350 and 400 pigs.

NSRRC models support numerous areas, including such specialties as somatic cell genome editing, reproductive biology, xenotransplantation, immunology, neurobiology, and heart valve transplantation; conditions like Fanconi anemia, congenital heart defect, pulmonary fibrosis, seizure, cancer, Alzheimer's disease, influenza, cardiovascular disease, liver disease, and hepatitis; and bone, eye, and ear studies. Dr. Whitworth emphasized that the animals are used for multiple projects to maximize the use of tissues.

The NSRRC provides services to numerous organizations and institutions, including the University of Missouri, Oregon Health & Science University, University of Pittsburgh, Columbia University, and University of Louisville. The NSRRC also supports several NIH Institutes, Centers, and Offices (ICOs). Highly distributed pig models include enhanced green fluorescent protein pigs (e.g., retinal stem cells, spinal cord regeneration), Cre-inducible cancer models, and xenotransplantation models (e.g., *GGTA1*, *hCD55*). Additional models are in development. Dr. Whitworth noted that the NSRRC recently received C06 funding to expand the facility.

The *Guide for the Care and Use of Laboratory Animals* specifies the number of swine per pen, based on the animal's weight and pen size. For larger animals, particularly swine, it is important that the configuration of the space allow the animals to turn around and move freely. Food troughs and water devices should be provided in sufficient numbers to allow ready access for all animals. Direction on temperature and relative humidity also is provided in the *Guide*. General guidance for animal facilities relates to structural strength, water and power, and storage. Other standards relate to feeding and watering.

Dr. Whitworth spoke with NSRRC's Director of Animal Care and Quality Assurance, as well as investigators, to determine shortfalls and opportunities related to extrinsic factors. Suggestions included improving animal welfare (e.g., proactive approach, experienced veterinary and animal care staff, maintenance of low animal stress levels, best feeding practices), optimizing the *Guide* requirements (e.g., funding for density studies, optimal enrichment), reliable phenotyping and characterization (e.g. third-party validation, standard rubric), and infrastructure (e.g., video monitoring equipment,

image-based health assessments, bioinformatics). The group proposed establishing specialty centers around NIH ICOs, as well as a pig transport center. Investigators could travel to perform their work in the best location, similar to the NPRCs. Other ideas include collaborations between animal science departments and medical centers, as well as the addition of pig facilities and staff.

Dr. Whitworth also pointed out that some inconsistency might be beneficial in research; pigs are outbred with large litters, robust performance, and high fertility. Miniature pigs are inbred, with small litters and infertility. She shared a perspective that if a model provides a predicted phenotype regardless of its environment, it is a reliable and reproducible model. She also noted the need for extension of funding periods and reasonable requirements for documentation.

Discussion

- Dr. Joseph Newsome emphasized the need for expert training on swine management and reproductive physiology for non-animal science programs that use these models. Dr. Whitworth agreed but noted that hands-on experience is needed, and biosecurity restrictions can impose challenges for access.

Presentations: Construction, Housing, and Caging That Impact Rigor and Reproducibility in Studies Using Large Animals

Impacts of NHP Enclosure Design on Welfare and Rigor and Reproducibility

Gregory Timmel, D.V.M., M.S., DACLAM, CNPRC

Dr. Gregory Timmel presented examples of NHP enclosure designs and their effects on rigor and reproducibility. He also discussed recent research on innovative enclosure designs. He shared examples of large outdoor enclosures, corn crib–style enclosures, indoor housing, and indoor–outdoor housing. He noted that these designs can be applied for different types of experiments and breeding practices. Dr. Timmel highlighted examples of environmental enrichment and considerations for management. He pointed out that all NHP species should be considered in this context, not only macaques. Additionally, smaller species can be easier to house socially in smaller areas.

A study was performed at the Emory National Primate Research Center (ENPRC) to determine the effects on animal welfare (e.g., behavior, health, stress) of providing young male rhesus macaques access to larger outdoor spaces. The animals showed strong preference for the outdoor space, were more physically active and played with one another, and had diminished cortisol levels. No effects were observed on other behaviors, alopecia, body condition score, or diarrhea cases. Additionally, no consistent patterns of response on cognitive bias testing were observed.

Dr. Timmel underscored the importance of NHP studies to assess the effects of housing on animal health and behavior. He also briefly highlighted a study on the effects of psychosocial stress on immune response during acute simian immunodeficiency virus (SIV) infection in a pigtail macaque model. Another study examined social housing status and attentive response in rhesus macaques. Dr. Timmel noted a study that reported the effect of single housing on innate immune activation in pigtail macaques infected with SIV.

In summary, factors to consider in NHP research include social housing, enclosure features (e.g., size, location, complexity), indoors versus outdoors, substrates, secondary enclosure schedules, natural versus artificial lighting, light cycle, behavioral enrichment, individual animal effect, and phenotype. He emphasized that much still is unknown, and more work in this area is needed.

Automated Feeding Stations Provide a Rigorous Tool for Enhancing Outcome Accuracy on Social Determinants of Nutritional Health and Breeding Colony Management in Monkeys
Kelly Ethun, D.V.M., Ph.D., DACLAM, ENPRC

Dr. Kelly Ethun discussed the use of automated feeding stations as a tool to study social determinants of nutritional health and novel colony management strategies. She explained that automated feeders have been used at the ENPRC for neurobehavioral–nutritional health translational studies (e.g., stress-induced eating) and for resource-related research. These studies would have been impossible without a noninvasive, automated method to accurately monitor and control the dietary environment of socially housed monkeys on an individual basis.

ENPRC's automated feeding systems have been used as a basis for high-throughput designs for use in outdoor corral-based feeding studies. This work was conducted in partnership with Research Diets, Inc. Improvements include a stainless-steel hopper, waterproof frame, Power over Ethernet computer and Category 6 cable, and a highly sensitive dispensing system that measures food pellets on a weighting platform with high accuracy. Dr. Ethun shared a video demonstrating the system's principle of operation.

The ENPRC field station houses more than 2,300 animals, primarily in large outdoor enclosures. The Center has purchased 32 commercial BioDAQ feeders to outfit eight compounds, which house about 750 animals. The feeders are a valuable resource to the Center and provide a modern alternative to conventional bin-feeding practices. Disadvantages of the bin hoppers include food waste, potential contamination, availability to vermin and other pests, and lack of accurate consumption monitoring. Animals fed from automated feeders, in contrast, waste less food, and intake can be quantified accurately.

Dr. Ethun noted that efficient social health surveillance methodologies can be employed to identify groups at risk for social instability before the onset of fighting. Potential consequences of unresolved conflicts include increased wounding and stress. Social stress resulting from group instability can lead to decreased reproductive success, decreased animal availability, increased variability in neurobehavioral and immunological processes, and decreased reproducibility. This new approach can enable automated and real-time monitoring. Dr. Ethun shared a case study of intrafamily aggression and daily caloric intake.

The Feeding Interaction Network (FIN) Project, funded by ORIP, involves the development of advanced computational approaches using temporal proximity feeding interaction data to enhance social health surveillance of rhesus breeding groups. FIN network analyses can be used to examine co-feeding patterns among group members and identify subgroups in feeding communities that are associated with changes in kinship and dominance structures. Management can use timely information to increase observations and develop strategies to intervene prior to escalation of aggression. New computational methods can be used to develop and validate community detection algorithms and machine learning models.

In summary, feeding behavior of socially housed monkeys provides useful information about nutritional health and the influence of socioenvironmental factors. Automated feeding data provide information about individual variability in caloric intake, feeding duration and timing, diet preference, and partner choice. Studies investigating feeding behavior of group-housed rhesus monkeys require high-throughput and modern equipment to ensure rigor and reproducibility. Modern primate research facilities need rigorous monitoring systems to optimize NHP well-being and foster the conduct of high-quality science.

Biocontainment Protocols for Improving Survivability of Severe Combined Immunodeficient (SCID) Pigs
Christopher Tuggle, Ph.D., Iowa State University

Dr. Christopher Tuggle discussed protocols for improving survivability in studies using SCID pigs. He explained that SCID pigs cannot reject xenografts of human cells. SCID pigs are used in cancer xenograft

studies, stem cell xenograft studies, and cell–drug interaction testing. Researchers are interested in exploring whether the SCID pig can be developed as an alternative biomedical model to rodents in such xenograft studies as well as in humanization (creation of a human immune system in the SCID pig). Attributes of a good animal model include accurate modeling of human disease or condition and routine availability for low cost, with minimal requirements for client husbandry expertise.

Dr. Tuggle has demonstrated that an ovarian cancer cell line can survive in a SCID pig model. In this work, the SCID pig model has allowed the development of a tumor similar to that in human patients. The SCID pig might be better than the SCID mouse for modeling certain markers. Human skin transplants also have been shown to survive on the SCID pig in a proof-of-concept study. Additionally, injection of human hematopoietic stem cells demonstrates substantial humanization at the neonatal stage.

In an ORIP-funded project, investigators developed three positive-pressure "bubbles" for SCID pigs. High-efficiency particulate air (HEPA)–filtered air flows out. Water is sterile filtered, ultraviolet treated, and acidified. Personnel wear Tyvek, double gloves, a hair net, and a surgical mask. The three bubbles are designated for entry, production, and fee for service. Husbandry initially was performed through snatch-farrow carrier gilts. This approach provides colostrum and a complex microbiota and is as close to natural as possible. However, reproduction is inefficient, and the system requires a large, high-risk investment. Biosecurity risks also are of concern.

The team now is exploring options for cesarean section to create gnotobiotic pigs that initially are raised in isolators and later are transferred to biocontainment. This approach decreases exposure of piglets to outside air during their most susceptible stage and is performed in a batch process. The approach also allows manipulation of different colostrum sources and microbiota types, which could help researchers move toward standardization. Disadvantages include pig fragility and the need to move to the bubble at 4–6 weeks for most projects. Additionally, large-animal expertise is needed.

Survivability was greatest for the snatch-farrow approach, although this approach—unlike the cesarean section—was used only in non-cloned pigs. Testing in this area is in progress. Dr. Tuggle explained that the major cause of death was early euthanasia due to poor health, generally resulting from sepsis. The source of bacteria is unknown, and more investigation in this area is needed. Treatments are provided based on clinical signs. Gut microbiome sequencing revealed that the number of bacteria is low, compared to process controls. As expected, the bacterial communities are not highly diverse. *Lactococcus* is the most abundant operational taxonomic unit across all samples, and *Enterococcus* is observed at later time points.

In summary, the combination of SCID and gnotobiotic condition might be uniquely difficult to raise. Widespread sepsis is caused by poor neonatal gut health, leading to lack of gut closure and sepsis by opportunistic bacteria. Cloned SCID pigs also might be uniquely susceptible. Complex microbiota and porcine colostrum might be required for reproducible husbandry of SCID pigs. Future plans include focusing on early gut health and gut closure, comparing the outcome of defined versus complex microbial populations, investigating immunoglobulin source and delivery, and comparing cloned and non-cloned pigs.

Gnotobiotic Pigs Infrastructure and Biological Model
Linda Saif, Ph.D., The Ohio State University

Dr. Linda Saif presented on infrastructure and biological models for gnotobiotic pigs. She first explained that germ-free pigs require a large-animal germ-free facility with temperature-controlled rooms, an air system with a central turbine unit and HEPA filtration, and positive-pressure isolators. Dr. Saif briefly outlined the layout of the facility, noting the inlet and outlet filters, housing isolator, isolator floor, and

enrichment toys. Extrinsic environmental factors include temperature, lighting, control of humidity, and space limitations. Extrinsic host factors include swine breed, disease status, litter size, and sterility.

Dr. Saif asserted that the pig has multiple advantages over other animal models (e.g., rodents, NHPs). Considerations include similarity to humans, ethical acceptance, high hygienic status, good animal compliance, sufficient sample material, good reproduction data, and established genetic modification. Maintenance costs of swine, however, remain a significant limitation. Dr. Saif noted that pigs can provide a dual model to study enteric viral infections and vaccines. Pigs are the only animal model that is susceptible to human rotavirus diarrhea and norovirus. Pigs are outbred and are anatomically, physiologically, and genetically similar to human infants. Additionally, extraneous enteropathogens, microbiota, and maternal antibodies are absent. For these reasons, the pig is a highly relevant model for mechanistic and translational research on human disease.

Germ-free piglets provide a unique model for studies of the microbiome, rotavirus infection, immunity, and vaccines. Goals and gaps include an approach for colonization with neonatal human fecal microbiota and probiotics, a model to understand the effects of the microbiome and probiotics on immunity and vaccines, and a model for evaluating why current rotavirus vaccines fail in field environments. Dr. Saif highlighted previous work demonstrating probiotic effects on rotavirus immunity and vaccines. Nutritional effects also have been demonstrated in pig models for vitamin A deficiency and protein malnutrition.

Gaps in the gnotobiotic pig model include lack of immunological tools and reagents, limited availability of gene expression microarray and proteome, limited knockout and genetically modified pigs, need for interagency partnerships, and need for federal support for germ-free large-animal facilities.

Discussion

- A participant asked whether the sex of experimenters could affect experimental outcomes in NHPs, similar to mice. Dr. Timmel replied that he was unaware of studies on this topic but that this could represent a potential variable.

- Dr. Cohen asked whether smaller automated feeders could be used in more conventional NHP facilities. Dr. Ethun responded that smaller feeders are commercially available.

- In response to a question from Dr. Lee, Dr. Saif explained that gnotobiotic pigs can be maintained in isolator bubbles for 8 weeks. Longer-term options are available if needed. The animals can be maintained in a calf isolator for 3 months.

Presentations: Equipment and Technology That Enhance Rigor and Reproducibility in Studies Using Large Animals

Markerless Motion-Capture Technology

Shannan Hall-Ursone, D.V.M., Southwest National Primate Research Center (SNPRC)

Dr. Shannan Hall-Ursone discussed the incorporation of gait and movement analysis technology to enhance clinical care and research outcomes. The specific aims of this project were to (1) adapt the Southwest Research Institute's markerless biomechanics technology to track baboons at Texas Biomedical Research Institute and (2) determine whether this technology could be used noninvasively to obtain information from animals that would electronically determine normal movement. The end goal was to use the analysis to identify abnormal movements using predetermined data and identify early signs of injury or disease. This information provides both clinical and research value.

The Human Performance Initiative was aimed at tracking joint angles and positions using monocular cameras, with no instrumenting of test subjects. The results of this study potentially could benefit two aspects of the 3Rs (i.e., replacement, reduction, and refinement) of animal research. If favorable results are obtained, reduction will be achieved, because the technology can aid in finding optimal candidates for study. Refinement can be achieved by using this technology to aid in early endpoints, as well as pain assessment. For clinical cases, understanding changes in the animal's movements that indicate pain will allow researchers to initiate treatment earlier, therefore increasing animal quality of life and welfare.

Dr. Hall-Ursone outlined the experimental setup, which included an enclosure with indoor and outdoor access, with cameras positioned in selected areas. Initial tasks included updating the neural network and annotation, identifying and labeling more than 300 images of baboons, designing and deploying a data-capture system for the baboon enclosure, adapting a human performance capture tool to fit the application, and capturing about 20,000 frames of data from seven baboons. Dr. Hall-Ursone shared a video recording using this approach.

Research applications of the technology include marmoset and baboon models for Parkinson's disease, a marmoset model for multiple sclerosis, and treadmill studies to characterize gait in baboons and marmosets. This project has fostered a strong partnership with the Southwest Research Institute, and grants currently are being pursued by two SNPRC scientists. Another investigator is determining how to modify the technology for use in marmosets. Dr. Hall-Ursone noted that a method for animal identification must be developed. Additionally, radiographic evaluation could help provide evidence for correlation between arthritis and movement.

Future tasks include using collected data to update baboon monitoring, correlating observed behavioral or physical changes with changes in posed data, and determining the technology's use in additional animal models and project applications.

Rigor and Reproducibility in Cognitive Behavior Without Social Isolation During Testing
Kathleen Grant, Ph.D., Oregon National Primate Research Center

Dr. Kathleen Grant spoke on the assessment of cognitive behavior in an animal model for voluntary alcohol self-administration. She explained that traditionally, cognitive behavior is assessed in NHPs in an isolated environment. She noted that lack of throughput is a limitation of this approach. She presented a new experimental setup, in which operant panels are embedded in quad housing cages for food and fluid intake and cognitive behavioral testing. Modifications include caging, electricity, cable management, and husbandry.

Advantages of the approach include precise measures of fluid and food intake; precise measures of timing; event-triggered chains of behavioral assessments; and lack of disruption due to relocation, which allows spontaneous sequencing of behaviors. Dr. Grant outlined the experimental model, which includes key timepoints in training and self-administration. Cognitive testing is performed via magnetic resonance imaging and hypothalamic-pituitary-adrenal axis function. Data can be analyzed and grouped via machine learning. She showed a representative video of individual differences in animal choices.

Dr. Grant shared data indicating that drinking behaviors can be predicted by baseline performance. Imaging studies indicate that the striatum, orbitofrontal and prefrontal cortices, and ventromedial prefrontal and superior temporal cortices play a role in determining performance. Performance can be improved through manipulation of the putamen with designer receptors exclusively activated by designer drugs (DREADDs).

Dr. Grant asserted that this approach is novel, efficient, replicable, predictive, sensitive, and longitudinal. Future directions include expansion into all housing environments. She concluded by emphasizing several

points: (1) cohort designs can test and reaffirm the rigor and reproducibility of scientific approaches, (2) allowing individual differences helps reveal predispositions to behavioral disorders mediated by neural circuitry, (3) throughput is essential for integration across data domains, and (4) a tissue and data repository procedure allows *ex vivo* synaptic recording and banking of brain areas and peripheral tissues.

Machine Learning–Enabled Pig Activity Monitoring
Gota Morota, Ph.D., Virginia Polytechnic Institute and State University

Dr. Gota Morota discussed recent work on machine learning and new technologies to perform monitoring in pigs. He first highlighted three components of genetics: phenotyping, genotyping and sequencing, and the phenotype–genotype relationship. The cost of genome sequencing has decreased substantially in recent years; as a result, phenotyping now is more expensive (i.e., in both money and labor) than genotyping.

Phenotyping has emerged as a major bottleneck in recent years. Phenotypes that are difficult to collect with current technologies include activity, behavior, social interaction, and repeated records. Real-time, continuous monitoring is critical in large animals (e.g., pigs). Available new systems include computer vision (e.g., videos, images) and wearable sensors.

New 3D depth-sensor cameras can be used to generate both color and dense-depth images, as well as other information. Computer vision systems for automated monitoring can record morphology (e.g., growth rate, body weight, body condition score) and activity (e.g., distance traveled; frequency of standing, sitting, or lying down; food and water intake).

Dr. Morota highlighted work focused on continuous monitoring of pig body weight from image data. He shared an example of a depth video. The images can be used to determine width, length, and height, which are used to calculate body volume; volume is highly correlated with body weight. Dr. Morota also discussed determination of pig activity, which involves tracking the same animal consistently across many frames and performing multi-object tracking. Trackers include sparse optical flow, multiple-instance learning, and channel and spatial reliability. Motion hotspots also can be detected.

Continuous monitoring of activity also can be achieved through the use of wearable sensors, which are attached to the animal's back with a harness. Dr. Morota briefly outlined the experimental setup. Data are annotated using Data Capture Lab software. Several behaviors—such as eating, lying down, walking, and standing—can be determined through machine learning performance comparison.

Dr. Morota concluded by outlining future directions for this work. He noted challenges, which include video data size and identification, battery duration, and the need for real-time monitoring. He suggested exploring ways in which computer vision and wearable sensors can be leveraged to help one another.

Automated Spatial and Nonspatial Memory Testing in Laboratory Pigs
Timothy Allen, Ph.D., Florida International University

Dr. Timothy Allen discussed his work on automating spatial and nonspatial memory testing in laboratory pigs. His laboratory is focused on the relationship between neurobiology and cognition. The group has used rodent models primarily, with a typical cross-species approach (i.e., moving directly between rodents and humans). He noted, however, that an additional animal model is needed to understand these systems. The group has developed pigs as a preclinical model for behavioral neuroscience.

The pig brain is about one-tenth the size of the human brain and is heavily gyrated, with long-distance networks and well-defined hippocampal formation. Previous studies suggest that pigs have strong spatial memory and can learn spatial tasks easily. To facilitate rigorous comparisons with rodent work,

Dr. Allen's group built a large, automated maze suitable for pigs. Tracking is performed in real time from a separate location. Dr. Allen shared representative videos and data from maze-based tracking experiments. He also highlighted the use of touchscreens to test cognition in pigs by assessing fixed and conditional associations. He shared a representative video of the animal performing the task. The generated data can be compared directly with results from experiments in humans.

The team also developed the "HogHat," which can be applied for neural recordings. Depth electrodes are implanted into the brain through the device for measurement of chronic pig neurophysiology. Additionally, spatial behaviors can be correlated to brain volumetrics through structural and diffusion-weighted neuroimaging.

Dr. Allen concluded by emphasizing that automated cognitive assessments can be performed reliably in pigs—with all the laboratory rigor available in rodents—using the automated maze and touchscreen setups. These setups allow rigorous comparisons with rodent and human tasks and facilitate pigs as a large-animal model for translational neuroscience. He emphasized that cognition is an important intrinsic factor, and laboratory tests will be needed to better understand this variable.

Discussion

- In response to a question from Dr. Matthew Jorgensen, Dr. Hall-Ursone clarified that the motion-capture system can identify and differentiate among individual animals.

- Dr. Cohen asked whether the motion-capture system can be used to capture abnormalities in animals prior to their use in experiments. Dr. Hall-Ursone commented that several efforts in this area are ongoing, and the technology could be applied for this use in the future.

- Dr. Ryan asked whether the animals in the cognition studies are separated from their pair-mates during study time or are singly housed during the entire experiment. Dr. Grant explained that the animals spend 2 hours per day with their partner and are separated during the rest of the period. She noted that the team is investigating other options that use radio-frequency identification technology, which could allow the pair-mates to stay together.

- Dr. Cohen asked Dr. Grant how the lack of relocation might affect study results. Dr. Grant responded that this approach is more efficient and highly replicable. She stated that she is interested in applying the tool to more complex housing environments.

- Dr. Grant remarked that low performers tend to be distracted by other factors in the room. She is interested in exploring attention deficit behaviors further. Mr. Alan Olzinski asked whether distracted animals might respond differently in isolation. Dr. Grant agreed that isolation likely would affect the results, but the team was interested in testing natural disposition.

- Dr. John Vanchiere wondered about the use of attention-deficit/hyperactivity disorder (ADHD) medications to help low performers. He noted that untreated ADHD might be related to future alcohol-use disorder. Dr. Grant was uncertain but noted that training of animals is likely to play a role in changing behavior. She noted that the field of medicine is moving toward specific circuitry manipulations and underscored the importance of more work in this area.

- Dr. Lee asked how many pigs can be tracked simultaneously. Dr. Morota replied that currently, the technology can accurately monitor as many as three pigs at once. Dr. Lee commented that for larger facilities, multiple cameras would be needed. In response to a follow-up question, Dr. Morota stated that the cost of the sensor is about $20–30, and the cost of the video camera is about $250–300.

- Dr. Lee asked about the size of the pig maze and touchscreen room. Dr. Allen noted that mazes are 17 feet long by 15 feet wide, and height is the greatest constraint. A hang-over version of the touch screen has been developed, so the devices can be moved as needed. He suggested that in the future, multiple pig touchscreen experiments could be performed simultaneously. The ideal weight limit is about 150 pounds; the group's heaviest pig in these experiments was about 220 pounds.

- A participant asked whether odor-based memories are addressed in repeated tests. Dr. Allen stated that the space is sanitized between animals, with a period of several hours between tests. The effects of odor cues cannot, however, be eliminated entirely.

- In response to a question from Dr. Lee, Dr. Allen stated that he has not observed sex differences in spatial memory. He noted that social dynamics play a role in results.

- Dr. Reginald Miller commented that the differences between adult and adolescent memory in smaller pigs should be considered. Dr. Allen noted that the tests can be used to determine changes in memory during development. The pig brain reaches its adult state by about 9–12 months. He noted that his team is collecting a data bank of pigs up to 6 years of age, and a substantial change in spatial performance has not been noted.

- Dr. John Hasenau asked Dr. Allen whether the HogHat studies can be performed in a social environment. Dr. Allen explained that the pig skull is thick and hard, which allows mounting of the device. Therefore, paired housing likely would be feasible, but he has not yet attempted to do so. Currently, the pigs are housed in neighboring pens.

Group Discussion and Summary

- Dr. Katherine Roe asked whether investigators who purchase animals through the NPRC system are provided a historical knowledge (e.g., rearing, housing, experimental history) of their animals. Dr. Kinnally responded that access to this information is dependent on various factors. She agreed that it would be a good idea to inquire about these factors and report them, if they are available. Dr. Lee added that these factors are challenging to obtain for pig research.

- Dr. Cohen asked the participants for their opinions on reporting of various extrinsic factors in publications. Dr. Grant commented that these factors (e.g., time since acquisition, age of acquisition, time in the experimental facility) are important to understand. She noted, however, that methods sections often are truncated in publications, and many journals discourage submission of supplemental materials. Medical records would be beneficial in experimental designs but likely would not be included in publications. Several participants commented that housing conditions are regularly reported. Dr. Whitworth noted that for pigs, the important factors are dependent on the experimental questions. Journals also have specific requirements.

- Dr. Fox inquired about addressing the history of NHPs imported from China. Dr. Cohen agreed on the importance and challenges of this issue. Dr. Saif noted that previous and current housing status is likely to affect the microbiome in NHPs. Dr. Fox added that this topic represents an important consideration that could be explored in a future workshop.

- Dr. Saif wondered whether animal models could be used to explore differences in susceptibility to COVID-19 and other diseases among socioeconomic groups. Dr. Cohen agreed that this topic would be interesting to explore, and NHPs have served as a valuable model for COVID-19 research. Dr. Grant added that Dr. Ethun's work on social status could provide opportunities for

further exploration in this area. Dr. Ethun noted that NHP social status has been used as a model for socioeconomic studies. She described studies examining changes in gene expression during rearrangement of social groups.

- Drs. Whitworth and Allen added that swine also establish a social hierarchy. Drs. Saif and Tuggle noted that field studies on this topic have been completed. Dr. Saif added that relevant variables (e.g., transport-induced stress) could be considered in this context.

- Several participants suggested that social rank be considered as an extrinsic factor. Dr. Allen cautioned that numerous factors might be present in these dynamics, and further investigation is needed. Dr. Ethun added that the overall effect of chronic stress might be more relevant. Dr. Grant noted that gestation in high-stress environments should be considered in the context of epigenetic changes. She added that protective effects have been reported.

- Dr. Fox asked about consideration of the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) 2.0 Essential 10 Checklist. Dr. Grant expressed support for the guidelines. She noted the need to address statistical requirements, particularly in the context of artificial intelligence. Dr. Ethun agreed on the importance of reporting extrinsic factors.

- Dr. Saif wondered about the importance of incorporating sex as a biological variable. Dr. Grant explained that in rhesus macaques, a spectrum of sexual maturity is present between 2–4 years of age. The brain continues to develop until 6–7 years of age. She noted that researchers often do not monitor these variables and therefore cannot report them. Dr. Saif also commented on the importance of reporting previous pregnancies and miscarriages in research.

- In response to a question from Dr. Fox, Dr. Saif underscored the importance of the microbiome on nutritional factors and the immune response. She noted that sterilization of pig diets is expensive. She added that she plans to refine and explore these dynamics in future studies. Dr. Cohen remarked that NHP researchers also are focused on characterizing the microbiome, including comparative studies of wild and captive animals. Most of these studies are focused on the microbiome in the context of HIV.

- Dr. Saif noted that companion animals tend to share microbiota with their owners. She wondered whether similar studies have been conducted in NHPs. Dr. Cohen was unaware of studies in this area. Dr. Fox remarked that Helping Hands: Monkey Helpers for the Disabled, Inc. might provide opportunities for study in this area.

- Dr. Roe asked about the feasibility of establishing a balance between controlling extrinsic factors while ensuring that experiments remain generalizable and translatable. Dr. Cohen agreed that every factor cannot be controlled fully, but thorough reporting can provide insight into which factors are most important. Dr. Grant added that many investigators are hesitant to modify their established systems. Dr. Timmel remarked that more research on these variables is needed. Such experiments are expensive to perform, and additional funding opportunities will be needed.

- Dr. Allen pointed out that considerations related to reproducibility necessitate both increased throughput and sample size; a balance between those two needs must be considered. Dr. Grant pointed out that NHP researchers have adapted their experimental designs over time to new advances in housing and enrichment. She noted the importance of ongoing flexibility in research.

Session Wrap-Up, Workshop Closing, and Adjournment

Dr. Li reminded the participants of NIH's dedication to rigor and reproducibility in biomedical and biobehavioral research, which was emphasized throughout the workshop. He briefly highlighted the previous session topics, co-chairs, and presenters. Dr. Li listed take-home messages from the workshop:

- Many variations exist among animal models, species, and strains of animals. Each component has specific characteristics, extrinsic factors, and needs for consideration.
- Researchers cannot standardize every extrinsic factor, because too many exist. More studies and discussions on this topic will be needed.
- Monitoring, recording, analyzing, and reporting will be needed.
- Current gaps include equipment for sensing, detecting, monitoring, real-time analyzing, and reporting. Other needs include newly designed fish tanks and uniform lighting in rodent cages.
- Reporting of extrinsic factors will help researchers increase transparency and will help other researchers manage those factors in their work.
- These efforts will entice more stakeholders to join the effort to improve rigor and reproducibility.

Dr. Li explained that the organizing committee will continue to meet after the workshop. Co-chairs of each group will develop a sub-report on each session; a summary of the overall workshop will be produced, and gaps might be suggested. ORIP's Division of Construction and Instruments (DCI) will analyze and identify gaps, inadequacies, or deficiencies in its infrastructure programs and will explore potential avenues to address gaps in monitoring, recording, and reporting. Dr. Li also noted that DCI manages construction and instrumentation programs.

In collaboration with other NIH ICOs, DCI will seek to promote awareness of, advocate support for, and work with other stakeholders to enhance rigor and reproducibility. Dr. Li emphasized that efforts from the scientific community will be needed to address these issues. He thanked the speakers, organizers, and participants for their engagement during the meeting.

Dr. Wang underscored ORIP's commitment to addressing the issue of rigor and reproducibility and emphasized that work in this area is ongoing. Dr. Wang adjourned the meeting.

Appendix A: Meeting Agenda

Session 3. Large Animals (Nonhuman Primates/Swine)
Virtual Meeting
September 30, 2022

12:00–12:10 p.m.	**Opening Remarks** *James Fox, D.V.M., M.S., DACLAM, Massachusetts Institute of Technology* *Xiang-Ning Li, M.D., Ph.D., 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)* *Guanghu (Jeff) Wang, Ph.D., M.B.A., ORIP, DPCPSI, OD, NIH*
12:10–12:40 p.m.	**Keynote Presentation: Precision Medicine—Targeting Individual Differences to Increase Rigor and Translatability** *Erin Kinnally, Ph.D., California National Primate Research Center (CNPRC)*
12:40–1:10 p.m.	**Keynote Presentation: Husbandry of Biomedical Swine Models** *Kristin Whitworth, Ph.D., National Swine Resource and Research Center*
1:10–2:30 p.m.	**Presentations: Construction, Housing, and Caging That Impact Rigor and Reproducibility in Studies Using Large Animals**
	Impacts of NHP Enclosure Design on Welfare and Rigor and Reproducibility *Gregory Timmel, D.V.M., M.S., DACLAM, CNPRC*
	Automated Feeding Stations Provide a Rigorous Tool for Enhancing Outcome Accuracy on Social Determinants of Nutritional Health and Breeding Colony Management in Monkeys *Kelly Ethun, D.V.M., Ph.D., DACLAM, Emory National Primate Research Center*
	Biocontainment Protocols for Improving Survivability of Severe Combined Immunodeficient Pigs *Christopher Tuggle, Ph.D., Iowa State University*
	Gnotobiotic Pigs Infrastructure and Biological Model *Linda Saif, Ph.D., The Ohio State University*
2:30–2:40 p.m.	**Break**
2:40–4:00 p.m.	**Presentations: Equipment and Technology That Enhance Rigor and Reproducibility in Studies Using Large Animals**
	Markerless Motion-Capture Technology *Shannan Hall-Ursone, D.V.M., Southwest National Primate Research Center*
	Rigor and Reproducibility in Cognitive Behavior Without Social Isolation During Testing *Kathleen Grant, Ph.D., Oregon National Primate Research Center*

	Machine Learning–Enabled Pig Activity Monitoring *Gota Morota, Ph.D., Virginia Polytechnic Institute and State University*
	Automated Spatial and Nonspatial Memory Testing in Laboratory Pigs *Timothy Allen, Ph.D., Florida International University*
4:00–4:30 p.m.	**Group Discussion and Summary**
4:30–4:50 p.m.	**Session Wrap-up**
4:50 p.m.	**Adjournment**

Appendix B: Participants List

Session 3. Large Animals (Nonhuman Primates/Swine)
Virtual Meeting
September 30, 2022

Leigh Allen, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH)
Timothy Allen, Florida International University
Yoko Ambrosini, Washington State University
Amanda Armijo, Massachusetts Institute of Technology
Matthew Arnegard, Office of Research Infrastructure Programs (ORIP), Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), Office of the Director (OD), NIH
Carmen Arsuaga, Nationwide Children's Hospital
Jill Ascher, Office of Scientific Resources, Office of Research Services, OD, NIH
Julia Bachman, National Institute of Neurological Disorders and Stroke (NINDS), NIH
Kate Baker, Tulane National Primate Research Center (TNPRC)
Ashley Barnes, ORIP, DPCPSI, OD, NIH
Doug Bartels, National Ferret Research and Resource Institute, The University of Iowa
Taylor Bennett, National Association for Biomedical Research
Skip Bohm, TNPRC
Francesca Bosetti, NINDS, NIH
Aleaya Bowie, Vanderbilt University Medical Center
Christopher Braunger, Washington National Primate Research Center
Jacqueline Brockhurst, Johns Hopkins University School of Medicine
Patricia Brown, Office of Laboratory Animal Welfare (OLAW), Office of Extramural Research (OER), OD, NIH
Monika Burns, Novartis
Ceara Byrne, Massachusetts Institute of Technology
Sarah Carratt, Seagen
Shreaya Chakroborty, National Institute on Aging (NIA), NIH
Anthony Chan, Center for Scientific Review (CSR), NIH
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