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The Vital Relationship Between Nutrition and Health in Zebrafish

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Abstract

In the relatively short span of four decades, the zebrafish (*Danio rerio*) has emerged as an increasingly important model organism for biomedicine and other scientific disciplines. As the scale and sophistication of zebrafish research expands, so too does the need to develop standards that promote the production and maintenance of healthy animals for experiments. A major, but long overlooked, contributor to fish health is nutrition. Historically, feeding practices for laboratory zebrafish have been designed to promote growth and reproductive function. However, as the field matures, it is becoming increasingly clear that the nutritional goals for these animals should evolve beyond basic production to the maintenance of clinically healthy research subjects. This review outlines weaknesses and limitations of current approaches and provides a justification for the development of defined standardized diets that will strengthen and facilitate the continued growth of the zebrafish model system.

Introduction

MODEL ORGANISMS HAVE been an essential component of biomedical and toxicological research for more than a century. Metazoan models include worms, flies, other insects and macrocrustaceans, frogs, birds, rodents, rabbits, dogs, pigs, and nonhuman primates. Within the last several decades, the zebrafish *(Danio rerio)* has evolved as one of the most important model organisms pursuant to the understanding of human health and disease.[1]

The acceptance and utility of zebrafish as a biomedical model have exceeded all expectations. Rapidly developing genomic, transcriptomic, proteomic, and metabolomic tools, along with advances in imaging technologies, have all been leveraged in the zebrafish with great success (e.g.,[2–4]). Indeed, it is now possible to utilize the fish to evaluate physiological, cellular, and molecular processes as related to the onset of human disease and its progression using methodologies that were never considered possible even 5 years ago. These developments have prompted many investigators to embrace zebrafish over traditional animal models. In fact, during the last 15 years, the National Institute of Health in the United States has invested over \$3.5 billion in zebrafish research as it relates to human health (Project Reporter.nih.gov; keyword: zebrafish). In short, the zebrafish model has arrived and is here to stay.

Despite the growing importance of the zebrafish to science, methods for its husbandry and management are still relatively poorly developed.[5] This situation makes it extremely challenging for scientists to standardize environmental conditions during experiments and puts zebrafish at a disadvantage relative to their considerably more defined mammalian counterparts. Perhaps the most prominent example of this environmental variation is in the area of nutrition.[6] Research in virtually all other models has historically promoted the importance of nutrition in outcomes related to development, health, disease, and response to toxic compounds in food and the environment.[7–9] Yet, there is a striking absence of literature that evidences a conviction to the role of nutrition as an important regulator of health and disease in zebrafish.

Scientists have been down this road before. Rodents were one of the first highly applied models for studying human health. It became obvious to all that standards in nutrition were necessary for productive research.[10] In the late 1960s and early 1970s, the American Institute of Nutrition convened committees to address nutritional requirements in the rat and related animal models. These meetings resulted in the recommendation of AIN-76 purified diets designed to evaluate the role of single nutrients, or nutrient classes, on animal performance, particularly as it related to human disease. This work was revisited in 1993 leading to the improved diets AIN-93. These diets represented dietary standards that would not only increase performance but also repeatability of experimental approaches among various laboratories. These purified diets (defined as *purified* using specific ingredients that have been highly purified to a single source nutrient or semipurified to a largely purified nutrient with a minimum of other nutrient inclusions) were effective, but not necessarily the diets that showed the best long-term outcomes in animal

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health. Thus, rodent ''chows'' were created and represented the use of *practical* ingredients (e.g., using corn meal from ground whole corn instead of corn starch or corn gluten [more purified ingredient forms derived from corn]). Unlike purified diets, many practical ingredients contain important micronutrients that enhance long-term growth and health performance (e.g., NIH-31 chow). Whether a purified diet or a chow, researchers needed to share the respective ingredient composition as ''open formulation'' so that nutritional similarities and variants among studies were easily documented.

The zebrafish research community must now undertake the same process. Critical to this endeavor is an understanding of both the limitations and weaknesses of the current system, as well as what the field hopes to achieve by creating defined standards for nutrition. This latter point is especially critical: what should be the nutritional goals for laboratory zebrafish?

Defining a "Healthy" Zebrafish

Given their obvious value for research, the health of laboratory colonies of zebrafish has undoubtedly received greater concern than ever before.[11–13] However, what exactly defines a zebrafish as healthy or unhealthy? The answer to this question is evolving, just as the usage of the model itself.

Traditionally, the general productivity of zebrafish observed in laboratories has also served as a proxy for their health. To put it simply, if the fish in a colony were surviving, breeding, and growing whereby experiments could be completed, then the fish were deemed to be ''healthy''.[12] This mindset has persisted over time, because acute disease-related mortalities in zebrafish research populations remain relatively (and thankfully) uncommon events. Today, especially in light of the rapidly growing sophistication and scale of experiments being conducted with the zebrafish model, such a simplistic worldview is no longer acceptable. Over the past several years, the field has shifted its focus on health away from a loose correlation with performance toward the identification, prevention, and elimination of specific pathogens in research populations and facilities. The approach has three basic goals: (1) to guard against large-scale mortality events arising from acute disease outbreaks, (2) to protect the occupational health of researchers who could be exposed to zoonotic agents, and (3) to better define the pathogen landscape of the experimental fish.

While each of these goals is important, the characterization of the pathogen landscape of the fish being used in experiments is perhaps most timely. An editorial recently published in *Nature* highlighted new guidelines set by the National Institutes of Health that have the goal of improving the reproducibility of funded experiments, including animal studies.[14] Historically, the zebrafish research community's chronic negligence of quantitatively monitoring the health status of laboratory populations is precisely one of the types of shortcomings that these new directives are designed to address and eliminate. This scenario is particularly relevant in the zebrafish world because most common pathogens that affect this species present as subclinical infections that may still impact the outcome of experiments in unexpected ways.[13] Indeed, the threat to reproducibility posed by this dynamic was dramatically highlighted in a recently published study by Spagnoli and coauthors,which showed that zebrafish subclinically infected with the common parasite *Pseudoloma neurophilia* exhibited an altered response to standard behavioral assays.[15]

It is essential then that the concept of fish health extends beyond simply the pathogen status of the animal, especially with respect to how health status relates to the definition of the experimental condition. Numerous environmental abiotic and biotic factors impact the health of the laboratory fish, including water chemistry, the physical environment, genetic background, and behavior. Of these, none is perhaps more pervasive than nutrition. The old adage of ''you are what you eat'' applies also to zebrafish, and any program that attempts to manage fish health must incorporate control over diet (nutrition) or run the risk of being incomplete or irrelevant.

If nutrition is accepted as a critical part of health management, it is instructive to consider the typical end goal of fish production in a research setting: to provide fish that are clinically healthy and representative of animals exhibiting normal physiological function.

Do Current Feeding Practices Promote Health in Laboratory Zebrafish?

In reality, it is not at all clear that feeding practices that are typically in use in zebrafish research settings today achieve the above stated goal of producing and maintaining ''representatively normal healthy'' research subjects. One primary reason for this is that the nutritional requirements of zebrafish are still largely unknown.[6] This is a direct result of a lack of emphasis placed on nutrition by the research community; in short, the subject has not received the attention or funding necessary to drive basic research in this area. The resultant information gap creates a challenge to determine whether or not the diets presently in use promote good health in subject fish.

Proper assessment becomes even more problematic if the metrics for success are not comprehensive. In fact, the only measures commonly used for good health are the absence of known pathogens (in some cases),[12] although this is rare if ever looked at in conjunction with the diet, and performance related indices such as survival, growth, and reproductive output.[16–18] While these factors are often correlated with health, the relationships are not always direct and/or positive in nature.

One way to broaden the evaluation of nutritional inputs on health is by looking at histology. Since 2000, over 2000 articles using zebrafish as a model for cancer have been published in the primary scientific literature (PubMed). Many of the histological markers associated with these cancers may have their origins in potential carcinogens found in undefined nonresearch grade diets.[19] In 2012, Spitsbergen *et al.* reported on the potential of diet and husbandry to influence tumor formation and histological spectra in zebrafish. They showed that diet can affect spontaneous tumor formation in zebrafish and that commercially formulated diets low in nitrosamine typically have lower incidences of ultimobranchial neoplasia. The authors also found that fish fed commercial diets containing sources of fish meal with elevated levels of nitrosamine had higher tumor incidences and a wider variety of histologic types of neoplasia. It is a relatively safe assumption that the commercial feed used for the fish in this study was chosen because it promoted growth and reproduction to at least some degree. This is a good example of how these metrics don't always actually promote good health, since the fish used in those experiments were certainly not healthy from a histological vantage point.

At the same time, there are several lines of evidence that clearly show these approaches are actually detrimental to the health of the fish themselves. One of the most notable weaknesses of current feeding practices for zebrafish is the reliance on live feeds. Live feeds are a vitally important component in the diet of most farmed fish species, especially during the first feeding stages of development.[20] In this respect, the zebrafish is similar. In fact, zebrafish culture relies on zooplankton as feed source to an even greater degree than many other cultured fish species, since the application of live feeds such as *Artemia* and brachionid rotifers often extends beyond the larval stages well into adult development.[21] Furthermore, nonconventional live prey species, most notably *Paramecium* spp., are also widely utilized as feed sources for zebrafish.[22,23]

Despite their prevalence, there are a number of potential pitfalls associated with the usage of these feeds, especially for fish in research settings. These pitfalls include variability in nutrient profile with the culture environment, limited availability, high consumable and labor costs, and the potential exposure of fish to non-nutritional factors (i.e., heavy metals or pesticides) that may be present in these organisms.[24,25]

Perhaps the most critical issue, especially as it relates to fish health, is the potential for live organisms to act as vector for pathogens.[26] Indeed, *Artemia* and rotifer cultures are veritable ''bacterial soups,'' which have been shown to support diverse microbial communities, including numerous species of *Vibrio*.[27–30] As these and other commonly utilized zooplankton species will indiscriminately feed on bacteria, it is entirely possible that they would then have infective potential if they ingested pathogens and then in turn were themselves ingested by fish. Documented cases of this dynamic are not limited to *Vibrio*. Peterson and coauthors showed that *Paramecium caudatum*, a commonly utilized prey item for first feeding zebrafish,[22,31] experimentally enhanced transmission of both *Mycobacterium chelonae* and *M. marinum* to zebrafish larvae.[32] Interestingly, these authors found much greater rates of infection in fish when the bacteria were bioencapsulated within *Paramecium* vs. direct exposure to the agent. This study clearly highlights the biosecurity risk associated with the use of live feeds.

The risk also extends to nonlive natural feeds, that is, frozen, freeze-dried, or otherwise processed post harvest. Oligochaete worms, several forms of which are frequently utilized in the hobbyist community as ''natural'' feeds for ornamental fish, have been shown to be a vector for myxozoan[33–35] and nematode parasites.[36]

Given the biosecurity risk associated with their use, efforts should be made to reduce or eliminate the use of live feeds in zebrafish research facilities. However, a major barrier to this is the efficiency loss associated with their removal from the diet.[12] Live diets are clearly superior to processed feeds during early larval stages of development.[37–40] Furthermore, there has been a recent movement, especially in Europe, to provide live prey items throughout the entire life cycle as a form of environmental enrichment.[41,42] Therefore, if live feeds are retained in the diet, the recommendation would be to subject these items to routine screening to reduce the risk of contamination by pathogens. Such an approach has been used at Boston Children's Hospital, where rotifers and algae paste (rotifer feed), along with the processed diets fed to adults,[16] are screened by PCR for a panel of the most common pathogens known to infect zebrafish.

Another major problem with current feeding practices is in the design of available diets. Most, if not all, diets in use in the zebrafish community are designed for commercial aquaculture species or ornamental fish. In particular, care should be exercised when extrapolating information from aquaculture species for the laboratory fish. Most aquaculture diets are formulated to (1) produce a fish that grows rapidly, often using metrics related to meat production and not necessarily long-term health, and (2) use least cost formulations, whereas cheaper and more available ingredient sources of nutrients are substituted for more expensive sources to reduce the cost of the feed. Furthermore, many commercially produced species are not reared to reproductive maturity, so reproductive outcomes that are often of critical importance in zebrafish research production settings are not considered. Thus, the goals of many food fish producers and associated science may be inherently different than those of a zebrafish researcher. The zebrafish research community should be less concerned with the cost of formulation than the nutrient content and the consequence of those dietary nutrients in relation to fish health. Nor can the focus be on rapid growth alone, but rather to optimize health while still achieving acceptable rates of growth.

Emerging data indicate that this is not being achieved with commercial diets. In their analysis, Watts *et al.* have just shown that zebrafish fed commercial feeds commonly utilized across the community had greatly increased adiposity when fed at normal daily rations (Abstract, Experimental Biology Conference, 2016). If diets that produce rapid growth are selected, then it should also be acknowledged that this may also lead to unconscious selection for genetics and epigenetics of rapid growth and any associated comorbidities. This could occur with little or no knowledge of the consequence to other metabolic processes, or how those processes affect disease onset and progression.

Finally, there is an overall lack of control over the ingredients going into the diets being used in research fish. It is very important to report and understand the composition of diets and the specific nutrients provided by all ingredients.[6] Not only is it critical to understand nutrient content but also many ingredients contain non-nutrient factors that can either promote or deleteriously affect animal health. Some of these factors are bioactive food compounds that are, in many cases, associated with promoting health. Resveratrol (associated with red grapes), sulforaphane (associated with broccoli), and epigallocatechin gallate (EGCG, associated with green tea) are just a few examples of compounds that can be found in some foods or ingredients. These compounds do not act as nutrients, but can affect the way in which nutrients are processed or used. In conjunction with the development of a standardized diet that will allow an understanding of the processes concerning disease onset and progression, the diet should not contain known bioactive food components.

Similarly, antinutritional factors in some ingredients can adversely impact health. These antinutritional factors may act to inhibit or even prevent the digestion and/or uptake of particular nutrients. Therefore, caution needs to be exerted in the choice of potential ingredients. Soybean meal, for example, can contain a trypsin inhibitor and as a result, there is rising use of fermented soybean meal in fish diets. Inhibitors of amylases used for carbohydrate digestion are commonly found in feedstuffs that originate from beans. In these cases, formulations may be based on ensuring that the level of the inhibitor is kept below a certain concentration. Oils derived from seeds that have a characteristically high lipid content may also contain lipase inhibitors.

Again, use of these ingredients is not prohibitive, but maximum levels may need to be established. Other compounds that are found in other potential ingredients and that have potential adverse effects on nutrient availability, consumption, and reproductive outcomes are phytic acid or phytate (the salt of phytic acid) and gossypol, found in cottonseed meal. Products such as glandless cottonseed meal eliminate the presence and toxicity of gossypol while providing an excellent source of protein. Addition of phytase to a diet can substantially reduce phytate's ability to reduce the availability of minerals for assimilation. Care must also be exercised to ensure that processing or source of the feedstuff does not concentrate pesticides that would be toxic at certain levels. Therefore, any standardized diet for zebrafish investigations must be formulated with the knowledge of potential bioactive food compounds and antinutritional factors in the choice of ingredients.

The lack of dietary standards is now further complicated by genetic modifications that have become so prevalent in the model. Tools that allow for relatively facile generation of transgenic other genetically-modified lines have made zebrafish an extremely powerful model. However, genetically modified fish may also vary in their energy and nutrient requirements, particularly when genes that are defined or hypothesized to have nutritional regulation in other species are targeted. Therefore, diet could possibly be used as an essential tool to further evaluate and understand the consequences of altered genetics.

The Path Forward

In 2014, the National Institutes of Health held a joint conference with the publishing community to focus on opportunities in the science publications to enhance rigor and further support research that is reproducible, robust, and transparent (www.nih.gov/research-training/rigor-reproducibility). Suggested guidelines included establishing best practice for animal research, including source, species, strain, sex, age, husbandry, and strain characteristics of transgenic animals. Husbandry includes procedures that can provide reader salient approaches used to propagate and rear the animals. This will include nutritional information. The current problem is that many articles do not provide the necessary nutritional information that would allow rigor to be evaluated and the experimental approach to be reproduced. Part of this shortcoming is based on the lack of nutritional training of animal research personnel, and thus, authors may ''not know what they don't know.''

Alternatively, authors may not realize that nutritional information is necessary or relevant, or they presume it doesn't really matter to their area of scientific inquiry. Since nutritional information is paramount for any discussion of health in an animal model, it cannot be minimized or ignored. Thus, it is time for community-based standards to be developed, with appropriate nomenclature and reporting standards.

Rigor and reproducibility in biomedical research have become a major focus at the NIH (www.nih.gov/research-training/rigor-reproducibility) and within the research community at large. In response to the renewed emphasis on ensuring reproducible results in NIH supported projects, the zebrafish community, with support from the NIH, USDA, and publishers within the scientific community, must address the evaluation of current logistical guidelines, nutritional requirements, reporting standards, and educational activities designed to improve experimental rigor and reproducibility of nutrition-dependent research and reporting in zebrafish. The *Nutrient Requirements of Laboratory Animals 4th Edition* (NRC) was last revised in 1995 and zebrafish were not included. In addition, ARRIVE guidelines for information related to nutrition reporting in animal preclinical studies are essentially absent. The research community would benefit greatly from a synthesis and inclusion of new information related to zebrafish nutrition and health.

The enhanced focus on rigor and reproducibility in animal model research, and specifically in zebrafish, will be essential for high-fidelity biomedical research. Appropriate, explicit, and well-developed standards in zebrafish nutrition will be a cornerstone in the foundation of rigorous experimental design and reproducibility in zebrafish research. To promote and enhance the functional utility of the zebrafish model, several tasks must be accomplished.

First, appropriate health markers specific to zebrafish must be identified. These markers could not only include behavior, animal growth, infection status, pathology, wound healing, and reproductive success but also use new molecular technologies that can use a systems approach to determining health, including markers of inflammation, immunity, oxidative stress, nutrient sensing, energetics, and cell function. Second, the daily nutritional requirements of zebrafish at all life stages need to be determined. This can only be accomplished if purified diets are developed to identify those specific nutrients important to normal physiological function. Rodent researchers began this approach over 50 years ago, and zebrafish research lags in this regard. Third, open formulation diets, whether purified or chemically-defined diets or practical chows, must be made available to the research community. Finally, reporting standards for publications should be established to promote the rigor and reproducibility of experimentation and allow meta-analysis for future evaluations.

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