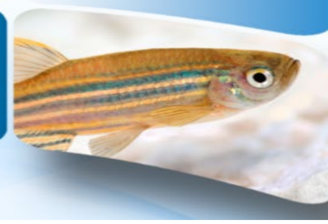

WORKSHOP REPORT

Defined Reference Diets for Zebrafish and Other Aquatic Biomedical Research Models: Needs and Challenges

Division of Program Coordination, Planning, and Strategic Initiatives,
Office of Research Infrastructure Programs,
National Institutes of Health

Bethesda, MD

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National Institutes of Health
Office of Research Infrastructure Programs

Defined Reference Diets for Zebrafish and Other Aquatic Biomedical Research Models: Needs and Challenges Workshop

Purpose of the Meeting:

Aquatic animal species, such as zebrafish (*Danio rerio*), are powerful models for studying human development, behavior, genetics, and disease. The ability to produce transgenic and mutant lines provides biomedical researchers with many options for developing models of human diseases and for developing relevant therapeutic approaches. Different facilities and laboratories use a variety of diets and feeding protocols to maintain these models. In many laboratories, zebrafish are reared with a combination of live feed (*ex vivo*) and/or one of many undefined commercial formulated diets. Commercial diets used in zebrafish husbandry differ significantly in ingredient and nutrient composition and often contain preservatives, lakes, dyes, antinutritional factors, or bioactive food compounds. Studies indicate that the length, weight, sexual maturation, fecundity, and mortality of zebrafish can vary significantly with different diets. Unfortunately, impacts of diet on zebrafish health and behavior and corresponding implications for zebrafish research outcomes are not well described. Currently, the daily dietary nutrient requirements of almost all nutrients have not been investigated. There is also no consensus among aquatic facilities, researchers, and commercial vendors on nutritional requirements at various life stages (*i.e.,* larval, juvenile, and adult) or for research applications to minimize husbandry variations among aquatic facilities or laboratories. Complicit in this lack of consensus is a community-wide lack of understanding of the role of nutrition in animal development, health, and research outcomes. To address this gap, the Office of Research Infrastructure Programs (ORIP) sponsored a workshop to bring together members of the zebrafish scientific community, with expertise in zebrafish and other aquatic and relevant models, for a day of discussion. The workshop attendants assessed the needs and challenges of developing defined reference diets and optimized feed management strategies that will support normal zebrafish development and physiology and will facilitate the analysis of phenotypes in a standardized nutritional environment. Standardization and education will promote rigor and reproducibility in some zebrafish studies and enhance the use of zebrafish and other aquatic models in biomedical research.

Objectives:

- Review diet development strategies, where available, in other biomedical model species.
- Assess the current nutrition status of zebrafish.
- Describe the need for defined diets for maintenance and experimental stocks, including assessment of life stage requirements.
- Discuss the potential impact of defined reference diets on genetic stocks used in biomedical research and on development, physiology, and expressivity of disease/mutant phenotypes.
- Identify obstacles and evaluate strategies that may lead to a successful consensus, acceptance, and implementation of defined reference diets among the different scientific community stakeholders.
- Define an educational approach to informing the community and associated partners (journals, organizations, granting agencies, etc.) about the need of reference diets.
- Determine whether/how the approach to develop a defined reference diet for zebrafish could be applied to other aquatic models, and animal models in general.

Location:

Office of Research Infrastructure Programs, 6701 Democracy Boulevard, 9th Floor Conference Room 987/989, Bethesda, Maryland.

Organizing Committee:

Stephen A. Watts (Chair), University of Alabama at Birmingham, AL

Diana Baumann, Stowers Institute for Medical Research, MO

Miguel Contreras, ORIP, NIH, Bethesda, MD

Willie McCullough, ORIP, NIH, Bethesda, MD

John Rawls, Duke University School of Medicine, NC

Thomas J. Smith, ORIP, NIH, Bethesda, MD

Lilianna Solnica-Krezel, Washington University School of Medicine, MO

Robyn Tanguay, Oregon State University, OR

Zoltan Varga, University of Oregon, OR

Desiree Von Kollmar, ORIP, NIH, Bethesda, MD

Sige Zou, ORIP, NIH, Bethesda, MD

INTRODUCTION TO THE WORKSHOP

Welcome

Stephanie Murphy, V.M.D., Ph.D., D.A.C.L.A.M., Department of Comparative Medicine, Office of Research Infrastructure Programs, NIH

Dr. Stephanie Murphy welcomed the participants and acknowledged her colleagues and the workshop organizing committee. She alluded to the September 2017 ORIP meeting—Zebrafish and Other Fish Models: Extrinsic Environmental Factors for Rigorous Experiments and Reproducible Results—that assembled various stakeholders to discuss experimental rigor and reproducibility in zebrafish and other fish models. The nutrition of zebrafish and other fish models was identified as an important factor and is the focus of this workshop, which is designed to assess the needs and challenges of developing and implementing reference diets and to promote rigor and reproducibility for zebrafish research and other aquatic models.

Defined Reference Diets for Zebrafish and Other Aquatic Biomedical Research Models: Needs and Challenges

Stephen Watts, Ph.D., Department of Biology, Aquatic and Marine Sciences, The University of Alabama at Birmingham

Dr. Stephen Watts thanked the NIH, organizing committee, speakers, collaborators, other stakeholders, and the Nutrition Obesity Research Center at University of Alabama at Birmingham (UAB). He highlighted the importance of zebrafish as a research model and acknowledged similarities between zebrafish and other animals. He reiterated that NIH's mission is to seek fundamental knowledge about the nature and behavior of living systems and apply this knowledge to enhance health, lengthen life, and reduce illness. Complex research tools and novel scientific discoveries require enhanced rigor and reproducibility, which are the cornerstones of scientific advancement. The challenges of establishing rigor and reproducibility include selecting the best statistical analyses, improving transparency, increasing reporting and data sharing, and creating appropriate guidelines. Best management practices and guidelines (i.e., a standardized reference diet) are obligatory to move zebrafish research forward.

Dr. Watts presented an historical overview of efforts toward developing standardized zebrafish diets. In 2010, the Zebrafish Husbandry Association decided that reference diets are needed. Addressing this need, Dr. Watts and his colleagues wrote a review on zebrafish nutrition. Experts convened at Experimental Biology 2016 to discuss nutrition and standardized diets for zebrafish. Recommendations from this conference include (1) determining an optimal standardized diet, (2) soliciting experts of other animal models that have standardized diets, and (3) leveraging existing databases on animal nutrition. The experts concluded that there is a critical need to identify dietary requirements for animal models and improve the quality of reporting to increase rigor and reproducibility.

Highlighting the objectives of the workshop, Dr. Watts mentioned that the participants will review diet development strategies for other model species, describe the need for defined diets for maintenance and experimental stocks while considering lifecycle requirements, and discuss the potential impact of defined reference diets on genetic stocks. Also important is to identify obstacles and evaluate strategies that may lead to a successful consensus, acceptance, and implementation of defined reference diets among diverse stakeholders. Other workshop objectives are to educate the research community and associated partners, as well as to understand how to leverage and apply the approach of developing a reference diet for other aquatic models.

SESSION 1. HISTORIC PERSPECTIVES, CURRENT DIETS, INGREDIENT CONSIDERATIONS, AND FEEDING MANAGEMENT IN THE HUSBANDRY OF ZEBRAFISH AND OTHER ANIMAL MODELS: A COMPARATIVE ANALYSIS

Session Chairs: Robyn Tanguay, Ph.D., Oregon State University; Stephen Watts, Ph.D., Department of Biology, Aquatic and Marine Sciences, University of Alabama at Birmingham

The History of Rodent Nutritional Research and the Development of Standardized Diets: Lessons Learned

Forrest Nielsen, Ph.D., U.S. Department of Agriculture

Dr. Nielsen presented the history of standardized diets and nutrition for rodent models. He acknowledged the efforts of Dr. Greenfield Briggs in developing the American Institute of Nutrition 93 (AIN-93) rodent diet. In 1971, Dr. Briggs and his colleagues noted the poor dietary design and inadequate nutritional content in research. Dr. Briggs recommended that the American Society for Nutrition create an AIN committee to establish nutritional guidelines for animal research. The AIN Committee on Standards for Nutritional Studies prepared a document describing the importance of good dietary environmental controls in experiments with animals. This document emphasized the need for diets with known components and acknowledged that commercial diets with variable, imprecise composition affect experimental results. Dr. Briggs described the various rodent diets. Open-formula diets provide precise percentages or quantities of each ingredient. Closed-formula diets explain the exact composition by type, and the amount of each ingredient is not disclosed. The nutrient composition of the NIH-07 open-formula rodent diet can vary despite the ingredients being listed. Purified diets are composed of consistently refined proteins, carbohydrates, and fat, with added minerals and vitamin mixtures containing appropriately pure components in precisely measured amounts. Purified diets are useful for replicative experiments and must contain open formulas.

Dr. Nielsen described the arduous process of developing the AIN-76 purified diet that met rodent nutrient requirements (e.g., growth, reproduction, lactation). Because of rancidity and vitamin K deficiency, AIN-76 was modified to AIN-76A. AIN-76, although a well-established guideline for 10 years, caused kidney calcification and sucrose-induced abnormalities. An AIN-76A diet reformulation workshop was convened in 1989 to discuss 10 dietary components, including amino acids, proteins, carbohydrates, fats, vitamins, macro- and trace minerals, and miscellaneous factors. The AIN-93G purified diet was developed to circumvent kidney calcification and address the concern about adding arsenic. Compared to AIN-76A, AIN-93G had less sucrose and more cornstarch, and corn oil and DL-methionine were replaced with soybean and L-cysteine, respectively. Changing the molar ratio of calcium and phosphorus eliminated kidney calcification. The final formulation and the use of AIN-93G were reported in a publication, which to date has more than 6,000 citations (Reeves et al, J Nutr. 1993 Nov;123(11):1939-51). Dr. Nielsen commented that the features of the AIN-93 diet are relevant today. He compared the nutrient composition (minerals and macronutrients) of the NIH-07 cereal-based diet with the AIN-93G formulation. NIH-07 contains more required minerals and vitamin content, whereas AIN-93G provides recommended levels of essential minerals. Boron is added to AIN-93G, which is also essential for zebrafish growth. Dr. Nielsen indicated that AIN-93 reduces experimental variability, allowing for data comparisons among laboratories. He commented on the importance of diet users knowing the proper environmental settings and storage conditions and practicing caution with mixing ingredients.

Discussion

In response to a question from Dr. William Ja, Dr. Nielsen indicated that the AIN-93M diet was formulated for rodent maintenance; there was no sex-associated difference between males' and females' response to this diet compared with others.

Dr. Steven Farber asked about the acceptability of the guidelines by the commercial diet vendors. Dr. Nielsen replied that Research Diets, Inc. and other vendors support these guidelines.

In response to a question from a participant, Dr. Nielsen explained that a commitment from individuals and community feedback are required to hasten development of a new diet.

Dr. Marc Tye wondered how many times a new diet must be tested. Dr. Nielsen replied that a new diet must be tested for possible oxidation, rancidity, and fat deterioration.

Dr. Zoltan Varga asked about reproducibility in isogenic strains. Dr. Nielsen speculated that there are likely different dietary requirements based on genetics.

Dr. John Rawls questioned whether open-defined diets exist for rodents. Dr. Nielsen said that there are open formulas of AIN-76 and AIN-93 available.

A participant pondered whether the NIH will stipulate mandatory use of defined diets. Dr. Cindy Davis replied that there is an extensive effort to increase nutritional reporting in science. Purified diets may not be suitable for certain research (e.g., microbiota). Diet standardization is important.

The Role of Nutrition Research in Developing Production Aquaculture: Applications to Zebrafish Culture
Ronald Hardy, Ph.D., Aquaculture Research Institute, University of Idaho

Dr. Hardy described the important nutrition-based parameters for aquaculture that could be applied to zebrafish culture. He noted that fish consumption has exceeded the per capita fish supply. Aquaculture is the fastest growing livestock sector globally because of vaccine development, genetic selection, government policies, and improved feed formulations resulting from nutritional requirement research. Dr. Hardy highlighted the historical development of aquaculture feeds. Illustrating the diversity of early fish feed production, Dr. Hardy outlined studies that established feed parameters. In a 1924 study, it was discovered that trout consumed 49 percent crude protein, which became the template for fish feed. From the 1920s to 1940s, feed was made from animal organs, carp and suckers, and dry blends. By 1953, a trout dry-mixture diet was formulated with 42 percent crude protein. Before 1950, feeds were developed empirically; there were no purified or semi-purified diets. Dr. John Halver -a fish nutritionist- created a semi-purified (casein-gelatin-dextrin) diet, which was used for determining the qualitative requirements of water-soluble vitamins for salmonids. Using this semi-purified diet, Dr. Halver determined fish nutrient requirements by measuring response variables in fish deficient in certain nutrients. Salmonids require 10 essential amino acids, omega-3 fatty acids, 15 essential vitamins, 10 required minerals, carotenoids, and energy. In 2011, the National Research Council (NRC) published the *Nutrient Requirements of Fish and Shrimp*. Despite these efforts, little is known about zebrafish nutrient requirements.

Dr. Hardy explained formulation changes in feeds. Digestible protein and dietary fat quantities have increased in trout feeds, which altered production. The food conversion ratio for salmon and trout have steadily decreased during the past 40 years. Dr. Hardy presented his “hierarchy of feeds,” illustrating the nutritional requirements for commercial feed production. The nutritional requirements for sustaining life, optimal growth, and health are unknown for zebrafish. Formulating nutritionally balanced feeds for zebrafish depends on knowing the nutritional requirements. Closed-formula zebrafish diets are commercially available, whereas open-formula diets (practical diets, semi-purified diets) are cited in the literature. He emphasized that there is insufficient information (e.g., source, chemical analysis, formulation, and ingredients) on experimental feeds. Current zebrafish diets vary in nutritional content among batches. Zebrafish studies should include (1) a commercial reference diet to facilitate comparisons among laboratories; (2) an open-formula, standard zebrafish diet; (3) feed details (i.e., formulation, proximate and energy composition), and (4) efforts to define nutritional requirements for zebrafish.

Discussion

A participant commented that several highly cited, published manuscripts have insufficient or poor nutritional requirement descriptions. Dr. Hardy speculated that the NIH could reach out to commercial vendors during the grant review process. He would like to see more conversations between aquaculture nutritionists and zebrafish communities.

Dr. Varga remarked that Dr. Stephen Ekker -Editor-in-Chief of the *Zebrafish* journal- is championing nutritional requirements for the zebrafish community. Publishing zebrafish research in nutrition journals is important. Dr. Hardy replied that this topic is published in toxicology journals. Dr. Farber added that much has been reported about zebrafish physiology without knowledge of optimal growth conditions. Novel funding mechanisms are needed to address this.

The History of Nutrition and Diet Development in *Drosophila*
William Ja, Ph.D., The Scripps Research Institute

As a comparative model system, Dr. Ja presented the development, nutrition, and dietary requirements for *Drosophila melanogaster* (also known simply as *Drosophila*). Understanding the role of nutrients that affect physiology and selecting best maintenance media are critical for *Drosophila* research. The lifecycle for *Drosophila* includes embryonic, larval, pupal, and adult stages. Within the last 100 years, media have evolved from fermented fruit to yeast, cornmeal, sugar, and agar formulations. Dr. Ja noted that there is no universally used standardized media, although there are several stock food recipes. He presented the methodology of dietary caloric restriction in *Drosophila*. Early work implemented dietary restriction by food dilution; it was assumed this process reduced caloric intake to extend life. Based on these and other reports, the assumption was that dilution reduces calories, which causes lifespan extension. Dr. Ja indicated that changing food concentrations alters food intake; e.g., *Drosophila* can compensate for a 50% decrease in food concentration by eating twice as much. Dietary restriction by food dilution changes food properties, prandial habits, microbial growth, and water intake. When water is supplemented to flies on a diluted diet, there is no impact on lifespan. These results showed that water intake is a crucial factor. Food dilution is no longer recommended for studying caloric and dietary restriction. A current approach implements yeast restriction.

Studies from Dr. Ja's laboratory demonstrate that acidic food increases palatability, mifepristone impacts feeding and lifespan, and microbes serve as a food source. The microbiota enhances *Drosophila* development under low-nutrient diet conditions. Microbial-mediated development occurs via upregulation of gut proteases, amino acid metabolism, and signaling pathways of target of rapamycin and insulin. His data demonstrated that lifespan on undernutrition diets can be rescued with a high-protein microbe diet. His laboratory confirmed the hypothesis that bacteria that best promote development grow better on *Drosophila* media. Various holidic media (entirely chemically defined ingredients) have been developed but are imperfect. One must consider when creating defined diets that amino acids are not palatable and increase osmotic pressure. He underscored that food intake measurements are critical for nutrition studies, including high resolution assays and positive controls to show the measurement assays are sufficiently sensitive. Regardless, the more relevant but not easily measured physiological parameter is nutrient bioavailability or uptake. Dr. Ja concluded by suggesting that there is some risk in standardization of diets or other experimental conditions since phenotypes may be context-specific and less broadly relevant. Improving transparency of research methods and reproducing phenotypes across diets or conditions may be a more valuable approach.

Discussion

In reply to a comment from Dr. Robert Geisler, Dr. Ja indicated that diet selection should be based on project goals (e.g., desired phenotypes).

In reply to a question from a participant, Dr. Ja mentioned that the importance of microbes in diets has been recognized since 1914. The standard medium is enough for sustaining development without adding microbes. Scientists have recognized the role of nature-derived bacteria in *Drosophila* growth and gut colonization.

Dr. James Minchin mentioned that carbohydrate-rich diets are not suitable for zebrafish and wondered whether this relates to palatability or absorption. Dr. Delbert Gatlin replied that, in comparison to other fish, zebrafish need less soluble carbohydrates for energy. Dr. Hardy added that this issue is associated with absorption and glucose transport.

The National Animal Nutrition Program (NANP): Relevance to Zebrafish and Laboratory Animal Models
Delbert Gatlin III, Ph.D., Texas A&M University

Dr. Gatlin talked about the NANP and its relevance to zebrafish research. The NANP was established in 2010 to provide an integrated and systemic approach to sharing, collecting, assembling, synthesizing, and disseminating scientific information and educational tools and to enabling development of animal nutrition technologies that will facilitate high-priority research across agricultural species. Dr. Gatlin expressed his expectation that the NANP will assist zebrafish researchers. The NANP addresses challenges, supports animal agriculture agencies, and fills research gaps in academic communities. Regarding funding and governance, the NANP is one of seven USDA national research support projects.

The NANP is structured into three committees—coordinating (led by Dr. Merlin Lindemann), feed composition (led by Dr. Phil Miller), and modeling (led by Dr. Mark Hanigan). The coordinating committee oversees and coordinates the member selection process and the feed composition and modeling committees, advises the National Academies, and provides a forum for addressing research support needs. The feed composition committee coalesces data and research resources related to feed composition, fosters communication, facilitates efficiencies and consistencies in data collection, and interfaces with and supports nutrient requirement revision committees. The feed committee is most relevant to the topic of zebrafish diet. The feed composition committee identifies assays or methods that are potentially beneficial for diet formulation. The modeling committee improves use of predictive technologies and tools to best utilize available platforms; works with researchers for sharing, combining, manipulating, and analyzing models and modeling information; and interfaces with and supports requirement revision committees. Dr. Gatlin noted that the NANP collaborates with the NRC on the development of nutrient requirement models and feed composition tables. Dr. Gatlin encouraged participants to peruse the NANP website (https://animalnutrition.org/). He highlighted another NANP effort -the Global Animal Nutrition Network- that provides additional information for nutrition researchers. He remarked that the NANP is a dynamic, current, and robust feedstuff database.

Discussion

In reply to a comment from a participant, Dr. Gatlin indicated that the website feed composition tables are updated weekly.

Dr. Farber wondered whether the feed committee addresses the biological complexity of lipids. Dr. Gatlin replied that the tables provide information on fatty acid and cholesterol composition; however, the detailed evaluations of all lipids are not yet available. Lipid fatty acid composition is outlined in the NRC publications.

The Role of Feed Management in Promoting Nutrition in Healthy Zebrafish

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

Mr. Lawrence presented important logistical aspects of feed management in zebrafish facilities. He outlined the zebrafish feeding paradigm that involves traditional and progressive approaches to nutrition. Traditional feeding methods focus on performance (egg growth and embryo production), whereas progressive feeding promotes performance and defines ingredients and quantity. Mr. Lawrence described prior feeding practices that are biologically based. He and his colleagues published an article demonstrating that feeding frequency does not impact zebrafish performance; however, the frequency may pose a challenge to the management of solid wastes generated from formulated feed. The design of standard housing tanks is inefficient for solid waste removal. He showed an example of *Artemia* waste entering a sump pump causing sludge accumulation. Sludge promotes the growth of adventitious microorganisms in the tank system. Feed delivery is challenging in the setting of hundreds of tanks housing zebrafish with varying lifecycles, density, and experimental states. The zebrafish community is moving toward progressive feeding methodologies, such as automated robotic approaches. Nutritional imbalance is another challenge to feed management. Excess nutrients in feed that promote rapid growth cause obesity and excess egg production in zebrafish that are in their maturation plateau growth phase. Mr. Lawrence described studies correcting for this nutritional imbalance that investigate the relationship between ration size and breeding interval in adult fish. Zebrafish were fed 1, 3, or 5 percent of their total body weight daily and bred at various time intervals. The relationship between the number of embryos and viability (fecundity) was evaluated. Zebrafish fed once daily had low fecundity. The goal is to establish higher fecundity and increased embryo viability. The proposed requirements for next-generation zebrafish diets include (1) defined and controllable ingredients, (2) production and maintenance of healthy fish, (3) practical delivery, (4) compatibility with current housing systems, and (5) for the diet to be specific to life-stage and experimental context.

Discussion

In reply to a comment from a participant, Mr. Lawrence specified that zebrafish were fed with an extruded pelleted diet that permits delivery of known quantities. Dr. Farber added that in his lab the preferred use of GEMMA Micro (Skretting Zebrafish) feed over flake diets is a result of its palatability and embryo viability.

Dr. Hardy wondered when zebrafish switch energy allocation from somatic growth to gonadal production. Mr. Lawrence replied that he is unaware of when that occurs; however, he speculated that when the fish are 3–6 months old and have reached a certain body weight (females, 0.5–0.8 grams; males, 0.4 grams), energy is allocated to reproduction. Dr. Minchin mentioned that the 2015 Leibold and Hammerschmidt PLoS One article "Long-term hyperphagia and caloric restriction caused by low- or high-density husbandry have differential effects on zebrafish postembryonic development, somatic growth, fat accumulation and reproduction" addresses this issue.

Mr. Lawrence indicated that altering the time of feed during the day is logistically prohibitive.

Feed and Ingredient Safety

Marc Tye, M.S., Zebrafish Core Facility, University of Minnesota

Mr. Tye presented important considerations for zebrafish feed and ingredient safety. He showed the adverse effects of chromium contamination on zebrafish embryo and larvae health. In 2016, staff members from the core facility at the University of Minnesota noticed a color change in yolk sacs and observed poor larval health. Researchers at the University of Utah also observed a yellow color caused by chromium in zebrafish feed. This contamination caused 90-100 percent mortality in 7- to 8-day-old embryos, severely impacting research. At the University of Minnesota, poor larval health was characterized by non-inflation of the swim bladder, misshapen yolk sac, and cardiac edema. Mr. Tye presumed maternal transfer of chromium onto offspring; thus, contamination did not occur from consumption. He presented studies demonstrating 70 milligrams (mg) of chromium per kilogram weight was present in the diet; this concentration was 30–40 times higher than other tested feeds. These results raised two fundamental questions: (1) What toxins and contaminants are of concern in a zebrafish diet? (2) What concentration levels are acceptable in zebrafish diets?

Addressing the first question, one must consider any compound that could affect research and contaminate feed. Water toxins and contaminants of concern are heavy metals, such as arsenic, chromium, and mercury. Heavy metals cause several developmental defects. Contaminants can be persistent organic pollutants, which include dichloro-diphenyl-trichloroethane, toxaphene, and glyphosate (also known as Roundup). Antimetabolites -trypsin inhibitors and phytic acid- cause adverse growth and metabolic issues. The phytoestrogen genistein induces apoptosis in hindbrain and the spinal cord, and biochanin A alters the sex ratio. Mycotoxins aflatoxin B_1 and ochratoxin B_1 are carcinogenic. Other factors of concern for feed include glucosinolates, oxidized fats, and non-nutritive additives. The European Directive 2002/32/EC of the European Parliament and of the Council of May 7, 2002, on Undesirable Substances in Animal Feed is a good first-step approach for addressing the second question. Nevertheless, more research is needed for determining sublethal effects of toxins and contaminants. The immune response, effect on individual tissues, apoptosis, gene expression, and tissue concentrations should be investigated. Researchers should be informed on the effects of toxins and contaminants and be provided with recommendations.

Discussion

A participant remarked that the toxicity level depends on the type of diet, which could alter the immune system.

Dr. Nielsen cautioned against stating that arsenic is toxic because this metal is inorganic under certain conditions (e.g., while in water). Mr. Tye agreed and added that hexavalent, not trivalent, chromium is toxic.

Zebrafish Nutrition in Europe

Robert Geisler, Ph.D., European Zebrafish Resource Center

Dr. Geisler presented a survey of zebrafish nutrition research in Europe. Out of 500 laboratories from the European Society for Fish Models in Biology and Medicine (EuFishBioMed) that were surveyed, 27 replied. Respondents came from France, Germany, Israel, Italy, Norway, Portugal, and Turkey. Results demonstrate that a wide range of zebrafish dry feeds are used. Skretting is used by approximately 50 percent of the respondent laboratories; 33 percent combine multiple brands of dry feed. Most laboratories use live feed supplemented with *Artemia*. Ninety-three percent combine dry and live feeds. Dr. Geisler explained the current European Zebrafish Resource Center (EZRC) feeding protocol. Zebrafish receive either caviar alone or supplemented with *Artemia*; older fish receive TetraMin flakes supplemented with *Artemia*. Most respondents deemed that feed variability is not an issue; however, several agree that this could present a problem. Many laboratories indicated that they use an internal standard for their feed. Two laboratories in Italy suggested an automated feeding method for flake-based diets. Most respondents believed that open-formulation, chemically defined feed is useful, especially for metabolism, toxicology, and ecotoxicology research. Supporters of this type of feed thought that commercial feed has too much protein and that increasing specific nutrients is better for reproduction. Opponents thought that live feed is better for health and less expensive than chemically defined diets. Sixteen laboratories agreed to test chemically defined feed; however, some institutions lacked the capacity to conduct such studies. When asked whether EuFishBioMed should participate to standardize feeds (e.g., contribute to a white paper), most respondents agreed but cautioned that one must first make quantitative comparisons of different feeding protocols across different facilities. Dr. Geisler guessed that EuFishBioMed lacks enough resources to perform these studies. EuFishBioMed published guidelines on zebrafish husbandry, which will help regulatory authorities and new laboratories.

Discussion

A participant commented that a chemically defined diet is not a natural ingredient diet. Dr. Geisler responded that this feed has individual purified components. Dr. Varga highlighted the importance of common knowledge and standardized terminology across countries. Dr. Minchin added that at his institution -the University of Edinburgh- most laboratories do not study zebrafish less than 5 days old. There will be much resistance toward changing their protocols. Dr. Geisler replied that laboratories should not be forced to change. The survey results are encouraging because most see a need for standardization. Dr. Farber indicated that the results point to the need for more scientific education regarding the relevance of zebrafish husbandry.

WORKING LUNCH. AQUATIC COMMUNITY SURVEY; CURRENT HUSBANDRY OF ZEBRAFISH

Diana Baumann, Ph.D., Reptile and Aquatics Facility, Stowers Institute for Medical Research; Zoltan Varga, Ph.D., Zebrafish International Resource Center, University of Oregon

Dr. Baumann presented the results of a survey of participants from the 13^{th} International Zebrafish Conference, as well as other zebrafish community members. Dr. Baumann outlined the responses of seven survey questions. The total number or percentage of respondents are represented in parentheses.

Question 1: *At what lifecycle do you use zebrafish for your research?*

- There were 244 respondents who used zebrafish; most researchers conduct their work on embryonic and larval stages. Researchers used these animals throughout developmental stages, which points to the diversity of studies.

Question 2: *What diet(s) do you feed your fish?*

- Respondents use *Artemia* (52); GEMMA Micro (34); Zeigler (29); rotifers (21); flake (17); Paramecia (14); Golden Pearls (14); ZM Fish Food and Equipment (7); SPAROS (4); Spirulina flake (4); Argent Hatchfry (3); or Special Diets Services (3). Infrequently used products included krill and caviar.

Question 3: *What made you chose these diets?*

- Experience (27); recommendations from others (24); cost, ease, and availability (16); food composition (13); other (13); or in-house trial (6).

Question 4: *How often are your fish fed per day?*

- Once (7.2%); twice (27.3%); thrice (14.4%); more than three times (5%); less often on weekends and holidays (23.7%); or combinations of above or other frequencies (22.3%). Additional determinants of feeding frequency involved the use of automated feeders, lifecycles, and breeder status versus maintenance.
- Most investigators fed according to the lifecycles, breeder status versus maintenance, and the availability of automated feeders.

Question 5: *How much are your fish being fed per single feed?*

- *Ad libitum* (7.9%); whatever they consume within a few minutes (38.1%); a controlled amount based on fish number per tank (19.1%); a controlled amount based on fish number per tank and an estimated percent body weight (9.5%); or combinations of the above depending on fish stock purpose (25.4%).
- The juveniles and breeders receive more feed.
- Rotifers are in polyculture.
- Investigators designed feed amounts based on 1–4 percent body weight.

Question 6: *Are you interested to participate in inter-laboratory research to determine the nutritional requirements of zebrafish?*

- Yes (17.5%); yes, but with appropriate support (57.1%); maybe (12.7%); or no (12.7%).

Question 7: *Are you interested to participate in inter-laboratory research to develop and test a basic, defined diet for zebrafish?*

- Yes (15.9%); yes, but with appropriate support (54%); maybe (22.2%); or no (7.9%).

Dr. Varga described the mission and activities of the ZIRC, a genetic resource center that acquires, maintains, and distributes wildtype and transgenic zebrafish for researchers. ZIRC emphasizes customer service, which leaves relatively little time for research discovery. The ZIRC consults with facilities regarding husbandry, animal health, and feeding. Food at the ZIRC is provided to fish based on lifecycle and purpose. Dry feeds include a larval dry feed mixture (Zeigler), juvenile mixtures (Zeigler and Golden Pearls), and master mixtures (Zeigler, Spirulina flake, Golden Pearl). Mixing dry feed compensates for potential deficiencies; provides necessary vitamins, trace elements, protein, fats, and pigments; and supports lifecycles. Dr. Varga commented that *Artemia* provides minimal nutritional value; therefore, it is a complementary dietary additive. Live feed comprises of Paramecia Reed Mariculture Rotifers (juveniles only, up to 10 days post fertilization), and *Artemia* (all older animals). Since 2018, the reporting of ingredients in Zeigler adult fish diets improved. Food-delivery methods at the ZIRC involve specialized spoons and feeding devices. Dr. Varga acknowledged that more optimization is needed to use these. He presented the results of a food testing study identifying which diet provides the most optimal growth. Male and female zebrafish were fed six different diets once, twice, or thrice daily for 2 months. Fecundity, gamete quality, weight curves, and growth curves were evaluated. The physical characteristics of certain feeds altered food access. Feed that floated and persisted in the water were more accessible and therefore promoted enhanced weight gain. The results will be used to develop ZIRC's future feeding strategy.

Discussion

Dr. Varga mentioned that the physical properties of food, such as its mass, particle size, or sink rate, play a role in the availability of food to zebrafish and weight gain may vary based on these food characteristics as well. Zebrafish gained the least amount of weight when feed was supplemented with *Artemia*. This is because *Artemia* provide little nutritional value and the *Artemia* feeding quasi "dilutes" the effect of nutrients provided with other feeds.

In response to a participant's query about the speed of consumption and nutrient availability, Dr. Varga indicated that the spoon provides consistent feed amounts for the larvae and adult fish based on zebrafish weight; however, animals are given more than needed. The appearance of sludge depends on the water volume and water turnover of the tank. Dr. Baumann agreed that overfeeding is a challenge and can cause deleterious effects.

Dr. Varga answered a question from Dr. Minchin by explaining that a staff veterinarian performs routine diagnostic evaluations (e.g., infection rate) on the zebrafish.

SESSION 2. THE IMPACT OF DIET VARIATION ON HEALTH AND EXPERIMENTAL OUTCOMES OF ZEBRAFISH AND OTHER AQUATIC MODELS

Session Chair: John Rawls, Ph.D., Duke Microbiome Center, Duke University School of Medicine

The Impact of Diet on Digestive Physiology Research in Zebrafish

Steven Farber, Ph.D., Department of Embryology, Carnegie Institution

Dr. Farber presented the effects of diet on zebrafish study of digestive physiology. A palatable, high-fat, egg-yolk-sac diet was fed to fish on an orbital rocker to address digestion questions. This experimental setup allows for synchronized food consumption. A gut filled with feed has a cloudy color because of the intracellular uptake of fatty acid lipid droplets. Dr. Farber explained the process of lipid metabolism in the intestines. In vertebrates, cytoplasmic lipid droplets provide a nutrient source for cells. Fatty acids are transported across enterocyte membranes into the endoplasmic reticulum and create a cytoplasmic lipid droplet. Lipoproteins assist in exporting fat to neighboring cells. Dr. Farber's laboratory investigates the mechanisms of fat export and transport by measuring the perilipins. To visualize perilipins (cytoplasmic lipid droplets), zebrafish were engineered for expression of enhanced green fluorescent protein (EGFP) at the PLIN 2 (perilipin 2) locus and red fluorescent protein (RFP) at the PLIN 3 (perilipin 3) locus. The goal is to learn how lipid droplets are digested by tracking PLIN proteins following a high-fat meal. More broadly, tracking over time could inform how lipid accumulation is regulated in vertebrates. Studies show that EGFP-PLIN 2 localize to the droplets 6 hours post feed, whereas PLIN 3-RFP is exported at this timepoint.

"Bad" cholesterol, known as low-density lipoprotein (LDL), correlates with the risk of cardiovascular disease (CVD). Apolipoprotein-B (ApoB) -a component of lipoprotein- is also a predictor of CVD. Dr. Farber's laboratory evaluates ApoB in zebrafish. To more sensitively image ApoB in the blood plasma, zebrafish were engineered to express the NanoLuc luciferase reporter at the ApoB locus. The use of ApoB-

NanoLuc permits for comprehensive beta-lipoprotein characterization in larvae after feeding. This is the first high-throughput screening imaging method to evaluate candidate anti-fat therapeutics. Dr. Farber presented data that showed that fasting lowers plasma LDL. Labeled perilipin and ApoB are tools to interrogate lipoprotein secretion and storage. These results are important for understanding how to restore the lipoprotein profile in zebrafish diets. To evaluate inflammation during lipid metabolism, zebrafish were engineered to express RFP at a macrophage-specific locus. These cells migrate to the vasculature during a feeding of a high-fat diet, which increases length and weight. The GEMMA Micro diet supplemented with surplus cholesterol produces lipid accumulation in the 10-day-old larval liver. The conundrum is knowing the baseline "normal" level of lipid droplets during disease.

Discussion

In reply to a comment from Dr. Ja, Dr. Farber acknowledged that he is interested in understanding the body fat content of fish in the wild. His laboratory used zebrafish under normal rearing feeding methods.

Dr. Varga surmised that fasting is a better method of controlling food intake. Dr. Farber replied that all feeding programs produce obese zebrafish.

Dr. Watts commented that GEMMA Micro is 19 percent fat (based on proximate analysis); certain trout feeds are as much as 30 percent.

Nutrition and Cancer—Approaches in Melanoma

Charles Kaufman, M.D., Ph.D., Department of Medicine, Department of Developmental Biology, Washington University of Medicine in St. Louis

Dr. Kaufman described the approach of studying nutrition to better address melanoma. Caloric restriction leads to longer life, improved metabolic parameters, and cancer reduction. Dr. Kaufman is interested in gene transcription, epigenetic changes, and oncometabolites. Cancer, in some cases, induces metabolites that alter gene expression and cause mutations. Therefore, metabolic pathway disruptions can lead to altered gene expression and cellular differentiation. Dr. Kaufman illustrated the process of cancer formation. Cancer initiation involves a single cell surrounded by cancer-prone cells undergoing certain changes that lead to malignancy. Understanding this process is critical for early intervention strategies. Mutations in the *BRAF* (proto-oncogene B-Raf) gene are targets for melanoma therapy. Using a zebrafish melanoma model (transgenic for mutated human *BRAF* [$BRAF^{V600E}$] and p53 deficiency in melanocytes), the epigenetic process of melanoma was evaluated. Dr. Kaufman noted that zebrafish are responsive to *BRAF* inhibitors that are used in humans. Melanoma tumor formation is monitored by evaluating the expression of the embryonic neutral crest marker, crestin. In a normal adult fish, crestin expression is silenced and then turned on during melanoma formation. Crestin-EGFP allows for visualization of melanoma tumor formation at the single-cell level. Tumor formation is preceded by patches of EGFP expression in melanocytes. Barriers to melanoma initiation involve reemergence of neural crest progenitor (NCP) identity, which could lead to cellular reprogramming and dedifferentiation. Dr. Kaufman hypothesized that super enhancers at the Sry-related HMg-Box gene 10 (*SOX 10*) play a role during initiation. In mice, it is believed that *SOX 10* is important for initiation and the turning on of crestin. His laboratory is searching for the upstream events during NCP identity and melanoma initiation by evaluating the epigenetic landscape.

Another focus of Dr. Kaufman's research is assessing the effect of feeding frequency on tumor onset. To do this, zebrafish were fed first with rotifer followed by a GEMMA Micro and rotifer mix, then GEMMA Micro alone. Fish fed four times daily compared with twice or once daily experienced a faster onset of tumor formation and had longer tails. Intermitted fasting experiments and examining the metabolic machinery and nutrients are important next-step approaches. Dr. Kaufman concluded by describing various perceived advantages and disadvantages of a more intensive feeding protocol.

Discussion

Dr. Farber presumed that increased feeding amplified cell division; therefore, cell number should normalize growth curves. Dr. Kaufman agreed and added that the number of melanocytes was not counted.

Physical Environmental Parameters Affecting Experiments: Neural/Behavioral Studies as an Example
John Rawls, Ph.D., Duke Microbiome Center, Duke University School of Medicine

Dr. Rawls explained how the zebrafish microbiome affects host nutrition and physiology and how dietary nutrients affect the microbiome. He presented studies that examined dietary fat and its role in the microbiome. Zebrafish were divided into control, high-fat, or low-fat diets. Gut and water microbiomes were collected at various developmental stages. He commented that the diets were co-developed by Zeigler Bros, Inc. and his laboratory. Low dietary fat increased body length only at 70 days post-fertilization; high-fat diets impaired somatic growth. The composition of the gut microbiome was identified using 16S ribosomal RNA sequencing, which revealed age-dependent inter-individual and inter-age variation in gut microbiome composition. Microbiota colonization in zebrafish results in increased intestinal absorption and metabolism of dietary fatty acids. The transcriptional response to high-fat liposome in the digestive tract is strongly dysregulated in the absence of microbiota. An in-depth analysis of these communities involved hierarchical clustering of operational taxonomic units (OTUS) for identifying putative assemblages. Ontogenetic differences in dietary fat influences the relative abundance of specific assemblages in zebrafish gut and water. The location (gut or water) of bacterial species assemblages depended on developmental stages. These results imply that dietary nutrients shape the compositions of and relationships between gut microbial communities and the surrounding water environment across the lifespan. Starvation during larval or adult stages results in even larger alterations in gut microbiome composition.

Dr. Rawls presented data that showed the effects of the microbiome on gut physiology. His laboratory examined how microbes affect the process of dietary fatty acid absorption into the gut enterocytes and are exported in chylomicrons or stored in lipid droplets. In collaboration with Dr. Farber's group, germ-free zebrafish were housed in gnotobiotic isolators or flaks, provided sterilized diet, and then colonized with selected microbes. *Exiguobacterium acetylicum* promoted dietary fatty acid uptake and formation of lipid droplets in the intestinal epithelium, with subsequent transport and metabolism in liver. A challenge to microbiome research is the development of a long-term gnotobiotic husbandry protocol. Dr. Rawls explained how his laboratory developed a feeding and colonization protocol addressing this challenge. The protocol involves the use of germ-free live feeds that unfortunately induced slow growth rates and poor survival in colonized animals. Another challenge is that germ-free animals eventually develop fatal brain edema. Gnotobiotic methods and microbial strain resources are available to interrogate host-microbiome-nutrient interactions in zebrafish.

Discussion

Dr. Kaufman wondered whether tanks that are housed next to each other have the same microbiome profile. Dr. Rawls replied that his experiments displayed a nontrivial amount of inter-tank microbiome variability. Experiments require multiple replicate tanks, and this would be important to consider in diet studies too.

Dr. Farber asked about the class of bacterial micronutrients that that are beneficial to the host. Dr. Rawls responded that there are different candidates (e.g., vitamins).

Obesity and Adipose Distribution in Relation to Nutrition
James Minchin, Ph.D., Centre for Cardiovascular Science, The University of Edinburgh

Dr. Minchin presented the genetic regulation of adipose tissue in zebrafish. Zebrafish adipose develops in various stages; diet affects this process. Dr. Minchin showed data from others demonstrating that zebrafish adipose accumulates lipid and is formed by adipocytes. Adipocytes are morphologically, functionally, and molecularly similar to mammalian white adipose. Zebrafish adipose lipid storage occurs in postembryonic stages of development; however, the day during which adipocytes first appear varies. This variation results from differences in growth rate and feeding frequency. Studies show that fish sizes vary during postembryonic stages. Twenty-day-old fish varied in total body weight and development, which must be considered when evaluating and selecting diets. Diet and environment strongly influence postembryonic development. Zebrafish grown at lower densities grow more because of high food availability. Fish grown at a higher density with greater food access have altered growth traits, demonstrating the effects of food availability on development. These results accentuate the need for standardized diets and husbandry practices reducing variability during postembryonic stages.

There are two phases of adipose distribution in zebrafish. Adipose develops internally (visceral and non-visceral) or subcutaneously (cranial, truncal, and appendicular). During the expansion of visceral adipose, body fat accumulates quickly. This rate decreases during the later stage of adipose growth (subcutaneous deposition). Dr. Minchin's laboratory assessed the effects of a high-fat diet (egg yolk) on adipose development. Zebrafish were fed their normal regimen and supplemented with 5 percent yolk at various timepoints. Somatic growth was unaltered; however, adipose storage increased. Diet supplementation during the visceral phase causes high-fat growth almost exclusively in the internal visceral tissues. When diet is supplemented at a later phase, the primary storage is subcutaneous. Under food restriction conditions, internal adipose tissues fail to fully replenish lipids during later stages. Thus, a high-fat diet differentially alters body fat distribution based on the phase.

Discussion

In response to a question from Dr. Rawls, Dr. Minchin replied that he does not know whether visceral fat causes metabolic dysregulation. Zebrafish initially store fat around the visceral organs, which may be beneficial to the animal.

Dr. Varga wondered about a possible relationship between adipose tissue and inflammation. Dr. Minchin indicated that high-fat diets cause early onset of inflammation in the adipose tissue.

Zebrafish Models for Obesity and Type 2 Diabetes Mellitus Research

Liqing Zang, M.D., Ph.D., Graduate School of Regional Innovation Studies, Mie University (Japan)

Dr. Zang discussed her laboratory's zebrafish models for obesity and type 2 diabetes. Two zebrafish facilities are located at Mei University-Medical Zebrafish Research Center and Zebrafish Drug Screening Center. Induced models and transgenic and mutant lines are used to model obesity in zebrafish. Dr. Zan's laboratory developed a diet-induced obesity (DIO) model (excess feeding with *Artemia*) that shares common pathophysiological pathways in obese mammals. Three-month-old zebrafish were fed thrice daily with *Artemia* at normally concentrations (5 mg of cysts per fish) or with excess amounts (60 mg of cysts per fish). After 8 weeks of feeding, both male and female fish had significantly increased body mass index and elevated plasma triglycerides. A quantifiable increase in adipose tissue was visible via three-dimensional micro-computed tomography.

Developing a diabetes model required creating hyperglycemic zebrafish. Six zebrafish feeds were tested, and the highest food intake occurred with the otohimeB2, BT-otohimeB2, and Zeigler diets. OtohimeB2 was selected to evaluate short-term intake. The maximum food intake in 5 minutes was 10 mg, whereas the maximum food intake in 1 hour was 15 mg. No difference in intake was observed between males and females. For continuous feeding at 1-hour intervals, females ate the exceeded maximum food daily intake (825 mg). To normalize feeding, an automated feeding system dispensed equivalent amounts. Hyperglycemia was observed in zebrafish that ate 120 mg of otohimeB2 six times daily. Body weight and fasting blood glucose were elevated in DIO animals. Because caloric restriction is the most common treatment for diabetes in humans, zebrafish underwent restriction, which caused reduction in fasting blood glucose as early as 2 weeks. Dr. Zang indicated that DIO fish were glucose intolerant and developed quantifiably increased insulin. Anti-diabetic therapeutics (metformin and glimepiride) reduced glucose in DIO zebrafish. Transcriptome analysis was performed by gene set enrichment analysis RNA sequencing.

Gene sets associated with insulin secretion in hepatopancreas of zebrafish were like those found in human type 2 diabetes. Ongoing projects in her laboratory include identifying and targeting therapeutic genes.

Discussion

In response to a question from a participant, Dr. Zang indicated that food intake is measured via two methods: (1) manually within 3 hours of placing food into the tank and (2) weighing the food onto a dry filter.

Dr. Rawls asked how the new diets induced hyperglycemia in the DIO model. Dr. Zang indicated that this had not been addressed in her studies. Dr. Watts added that diets from laboratory induced hyperglycemia if animals were overfed.

Nutrition and Toxicological Considerations
Robyn Tanguay Ph.D., Department of Environmental and Molecular Toxicology, Oregon State University

Dr. Tanguay spoke about nutritional and toxicological considerations for zebrafish diets. Creating a defined diet is challenging because the components needed for optimal zebrafish health are unknown. Diet performance in recirculating systems is not studied; more education on diet is needed. Long-term measurements of the source, quality, stability, and half-life of feed components are critical for diet standardization. Dr. Tanguay described a case study evaluating defined diets on growth and production. Wildtype zebrafish with greater genetic diversity than typical inbred strains were fed vitamin E-deficient, defined, or commercial feed. Adults were spawned at 3 months and embryos were collected. Vitamin E-deficient feeds caused arrested development and death in offspring.

Dr. Tanguay highlighted a change of the feeding program at the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. As of 2010, small live feeds no longer were offered at this facility. A change in diet was implemented because *Artemia* has inconsistent quality and is a potential source for biosecurity breaches and toxicants. Commercial feeds were removed because of contaminants, along with processing and product inconsistency. The goals of this change included reducing labor cost, improving embryo survival and production, and minimizing biosecurity challenges. A study addressing these goals involved the testing of various diets: SARL (laboratory control), GEMMA Micro, Larval AP-100 (Zeigler), or a Larval AP-100 and *Artemia* mixture. The survival of wildtype zebrafish was evaluated; fish were spawned at 10- to 14-day intervals, and the cumulative number of embryos was recorded. More embryos were produced, larvae survived the longest, and weight gain in the adults was enhanced using the GEMMA Micro diet. This diet produced female fish with a higher body condition factor. These results demonstrate that removal of *Artemia* from the program, in addition to the absence or other live feeds, was beneficial to the animals. Biosecurity potential, labor, and cost were minimized by using different feed.

Bioaccumulation of food contaminants is a major challenge with diets. In separate studies, feeds were evaluated for the presence of toxicants that could modulate development (e.g., pesticides, industrial chemicals, plastics). Toxic compounds eaten by the parents are delivered to the egg and negatively affect embryo development. Dr. Tanguay presented examples from the literature demonstrating that mercury contamination is difficult to remove from the fish body and epigenetically modified offspring.

Discussion

A participant asked why casein, egg whites, and wheat gluten were selected ingredients for the defined diet. Dr. Tanguay answered that these components were selected after receiving advice from a nutritionist, and they do not contain known contaminants.

Diet and Lifecycle: What We Need to Know
Louis D'Abramo, Ph.D., Department of Biology, University of Alabama at Birmingham

Dr. D'Abramo informed the participants about how diet affects the zebrafish lifecycle. He described his previous experience studying and developing defined reference diets for zebrafish. Managing nutritional physiology of different lifecycles is important for establishing greater confidence, comparative value, and efficient progress in research. The adult, juvenile, and larval stages are targets for diet development. Growth is a fundamental physiological process, dietary protein influences body size. The requirements for protein differ according to developmental stage in other fish models. Dr. D'Abramo recommended supporting foundational application of existing knowledge by leveraging published protein requirements for other fish. Knowledge of nutritional requirements is important for developing standardized diets. These requirements, which change throughout the lifecycle, must account for the physical aspects of the feed, palatability, and nutrient sources. He referred to Dr. Watt's study that evaluated zebrafish responses to an array of formulated diets. Male and female zebrafish -28 days post fertilization- were fed *ad libitum* with five commercially available or two experimental diets for 9 weeks. By 9 weeks, animals fed with experimentally defined diet A or B weighed 68-279 mg (females) and 72-181 mg (males). Therefore, experimental diets promoted growth rates comparable to undefined commercial feeds. Commercial diets differ by form, nutrient and energy content, nutrient source, and availability. These differences influence physiological processes; thus, controlling these parameters is important for growth. Control is achieved by identifying at least two readily available food sources that are highly digestible, balanced in amino acids, and not subject to nutrient composition fluctuations.

Based on aquaculture research, Dr. D'Abramo explained the specific nutrient needs per lifecycle. Larvae require phospholipids, long-chain poly-unsaturated fatty acids, high protein, and lipid energy sources. Juvenile adults require phospholipids and predominantly lipid-based energy sources. Brooder fish need high protein and lipid content; vitamins C, E, and B6; and long-chain poly-unsaturated fatty acids. Diet standardization involves (1) knowledge of nutrition for developing standard reference diet(s) during developmental stages, (2) formulated diets through a commercial manufacturer for testing, and (3) fundamental acceptance and use of standard reference diets by the scientific community. Dr. Watts' standardized zebrafish feed could serve as a reference diet.

Discussion

Dr. Ja asked about the relevance of feeding fish *ad libitum* for 9 weeks. Drs. D'Abramo and Watts clarified that feed was incrementally delivered for a specified timeframe, not 24 hours daily for 9 weeks.

SESSION 3. GROUP DISCUSSION, AND SUMMARY

Session Chairs: Organizing Committee

Dr. Watts emphasized the critical value of the zebrafish model in biomedical research while highlighting a substantial knowledge gap of approximately 50 years in zebrafish nutrition compared to other research models. Acknowledging the importance of nutrition education in enhancing the zebrafish model's potential, Dr. Watts convened a discussion to formulate a comprehensive plan for well-defined reference diets. Participants engaged in a comprehensive discussion and provided a series of recommendations aimed at addressing this crucial issue.

Developing Technology for Feed Management: Participants discussed the development of quantifiable food delivery systems, i.e., tools and devices, to ensure precise and consistent food delivery. They also considered the implications of shared versus separate water systems, cost-efficiency, and measures to reduce cross-contamination among tanks. Additional research in this area is warranted.

Improving Quality Control of Feed and Education: The participants discussed addressing the perceived lack of experimental controls in zebrafish nutrition research. They emphasized the need for more outreach and education within the zebrafish research community regarding the impact of diet on diverse phenotypes, drawing insights from other animal systems. Clear goals for nutrition education were identified, recognizing distinct target audiences with varying levels of interest in nutrition research.

Exploring Nutritional Requirements of Zebrafish: The discussion revolved around building on knowledge from other species, performing further research, and defining required daily requirements for macro- and micronutrient content. Participants emphasized the need for research into identifying indicators of overfeeding, developing assays for measuring feeding behavior, digestive efficiency, and physiological outcomes. The group discussed actions regarding existing reference diets and determining which diets support normal zebrafish physiology, including examining reference fish strains.

Determining Desired Health Outcomes. The group focused on evaluating intergenerational health outcomes such as body fat, length, body weight, specific growth rate, viable embryo production, and fecundity. The community also needs to develop a matrix of molecular markers for estimation of health-based parameters.

Overarching Goals. The discussion highlighted the importance of establishing reference diets to facilitate the study of individual dietary components and support nutrition research in aquatic model organisms. Additionally, the participants emphasized the need to investigate the effects of diet on disease susceptibility.

Creating Goals for Diet Development (1–5 Years). The participants proposed a set of goals for diet development over the next 1-5 years. These included gathering input from the zebrafish community, achieving good growth and reproduction while avoiding complications, comparing different diets, and creating nutritionally complete diets with minimal complicating factors. They discussed protein-energy ratios, purified diets, feeding frequency, partnerships between commercial vendors and users, open diet formulations, terminology development, and increasing rigor and reproducibility in research.

Other Discussion Points. Additional topics included the development of an NIH-funded center facility for diet analysis, exploring uniform food presentation methods using microcarrier techniques, and the importance of considering water quality and mineral concentrations. Dr. Watts emphasized the opportunity for the zebrafish community to learn from rodent and aquaculture research.

In conclusion, the discussion highlighted the pressing need for collaborative efforts, interdisciplinary approaches, and funding mechanisms to advance zebrafish nutrition research. The aim is to bridge the significant knowledge gap and enhance the zebrafish model's suitability for translational biomedical research by improving dietary standards and understanding nutritional requirements.

INCREASING RIGOR AND REPRODUCIBILITY: KNOWLEDGE GAPS AND CONSIDERATIONS FOR FEED AND FEED MANAGEMENT

Organizing Committee

General Workshop Considerations:

The Workshop concluded that the zebrafish and other aquatic models are important pre-clinical models of human health. Although a variety of commercial diets are available, dietary requirements are largely lacking when compared to other cultured food fish or animal models such as rodents. The value of the zebrafish and other aquatic models can be greatly enhanced with an increase in understanding of nutrition and nutrient allocation as they relate to development, growth, metabolic health, and reproduction success. These outcomes can be greatly enhanced with the availability of open formulation 1) colony maintenance diets, and 2) standard reference diets.

Identified Knowledge Gaps:

Workshop attendants and the Organizing Committee recognize several knowledge gaps that need to be considered for zebrafish and other aquatic model organisms. To address these knowledge gaps future studies are needed to address the following:

1. *Functional Morphology of Nutrient Acquisition*. For any biomedical model, an understanding of nutrient procurement, including anecdotal and observational data (field and lab) on feeding behavior, the anatomy of the feeding apparatus, and the overall functional morphology of the digestive system is needed. With this information, understanding the dynamic process of feeding

physiology can begin. This does not exclude the role of dissolved organic and inorganic material as sources of nutrition.

2. *Feeding Physiology*. Dynamic physiological attributes can be evaluated by examining a sequential series of related processes, starting with feed intake (those processes related to ingestion) and including information on feed attractants and deterrents, food size, shape, hardness, and other physical and chemical attributes; nutrient processing (those processes related to digestion) including the physiology of digestion at the organ, tissue and cellular levels; nutrient transport across the digestive epithelium, associated endocrine and immune responses; and nutrient/gut/brain interactions. Also, an understanding of the role of the microbiome in digestive physiology and health is needed. Finally, an understanding of waste production must be evaluated. Egestion and associated physiological processes are required to fully understand digestibility and efficiency. Waste production will also affect water quality parameters and, by extension, animal health.

3. *Physiological State of the Model*. Ingestion, digestion and egestion are most likely influenced by the physiological state of the organism. Organismal nutrition will be influenced by body size, age, reproductive state, level of stress, and the nutritional history of the organism. Furthermore, the culture environment, including physical factors such as temperature, salinity, hydrodynamics, light, water chemistry, and their interactions will affect nutritional requirements. Finally, there is a need to recognize that bioactive food components can not only affect digestive and metabolic processes but can affect nutrient allocation and overall health.

4. *The Nutritional Requirements of the Zebrafish and Other Aquatic Model Organisms Are Poorly Understood*. Whereas the natural diet of zebrafish has been previously explored and determined in the field, the specific molecular composition of this diet is not well explored. Importantly, it is unclear whether a standard reference diet developed in zebrafish will work in other freshwater fish models, or if species specific diets will have to be developed for several aquatic species. Key areas needing research in all aquatic model organisms include understanding the amino acid composition essential for growth, maintenance, and breeding, the fatty acid/lipid composition crucial for these same aspects, and the trace minerals and vitamins necessary. Furthermore, there is a need to investigate the optimal dietary balance of amino acids, lipids, vitamins, and micronutrients in the diet. Additionally, exploring differing dietary needs between larvae and adults, especially for optimal growth and breeding, is essential. Lastly, investigating dietary requirements for sex determination, considering genetic and environmental factors, is also crucial.

5. *There Is A Knowledge Gap Regarding Testing Standards for Nutrition Research*. While aquaculture has established best practices for controlling dietary intake and measuring outcomes, these standards have not been rigorously applied in zebrafish and aquatic model organism research. Currently, various methods exist for providing food and measuring outcomes, but they lack consistency. Some methods, like feeding to satiation, are vaguely defined. Others are semiquantitative but cannot account for uneaten food washed out in recirculating filtration systems. Some approaches are highly labor-intensive, involving manual feeding of individual fish, which negates some advantages of aquatic models. The community needs to research and establish a standardized method for controlling dietary intake and measuring outcomes consistently across laboratories. These procedures have yet to be researched and adopted. Additionally, the impact of the aquatic environment on diet uptake and conversion remains poorly understood. For instance, it is unclear whether changes in water chemistry accelerate the leaching of nutrients from provided diets before ingestion or affect the uptake of nutrients by the fish. Similarly, research is needed to determine whether water flow and other environmental parameters should be standardized or if feeding tests should be conducted under static water conditions for improved food uptake control.

6. *The Influence of Diet on the Microbiome and Microbiome Dependent Genetic Pathways Is Poorly Understood*. More research is needed to better understand which research fields would benefit specifically from a standardized reference diet. To achieve this, the influence of diet on gut

microbiome composition and function attributes related of metabolic pathways that promote and regulate normal development and physiology needs to be studied on organismal, cellular, and molecular levels. A set of microbiomes, genetic, and husbandry criteria needs to be researched and determined to establish a baseline standard for a "normal" microbiome against which diet research and dietary requirements can be compared.

7. *Few Pilot Tests Have Been Undertaken Using the Larval and Adult Diets Developed in the Watts Laboratory*. Most studies focus on short-term outcomes, such as growth and breeding performance over several weeks. More research is needed to better understand the transgenerational effects of diets on fish populations. This means, long-term investigations are necessary to explore the role of nutrients on subsequent generations through genetic selection, genetic imprinting, microbiome composition in the gut, and the environment, among other factors.
8. *Aquatic Model Organisms Are Maintained in Closed Populations, Such As Recirculating Water Filtration Systems and Static Tanks*. The impact of diets, including reference standard diets, on biofilter microbial populations, tank biofilm, and aquatic colony health, is virtually unexplored. Therefore, future research also needs to consider the impact of different diets on the fish environment and impact on aquatic facilities including cleaning and maintenance of filter material, soilage of tanks, and Nitrogenous waste load of system water and water exchange rates.
9. Virtually nothing is known about whether knowledge gained from dietary requirements and nutrition research of zebrafish and other aquatic model organisms can be translated to larger scale aquaculture and the modeling of human nutritional diseases and conditions. Diet and nutritional research are needed, for instance, with zebrafish strains that model human metabolic diseases.
10. Due to several aquatic species involved and the interdisciplinary collaboration effort envisioned, an information exchange platform is needed to connect nutritional research on aquatic model organisms, capturing research information and offering education and outreach opportunities. While there is a recognized need for developing standard reference diets in general, there are currently no means to better understand the specific requirements of individual biomedical disciplines within the aquatic research community that rely on more specialized standard reference diets to varying degrees. Knowledge of these specific community needs will assist in the development of more specialized diets in the future, in addition to addressing the immediate needs identified above, which include supporting the growth and rearing of juveniles and the maintenance and breeding of adults.

The above insights acknowledge the requirements and complexities of digestive processes in a number of aquatic models. Comparisons and contrasts among the various models will be of immense value in understanding essential processes related to overall digestion and ultimately animal and human health. Fortunately, many questions related to husbandry can be answered based on our knowledge of multiple species in culture. Of pressing importance is the choice of a diet to support the overall health and ultimately propagation and domestication of many of these species. For this reason, most of our initial attention may focus on development of the diet itself.

Open formulation diets will be a requirement for biomedical animal models. These diets were established in rodents models several decades ago and have been used successfully in thousands of studies. For aquatic models, these diets would include a colony maintenance diet and one or more standard reference diets. A colony maintenance diet contains semipurified, practical, and/or natural ingredients. These diets are not chemically defined but contain well characterized ingredient sources that promote good health and reproductive attributes. Colony maintenance diets are generally for typical animal maintenance and holding/propagation protocols and are not usually used in experiments where nutrition is a variable. The second class of diets are standardized reference diets. These diets are composed of chemically defined ingredients, most of which are purified. Bioactive food compounds are not present in these diets, and they allow for reproducibility among experiments and laboratories.

FINAL COMMENTS AND ADJOURNMENT

Dr. Watts thanked the participants and meeting organizers and remarked that participants learned a great deal from the workshop. The workshop outcomes and recommendations will be outlined in a report that participants will be able to review before it is finalized and sent to the NIH for review. The NIH will identify the methods of addressing knowledge gaps. Dr. Watts adjourned the meeting at 5:16 p.m.

APPENDIX

1. Workshop Agenda

7:30 - 8:00 **On-Site Registration**

8:00 - 8:25 **Introduction and Welcome**
Stephanie Murphy, Division of Comparative Medicine Director, ORIP, NIH
Stephen Watts (OC Chair), University of Alabama at Birmingham, AL

8:25 - 12:00 **Session 1: Historic perspectives, current diets, ingredient considerations, and feeding management in the husbandry of zebrafish and other animal models: a comparative analysis.**
Session Chairs: Robert Tanguay, Oregon State University, and Stephen Watts, University of Alabama at Birmingham

Speakers:

8:25 am: ***The history of rodent nutritional research and the development of standardized diets: lessons learned.*** Forrest Nielsen, Grand Forks Human Nutrition Research Center, U.S. Department of Agriculture

9:05 am: ***The role of nutritional research in developing production aquaculture: applications to zebrafish culture.*** Ronald Hardy, Aquaculture Research Institute, University of Idaho

9:45 am: ***The history of nutrition and diet development in Drosophila and related insects.*** William Ja, Scripps Research Institute

10:15 - 10:30 Break[1]

Speakers:

10:30 am: ***The National Animal Nutrition Program: Relevance to zebrafish and lab animal models.*** Delbert Gatlin, Wildlife and Fisheries Sciences, Texas A&M University

11:00 am: ***The role of feed management in promoting healthy nutrition in zebrafish.*** Chris Lawrence, Boston Children's Hospital, Harvard University

11:20 am: ***Feed and ingredient safety: what we should consider and how we move forward.*** Mark Tye, Zebrafish Core Facility Manager, University of Minnesota

11:40 am: ***Nutrition as a variable in European research communities.*** Robert Geisler, Karlsruhe Institute of Technology, Germany; European Zebrafish Resource Center

12:00 - 12:10 Break[1]

12:10 - 1:00 **Working Lunch.** ***Aquatic Community Survey / Current husbandry of zebrafish.*** Diana Baumann, Stowers Institute, and Zoltan Varga, ZIRC/University of Oregon

1:00 - 3:35 **Session 2: The impact of diet variation on health and experimental outcomes of zebrafish and other aquatic models.**
Session Chair: John Rawls, Duke University School of Medicine

Speakers (15 min presentation and 5 min discussion):

1:00 pm: ***The impact of diet on digestive physiology research in zebrafish.*** Steven Farber, Carnegie Institution for Science

1:20 pm: ***The impact of diet on host microbiome.*** John Rawls, Duke University School of Medicine

1:40 pm: ***Obesity and adipose distribution in relation to nutrition.*** James Minchin, University of Edinburgh, Scotland

2:00 pm: ***Nutrition and cancer***. Charles Kaufman, Washington University School of Medicine

2:20 pm: ***Zebrafish models for obesity and type 2 diabetes mellitus research.*** Liqing Zang, Mie University, Japan

2:40 pm: ***Nutrition and toxicology.*** Robert Tanguay, Oregon State University

3:00 pm: ***Diet and life stage: what we need to know.*** Louis D'Abramo, Past-President World Aquaculture Society, University of Alabama at Birmingham

3:20 - 3:35 Break[1]

3:35 - 4:45 Session 3: Group discussion, and summary
Session Chairs: Committee members.

4:45 - 5:00 Final comments and adjourn.

2. Addendum (January 2024)

The following is a brief update of several outcomes (pilot projects, activities, and events) that were implemented as a consequence of the Workshop reported above. The delay of this report was due to personal and family health-related issues of the Organizer Committee Chair, followed by the COVID-19 pandemic.

2.1. Pilot Projects

In response to the workshop "Defined Reference Diets for Zebrafish and Other Aquatic Biomedical Research Models: Needs and Challenges" held in Bethesda, MD on July 30, 2018, ORIP provided administrative supplements ($278K, total amount) in FY19 (08/07/2019) to support pilot studies of a reference diet at three different fish labs, coordinated by the Zebrafish International Resource Center. Awards were provided to the following parent grants:

- P40OD011021 Zebrafish International Resource Center (ZIRC), University of Oregon, Eugene, OR.
- R24OD011120 Xiphophorus Genetic Stock Center (XGSC), Texas, United States, San Marcos, TX.
- R24OD010998 (OSU) Michael Kent's pathology laboratory, Oregon State University, Corvallis, OR

The workshop discussion highlighted the need of test the basic qualities of a candidate refence diet for fish husbandry in biomedical research. It was proposed studies to compare 3 diets (a facility-specific control diets, a commercially available GEMMA Micro diet, and the Laboratory-formulated Standard Reference Diet/Watts's Diet) using 3 fish species (zebrafish, *Xiphophorus* and medaka) across 3 distinct fish facilities (listed above).

ORIP took advantage of this unique opportunity and -as mentioned- provided funding support for pilot studies at ZIRC (Zoltan Varga (Director), Monte Westerfield (PI) and Katrina Murray (Veterinarian)) that collaborated and coordinated with two other laboratories, one at the Oregon State University (Michael Kent (Pathology) and Thomas Sharpton (Microbiomes)) and another at the *Xiphophorus* Stock Center (Ronald Walter (Director)) to test a proposed reference diet and two other commercially available diets currently used in the aquatic community to grow larva/juvenile and to maintain adult fish. This research consortium pilot studies were meant to establish a cross-platform analysis of fish food and its role for fish fecundity and growth, which represent a subset of key performance requirements for most biomedical fish facilities. The project's goal was to provide data comparing currently used diets vs a reference diet (of well-defined nutritional content and ingredients, i.e., Dr. Steve Watts' diet) on several fish endpoints: growth of juveniles, breeding frequency, clutch size, fertilization rates, embryonic development, early survival rates, and impact on non-infectious diseases, common pathogens, and gut microbiome. The project would help to develop a reference diet for fish husbandry and fill the gap on the knowledge of the nutritional requirements for zebrafish and other small fish. The expectation is that this project had the potentially to get the aquatic community attention to improve rigor and reproducibility of research in zebrafish and other fish for biomedical research.

Outcomes from those pilots' studies are the following published manuscripts:

- Russo C, Drewery M, Chang CT, Savage M, Sanchez L, Varga Z, Kent ML, Walter R, Lu Y. Assessment of Various Standard Fish Diets on Growth and Fecundity of Platyfish (*Xiphophorus maculatus*) and Medaka (*Oryzias latipes*), Zebrafish. 2022 Oct;19(5):181-189. PMC9595639. Tweet.
- Soria E, Russo C, Carlos-Shanley C, Drewery M, Boswell W, Savage M, Sanchez L, Chang C, Varga ZM, Kent ML, Sharpton TJ, Lu Y. Assessment of various standard fish diets on gut microbiome of platyfish *Xiphophorus maculatus*. J Exp Zool B Mol Dev Evol. 2024 May;342(3):271-277. PMC10962282. Tweet.
- Sieler M Jr, Al-Samarrie CE, Kasschau KD, Varga ZM, Kent ML, Sharpton TJ. Common laboratory diets differentially influence zebrafish gut microbiome's successional development and sensitivity to pathogen exposure. Res Sq. [Preprint]. 2023 Feb 2:rs.3.rs-2530939. doi: 10.21203/rs.3.rs-2530939/v1. PMC9915791.

- Sieler M Jr, Al-Samarrie CE, Kasschau KD, Varga ZM, Kent ML, Sharpton TJ. Disentangling the link between zebrafish diet, gut microbiome succession, and *Mycobacterium chelonae* infection. Anim Microbiome. 2023 Aug 10;5(1):38. PMC10413624. Tweet.

2.2. Webinar

The original plan was to have session update of the Diet's Workshop at the 10th Aquatic Models of Human Disease (AQMHD) Conference to be held in 2020. Due to COVID-19 Pandemic, the AQMHD Conference was postponed twice (2020, 2021) until 2022. To keep the aquatic community engaged, two Special Webinars - "Aquatic Models Genomics", October 7-8, 2021, and "Emerging Aquatic Models & Husbandry", February 17-18, 2022, were organized. The second webinar series included a session on Friday, February 18 (10am-12:30pm EST) that was entitled "***Report of a multiplatform candidate reference diet test for biomedical fish models***", and included the participation of the following ORIP Grantees (Tweet):

- Zoltan Varga, Director, Zebrafish International Resource Center
- Yuan Lu, Associate Director, *Xiphophorus* Genetic Stock Center
- Michael Kent, Professor Department of Microbiology

2.3. Update session of the Diet's Workshop at the AQMHD Conference

An update session of the Diet's workshop was organized by ZIRC at the 10th AQMHD Conference (October 6-10, 2022, Marine Biological Laboratory Woods Hole, MA), entitled "Aquatics Nutrition and Reference Diet Development". The session started with a video-recorded introduction to the topic by

Dr. Stephen Watts (University of Alabama; "*Introduction to Nutrition and Standard Reference Diet*") followed by the following reports on ORIP supported pilot projects:

- Dr. Zoltan M. Varga: "*Study Overview and Reference Standard Diet Testing at the ZIRC*"; ZIRC, University of Oregon, Eugene, OR.
- Dr. Yuan Lu: "*Assessment of Various Standard Fish Diets on Growth, Fecundity, and Microbiome of Xiphophorus maculatus (Platyfish) and Oryzias latipes (Medaka)*"; XGSC, Texas State University, TX.
- Dr. Michael Kent ("*Impact of Diet on Growth and Disease Susceptibility in Zebrafish*"; Oregon State University, Corvallis, OR.
- Dr. Thomas Sharpton: "*The Impact of Diet on the Zebrafish Gut Microbiome*"; Oregon State University, Corvallis, OR.

After these presentations, there was a Discussion Session on "*Community Feedback and Community Needs - Nutrition and Development of Standard Reference Diets*" (a summary of the session was reported in the reference cited below).

The following manuscript summarizing the workshop's session has been published:

- Sharpton TJ, Lu Y, Kent ML, Watts SA, Varga ZM. Tenth Aquatic Models of Human Disease Conference 2022 Workshop Report: Aquatics Nutrition and Reference Diet Development. Zebrafish. 2023 Dec;20(6):243-249. PMC10733753. Tweet.

2.4. ORIP's Small Business Program (SBP) Awards

ORIP's SBP is currently supporting the STTR grant R42 OD034188 entitled "*Development and Production of Standardized Reference Diets for Zebrafish Research*". The grant proposed to optimize and implement high-quality standard reference diets for the Zebrafish, an animal model that is important for enhancing the knowledge of human health and the development of vertebrate organisms (ORIP's SBIR grant 1R43 OD026460-01A1 (with the same title indicated above) was transferred to NIMH (R41 MH119793) that funded the grant's phase I (proof-of-concept) in 2020).

The following manuscript has been published acknowledging the support of the R41 grant:

- Watts SA, D'Abramo LR. Standardized Reference Diets for Zebrafish: Addressing Nutritional Control in Experimental Methodology. Annu Rev Nut. 2021 Oct 11:41:511-527. PMC8582320.

In Summary, the Diet's Workshop created a unique opportunity for ORIP to support pilot studies that have resulted in 4 publications (and one preprint) so far; provided Small Business Program support to develop and bring to the market place Standardized Reference Diets for Zebrafish Research; and created opportunities for organizing two events to reach out to the aquatic community (a webinar and a workshop) to provide updates of the pilot projects results and continue conversations about the need to harmonize one of most important extrinsic factors that affect fish husbandry, diets which are recognized as one of the main extrinsic factors that may have an impact on animal research. As such, program will continue to promote this priority topic through some of the following steps as opportunities arise:

- Engage the wider community to promote testing the reference diet in comparison to their current routine facility feeds.
- Highlight preliminary studies results, that have shown that other aquatic communities may need to develop their own specialized diet, as the caser *Xiphophorus*/Platyfish.
- Develop an initiative to accelerate studies of reference diets for aquatic models' research.